The cannabinoid CB2 receptor as a target for the treatment of neuropathic pain

Philip William Brownjohn

A thesis submitted for the degree of Doctor of Philosophy

Department of Pharmacology and Toxicology

University of Otago
Abstract

Cannabinoids are cannabis-like drugs that are moderately efficacious in the treatment of some types of chronic pain, including pain of a neuropathic origin. Limiting their therapeutic application, however, are adverse psychoactive effects as a result of cannabinoid receptor activation in the central nervous system (CNS). Cannabinoids act via two receptors: cannabinoid receptor I (CB1) and cannabinoid receptor II (CB2), which are respectively known as the central and peripheral cannabinoid receptors. While CB1 receptors are thought to mediate the antinociceptive effects of cannabinoids, they are also responsible for the adverse effects. Selective targeting of CB2 receptors has shown promise as a treatment in neuropathic pain models in animals, without the associated psychoactivity seen following CB1 receptor activation, however the cellular mechanisms involved have not been elucidated. While initially considered a peripheral receptor, recent evidence has suggested that CB2 receptors may be upregulated in the CNS following neuropathic pain. Using a well established rodent model of neuropathic pain, this study aimed to assess the efficacy of CB2 selective agonists in the treatment of allodynia (pain in response to a normally innocuous stimulus), and investigate the presence and function of CB2 receptors in the spinal cord, a key structure in nociceptive transmission and processing.

In the chronic constriction injury (CCI) model of sciatic neuropathy, CB2 selective agonists were efficacious when delivered systemically at high doses, but not when delivered by intrathecal cannulation to the spinal cord. Using immunohistochemistry and Western blot, labeling was detected for CB2 receptors in the superficial dorsal horn of the spinal cord, which was not modulated by CCI surgery or drug administration. Attempted validation of the antibody used in these approaches, however, indicated that this antibody was not specific for CB2 protein, and detects at least one unspecified cytosolic protein in addition to CB2. These findings cast doubt on the
validity of this primary antibody which has been widely used, both in these immunohistochemistry and Western blot studies, as well as in previous reports of CB2 receptor protein expression. To circumvent this issue, a functional receptor assay, the [$^{35}$S]GTPγS assay, was employed to assess the presence of functional CB2 receptors in the spinal cord. Employing this assay on membrane preparations and tissue slices in situ, no evidence for functional CB2 receptors was found in sham or neuropathic spinal cords.

This study found that while efficacious in the treatment of neuropathic pain in this model, CB2 selective agonists are not acting via spinal CB2 receptors. Furthermore, no evidence was found for functional CB2 receptors above the threshold for detection in the healthy or neuropathic spinal cord, suggesting that spinal CB2 receptors are not a rational target for the treatment of neuropathic pain following peripheral nerve injury.
Acknowledgements

This long and rewarding journey has only been possible because of the advice and support I received from so many along the way.

First and foremost I would like to thank my primary supervisor Dr John Ashton, who has been a source of invaluable guidance and support throughout the last 3 years. I sincerely appreciate his commitment, and his providing me the opportunity to be involved in such a rewarding project. His approach to science and supervision have inspired me, and driven me to develop as a scientist, and for this I cannot thank him enough. Thanks also goes to my co-supervisors Professor Paul Smith, Associate Professor Steve Kerr, and, until recently, Professor George Lees, who all provided helpful and enlightened suggestions at meetings that have improved this project dramatically. I also wish to specially acknowledge Professor Paul Smith, who provided statistical advice throughout the project.

Integral to the successful establishment of the surgical model, I wish to draw attention to Dr John Schofield, the University of Otago veterinarian for his surgical expertise. Both his commitment to animal welfare and his pragmatic approach to research are to be commended. For their expertise with spinal anatomy and dissection in the early stages of the project, I wish to thank Dr Toyokazu Tsuchihara and Associate Professor Dorothy Oorschot.

From the University of Auckland, I would like to thank Associate Professor Michelle Glass and her laboratory, namely Dr Megan Dowie, Dr Scott Graham and Dr Catherine Goodfellow. While providing advice from afar, visits to their laboratory have been stimulating and enlightening. From the University of New Mexico, special thanks go to Assistant Professor Erin Milligan and her team, particularly Jenny Wilkerson and Professor James Wallace. It was while undertaking a research trip to their institution in 2009 that I learnt the behavioural testing paradigm and intrathecal delivery technique that have formed a core part of this project. Also at the University of New Mexico, Dr Dan
Savage provided some excellent advice regarding functional cell assays. From the Hebrew University of Jerusalem, I would like to acknowledge Raphael Mechoulam and Lital Magid who synthesised and generously provided us with the cannabinoid compound HU910. Similarly, I would like to thank our collaborator, Professor Ken Mackie, at Indiana University. While providing the knockout tissue for this project, he was also kind enough to host me at his institution, where he, Professor Andrea Hohmann and Assistant Professor Heather Bradshaw provided invaluable advice at a key stage in this project. From the Centre for Protein Research at the University of Otago, I would also like to sincerely thank Dr Torsten Kleffmann, who went above and beyond with his commitment to the mass spectrometry work in this project.

From the Ashton laboratory, I would like to especially thank Jack Rivers and Simran Maggo, who have been there from the beginning, for their good humour and support, as well as Therri Hoffman and Courtney Breen. And from the Department of Pharmacology and Toxicology, I would like to thank the academic and general staff, as well as the students, for all the help they have given me.

Finally, I would like to thank my family, and my partner Alice, whose unwavering love and support have helped me through this challenging project over the past 3 years.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>I</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>III</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>V</td>
</tr>
<tr>
<td>List of Tables</td>
<td>VII</td>
</tr>
<tr>
<td>List of Figures</td>
<td>VIII</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>X</td>
</tr>
</tbody>
</table>

1 Introduction

1.1 Neuropathic pain
1.2 Cannabinoids
1.3 Cannabinoid ligands
1.4 Cannabinoids as a treatment for neuropathic pain
1.5 Targeting the CB2 receptor
1.6 Target of CB2 selective ligands
1.7 Aims of the current study

2 Establishment and validation of the CCI model

2.1 Introduction
2.2 Experimental aims
2.3 Methods
2.4 Results
2.5 Discussion

3 The effect CB2 receptor agonism on allodynia in the CCI model

3.1 Introduction
3.2 Experimental aims
3.3 Methods
3.4 Results
3.5 Discussion

4 CB2 receptor protein expression in the CCI model

4.1 Introduction
4.2 Experimental aims
4.3 Methods
4.4 Results
4.5 Discussion
4.6 Conclusions

5 CB2 receptor function in the CCI model
  5.1 Introduction
  5.2 Experimental aims
  5.3 Methods
  5.4 Results
  5.5 Discussion
  5.6 Conclusions

6 Discussion
  6.1 Summary of findings
  6.2 CB2 selective agonists for the treatment of pain
  6.3 Preclinical pain testing
  6.4 CB2 receptor expression in the CNS
  6.5 Suppression of microglia as a treatment strategy for neuropathic pain
  6.6 Final conclusions

7 References

8 Appendix 1 – Publications arising from this thesis
9 Appendix 2 – Densitometry saturation plots
10 Appendix 3 – The effects of cannabinoid vehicles on allodynia
11 Appendix 4 – Toxicology of chronic GW405833 administration
List of Tables

Table 1 *Functional and physical characteristics of sensory afferent fibers.* 4
Table 2 *Preclinical evidence for CB2 selective agonist anti-nociception in rodents.* 41
Table 3 *Preclinical evidence for a spinal location of CB2-mediated anti-nociception.* 45
Table 4 *Organ weights of Wistar rats following daily administration of GW405833 or vehicle.* 238
List of Figures

Figure 1.1 Primary afferents synapse in the dorsal horn of the spinal cord. 7
Figure 1.2 The spinothalamic and spinoparabrachial ascending pain pathways. 9
Figure 1.3 Representative cannabinoid ligand structures. 28
Figure 1.4 Sites of cannabinoid CB1 mediated antinociception. 37
Figure 1.5 Proposed sites of cannabinoid CB2 mediated anti-nociception in neuropathic pain. 46

Figure 2.1 Schematic representation of rodent neuropathic pain models. 49
Figure 2.2 CCI induces bilateral mechanical allodynia in the hindpaws of the rat. 55
Figure 2.3 Gliosis in the lumbar spinal cord following CCI or sham surgery. 56
Figure 2.4 Microglial encapsulation of motor neurons in the ipsilateral ventral horn of CCI treated rats. 57

Figure 3.1 Timeline of drug intervention and behavioural testing in CCI treated rats. 70
Figure 3.2 Effect of systemically (i.p.) administered CB2 selective agonist GW405833 on CCI-induced mechanical allodynia in the rat. 75
Figure 3.3 Effect of spinally (i.t.) delivered CB2 selective partial agonist GW405833 on CCI-induced mechanical allodynia in the rat. 76
Figure 3.4 Delivered i.t., the CB2 selective agonists GW405833, JWH-133 and HU910 have no significant effect on mechanical allodynia in CCI treated rats. 77
Figure 3.5 I.t. administration of WIN 55,212-2 ameliorates CCI-induced mechanical allodynia. 78
Figure 3.6 Comparison between methods of intrathecal delivery. 90

Figure 4.1 CB2 receptor immunolabeling with the Cayman Chemical CB2 antibody. 113
Figure 4.2 Quantitative analysis of CB2 receptor immunolabeling in the superficial dorsal horn of sham and CCI treated rats. 114
Figure 4.3 Phenotyping of CB2 immunolabeled cells. 115
Figure 4.4 Western blot analysis of CB2 receptor labeling with the Cayman Chemical antibody. 117
Figure 4.5 CB2 receptor immunocytochemistry with the Cayman Chemical CB2 antibody. 118
Figure 4.6 CB2 receptor immunolabeling in wildtype and CB2 knockout mouse tissue with the Cayman Chemical CB2 antibody. 119
Figure 4.7 Differential preparation of Western blot samples for mass spectrometry alters the banding pattern detected by the Cayman Chemical CB2 antibody.

Figure 4.8 Mass spectrometric analysis of the CB2 receptor protein in rat CB2 over expressing CHO cells.

Figure 4.9 Knockout images with corresponding normalized optical density histograms from Wotherspoon et al. (2005).

Figure 5.1 The $[^{35}\text{S}]$GTPγS assay.

Figure 5.2 Ligand stimulated $[^{35}\text{S}]$GTPγS binding in healthy spinal tissue.

Figure 5.3 JWH-133 stimulated $[^{35}\text{S}]$GTPγS binding in spinal membrane homogenates from CCI and sham treated rats.

Figure 5.4 $[^{35}\text{S}]$GTPγS autoradiography in spinal cord sections from sham and CCI treated rats.

Figure 5.5 Stimulated $[^{35}\text{S}]$GTPγS autoradiography in the spinal cord dorsal horn of sham and CCI treated rats.

Figure 9.1 Densitometry saturation curve for Western blot experiments.

Figure 9.2 Densitometry saturation curve for $[^{35}\text{S}]$GTPγS experiments.

Figure 10.1 The effect of vehicle and administration paradigms on the reversal of CCI-induced mechanical allodynia.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonyl glycerol</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin biotin complex</td>
</tr>
<tr>
<td>Abn-CBD</td>
<td>Abnormal cannabidiol</td>
</tr>
<tr>
<td>ACPA</td>
<td>Arachidonylcyclopropylamide</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AMG</td>
<td>Amygdala</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid sensing ion channel</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>BPA</td>
<td>Brachial plexus avulsion</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cap-SMH</td>
<td>Capsaicin-induced secondary mechanical hyperalgesia</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid 1 receptor</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid 2 receptor</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injury</td>
</tr>
<tr>
<td>CE</td>
<td>Cremophor® EL ethanol vehicle</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freud’s adjuvant</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COR</td>
<td>Cortex</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CST</td>
<td>Complete sciatic transection</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DABNi</td>
<td>Diaminobenzidine nickel</td>
</tr>
</tbody>
</table>
DADLE  [D-Ala^2, D-Leu^5]-encephalin
DAMGO  [D-Ala^2, N-MePhe^4, Gly-ol]-encephalin
DCE   DMSO, Cremophor, ethanol vehicle
dH_2O  Distilled water
DMSO  Dimethyl sulfoxide
DPCPX  8-cyclopentyl-1,3-dipropylxanthine
DPX   Di-n-butyl phthalate in xylene
DRG   Dorsal root ganglion/ganglia
DTEA  Docosatetraenylethanolamide
DTT    Dithiothreitol
EAE    Experimental autoimmune encephalomyelitis
EDTA  Ethylenediaminetetraacetic acid
EGTA  Ethylene glycol tetraacetic acid
ENaC  Epithelial sodium channel
FAAH  Fatty acid amide hydrolase
FDA   Food and drug administration
GABA  Gamma-aminobutyric acid
GDP   Guanosine-5'-diphosphate disodium salt
GFAP  Glial fibrillary acidic protein
GM-CSF Granulocyte macrophage-colony stimulating factor
GPCR  G protein coupled receptor
GTP  Guanosine triphosphate
[^35S]GTP\gamma S[^35S radiolabeled guanosine 5'-O-[gamma-thio]triphosphate
GTP\gamma S  Guanosine 5'-O-[gamma-thio]triphosphate
HCl   Hydrochloric acid
HEK  Human embryonic kidney
HIV   Human immunodeficiency virus
HLEA  Homo-\gamma-linolenylethanolamide
HPLC  High performance liquid chromatography
HRP  Horse radish peroxidase
HYP  Hypothalamus
IASP  International Association for the Study of Pain
Iba1 Ionized calcium binding adapter molecule 1
i.c.v. Intracerebral ventricular
IgG Immunoglobulin G
IHC Immunohistochemistry
IL-1β Interleukin 1 beta
IL-6 Interleukin 6
INFγ Interferon gamma
iNOS Inducible nitric oxide synthase
i.p Intraperitoneal
i.t. Intrathecal
i.v. Intravenous
kD Kilodalton
KO Knockout
L4 – L6 Lumbar spinal region 4 – 6
LC-MS/MS Liquid chromatography tandem mass spectrometry
LPI Lysophosphatidylinositol
LPS Lipopolysaccharide
MAGL Monoacylglycerol lipase
MAPK Mitogen-activated protein kinase
mRNA Messenger ribonucleic acid
MS Multiple sclerosis
MS Mass spectrometry
NAc Nucleus accumbens
NADA N-arachidonoyl-dopamine
NeuN Neuronal nuclei
NeupSIG Neuropathic pain special interest group
NGF Neural growth factor
NIH National Institutes of Health
NMDA N-methyl D-aspartate
NNT Number needed to treat
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus raphe magnus</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting compound</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OX-42</td>
<td>Integrin αM</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>PB</td>
<td>Parabrachial</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHARMAC</td>
<td>Pharmaceutical Management Agency</td>
</tr>
<tr>
<td>PHN</td>
<td>Post herpetic neuralgia</td>
</tr>
<tr>
<td>PNS</td>
<td>Post nuclear supernatant</td>
</tr>
<tr>
<td>PSL</td>
<td>Partial sciatic ligation</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIM</td>
<td>Selective ion monitoring</td>
</tr>
<tr>
<td>SIN</td>
<td>Sciatic inflammatory neuritis</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Silencing ribonucleic acid</td>
</tr>
<tr>
<td>SNI</td>
<td>Spared nerve injury</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve ligation</td>
</tr>
<tr>
<td>SNRI</td>
<td>Selective noradrenalin reuptake inhibitor</td>
</tr>
<tr>
<td>SNT</td>
<td>Spinal nerve transection</td>
</tr>
<tr>
<td>SRM</td>
<td>Selective reaction monitoring</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>STOPNET</td>
<td>The Study of the Prevalence of Neuropathic Pain</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>THA</td>
<td>Thalamus</td>
</tr>
<tr>
<td>THC</td>
<td>Δ⁹-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential subfamily A, member 1</td>
</tr>
<tr>
<td>TRPM8</td>
<td>Transient receptor potential subfamily M, member 8</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid receptor</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris buffered saline with Triton X-100</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VPL</td>
<td>Ventral posterior nucleus</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range neuron</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Neuropathic pain

1.1.1 Introduction

Neuropathic pain is a pathological condition involving damage to peripheral or central nerve pathways, resulting in a persistent state of spontaneous pain, and/or a hypersensitivity to painful and innocuous stimuli. The defining characteristics of neuropathic pain are the subject of extensive debate. A special interest group of the International Association for the Study of Pain (IASP) with a focus on neuropathic pain (NeuPSIG) has redefined the condition as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treede et al., 2008).

The underlying etiologies of neuropathic pain are varied, yet exhibit similar clinical hallmarks. Some of the more common causes of peripheral neuropathic pain are: painful diabetic neuropathy, caused by hyperglycemic nerve degradation; post herpetic neuralgia (PHN), resulting from herpetic skin lesions; trigeminal neuralgia, an often idiopathic neuropathy of the trigeminal nerve; postsurgical/traumatic injury; phantom limb pain following amputation; multi-factorial consequences of human immunodeficiency virus (HIV) infection; and cancer, either as a result of tumor compression of peripheral nerves, or chemotherapy-induced nerve damage. Central neuropathic pain may arise as a direct result of damage to the spinal and central pain pathways, specifically as a result of stroke, spinal cord injury (SCI) and multiple sclerosis (MS) (Bennett, 2007).
1.1.2 Prevalence

Large scale population studies have only recently been conducted to accurately gauge the prevalence of neuropathic pain in the general population. The first study of this kind, carried out in the UK, reported a 48% prevalence of chronic pain, and an 8.2% prevalence of pain predominantly of neuropathic origin (Torrance et al., 2006). The Study of the Prevalence of Neuropathic Pain (STOPNET) estimated the prevalence of chronic pain at 31.7%, and of chronic pain with neuropathic origin at 6.9% in the general French population (Bouhassira et al., 2008). An Austrian study reported a comparatively low 3.3% prevalence of neuropathic pain, although the authors acknowledge that the use of an internet based survey may have reduced the number of older participants, and thus resulted in an underestimate (Gustorff et al., 2008). Varying estimations of prevalence could also be attributed to the lack of standardized definitions of conditions, and assessment criteria.

Certain underlying conditions can predispose some to neuropathic pain, causing an increased incidence and prevalence of neuropathic pain in specific patient populations. In patients with Herpes zoster, the incidence rate of PHN 3 months post infection has been reported in prospective analyses to be 7.2% (Helgason et al., 2000), and even as high as 27% (Scott et al., 2003). In mixed diabetics, painful peripheral neuropathy has a reported prevalence of 16.2% (Daousi et al., 2004), and in type 2 diabetics, painful diabetic peripheral neuropathy has a prevalence of 26.4% (Davies et al., 2006). HIV related neuropathy can affect anywhere from 1.2 to 69.4% of HIV positive patients (Ghosh et al., 2011). A recent epidemiological study was the first of its kind to directly measure cancer related neuropathic pain, and found a prevalence of 10% in patients from the disease and associated chemotherapy (Garcia de Paredes et al., 2010). Patients with MS are also particularly at risk of developing central neuropathic pain, with a prevalence estimated to be 28% in this patient population (Boneschi et al., 2008). Of particular note is the high prevalence of phantom limb pain in amputees and those with SCI: it has been reported that 70 – 80% of patients experience phantom
limb pain of varying intensity following limb amputation (Sherman et al., 1984; Ehde et al., 2000; Ephraim et al., 2005), and up to 75% of patients with spinal cord injury suffer from pain of neuropathic origin in the 5 years post injury (Siddall et al., 2003).

The socio-economic implications of neuropathic pain are immense. An estimation of direct healthcare costs of neuropathic pain was generated in an analysis of insurance claims in the United States, which found a 3-fold increase in annual healthcare costs in patients with neuropathic pain compared to matched controls ($US17355 versus $US5715, respectively) (Berger et al., 2004). A similar study in Canada found annual healthcare costs more than doubled for patients suffering neuropathic pain compared to age matched controls, from $CAN1846 to $CAN4163 (Lachaine et al., 2007). Co-morbidities such as anxiety and depression are particularly common in sufferers (Gustorff et al., 2008), putting further strain on the health system. Further costs arise from lost employment, and studies have indicated a significant loss of working productivity in patients suffering neuropathic pain (Meyer-Rosberg et al., 2001; McDermott et al., 2006; Galvez et al., 2007). Neuropathic pain is thus a serious condition involving both a reduction in quality of life for sufferers, and an economic cost to society. The optimization of therapy and the development of improved compounds to treat this condition are therefore paramount to easing this social and economic burden.

1.1.3 Nociception

The IASP describes the subjective perception of pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1979). Pain has developed as an evolutionary response to perceived or potential damage to bodily tissues as an essential protective mechanism, and is a complex experience that involves the
sensation and transduction of noxious stimuli, termed nociception from the Latin word “nocere” meaning “to harm”, as well as an emotional component.

1.1.3.1 Nociceptors

In the early 20th century, Sir Charles Sherrington determined that pain was a specific nociceptive response, and first proposed the theory of the nociceptor, a pain sensing neuron characteristically distinct from other sensory fibers, in his seminal 1906 work “The Integrative Action of the Nervous System” (Kusurkar, 2004). In the past century, extensive work has characterized nociceptive transmission and the sensation of pain, and the molecular components involved. There are two major types of nociceptor, termed Aδ and C fibers, which are subsets of primary sensory afferents, a group which also includes Aα and Aβ fibers, which transmit innocuous stimuli. The sensory properties of these sensory fibers are outlined in Table 1.

Table 1 Functional and physical characteristics of sensory afferent fibers. Adapted from Julius et al. (2001) & (Beaulieu et al., 2010).

<table>
<thead>
<tr>
<th>Primary afferent type</th>
<th>Diameter (µm)</th>
<th>Myelinated</th>
<th>Conduction rate (m/s)</th>
<th>Thermal threshold (°C)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aα and Aβ</td>
<td>6 – 12</td>
<td>Heavily</td>
<td>35 – 75</td>
<td>n/a</td>
<td>Proprioception, light touch</td>
</tr>
<tr>
<td>Aδ Type I</td>
<td>1 – 5</td>
<td>Lightly</td>
<td>5 – 30</td>
<td>53</td>
<td>Nociception (mechanical, chemical, high threshold heat)</td>
</tr>
<tr>
<td>Aδ Type II</td>
<td>1 – 5</td>
<td>Lightly</td>
<td>5 – 30</td>
<td>43</td>
<td>Nociception (mechanical, chemical, moderate threshold heat)</td>
</tr>
<tr>
<td>C</td>
<td>0.2 – 1.5</td>
<td>No</td>
<td>0.5 - 2</td>
<td>43</td>
<td>Nociception (mechanical, chemical, moderate threshold heat), itch</td>
</tr>
</tbody>
</table>
As large fiber diameter and myelination both improve transduction speeds, light touch and
proprioception mediated by large diameter, heavily myelinated Aα and Aβ fibers is transmitted
quickly, and is sensed before pain. Nociception is transmitted more slowly via lightly myelinated,
medium diameter Aδ fibers, and unmyelinated small diameter C fibers. Differential transmission
speed through these two fiber types leads to ‘first’ and ‘second’ phases of pain, characterized by an
initial acute, sharp pain, before a diffuse, dull pain (Julius et al., 2001).

Nociceptive receptors respond to a host of exogenous and endogenous chemical signaling
molecules. The transient receptor potential (TRP) channels are cation channels that function as the
parent receptors for a number of exogenous noxious chemical irritants: the TRP vanilloid receptor
(TRPV1) is the receptor for capsaicin, the spicy component of chili peppers and piperine, a
component of black pepper; TRPA1 is the receptor for allyl isothiocynate in mustard oil and wasabi,
and cinnamonaldehyde in cinnamon; TRPM8 is the “minty cool” or menthol receptor also activated
by eucalyptol (McKemy, 2005). The TRP channels have also been described as key molecular targets
of temperature sensation, including in the noxious range. TRPV1 is widely expressed on C fibers and
type II Aδ fibers, and is activated upon noxious heat (> 43°C). TRPV2 on the other hand, is only
expressed on type I Aδ fibers, and is activated at a higher heat threshold (> 53°C), explaining the
differential response of type I Aδ fibers and type II Aδ and C fibers to temperatures in the noxious
range (Caterina et al., 1999). At the other end of the scale, TRPA1 is thought to be responsible for
the transmission of noxious cold stimuli (< 17°C), although reports have been conflicting (Chung et
al., 2011). The molecular basis for noxious mechanical sensation has not been elucidated, and has
been the focus of intense research. It is generally accepted that the molecular target is a mechano
sensitive cation channel, and work with genetic models has suggested as potential candidates the
proton sensitive acid sensing ion channels (ASICs), members of the polymodal TRP channel family
and epithelial sodium channels (ENaC) (Im et al., 2011), although the relative contributions of each
have yet to be elucidated.
1.1.3.2  Nociceptive transmission

Primary afferents are unique neurons in that they have a pseudo-unipolar arrangement, with two axons originating from a single cell body. The cell bodies lie in the dorsal root ganglia (DRG), which are nestled in the intervertebral foramen alongside the spinal canal, and while one axon extends to the periphery, the other innervates the dorsal horn of the spinal cord, with the whole arrangement transmitting peripheral sensory information to the central sensory pathways. The superficial dorsal horn of the spinal cord where these afferents synapse is termed the “gate” (Melzack et al., 1965), so named for its “gate keeping” role in primary processing of sensory transmission. Once processed, sensory information can be acted upon locally, as in reflex circuits, or can be transmitted to higher brain centers where the affective and discriminative nature of pain is encoded.

The spinal cord gray matter is delineated by molecularly determined laminae, termed Rexed’s laminae. The dorsal horns of the gray matter, consisting of laminae I – VI, integrate afferent sensory information, including nociception, while the ventral horns are key components of the efferent motor pathways (Noback et al., 2005). The particular modality of a primary afferent determines which lamina the fiber will terminate in, and in turn determines how the stimulus is integrated and perceived. While nociceptive Aδ and C fibers synapse primarily in the superficial laminae I – II, Aβ touch fibers synapse in deep lamina III – IV (Todd, 2002). Figure 1.1 outlines the delineation of the gray matter by laminae, and the location of primary afferent termination by fiber type.
Primary afferents, also known as first order neurons, synapse with second order neurons in these laminae, which come in two forms: nociceptive-specific neurons, which as their name suggests, deal exclusively with input from nociceptive Aδ and C fibers; and wide dynamic range (WDR) neurons, which encode input from both sensory and nociceptive fibers (Beaulieu et al., 2010). These neurons decussate to the contralateral white matter, and then transmit to the brain via many pathways. Essential for nociception are the spinothalamic and spinoparabrachial pathways, which are outlined in Figure 1.2. Second order spinothalamic tract neurons originate from laminae I and IV – VI, and synapse with tertiary neurons in the lateral thalamus. These fibers then project to the
somatosensory cortices, and are associated with the sensory side of pain sensation, that is modality, strength and location of incoming pain signals. On the other hand, second order spinoparabrachial neurons project to the parabrachial structure of the brainstem, where they synapse with tertiary neurons which then project to the amygdala and hypothalamus, regions associated with the affective and emotional side of pain (Hunt et al., 2001).

1.1.3.3 Endogenous pain inhibition

Modulation of pain transmission throughout the various pathways ultimately determines the perceived level of pain by the organism. At the spinal level, dense populations of inhibitory and excitatory interneurons in the dorsal horns regulate the activity of second order pain neurons, and are themselves modulated by the extracellular environment, and the activity of primary sensory and nociceptive afferents. Most notably in this regard, the activation of Aβ touch fibers results in a recruitment of inhibitory interneurons in the dorsal horn, which in turn temporarily inhibits nociceptive responses from Aδ and C fibers at the same level, a process that forms the basis of the dorsal “gate” theory of pain regulation (Melzack et al., 1965). Descending inhibition involving the cannabinoid, serotonergic, noradrenergic and opioid systems is regulated by nuclei in the brainstem, namely the periaqueductal gray (PAG) and nucleus raphe magnus (NRM), which are themselves modulated by input from the ascending pain pathways (Heinricher et al., 2009).
Figure 1.2 The spinothalamic and spinoparabrachial ascending pain pathways. The spinothalamic pathway (red) sends nociceptive information to areas of the CNS concerned with sensation and discrimination, while the spinoparabrachial pathway (blue) sends nociceptive information to areas of the CNS that process the affective component of pain. Dashed lines indicate ascending axon tracts in the spinal cord. AMG = Amygdala; COR = Cortex; HYP = Hypothalamus; PB = Parabrachial area; THA = Thalamus.
1.1.4  Molecular mechanisms of neuropathic pain

Damage to peripheral nerves initiates a cascade of events that results in a pain state which can persist long after the initial insult has subsided. These events begin locally at the site of damage, in the form of inflammatory and peripheral neuronal changes, which in turn lead to changes in synaptic plasticity and inflammation in the central pain pathways, and ultimately an alteration of pain transmission and perception. Classical hallmarks of neuropathic pain include increased responsiveness to painful stimuli, termed hyperalgesia, a sensation of pain in response to normally innocuous stimuli, termed allodynia, and spontaneous pain in the absence of stimuli.

1.1.4.1  Peripheral sensitization following nerve injury

Peripheral nerve injury results in a cascade of events, driven by local inflammatory mediators, which prime nociceptor afferents to fire at a lowered threshold and/or increased rate. Neutrophils dominate the initial response to nerve lesion, releasing chemoattractants that drive the recruitment and activation of resident and circulating macrophages (Scholz et al., 2007). These immune cells, in combination with supporting Schwann cells and the damaged afferents themselves, release a host of inflammatory mediators, termed an “inflammatory soup” (Dray, 1994). This “soup” of mediators contains prostaglandins, serotonin, adenosine, cytokines and bradykinin, as well as neurotrophic factors such as neural growth factor (NGF), which act to directly sensitize peripheral nerve terminals (Dray, 1995). This sensitization is driven by the activation of kinases which phosphorylate ion channels, and eventually gene regulation by neurotrophic factors which directly affects the expression of nociceptive receptors and ultimately the excitability of the afferents. This can result in firing at a reduced threshold, or even ectopic firing in the absence of stimulation (Woolf et al., 2000).
These peripheral changes in firing characteristics can ultimately change the nature of neuronal activity in the CNS.

1.1.4.2 Central sensitization following nerve injury

An increase in the firing rate of C fiber afferents following nerve injury drives the initial phase of central sensitivity in the spinal cord. Tetanic firing of C fibers, even at low frequency, induces increased glutamate and excitatory neurotransmitter release from afferent terminals, which activate postsynaptic N-methyl,D-aspartate (NMDA) receptors on second order neurons of the dorsal horn. This in turn drives cellular and structural changes, including changes in gene expression, which increase synaptic strength of these nociceptive pathways, a process termed “wind-up”, which parallels the well known process of long term potentiation in CNS synaptic plasticity (Suzuki et al., 2005).

While wind up may account for a hypersensitivity to pain, it does not readily explain the phenomenon of allodynia, where normally innocuous stimuli produce painful responses. As touch and nociceptive afferents are functionally distinct, a central mechanism involving altered integration of sensory information is implied. One popular theory proposes that the reorganization of neuronal afferent terminals in the dorsal horn could potentially result in the miscoding of sensory information in terms of modality (Woolf et al., 1992). It was demonstrated that following peripheral nerve injury, low threshold mechanoreceptor Aβ fibers, which normally terminate in lamina III and IV and convey innocuous touch, sprout fibers into lamina II where nociceptive input from C fibers is encoded (Woolf et al., 1992). Providing functional evidence for this hypothesis, it was confirmed that following sprouting, a subset of terminating afferents in laminae II were now activated by low threshold mechanical stimuli (Kohama et al., 2000). This theory was attractive, as it seemed to neatly
explain the complex phenomenon known as allodynia. However, more recent work has challenged the extent and significance of neuronal sprouting in the dorsal horn following nerve injury (Hughes et al., 2003), and it is now proposed that decreases in the synaptic strength of inhibitory GABAergic and glycinenergic interneurons may result in an unabated spread of excitation from deep laminae processing innocuous stimuli to superficial laminae processing nociceptive input, thus resulting in a miscoding of sensory information (Schoffnegger et al., 2008).

1.1.4.3 Role of glia in central sensitization

In addition to changes in neuronal reorganization in the CNS, it is now widely accepted that glia play an essential role in the development and maintenance of neuropathic pain. While initially thought to play a passive supportive role in relation to neurons, more recent work has shown glia play active roles in neural activity (Vernadakis, 1996; Hatton, 1999). There are two classes of glia in the CNS: macroglia and microglia. Macroglia are comprised of oligodendrocytes, which myelinate neurons; and astrocytes, which share a close relationship with neurons in terms of nutrient support and neuronal activity (Haydon, 2001). Microglia on the other hand have a diverse role to play in CNS physiology and pathology: in the healthy CNS, microglia are proposed to support and maintain synaptic connectivity, while continuously scanning and surveying the microenvironment for pathological changes that induce rapid microglial activation. Once activated, microglia perform the role of a macrophage immune cell in the CNS (Graeber, 2010).

Peripheral nerve injury is associated with an increase in spinal microglial and astrocytic activation, a profile which matches the time course of alterations in pain behavior (Garrison et al., 1991; Colburn et al., 1997; Mika et al., 2009). It has been demonstrated in animal models that inhibition of glial activation and signaling attenuates the development of neuropathic pain (Meller et al., 1994).
Specific inhibition of microglial activation has the same effect, suggesting that microglia may play a more important role than astrocytes in this process (Ledeboer et al., 2005; Mika et al., 2009). The mechanisms by which microglia may contribute directly to altered neuronal function have not been fully elucidated, however an unspecified effect of pro-inflammatory cytokines is suspected, as intrathecal administration of specific cytokines is associated with altered dorsal neuron activity and the development of neuropathic pain behaviors in healthy animals (Reeve et al., 2000; Xu et al., 2010). Another intriguing possibility is that brain-derived neurotrophic factor (BDNF), released from activated microglia, acts on lamina I neurons to cause a down regulation in chloride potassium symporter 5, and subsequent shift in anion reversing potentials, switching GABAergic and glycinergic mediated currents from inhibitory to excitatory (Coull et al., 2005).

One of the intriguing features of peripheral nerve injury is the regular incidence of “mirror-image” pain, in which a unilateral nerve injury can result in the development of bilateral pain and hypersensitivity. The inhibition of glia is able to attenuate the development of mirror-image pain in neuropathic animals (Milligan et al., 2003) strongly suggesting a role for glia in this largely unexplained phenomenon.

### 1.1.5 Evidence base for current treatment strategies

Current pharmacotherapies used in the treatment of neuropathic pain cover a broad range of drug classes, which act at a multitude of sites within the pain pathway. In the periphery, topical anesthetics reduce sensory transmission in sensory afferents, primarily via blockade of depolarizing sodium channels (Amir et al., 2006), while topical capsaicin patches induce the same effect through desensitizing blockade of TRPV1 channels on afferent fibers (Winter et al., 1995). At the spinal level, classical anticonvulsants such as lamotrigine directly inhibit depolarizing sodium currents, resulting
in a decreased excitatory neurotransmitter release from terminating sensory afferents; while
atypical anticonvulsants such as Gabapentin and pregabalin induce similar effects via inhibition of
calcium influx in the same neurons (Jensen et al., 2009). Similarly at the spinal level, opioids act via
presynaptically located inhibitory GPCRs to reduce excitatory neurotransmitter release in the spinal
cord, while also activating descending inhibitory pathways in supraspinal centers (Bushlin et al.,
2010). Acting chiefly supraspinally, antidepressants increase levels of serotonin and noradrenalin at
synaptic junctions via selective inhibition of reuptake pumps, which also act to stimulate descending
inhibitory pathways (Dharmshaktu et al., 2012).

Anticonvulsants, including the widely studied calcium channel α2-δ ligands Gabapentin and
pregabalin, are now at the forefront of neuropathic pain treatment. In a comprehensive meta-
analysis, Gabapentin was determined to be more efficacious than placebo in a range of neuropathic
conditions (Moore et al., 2011). This is not the case for all neuropathies, however, as evidenced by a
lack of treatment effect on pain scores in SCI (Rintala et al., 2007) and chemotherapy induced
neuropathy (Rao et al., 2007). Similarly, an analysis by Moore et al (2009) of 19 randomized
controlled trials (RCTs) of pregabalin reached similar conclusions, suggesting that although no
evidence for the use of pregabalin existed in acute or chronic nociceptive pain conditions, sufferers
of neuropathic pain associated with PHN, diabetic neuropathy, central neuropathy and fibromyalgia
benefited considerably from pregabalin (Moore et al., 2009).

Antidepressants have been widely used in the treatment of neuropathic pain, often with mixed
results. In a triple crossover design, the tricyclic antidepressant (TCA) amitriptyline was significantly
more efficacious in the treatment of SCI associated neuropathic pain than Gabapentin or an active
placebo (Rintala et al., 2007), was more efficacious than placebo in the treatment of painful diabetic
neuropathy (Max et al., 1992) and phantom limb pain (Wilder-Smith et al., 2005) and had a
significant morphine sparing effect in patients with neuropathic cancer pain compared to placebo
(Mishra et al., 2011). However in a parallel design RCT, amitriptyline was not efficacious in treatment
of SCI associated chronic pain of mixed origin (Cardenas et al., 2002), and was ineffective in the treatment of HIV induced neuropathy (Kieburtz et al., 1998). Selective noradrenalin reuptake inhibitors (SNRIs) appear to have shown promise in diabetic neuropathy, with both venlafaxine (Rowbotham et al., 2004) and duloxetine (Raskin et al., 2005) reducing pain scale scores in randomized placebo controlled clinical trials during chronic treatment. Selective serotonin reuptake inhibitors (SSRIs), by comparison, have shown little (Otto et al., 2008) or no (Max et al., 1992) effect on pain scores in controlled trials of painful diabetic neuropathy.

As topical capsaicin and lidocaine formulations act via peripheral nerve pathways, these treatments are only efficacious in the treatment of some peripheral neuropathies. While a wealth of evidence exists for the efficacy of topical lidocaine in the treatment PHN (see Wolff et al., 2011), this treatment has thus far shown limited benefit in patients with post surgical neuropathic pain (Cheville et al., 2009) and neuropathies of varying origin (Ho et al., 2008). A high dose capsaicin dermal patch, developed by NeurogesX, has shown reasonable efficacy in the treatment of post herpetic neuralgia (Backonja et al., 2008) and painful HIV neuropathy (Simpson et al., 2008), and is active for up to 12 weeks following a single application. The benefit of these types of therapeutics is the limitation of adverse side effects. Most adverse events are mild local reactions, as systemic exposure is limited.

Opioids are regularly employed in the management of acute pain, but clinical evidence has also indicated their use in neuropathic pain conditions. A comprehensive Cochrane review revealed that intermediate length treatment (8 days – 10 weeks) of patients with neuropathic pain conditions with typical opioids was efficacious in the reduction of neuropathic pain behaviors compared to placebo (Eisenberg et al., 2006). Specifically, morphine, oxycodone, methadone and levorphanol reduced spontaneous pain in diabetic neuropathy, PHN, phantom pain, and mixed neuropathy significantly, by an average of 13 points on the visual analogue scale (Eisenberg et al., 2006).
Limiting the upper therapeutic dose of many of these drugs are the adverse side effects, particularly apparent with opioids. In theory, combination poly-pharmacy should allow a reduction in dose to avoid adverse events while maintaining or improving efficacy beyond traditional monotherapy. Several combinations with Gabapentin have been studied in RCTs, and compare favorably to monotherapy. Morphine and Gabapentin reduced pain to a greater degree when given in combination than when given individually in the treatment of PHN and diabetic neuropathy, despite lower individual dosages (Gilron et al., 2005). Similarly, the addition of oxycodone to Gabapentin treatment improved pain scores by one third in patients with painful diabetic neuropathy (Hanna et al., 2008). Combination therapy has also been employed with TCAs, and Gilron et al. have shown that combination therapy with Gabapentin and nortriptyline is clinically superior to monotherapy in patients with PHN and diabetic neuropathy (Gilron et al., 2009). In an example of single drug poly-pharmacy, tramadol is an atypical opioid with additional actions on serotonin and noradrenaline transporters that is commonly used to treat neuropathic pain (Otari et al., 2011). In clinical trials, tramadol was able to reduce pain intensity scores in patients with SCI (Norrbrink et al., 2009), diabetic neuropathy (Freeman et al., 2007), PHN (Boureau et al., 2003) and phantom limb pain associated with limb amputation (Wilder-Smith et al., 2005) when compared with placebo.

1.1.6 Recommendations

Several thorough meta-analyses have been conducted to assess the efficacy of various treatments in neuropathic conditions. These aim to provide up to date evidence-based recommendations to clinicians for the treatment of neuropathic pain of mixed origin. Although the origin of neuropathic pain is often a determining factor in treatment selection, considerable diversity between patients
with the same painful conditions means optimum treatment regimes and dose titration require tailoring to the individual.

From RCTs, Finnerup and colleagues (2005) calculated the number needed to treat (NNT) to obtain at least 50% pain relief for a number of therapeutics across all tested neuropathic pain conditions. Opioids and TCAs were among the more efficacious with a NNTs of 2.5 and 3.1, respectively, followed by tramadol at 3.9, Gabapentin and pregabalin at 4.7, and SNRIs at 5.5. Topical lidocaine had a NNT of 4.4, while capsaicin showed only relevant efficacy for PHN, with a NNT of 3.2 (Finnerup et al., 2005). The European Federation of Neurological Societies constructed a set of evidence based guidelines in 2006. They recommend as first line treatments TCAs, Gabapentin and pregabalin, and as second line treatments, selective noradrenalin reuptake inhibitors (SNRIs), lamotrignine and tramadol (Attal et al., 2006). In 2010 the guidelines were updated to include carbamazapine for trigeminal neuralgia, and topical lidocaine and capsaicin for PHN as first line treatments, and recommended cannabinoids or combination therapy for refractory patients (Attal et al., 2010). A consensus statement from the Canadian Pain Society in 2007 made many of the same recommendations: TCAs, Gabapentin and pregabalin were once again recommended as first line treatments, followed by SNRIs and topical lidocaine for PHN second, tramadol and opioids third, and cannabinoids, methadone and alternative anticonvulsants for refractory patients (Moulin et al., 2007). In New Zealand, no specific clinical guidelines exist for pharmacotherapeutic intervention in neuropathic pain, although a government funding body, the Pharmaceutical Management Agency (PHARMAC), recommends, by way of government subsidy, initial treatment with TCAs, before the implementation of Gabapentin as a second line therapeutic in case of a lack of efficacy or intolerability (Wilson et al., 2011). Furthermore, a topical capsaicin based cream is specifically indicated in cases of post herpetic neuralgia or diabetic peripheral neuropathies (Wilson et al., 2011).
Although an extensive range of therapeutic strategies exists for the treatment of neuropathic pain, the clinical and epidemiological evidence suggests that many patients may either be refractory to treatment, or may obtain only partial relief of symptoms with the current treatment strategies. High NNT values for almost all of the current treatments (Finnerup et al., 2005) suggest that many patients are not responsive to treatment, and it has been further estimated that 40-60 % of patients remain symptomatic following therapeutic intervention (Dworkin et al., 2003). These figures all point to a considerable need for new therapeutics to treat this condition.
1.2 Cannabinoids

1.2.1 Introduction

Cannabinoids are compounds that are either derived from cannabis, or mimic the physiological effects of cannabis. Cannabinoids can be broadly grouped by their source: those produced by the cannabis plant, termed phytocannabinoids; those produced by animals, including humans, termed endocannabinoids; and those designed synthetically to mimic naturally occurring phyto- and endocannabinoids.

While the cannabis plant has been used for millennia in the treatment of a multitude of diseases, it has only been with the development of purified compounds and characterization of the endocannabinoid system in the body that the potential for cannabinoids as a treatment for chronic pain has been fully realized. While the therapeutic potential of cannabinoids in neuropathic pain has remained low in clinical trials thus far, it is hoped that a greater understanding of cannabinoid pharmacology, coupled with the development of new compounds, could lead to the wider implementation of cannabinoids in the management of pain.

1.2.2 Early history

There are three known species of plant that produce phytocannabinoids: Cannabis sativa, C. indica, and C. ruderalis. Originating in Central and South Asia, the medicinal properties of cannabis have been documented in medicine for thousands of years. Earliest reports of medicinal use originate from China and India, where early pharmacopeias and medical guides describe cannabis as
analgesic, hypnotic, anaesthetic, anti-inflammatory, antibiotic, antiparasitic, antispasmodic, appetite stimulant, diuretic, aphrodisiac and antitussive, highlighting the myriad medical applications of this diverse flora (Zuardi, 2006).

Despite its long history in medicine, it took until the mid 19th century before the study into the pharmacological properties of cannabis was initiated, by Dr William O'Shaughnessy. By the turn of the 19th century, the first cannabinoid, termed cannabinol, was isolated and purified by Wood Spivey and Easterfield (Wood et al., 1899), and three decades later, the chemical structure elucidated (Cahn, 1932). It was in the 1960s, however, that Raphael Mechoulam and colleagues described the structure of Δ9-tetrahydrocannabinol (THC), the principle component of cannabis that is responsible for its well known psychotropic effects (Mechoulam et al., 1965; Mechoulam, 1970; Mechoulam et al., 1970). Elucidation of the constituents of cannabis continued through the proceeding years, and at present, nearly 70 phytocannabinoids have been identified in C. sativa.

1.2.3 The endocannabinoid system

The endocannabinoid system, comprised of endogenously produced cannabinoids, their synthesis and degradation pathways, and ultimate molecular targets, plays a large part in homeostasis and normal physiological function, as well as many pathological conditions. Evidence of endocannabinoid system involvement has been reported in pain and inflammation, food and energy metabolism and various neuropathologies, in addition to the immune, cardiovascular and hepatic systems (for a review see Di Marzo, 2008).
1.2.3.1 Cannabinoid receptors

Up until the late 1980s, cannabis and cannabinoids were thought to exert their psychological effects via the disruption of neuronal membranes. Using a tritium-labeled synthetic cannabinoid, CP55,940, however, Devane et al. (1988) described high affinity binding sites in the rat CNS, suggesting a molecular target for cannabinoids (Devane et al., 1988). Future studies confirmed this, with the cloning of the cannabinoid 1 (CB1) receptor in the rat (Matsuda et al., 1990), and in the human, where levels were greatest in the CNS, and minimal in the periphery (Gerard et al., 1991). In situ radiographic studies in the rat with tritiated CP55,940 found CB1 present at high density in the substantia nigra, globus pallidus, cerebellum, hippocampus, cerebral cortex and striatum, yet relatively low densities in the brainstem and thalamus (Herkenham et al., 1990), findings that correlate well with a parallel study in the human CNS (Glass et al., 1997). This pattern of distribution perhaps explains why cannabis produces catalepsy and memory deficits, yet exhibits only mild acute toxicity compared to other drugs of abuse (Gable, 2004). Expression of CB1 has since been reported in human tonsils, spleen, ovaries, uterus, prostate, lung, heart, adrenal gland (Galiegue et al., 1995), and sensory nerve fibers (Stander et al., 2005), although the peripheral expression of CB1 occurs at significantly lower levels than CB1 in the brain.

Shortly following the initial discovery of CB1, a second cannabinoid receptor, CB2, was identified and cloned in rat spleen macrophages by Munro and colleagues, (1993). This paralogue shares only 44% protein homology with CB1 (Munro et al., 1993). Regionalization studies by Galiegue et al. (1995) revealed a high expression of CB2 mRNA transcripts in immune cells and immune cell rich tissues, namely the tonsils, spleen, and thymus, at comparable levels to CB1 transcripts in the brain. Unlike CB1, however, there appeared to be an absence of CB2 transcripts in the rat CNS (Munro et al., 1993; Galiegue et al., 1995; Ibrahim et al., 2003), indicating a peripheral immune localization of CB2, which explains the anti-inflammatory and immune suppressing properties of cannabis and
cannabinoids. Due to their distinctive expression patterns in the body, CB1 has traditionally been referred to as the central cannabinoid receptor, while CB2 has been referred to as the peripheral or immune cannabinoid receptor.

More recently, the idea that CB2 is a strictly peripheral cannabinoid receptor has been challenged, with many studies now indicating that the CB2 receptor may be present in the CNS. However the conditions under which CB2 may be expressed centrally and the cell types potentially expressing the receptor are a hot topic of debate. The presence of CB2 in central immune cells, specifically microglia, has been readily reported in a variety of conditions, and the expression of CB2 on neurons themselves has also been described by a few groups. Constitutive expression of CB2 has been reported in resting perivascular microglia (Nunez et al., 2004; Ashton et al., 2006), and pathological upregulation has been observed in activated microglia in Alzheimer’s disease (Benito et al., 2003), multiple sclerosis (Benito et al., 2007), animal models of HIV-1 (Benito et al., 2005), and ischemic stroke (Ashton et al., 2007b). More controversially, CB2 expression has also been reported in healthy CNS neurons (Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006).

Cannabinoid receptors are 7 transmembrane G protein coupled receptors (GPCRs), generally linked to Gi/o proteins. The activation of CB1 and CB2 by cannabinoids results in an inhibition of adenylyl cyclase, and a subsequent reduction in intracellular cyclic adenosine monophosphate (cAMP), thus modulating protein kinase A (PKA) phosphorylation and gene regulation (Lutz, 2002). Cannabinoid receptor activation is also associated with mitogen-activated protein kinase (MAPK) pathway modulation, which has an essential role in key cellular processes such as cell differentiation, and has been proposed as a mechanism behind CB2-mediated immunosuppression (Demuth et al., 2006). Receptor signaling via CB1 has been more extensively studied than CB2 receptor signaling, and is arguably more complex. Neuronal CB1 receptors are located presynaptically, and their activation is associated with an inhibition of neurotransmitter release. In addition to cAMP mediated effects, CB1 activation is associated with the stimulation of inwardly rectifying potassium channels, and the
inhibition of voltage sensitive calcium channels, which reduces presynaptic neuronal excitation, and the chance of neurotransmitter release (Turu et al., 2010).

Recent evidence suggests that there may be additional cannabinoid-like GPCRs, distinct from CB1 and CB2, which share some pharmacological similarity with their cloned and relatively well characterized relatives. Three of these receptors, the orphan GPCRs GPR18, GPR55 and GPR119, interact with atypical cannabinoids. Cloned as an orphan receptor, GPR18 is expressed in spleen and testis (Gantz et al., 1997), and while its natural ligand is proposed to be a metabolite of an endocannabinoid, termed N-arachidonyl, this receptor has also been shown to interact with THC (McHugh et al., 2011). GPR55 is widely expressed in the CNS and peripheral tissues, and despite sharing only 13.5 and 14.4% homology with CB1 and CB2, respectively, binds some cannabinoids (Sharir et al., 2010). Although the natural ligand for GPR55 appears to be a lysophospholipid, lysophosphatidylinositol (LPI) (Oka et al., 2007), it has been tentatively termed a cannabinoid receptor based on its stimulation by THC and the synthetic cannabinoids methanandamide and JWH-015 (Lauckner et al., 2008). While the role of GPR55 has not been thoroughly characterized, its expression in DRG neurons (Lauckner et al., 2008) and apparent role in neuropathic and inflammatory hyperalgesia (Staton et al., 2008) suggests it may play an essential part in the pain pathway. There is also evidence that another orphan receptor, GPR119 is a cannabinoid receptor: expressed mainly in pancreatic tissue (Fredriksson et al., 2003), this receptor is thought to be the target of the cannabinoid-like fatty acid derivative oleylethanolamide, which has a role in food intake (Overton et al., 2006).The complex pharmacology and response profiles of these receptors have hampered characterization and classification, and as such, they are only considered putative cannabinoid receptors at this stage. In addition to GPCRs, some endogenous cannabinoids have been shown to interact with TRPV1 (Zygmunt et al., 1999; Smart et al., 2000; Huang et al., 2002), which is a member of the ligand-gated ion channel family commonly associated with pain and noxious stimuli sensation.
In the CNS, a CB1-independent effect of cannabinoid agonists has been described in hippocampal electrophysiology studies and whole brain radio ligand binding studies in both wild type and CB1 knockouts (Breivogel et al., 2001; Hajos et al., 2001). As the presence of CB2 in the CNS has not been thoroughly corroborated, the idea of a distinct neuronal non-CB1/CB2 GPCR has been suggested.

1.2.3.2  Endocannabinoids

With the confirmation that exogenous cannabinoids were acting via specific receptor-mediated pathways to elicit their effects, it was proposed that endogenous cannabinoid ligands, or endocannabinoids, may occur naturally in the body. The laboratory of Raphael Mechoulam isolated and characterized the first endocannabinoid, arachidonylethanolamide, from the porcine brain, and termed it “anandamide”, translated from the Sanskrit term ananda, meaning bliss (Devane et al., 1992). When delivered systemically to mice, anandamide produced effects similar to THC (Fride et al., 1993). Shortly after this discovery, a second endogenous cannabinoid was described. The compound, 2-arachidonyl glycerol (2-AG), was initially isolated from intestinal tissue of the canine, and preliminary data suggested a highly variable, yet somewhat lower affinity of 2-AG for both CB1 and CB2 than anandamide, though with similar behavioural effects (Mechoulam et al., 1995). In 1997, Stella and colleagues reported the discovery and characterization of 2-AG in the rat brain, and reported that 2-AG had a similar potency and affinity for CB1 receptors as anandamide, but was present in levels 170 fold greater (Stella et al., 1997).

Unlike most classical neurotransmitters, anandamide and 2-AG are enzymatically synthesized on demand from phospholipid precursors (Sugiura et al., 2006; Okamoto et al., 2007). Both are highly lipophilic, and rapidly cross plasma membranes to interact with cannabinoid receptors, before rapid cellular uptake, thought to mediated via selective transporter proteins (Hillard et al., 2003; Hajos et
al., 2004; McFarland et al., 2004). Dedicated enzymes then hydrolyze sequestered endocannabinoids: anandamide is enzymatically degraded by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1995; Cravatt et al., 1996), while the principal pathway of 2-AG hydrolysis is catalyzed by monoacylglycerol lipase (MAGL) (Goparaju et al., 1999). Both enzymes are found throughout the body, with notably high levels of expression in the CNS (Giang et al., 1997; Karlsson et al., 1997).

Aside from anandamide and 2-AG, a number of additional putative endocannabinoids exist. Docosatetraenylethanolamide (DTEA), and homo-γ-linolenylethanolamide (HLEA), were discovered in the porcine brain (Hanus et al., 1993), and were shown to exhibit partial agonist activity at the CB1 receptor in vitro and in vivo (Barg et al., 1995). Noladin ether acts as a partial agonist at CB1, and a possible full agonist at CB2 (Hanus et al., 2001; Shoemaker et al., 2005). Virodhamine has been reported in the rat brain and periphery, at comparable levels to anandamide, and has been shown to act as an antagonist at the CB1 receptor in vivo, and as a full agonist at CB2 (Porter et al., 2002). And finally N-arachidonoyl-dopamine (NADA) is an endovanilloid, acting at TRPV1, as well as an endocannabinoid with agonist activity at CB1, and is present in the rat brain (Huang et al., 2002).
1.3 Cannabinoid ligands

1.3.1 Cannabinoids

Cannabinoids can be grouped into classes based on their source and structure: phytocannabinoids derived from *C. sativa*; bicyclic and tricyclic synthetic analogues of phytocannabinoids; aminoalkylindoles; diarylpyrazoles; and the endocannabinoids derived from arachidonic acid. The structural attributes of these 5 distinct classes are illustrated with several examples in Figure 1.3.

The first group of cannabinoids to be described was the phytocannabinoids, derived from *C. sativa* (Figure 1.3A). This group contains nearly 70 tricyclic terpenophenols with cannabimimetic activity and high lipophilicity (Brownjohn *et al.*, 2011). Members of the group include cannabinol, the first cannabinoid chemically elucidated; THC, responsible for the chief psychoactive effects of cannabis; and cannabidiol, devoid of psychoactive effects, but medicinally relevant. Dronabinol, a compound used clinically in human conditions, is a synthetic *trans* isomer of THC, and is also a member of this group.

The second group constitutes the bicyclic and tricyclic analogues of the phytocannabinoids, which, although sharing a similar structure to their natural counterparts (Figure 1.3B), are synthetically produced. These were the first of the synthetic cannabinoids to be developed, and have been instrumental in the characterization of the endocannabinoid system, and the development of cannabinoid therapeutics. The lead compound of a group developed in the 1970s by Pfizer was CP55,940. It is a bicyclic analogue of THC, but has a much greater affinity for cannabinoid receptors, and is less lipophilic, than the natural compound – key attributes in early radiolabeled binding studies describing the distribution and functionality of the cannabinoid receptors (Herkenham *et al.*, 1990; Glass *et al.*, 1997). Knowledge of the receptor structure and binding pockets of the
cannabinoid receptors has allowed the development of ligands with selective binding profiles. Raphael Mechoulam, the father of modern cannabinoid research, has developed a series of bicyclic and tricyclic ligands with varying selectivity, including the non-selective but highly potent HU210, and the CB2 selective HU308 (Hanus et al., 1999) and HU910 (Horvath et al., 2011), all named for Hebrew University where his research team is situated. Research by John W. Huffman in particular has led to the development of a series of CB2 selective ligands bearing his name, including JWH-133, a tricyclic THC analogue with 200 fold selectivity for CB2 over CB1 (Huffman et al., 1999) (Figure 1.3B).

A structurally different class of cannabinoids makes up the third group termed the aminoalkylindoles (Figure 1.3C). Bearing little structural similarity to any other class of cannabinoid, this group consists of high potency ligands, the most well studied of which is WIN55,212-2. A full agonist at both cannabinoid receptors, this compound has marginal selectivity for the CB2 receptor, and derivatives have been synthesized with improved selectivity for the CB2 receptor, namely GW405833 (Gallant et al., 1996), a partial agonist, and JWH-015 (Showalter et al., 1996), which are both used extensively in preclinical research. Alexandros Makryannis of North Eastern University has contributed greatly to the CB2 field with the development of the WIN55,212-2-derived “AM” compounds, the most notable of which is AM1241, with a putative 340 fold selectivity for CB2 over CB1 (Malan et al., 2001).

The development of the receptor subtype selective antagonists SR141716 (CB1) and SR144258 (CB2) by Sanofi-Aventis (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998) led to the formation of the fourth class of cannabinoids, the diarylpyrazoles (Figure 1.3D). This group now contains the CB1 selective AM251 (Gatley et al., 1996) and the CB2 selective AM630 (Ross et al., 1999), both developed by Alexandros Makriyannis. All four of these compounds have been utilized extensively in the characterization of cannabinoid receptor subtype contribution to physiological and pathological processes, as well as in determining the selectivity of cannabinoid ligands. Furthermore, the CB1
selective SR141716 was marketed clinically by Sanofi-Aventis as Rimonabant (Acomplia™) in the treatment of obesity, before it was withdrawn following adverse psychiatric effects, including an increased risk of suicide (Sanofi-Aventis, 2008).

Figure 1.3 Representative cannabinoid ligand structures. (A) Phytocannabinoids Δ9-THC, cannabinol, cannabidiol; (B) Classical synthetic analogues of the bicyclic (CP55,940) and tricyclic (HU910, JWH-133) type; (C) Aminoalkylindoles WIN55,212-2, GW405833; (D) Diarylpyrazoles SR141716, SR144528; (E) Endocannabinoids anandamide, 2-arachidonylglycerol (2-AG).
Members of the fifth group of cannabinoids, the endocannabinoids, are structurally unrelated to the four other groups (Figure 1.3E). They are derived from arachadonic acid, and contain long hydrophobic alkyl chains. Both anandamide and 2-AG were discovered by Mechoulam and colleagues in the 1990s following the characterization of the cannabinoid receptors. Their structures differ only in that anandamide contains one hydroxyl and an amide head group, while 2-AG contains two hydroxyls and an ester head group.

1.3.2 Other natural cannabinoid-like ligands

Other than the well characterized phytocannabinoids from *C. sativa*, several other natural ligands have been putatively described as cannabinoid ligands. A group of alkylamides in *Echinacea* may elicit some of their well characterized effects via CB2 modulation. It was first reported that a subset of *Echinacea*-derived N-isobutyl amides were able to inhibit LPS-induced pro-inflammatory cytokine expression via a CB2-dependent mechanism in human monocytes (Gertsch et al., 2004). Further *in vitro* investigation revealed that two purified constituents of *Echinacea* bound to human cannabinoid receptors, with greater affinity for CB2 over CB1 (Raduner et al., 2006). Although initial *in vitro* reports of these extracts appear promising, *in vivo* studies of these compounds are yet to be undertaken in relation to their cannabimimetic effects.

Similarly, β-caryophyllene, a constituent of a wide diversity of flora, including cannabis, has been putatively described as a cannabinoid receptor ligand. *In vitro*, it has an affinity for the CB2 receptor in the nanomolar range, inhibiting cAMP production and increasing intracellular calcium currents. *In vivo*, β-caryophyllene reduced carrageenan-induced paw inflammation in wildtype, but not CB2 knockout mice, indicating a robust CB2-dependent anti-inflammatory effect (Gertsch et al., 2008).
1.4 Cannabinoids as a treatment for neuropathic pain

1.4.1 Cannabis

The majority of the evidence suggesting a beneficial effect of cannabis on neuropathic pain comes from epidemiological studies, and surveys of specific patient populations. In Canada, where the use of medicinal cannabis is legally tolerated, 15% of chronic pain patients surveyed in a recent study reported use of cannabis to relieve pain, with 38% of those patients using at least once daily (Ware et al., 2003). Within specific patient populations, high proportions of cannabis users have been reported. In patients suffering MS, 29% had used cannabis for pain relief (Chong et al., 2006); in HIV patients, 27% had used cannabis for HIV-related pain (Woolridge et al., 2005); and in patients following SCI, 23% had tried cannabis as an alternative medication (Cardenas et al., 2006). Pain conditions rank highly in the reasons given for medicinal cannabis use: In a study of medicinal cannabis users in the UK, 25% of patients used cannabis for chronic pain, and 19% for neuropathic pain (Ware et al., 2005).

There are relatively few clinical trials investigating the effect of smoked cannabis on pain, although some do exist. In two recent randomized, double-blind, placebo-controlled trials (RCTs) investigating smoked cannabis, approximately 50% of HIV-neuropathic patients in each trial smoking cannabis daily recorded a clinically significant 30% reduction in pain scores (Abrams et al., 2007; Ellis et al., 2008). Some studies, however, indicate that cannabis has mixed effects on acute pain. In healthy volunteers, Hill et al (1974) report an increased sensitivity to, and decreased tolerance of, painful electrical stimuli following smoked cannabis when compared to THC devoid cannabis (Hill et al., 1974). Similarly, Wallace et al., (2007a) found that while modest THC content (4%) cannabis cigarettes significantly reduced capsaicin-induced hyperalgesia in healthy volunteers, higher THC content (8%) cigarettes resulted in an increased hyperalgesic response. The proposed mechanism of
this counter-intuitive cannabinoid-induced hyperalgesia is the activation of CB1 receptors expressed on inhibitory glycinergic and GABAergic interneurons, which in turn reduces levels of these inhibitory neurotransmitters (Pernia-Andrade et al., 2009). The contrasting results of cannabis in acute and chronic pain states suggest that cannabis may be more effective at relieving the latter rather than the former.

Although smoked cannabis may be beneficial in some pain syndromes, smoking as a form of drug delivery is not an acceptable method, due to the well documented adverse health effects, psychoactivity, and varied bioavailability via the inhaled route. The use of standardized cannabinoid preparations is thus a more desirable approach for the investigation and implementation of cannabinoids in pain treatment. Although the bioavailability of orally delivered cannabinoids is also highly variable (Grotenhermen, 2003), standardized cannabinoids preparations designed for delivery via alternative routes, with more predictable pharmacokinetics, are also under development, and will be briefly discussed below.

1.4.2 Standardized cannabinoids

There are currently three licensed synthetic cannabinoid preparations in clinical use internationally: Dronabinol, Nabilone and Sativex. These formulations and others are discussed below.

1.4.2.1 Dronabinol

Dronabinol is a synthetic (-)-trans-isomer of THC that was approved by the Food and Drug Administration (FDA) in 1985. It is marketed as Marinol® by Solvay Pharmaceuticals suspended in
sesame oil in oral capsules, and as Namisol® by Echo Pharmaceuticals as an emulsified preparation in
oral tablets, using a patented delivery technology. While licensed primarily for the treatment of
chemotherapy-induced emesis and HIV-induced wasting, recent clinical trials have shown positive
results in the use of dronabinol to treat chronic or neuropathic pain. In clinical trials, dronabinol was
able to improve pain scores in patients with stable chronic pain (Notcutt et al., 2004) and
fibromyalgia (Schley et al., 2006), and was able to improve pain and quality of life scores in patients
suffering MS related pain (Zajicek et al., 2003; Svendsen et al., 2004), effects that were long lasting
in a 12 month follow up (Zajicek et al., 2005). Conversely, it has also been reported that dronabinol
has minimal effect in chronic refractory neuropathic pain (Clermont-Gnamien et al., 2002; Attal et
al., 2004), although it must be noted that these trials involved very small cohorts, and were not
placebo-controlled.

1.4.2.2 Nabilone

Nabilone is a synthetic, potent analogue of THC that was first described over 30 years ago
(Lemberger et al., 1975). Marketed as an oral capsule, Cesamet®, by Meda Pharmaceuticals, it is
currently approved for use in the UK, USA, Canada and Mexico in the treatment of chemotherapy-
associated emesis. Early case studies suggested that off-label use of nabilone improved chronic non-
cancer pain (Berlach et al., 2006), a finding corroborated by a placebo controlled trial of nabilone as
an add-on treatment in patients with intractable chronic pain (Pinsger et al., 2006). RCTs have also
confirmed that nabilone, given as an adjuvant therapeutic, reduces pain scores in spasticity related
pain (Wissel et al., 2006) and fibromyalgia (Skrabek et al., 2008).
Perhaps the most promising of the current cannabinoid therapeutics is Sativex®, an oral spray containing a 2.7:2.5 ratio of THC:cannabidiol and marketed by GW Pharmaceuticals. Sativex is derived from standardized *C. sativa*, and has been approved for use in the treatment of MS associated neuropathic pain since 2005 in the UK, Spain, Canada and NZ, and as an adjuvant analgesic in terminal cancer pain since 2007 in Canada. Preliminary reports indicated that Sativex was primarily useful in treating muscle spasms and sleep disturbance associated with MS and other neuralgic disorders (Wade *et al.*, 2003). More comprehensive trials indicated that Sativex is effective in treating MS related spasticity in a 6 week randomized double blind placebo-controlled, followed by a 4 week open labeled period (Wade *et al.*, 2004), and that long-term extension of Sativex use in these patients was relatively well tolerated, with a maintained level of efficacy (Wade *et al.*, 2006). It has also proven efficacious in the management of MS and neurogenic chronic pain (Rog *et al.*, 2005). In the treatment of mixed neuropathic pain, Sativex was able to reduce pain scores and improve sleep disturbance in patients suffering brachial plexus avulsion (Berman *et al.*, 2004), and significantly reduced pain scores in patients suffering neuropathic pain of varying etiologies, while maintaining efficacy and a tolerable side effect profile over the course of a year (Nurmikko *et al.*, 2007).

Due to their lipophilic nature, cannabinoids have a pharmacokinetic profile that varies depending on the route of delivery. Smoked cannabis produces very high plasma concentrations of THC within a short time frame, which increases efficacy but also intoxication. In a comparative study, smoked cannabis resulted in peak plasma concentrations of THC ten times greater than an equivalent dose delivered by oromucosal Sativex (Robson, 2005). Furthermore, although scant epidemiological evidence exists on the detrimental respiratory effects of smoked cannabis, the presence of carcinogens in cannabis smoke (Novotny *et al.*, 1982) suggests that this method of cannabinoid
delivery is not ideal for chronic medical use. On the other hand, while the oral delivery of cannabinoids like dronabinol and nabilone produces a long duration of action, delivery via this route results in a slow absorption rate and a low bioavailability, owing to extensive first pass metabolism (Grotenhermen, 2003). Sativex attempts to find middle ground by using an oromucosal delivery technique to deliver a measured dose of THC:CBD, thus avoiding the intoxicating high and adverse effects of smoked cannabis, and improving the bioavailability compared to oral formulations.

1.4.2.4 Efficacy

Several meta-analyses and reviews have attempted to quantify the efficacy of cannabinoids in pain treatment.

An analysis by Iskedjian et al (2007) of 6 published trials of cannabinoids on MS pain found that cannabinoids had a significant pain relieving effect in this population. Specifically, pooling of the effects of dronabinol, cannabidiol and Sativex produced a 1.6 point reduction in pain as measured on an 11 point scale, compared to a 0.8 reduction with placebo. As baseline scores were 5.5 – 7.7 out of 11, a 1.6 point drop corresponds to an approximately 24% reduction in pain. Furthermore, the authors suggest that pain relief may differ substantially between patients, with “cannabinoid responders” obtaining much greater than the average pain relief (Iskedjian et al., 2007). Ashton and Milligan (2008) analyzed clinical trials involving cannabinoids and neuropathic pain published in the past 10 years. From 18 studies, the authors identified only 3 studies in which cannabinoids had no beneficial effect on pain, two of which were not placebo-controlled, and contained low numbers of trial participants. The authors therefore concluded that cannabinoids have a place in the treatment of neuropathic pain, particularly in patients refractory to other treatments (Ashton et al., 2008a). In a recent meta analysis of neuropathic pain treatments, Finnerup et al. (2010) describe a modest
effect of cannabinoids on central MS and peripheral neuropathic pain, but have only limited effects in diabetic polyneuropathy (Finnerup et al., 2010).

Campbell et al (2001) reviewed clinical trials of cannabinoids in all types of pain, and found that in two trials of cannabinoids in acute pain, THC and nabilone were no more effective than codeine, despite greater adverse effects (Campbell et al., 2001). Two more recent RCTs found that nabilone was ineffective in acute pain: it induced no change in reported pain levels in volunteers in an experimental model of heat pain (Redmond et al., 2008), and actually worsened pain significantly compared to placebo in a trial for post operative pain (Beaulieu, 2006). This also correlates well with studies involving smoked cannabis, which suggest no effect, or a worsening of pain in acute situations (Hill et al., 1974; Wallace et al., 2007a).

1.4.2.5 Adverse events

Most of the studied trials above contain information on adverse events. The most common of these are mild, and include drowsiness, ataxia, euphoria and dizziness, which are comparable with existing psychoactive pain medications. In general, the cannabinoids tested in the above studies were described as “well tolerated”. In a meta-analysis of adverse events, Wang et al (2008) analyzed 321 studies over the past four decades for safety and tolerability, with an emphasis on adverse events. They report that the incidence rate of mild adverse events was significantly higher in the treatment group compared to the placebo group, with a risk ratio of 1.86 (10.37 versus 6.87 events/person-year). For serious adverse events, however, there was no significant difference between groups, with a risk ratio of 1.04 for the treatment group (Wang et al., 2008).

Two studies have investigated the adverse effects associated with the long term use of cannabinoids, which is essential for the treatment of a chronic condition. Wade et al (2006)
investigated long term Sativex use in MS patients, and found mild side effects the most common, and although several adverse effects were noted, the underlying cause may have been MS, which was a risk factor in many of the reported serious effects (Wade et al., 2006). Similarly, Zajicek et al (2005) investigated the long term use of dronabinol and cannabis extract on MS over 12 months, and found similar rates of mild and serious adverse events in the treatment and placebo groups (Zajicek et al., 2005). Specific to Sativex, some concern has been raised that extended use may cause mucosal damage, thought to be due to the ethanol solvent. One study has reported the appearance of white mucosal lesions in patients using Sativex over extended periods (Scully, 2007).

While cannabinoids are well tolerated at moderately efficacious doses, there is evidence to suggest that increasing potency of ligands in order to achieve greater efficacy is also associated with a marked increase in adverse effect frequency and severity. For example, levonantradol, a THC analogue many times more potent that its parent compound (Little et al., 1988), was shown to be effective in the alleviation of acute post operative pain (Jain et al., 1981), although multiple dosing in this efficacious range was associated with prohibitive side effects (Joss et al., 1982). In order to improve the therapeutic ratio of cannabinoids for clinical use, an understanding of the mechanisms behind cannabinoid mediated antinociception is required.

1.4.3 Mechanisms of cannabinoid mediated antinociception

Cannabinoid compounds currently in clinical use are non-selective, and thus activate both CB1 and CB2 cannabinoid receptors. CB1 receptors are expressed in various structures within the nociceptive pathway under physiological conditions, as shown in Figure 1.4, and are thus in prime positions for the modulation of nociceptive signaling at various stages of transmission. In the periphery, CB1 receptors have been described in nociceptive primary afferents (Stander et al., 2005; Walczak et al.,
Figure 1.4 Sites of cannabinoid CB1 mediated antinociception. Shown in red are the structures within the nociceptive pathway known to play a role in cannabinoid mediated antinociception, likely via cannabinoid CB1 receptor activation. AMG = amygdala; DRG = dorsal root ganglia; PAG = periaqueductal grey; RVM = rostral ventrolateral medulla; THA = thalamus.
38
2005), and their cell bodies, resident in DRGs (Hohmann et al., 1999a; Hohmann et al., 1999b; Ahluwalia et al., 2000). Within the spinal cord, CB1 receptors are highly expressed in the dorsal horns (Herkenham et al., 1990; Ibrahim et al., 2003), particularly post-synaptically in spinal interneurons (Farquhar-Smith et al., 2000), consistent with the well documented efficacy of spinally administered cannabinoids (Lichtman et al., 1992; Smith et al., 1992).

Supraspinally, cannabinoid mediated antinociception and suppression of neuronal firing has been documented in the thalamus (Martin et al., 1996) and amygdala (Martin et al., 1999), key structures in the ascending pain pathways, and known to contain CB1 receptors (Herkenham et al., 1990; Glass et al., 1997). Cannabinoid mediated antinociception has also been documented in descending inhibitory pathway structures. Microinjections of cannabinoids into the periaqueductal grey and the rostral ventrolateral medulla (RVM) result in analgesia (Martin et al., 1999), and stress-induced, endocannabinoid mediated analgesia is CB1 receptor dependent in these structures (Suplita et al., 2005).

1.4.3.1 Summary of cannabinoids as a treatment for pain

Taken together, there is a large body of evidence to suggest that cannabinoids may be effective in the treatment of some pain states, particularly those with a chronic element. However, while adverse effects are usually mild, there is evidence to suggest that the efficacy of cannabinoids is limited by more serious adverse effects at high doses. The antinociceptive and adverse effects of cannabinoids are thought to be mediated largely by cannabinoid CB1 receptors.
1.5 Targeting the CB2 receptor

There has been considerable interest in developing CB2 selective agonists for the treatment of chronic pain, as these compounds may have the potential to provide analgesia whilst circumventing undesirable CB1-mediated central side effects associated with non-selective cannabinoid administration. Currently almost all evidence for the use of CB2 selective agonists in painful conditions is preclinical, and has been obtained using animal models. Models of acute, inflammatory and neuropathic pain have been developed in rodents to mimic painful conditions in humans, in order to study the pathology of pain, as well as potential treatments.

1.5.1 Rodent models of pain

Acute pain paradigms in animals involve the assessment of mechanical thresholds and thermal latencies in the nociceptive range, generally in the hindpaws of naïve animals. Analgesic drugs will increase nociceptive thresholds above baseline values. On the other hand, chronic pain paradigms involve the assessment of the same outcomes in animals following lesioning of peripheral or spinal nerves (neuropathic pain), or the induction of inflammation with compounds such as Complete Freund’s Adjuvant (CFA), carrageenan, or formalin (inflammatory pain). Induction of pain in these models is associated with varying pain response profiles, but often includes hyperalgesia - a reduction in nociceptive thresholds from baseline - and in some cases, allodynia - a painful response following normally innocuous stimulation outside of the nociceptive range. As these responses are two of the most frequently reported symptoms of neuropathic pain in humans, the use of these models allows the analysis of the efficacy of experimental drugs in animals before the advent of clinical testing. As cannabinoids appear to have a greater efficacy in chronic rather than acute.
conditions in humans, the analysis of cannabinoid efficacy in animals is often performed in chronic, rather than acute paradigms.

1.5.2 Antinociceptive efficacy of CB2 selective agonists in rodents

CB2 selective agonists have shown antinociceptive efficacy in acute and chronic pain paradigms in rodent models. Table 2, below summarizes the antinociceptive effects of a number of CB2 selective agonists in a range of rodent acute, inflammatory and neuropathic pain conditions.

One of the most attractive properties of CB2 selective agonists are their lack of psychoactive adverse effects at therapeutic doses in preclinical testing. In rodents, behavioural testing is used to assess the CB1 mediated central effects of cannabinoids. Researchers generally use one or a combination of the “tetrad” of tests developed by Little et al (1988), which determine CB1 mediated central effects, and consist of analgesia (as assessed by an increased latency in the heat induced tail flick test), catalepsy, sedation and hypothermia (Little et al., 1988). A comprehensive analysis by Valenzano et al (2005) found that doses of GW405833 within the anti-hyperalgesic range, up to and including 30 mg/kg, had no effects on ataxia, catalepsy or anxiety or tail flick. At higher doses, however, 100 mg/kg GW405833 had significant effects on all of these behavioural outcomes, consistent with central CB1 receptor activation (Valenzano et al., 2005). Similarly Yao et al (2008a, 2008b) determined that the CB2 selective compounds A-796260 and A-836339 did not impair motor activity at analgesic doses (Yao et al., 2008a; Yao et al., 2008b), and Malan et al (2001) describe the same for AM1241 (Malan et al., 2001). These results suggest that CB2 selective agonists are efficacious without the psychoactivity seen following non-selective cannabinoid administration.
Table 2 Preclinical evidence for CB2 selective agonist anti-nociception in rodents. BPA = Brachial Plexus Avulsion; Cap-SMH = capsaicin-induced secondary mechanical hyperalgesia; CCI = Chronic Constriction Injury; CFA = Complete Freund’s Adjuvant; PSL = Partial Sciatic Ligation; SNI = Spared Nerve Injury; SNL = Spinal Nerve Ligation; SNT = Spinal Nerve Transection.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pain model</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-796260</td>
<td>Arthritis</td>
<td>↑ hind limb grip force</td>
<td>(Yao et al., 2008b)</td>
</tr>
<tr>
<td></td>
<td>CCI</td>
<td>↓ mechanical allodynia</td>
<td>(Yao et al., 2008b)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ thermal hyperalgesia</td>
<td>(Yao et al., 2008b)</td>
</tr>
<tr>
<td></td>
<td>Post surgical pain</td>
<td>↓ mechanical alldynia</td>
<td>(Yao et al., 2008b)</td>
</tr>
<tr>
<td>A-836339</td>
<td>Cap-SMH</td>
<td>↓ mechanical hyperalgesia</td>
<td>(Yao et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td>CCI</td>
<td>↓ mechanical allodynia</td>
<td>(Yao et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td>CCI</td>
<td>↓ mechanical allodynia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ thermal hyperalgesia</td>
<td>(Yao et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ thermal hyperalgesia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Post surgical pain</td>
<td>↓ mechanical alldynia</td>
<td>(Yao et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ mechanical alldynia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td>AM1241</td>
<td>Acute</td>
<td>↓ thermal nociception</td>
<td>(Ibrahim et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Acute</td>
<td>↓ thermal nociception</td>
<td>(Ibrahim et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Acute</td>
<td>↓ thermal nociception n/c mechanical nociception</td>
<td>(Rahn et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ mechanical alldynia</td>
<td>(Lozano-Ondoua et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ spontaneous pain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ mechanical allodynia</td>
<td>(Saghafi et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ mechanical alldynia</td>
<td>(Curto-Reyes et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ thermal hyperalgesia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemotherapy</td>
<td>↓ mechanical alldynia</td>
<td>(Rahn et al., 2007)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td>↓ mechanical hyperalgesia</td>
<td>(Rahn et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Formalin test</td>
<td>↓ licking and flinching</td>
<td>(Beltramo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Post surgical pain</td>
<td>↓ mechanical alldynia</td>
<td>(LaBuda et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>SNI</td>
<td>↓ mechanical hyperalgesia</td>
<td>(Gutierrez et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ alldynia</td>
<td>(Ibrahim et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ thermal hyperalgesia</td>
<td>(Beltramo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>Reduced tactile alldynia</td>
<td>(Naguib et al., 2008)</td>
</tr>
<tr>
<td>Compound</td>
<td>Model</td>
<td>Effects</td>
<td>References</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
<td>----------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>AM1714</td>
<td>Acute</td>
<td>↓ thermal nociception, n/c mechanical nociception</td>
<td>(Rahn et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ mechanical hyperalgesia</td>
<td>(Rahn et al., 2008)</td>
</tr>
<tr>
<td>GW405833 (L768242)</td>
<td>Acute</td>
<td>↓ thermal nociception</td>
<td>(Valenzano et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ thermal nociception</td>
<td>(Whiteside et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ mechanical alldynia</td>
<td>(Hu et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>CCI</td>
<td>↓ mechanical hyperalgesia</td>
<td>(Valenzano et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ mechanical hyperalgesia</td>
<td>(Beltramo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Formalin test</td>
<td>↓ licking and flinching</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSL</td>
<td>↓ mechanical hyperalgesia</td>
<td>(Valenzano et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>PSL</td>
<td>↓ mechanical alldynia</td>
<td>(Whiteside et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ mechanical alldynia</td>
<td>(Beltramo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>SNT</td>
<td>↓ mechanical alldynia</td>
<td>(Leichsenring et al., 2009)</td>
</tr>
<tr>
<td>JWH-015</td>
<td>BPA</td>
<td>↓ mechanical alldynia</td>
<td>(Paszcuk et al., 2011)</td>
</tr>
<tr>
<td>MDA7</td>
<td>Chemotherapy</td>
<td>↓ mechanical alldynia</td>
<td>(Naguib et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ mechanical alldynia</td>
<td>(Naguib et al., 2008)</td>
</tr>
</tbody>
</table>
1.6 Target of CB2 selective ligands

Whilst a wealth of evidence suggests that CB2 agonists are anti-allodynic and anti-hyperalgesic in preclinical models of inflammatory and neuropathic pain when delivered systemically, it is currently not known where in the pain pathway these drugs may be acting.

1.6.1 Evidence for peripheral mechanisms

Administration of AM1241 into the ipsilateral but not contralateral hindpaw reverses CFA-induced thermal hyperalgesia (Hsieh et al., 2011), and inhibits the maintenance of mechanical allodynia and hyperalgesia (Gutierrez et al., 2007), and thermal hyperalgesia in the carrageenan model (Quartilho et al., 2003). Furthermore, the CB2 agonists AM1241 (Quartilho et al., 2003) and GW405833 (Clayton et al., 2002) are able to reduce paw edema associated with carrageenan induced inflammatory pain. Taken together, these results are highly suggestive of a local, peripheral site of action of CB2 agonists in nociceptive and inflammatory pain. Consistent with the presumed role of the CB2 receptor in inflammation (Buckley et al., 2000), these results could be due largely to suppression of the local immune response that is an essential component of both inflammatory and neuropathic pain.

There is also some evidence to suggest a direct effect of CB2 selective agonists on neural transmission, independent of inflammation. Malan et al (2001) showed that intraplantar AM1241 can increase thermal nociceptive thresholds in naïve rats, in the absence of inflammation. Furthermore this systemic AM1241-induced increase in naïve thermal thresholds was antagonized by ipsilateral, but not contralateral intra paw CB2 antagonist administration (Malan et al., 2001), highly suggestive of an action on peripheral nociceptors. Circumstantial evidence for a direct effect
on nociceptors in naïve animals is the fact that THC is still efficacious in the tail flick test of thermal nociception in CB1 receptor knockout, naïve mice (Zimmer et al., 1999), a test previously thought to be a classical sign of CB1 mediated analgesia (Little et al., 1988). This hypothesis is also supported by the electrophysiological work of Elmes and colleagues, who reported that intraplantar administration of the CB2 agonist JWH-133 was able to attenuate innocuous and noxious stimuli-induced firing of WDR neurons in the lumbar dorsal horn of naïve, SNL and carrageenan rats, and inhibited carrageenan induced increases in WDR receptive field (Elmes et al., 2004). This is also in concurrence with the results of another group, who found that carrageenan-induced increases in WDR activity were suppressed by intrapaw co-administration with AM1241, a result due to a decrease in C fiber activation (Nackley et al., 2004). Although AM1241 did not significantly alter spontaneous firing in these neurons prior to carrageenan administration, there was a strong trend towards an AM1241 induced decrease (Nackley et al., 2004). At the level of the primary afferent cell bodies, Sagar et al (2005) showed that capsaicin-induced increases of intracellular calcium in cultured DRG neurons from sham and SNL neuropathic rats were attenuated by pre-incubation with the CB2 selective agonist JWH-133 (Sagar et al., 2005).

1.6.2 Evidence for spinal mechanisms

More recently, pharmacological investigations into the site of action have focused on potential central mechanisms. Specifically, a host of studies in recent times have detailed the efficacy of CB2 agonists delivered spinally, via intrathecal injection, on nociception in several models of chronic pain. A complete summary of the recent findings of intrathecal CB2 agonist administration are presented in Table 3.
Table 3 Preclinical evidence for a spinal location of CB2-mediated anti-nociception; studies involving intrathecal administration of CB2 selective agonists. BPA = Brachial Plexus Avulsion; CFA = Complete Freund’s Adjuvant; PSL = Partial Sciatic Ligation; SNL = Spinal Nerve Ligation; SNT = Spinal Nerve Transection.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Model</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-836339</td>
<td>CFA</td>
<td>↓ thermal hyperalgesia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ mechanical allodynia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td>AM1241</td>
<td>Cancer</td>
<td>↓ mechanical allodynia</td>
<td>(Curto-Reyes et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ mechanical allodynia</td>
<td>(Curto-Reyes et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>↓ thermal hyperalgesia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>↓ mechanical allodynia</td>
<td>(Hsieh et al., 2011)</td>
</tr>
<tr>
<td>JWH-015</td>
<td>BPA</td>
<td>↓ mechanical allodynia</td>
<td>(Paszczuk et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>↓ mechanical allodynia</td>
<td>(Gu et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Post surgical pain</td>
<td>↓ mechanical allodynia</td>
<td>(Romero-Sandoval et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>SNT</td>
<td>↓ mechanical allodynia</td>
<td>(Romero-Sandoval et al., 2008)</td>
</tr>
<tr>
<td>JWH-133</td>
<td>CFA</td>
<td>↓ mechanical allodynia</td>
<td>(Curto-Reyes et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Formalin test</td>
<td>n/c flinching</td>
<td>(Yoon et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>PSL</td>
<td>↓ mechanical allodynia</td>
<td>(Yamamoto et al., 2008)</td>
</tr>
</tbody>
</table>

The apparent efficacy of centrally delivered CB2 agonists is an interesting proposition, as the CB2 receptor does not appear to exist in the naïve CNS. For instance, CB2 receptor mRNA transcripts were initially reported to be absent from the naïve CNS (Munro et al., 1993; Galiegue et al., 1995), and CP55,940 binding is abolished in the brain and spinal cord of CB1 receptor knockout mice (Herkenham et al., 1990; Zimmer et al., 1999; Ibrahim et al., 2003). More recently, however, the idea that the CB2 receptor is absent from the CNS has been challenged, with a number of studies describing anything from inducible central CB2 receptor expression under certain pathologies.
(Benito et al., 2003; Ashton et al., 2007b; Benito et al., 2007; Benito et al., 2008), including pain (Zhang et al., 2003; Wotherspoon et al., 2005; Beltramo et al., 2006; Hsieh et al., 2011) to widespread neuronal expression in the CNS of healthy animals (Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006). The central expression of CB2 is thus hotly debated, and there is no consensus on when and where the CB2 is expressed, and indeed if it is even present in the CNS at all (Atwood et al., 2010). Figure 1.5 summarizes the known and proposed sites of CB2 receptor expression that may underlie CB2 selective agonist mediated antinociception in neuropathic pain.

Figure 1.5 Proposed sites of cannabinoid CB2 mediated anti-nociception in neuropathic pain. Shown in red are the structures within the nociceptive pathway unequivocally known to contain cannabinoid CB2 receptors. Shown in blue are the structures within the nociceptive pathway proposed, but not conclusively proven, to contain CB2 receptors. DRG = dorsal root ganglia; THA = thalamus.
1.7 Aims of the current study

In summary, a promising new therapeutic approach to the treatment of neuropathic pain is the use of CB2 selective agonists. These compounds appear to maintain the therapeutic efficacy of cannabinoids, whilst circumventing undesirable CB1-mediated central side effects, in multiple animal models of neuropathic pain. It is not known, however, at which point in the pain pathway these agonists are exerting their effects. It is hoped that a deeper understanding of CB2 mediated mechanisms of neuropathic pain treatment may lead to the development of more targeted pharmacological agents for this undertreated condition.

With these considerations in mind, the current study aims to:

1) Establish and characterize the chronic constriction injury (CCI) model of sciatic nerve injury induced neuropathic pain at the University of Otago.

2) Using the CCI model, assess the efficacy of CB2 selective agonists in the treatment of mechanical allodynia associated with peripheral nerve injury, and determine if the effects of these compounds are mediated via peripheral or spinal mechanisms.

3) Assess CB2 receptor protein levels in the rat spinal cord, and quantify any changes in protein following peripheral nerve injury.

4) Assess the functionality of CB2 receptors in the rat spinal cord, and measure changes in CB2 receptor functionality following sciatic nerve injury.
2 Establishment and validation of the CCI model

2.1 Introduction

The development of animal models of neuropathic pain has not only allowed for the preclinical assessment of potential treatments, but has also advanced the understanding of underlying molecular mechanisms. A range of models now exist, all ultimately derived from the initial description of neuropathic-like pain in rats, consisting of self-mutilation of the foot after transection of the sciatic nerve in the hindlimb of the rat (Wall et al., 1979). This observation, termed autotomy by the authors, is due to sensory loss, parallel to the condition of anaesthesia dolorosa in man, and thus was proposed as a model of neuropathy (Wall et al., 1979). Several years later, Bennett and Xie (1988) described a similar lesioning of the sciatic nerve, with a loose ligation rather than a transection leaving some sensory fibers intact. This model, termed the chronic constriction injury (CCI) model, produced a wide range of symptoms that parallel neuropathic pain in man, namely alldynia, hyperalgesia and some signs of spontaneous pain (Bennett et al., 1988). A variation on CCI is the partial sciatic ligation (PSL) model, involving a tight ligation of a portion of the sciatic nerve, and generates a similar pathology to CCI (Seltzer et al., 1990).

Lesioning of the sciatic nerve in the CCI and PSL models is quick and simple to perform, and this contributes to the popularity of these methods in the neuropathic pain field. Common alternative models involve lesioning of (1) sciatic nerve branches as in the spared nerve injury (SNI) model (Decosterd et al., 2000) (2) spinal nerves as in the spinal nerve ligation (SNL) (Kim et al., 1992) and spinal nerve transection (SNT) (Martin et al., 2003) models. Location of the lesions in the various models is shown in Figure 2.1. While each model has advantages and disadvantages, the CCI model
was chosen in the current study as it is cost effective, easy to perform, long lasting, and produces less autotomy than more severe sciatic transection, and is ethically favored at this institution where these experiments were performed.

2.2 Experimental aims

The initial work here describes the successful establishment of the CCI model of neuropathic pain in the rat, in order to investigate the CB2 receptor in the spinal pain pathway. The development of mechanical allodynia in the hindpaws was measured with Von Frey hair filaments, and the pattern of gliosis in the spinal cord was characterized and compared to existing literature. Additionally, rats were monitored for adverse events, and the effects of surgery technique and housing on these factors were recorded.
2.3 Methods

2.3.1 Animals

All experiments conducted at the University of Otago were approved by the Animal Ethics Committee at the University of Otago, under guidelines set down for the ethical and humane use of animals in research under the United Kingdom Animals Act 1986. Male Wistar rats (200-350g) were obtained from the Taieri-Hercus Resource Unit (Dunedin, New Zealand). Prior to surgical manipulation, rats were housed for at least 5 days in a 12 hour light/dark cycle with \textit{ad libitum} access to food and water.

2.3.2 Chronic constriction injury

Surgical procedures on the sciatic nerve were conducted as described by Bennett and Xie (1988) under aseptic conditions. Animals were induced and maintained under 2.5% halothane anaesthesia (Nicholas Piramal Ltd., India) in oxygen, with adequate depth determined by lack of pedal withdrawal reflex. \textit{Pars cranialis} and \textit{pars caudalis} of \textit{biceps femoris} were separated by blunt dissection to expose the underlying sciatic nerve. Four sutures (4/0, 2 metric chromic gut; Ethicon, NJ, USA), 2mm apart, were loosely tied around the exposed sciatic nerve with no occlusion of the nerve process. The wound was closed in layers, and the animal allowed to recover. For acute post operative analgesia, buprenorphine (Reckitt Benckiser, UK) was administered peri-operatively, and then post operatively 12 and 24 hours post surgery (0.1 mg/kg s.c.). This analgesic protocol has previously been shown to have no effect on the later development of neuropathic pain behaviors.
Sham surgery was performed as for CCI surgery, but the sciatic nerve was not ligated with suture.

2.3.3 **Von Frey Hair Analysis**

The following test procedure and statistical analysis have been adapted from previously described methods (Milligan *et al.*, 2000). Behavioural testing was undertaken one day prior to (baseline), and then 3 and 10 days post surgery. On the day of testing, animals were placed in a mesh wire cylinder on an elevated wire mesh flooring (1.5 mm bars, 14 mm spacing) in a darkened room, lit minimally with red light, and allowed to habituate for 20-30 min prior to testing. A logarithmically graded series of Von Frey hair monofilaments (North Coast Medical, Morgan Hill, CA) were presented to the left and right hindpaws, and the response pattern used to estimate 50% paw withdrawal thresholds. The hairs presented had the following log force (10,000 X g) intensities: 3.61 (0.407 g), 3.84 (0.692 g), 4.08 (1.202 g), 4.17 (1.479 g), 4.31 (2.041 g), 4.56 (3.630 g), 4.74 (5.495 g), 4.93 (8.511 g), 5.07 (11.749 g), and 5.18 (15.136 g).

Using a modified form of the up/down testing procedure (Dixon, 1980), filaments were applied alternately to the left and right ventral plantar aspects of the hind paws at a perpendicular angle for 8 seconds; a withdrawal considered as a paw flinch upon presentation or sustained application of a given monofilament. Testing began with the 2.041 g monofilament, which was presented three times, 30 seconds apart. In the presence of two or more paw withdrawals at this stimulus, the lowest strength monofilament (0.407 g) was presented. In the absence of two or more withdrawals with the 2.041 g monofilament, the next strongest monofilament (3.630 g) was presented. With the exception of the 2.041 monofilament, which was tested three times, each stimulus was presented once initially, then twice more only if a withdrawal was elicited with the first presentation. Once
three withdrawals were elicited from the same monofilament, no further stimuli were presented, and this was considered threshold. Presentation of monofilaments continued in ascending order until this threshold was reached. To avoid tissue damage in hypoalgesic animals, the upper cut-off value was assigned in the case of an absence of a response to the highest stimulus (15.136 g). All single withdrawals were recorded for analysis, but testing was only complete once threshold had been reached i.e. two withdrawals at a given stimulus intensity.

Paw withdrawal response rates were fitted with a Gaussian integral psychometric function using a maximum-likelihood fitting method in order to generate 50% paw withdrawal thresholds. Converting the threshold values using this method creates a continuum of values, suitable to analyze parametrically. The computer program used to convert this data, PsychoFit, was created by Professor Lewis O. Harvey Jr. at the University of Colorado, and is available as a freeware download (http://psych.colorado.edu/~lharvey/html/software.html).

2.3.4 Immunohistochemistry

Immunohistochemistry methods are described in detail in Chapter 4. Briefly, 10 days following CCI or sham surgery, animals were sacrificed, and the L4 – L6 lumbar spinal cord excised and snap frozen or paraffin embedded for immunohistochemical analysis. Tissue was sectioned and incubated with antibodies raised against ionized calcium binding adapter molecule 1 (Iba1; Abcam, MA, USA) or glial fibrillary acidic protein (GFAP; Abcam, MA, USA), markers for microglia and astrocytes respectively.
2.3.5  **Statistical analysis**

Time courses for paw withdrawal thresholds were analyzed with a 2-way repeated measures ANOVA, with time as one factor and surgery as the other, with Bonferroni post hoc tests in Prism®, Version 5.01 (GraphPad Software, CA, USA).
2.4 Results

2.4.1 Mechanical allodynia in the CCI model

Behavioural data from animals used across all experiments within this study were pooled to allow a meta-analysis of the development of mechanical allodynia in the CCI model. Analyzed by 2-way repeated measures ANOVA, CCI but not sham surgery induced mechanical allodynia in the rat, evidenced by a significant reduction in paw withdrawal threshold in the ipsilateral and contralateral hindpaws ($F = 248 (1, 363); p < 0.0001$ and $F = 139 (1, 363); p < 0.0001$, respectively) (Figure 2.2). Bonferroni post hoc analysis revealed that paw withdrawal thresholds were significantly different between sham and CCI treated rats both 3 and 10 days post surgery, both ipsilateral and contralateral to injury ($p < 0.001$ for each comparison). Allodynia was marked 3 days post surgery, and was fully established by the 10 day time point, at which point either pharmacological intervention or tissue harvesting for molecular analysis took place. Withdrawal thresholds in the ipsilateral paw at the 10 day time point were approximately 50% lower than thresholds in the contralateral paw, correlating with a greater ipsilateral mechanical allodynia.
2.4.2  Spinal gliosis

Microglial and astrocytic activation was observed in the spinal cord of CCI rats (Figure 2.3). Iba1, a marker for microglia, was sparsely expressed throughout sham spinal cords, and was strongly upregulated in the ipsilateral dorsal and ventral horns in CCI treated spinal cords, correlating with microglial activation (Figure 2.3A, B). The morphology of microglia differed from mostly ramified in sham, to mostly amoeboid in CCI spinal cords, indicating a phenotypic change from resting to activated (Figure 2.3B, C). Immunolabeling with GFAP revealed a presence of activated astrocytes in the ipsilateral dorsal horn of CCI treated rats, in a similar pattern to the dorsal population of activated microglia (Figure 2.3D, E).
Figure 2.3 Gliosis in the lumbar spinal cord following CCI or sham surgery. Microglia, labeled with Iba1, are sparsely scattered throughout the sham spinal cord (A), while high concentrations of activated microglia are present in the ipsilateral dorsal and ventral horns 10 days following CCI (B, arrows). The appearance of microglia changes from ramified while resting in the sham spinal cord (C, inset), to amoeboid when activated in the CCI spinal cord (D, inset). Mild astrocytic activation, visualized with GFAP immunolabeling, is present in the ipsilateral dorsal horn of CCI treated spinal cords 10 days post surgery (E, arrows, F). Scale bars equal to (A, B, E) 400 μm, (C, D, F) 100 μm, and (C, D insets) 20 μm.
There were two distinct populations of activated microglia in CCI spinal cords, both ipsilateral to injury. One of these populations was in the dorsal horn, from approximately lamina I to lamina V, at the termination point of sensory afferents. The second population was detected in lamina IX of the ventral horn, where the cell bodies of efferent alpha motor neurons reside (Figure 2.4A). Upon closer examination of the ventral population, it was seen that these activated microglia form mantles or baskets around the alpha motor neuron cell bodies present in this lamina (Figure 2.4B, C). The encapsulation is extensive, and appears to affect nearly all motor neurons within the ventral microglial population.

![Figure 2.4 Microglial encapsulation of motor neurons in the ipsilateral ventral horn of CCI treated rats. Activated microglia are present in the dorsal and ventral horns of the ipsilateral spinal cord 10 days following CCI surgery, as shown by Iba1 immunolabeling (A). Activated microglia (Iba1 +ve, black) encapsulate large motor neurons (NeuN +ve, brown) in the ventral horn, forming mantles around the soma (B, C). Scale bars equal to (A) 400µm, (B) 50 µm, (C) 15 µm.](image)
2.4.3 Adverse events associated with surgery

The incidences of adverse events from animals used across all experiments within this study were pooled. Red tears, or chromodacryorrhea, was seen in 9 CCI treated rats out of 173 (5%), and 1 sham rat out of 44 (2%). Mild nasal chromodacryorrhea was seen in 4 CCI treated rats (2%), but no sham treated animals. In all cases, symptoms were present 24-48 hours following surgery, and resolved shortly thereafter with no intervention required.

Self mutilation of the hindpaw, or autotomy, of any severity was present in 12 out of 173 CCI treated rats (10%), and was absent from all sham treated animals. In the 3 milder cases, animals gnawed claws of the affected hindpaw to the stumps, which caused mild bleeding. In these cases, the affected paw was cleaned, and further monitoring revealed no further damage requiring intervention. In the more severe 9 cases, animals were euthanized immediately. All cases of autotomy appeared within 30 hours of surgery.

In addition to autotomy of the hindpaw, some self mutilation at the wound site was seen in CCI, but not sham treated rats. This wound mutilation was pronounced when latex instead of nitrile gloves were used during surgery. Over a brief period of latex glove use, 4 out of 8 animals (50%) exhibited signs of self mutilation by gnawing at the wound site, whereas only 4 cases were seen in the remaining 165 animals (2%). Nitrile gloves were thus employed for the remainder of the study. Mild edema was also observed in the ipsilateral hindpaw in a proportion of CCI treated animals, but was absent in all sham animals. It was noted that a change in bedding from pine shavings to heat treated corncob bedding increased the incidence of edema considerably. With pine shaving bedding, 9% of animals exhibited edema, whereas corn cob bedding increased the incidence rate to 45%. In addition to an increased incidence rate, swelling intensity also appeared to increase, although this was not
quantified. Once this was discovered, bedding was changed back to pine shavings to avoid the potentially confounding effect of edema.
2.5 Discussion

The results of this study detail the successful replication of the CCI surgery as described by Bennett and Xie (1988). Mechanical allodynia developed in the ipsilateral and contralateral hindpaws of CCI but not sham treated animals, within 3 days post surgery, with a stable level achieved at the 10 day time point. Both astrocytes and microglia were activated in the ipsilateral dorsal horn of the spinal cord following CCI but not sham surgery. Activation of microglia resulted in an altered morphology from mostly ramified in sham spinal cords, to mostly amoeboid in CCI spinal cords. An additional population of activated microglia was observed in the ventral horns of CCI treated spinal cords, some of which encapsulated large alpha motor neurons to form a mantle. Moderate incidences of adverse events were observed, in line with the original description of the model (Bennett et al., 1988), some of which were able to be minimized by controlling surgical and housing factors.

2.5.1 Pain behaviors

Behavioural data from all experiments was pooled, allowing a meta-analysis of paw withdrawal threshold at baseline, and 3 and 10 days post surgery for all surgery treated and behaviorally assessed animals. Paw withdrawal thresholds of sham and CCI treated rats were not significantly different at baseline, but differed significantly by 3 and 10 days post surgery in both ipsilateral and contralateral hindpaws (p<0.001 CCI versus sham, 3 and 10 days post surgery), indicating that mechanical allodynia, a well established hallmark of the CCI model (Attal et al., 1990; Kim et al., 1997; Dowdall et al., 2005), was fully established at both time points. In the first description of the model by Bennett and Xie (1988) the authors reported that mechanical allodynia was not detected, although it was noted that the testing methods employed were probably inadequate for
determining this, and it was in fact likely that mechanical allodynia was an element in this model (Bennett et al., 1988). It has previously been reported that mechanical allodynia develops in the CCI model over several days, and is stable for several weeks before a slow decline back to baseline levels (Kim et al., 1997; De Vry et al., 2004; Dowdall et al., 2005), making the 10 day time point in the current study a rational choice for the assessment of evoked pain behavior. Rapid onset of mechanical allodynia is also a common feature of other rodent models of neuropathic pain, and has consistently been reported in the PSL (Seltzer et al., 1990), SNL (Choi et al., 1994), SNI (Decosterd et al., 2000) and CST (Dowdall et al., 2005) models.

Of particular interest was the development of allodynia contralateral to injury. Reports first describing the CCI (Bennett et al., 1988), SNI (Decosterd et al., 2000), and SNL (Kim et al., 1992) models failed to detect any bilateral allodynia or hyperalgesia. However, bilateral allodynia has since been reported in the sciatic inflammatory neuritis (SIN) model of inflammatory neuropathic pain (Chacur et al., 2001), and several models of neuropathic pain, namely PSL (Seltzer et al., 1990), SNI (Li et al., 2006), and the CCI model (Attal et al., 1990; Paulson et al., 2000; Paulson et al., 2002; Spataro et al., 2004; Honore et al., 2006; Grace et al., 2010). This is a desirable element of an animal model of neuropathic pain for at least two reasons. First, the existence of mirror image pain has been documented in humans; specifically in complex regional pain syndrome (Veldman et al., 1996; Maleki et al., 2000). Secondly, and importantly for the objectives of this project, this phenomenon is probably centrally mediated (Koltzenburg et al., 1999), meaning that mechanisms and treatment of central sensitization, an essential part of the development and maintenance of neuropathic pain in humans, may be studied in detail.

In addition to mechanical allodynia, numerous evoked and spontaneous pain behaviors have been described and characterized in rodent models of peripheral nerve injury. As with mechanical allodynia, mechanical hyperalgesia is a universal feature of rodent pain models and tends to develop over the course of several days, with severity greatest in the PSL model (Dowdall et al., 2005).
Similarly, response to cold chemical stimulation with acetone develops to a comparable degree over a similar time course in all tested models (Kim et al., 1997; Dowdall et al., 2005). Pain evoked by thermal stimulation, however, is markedly variable between models. While nerve ligation induces rapid and intense cold and hot allodynia, transection induces delayed onset mild cold allodynia, with no or only transient hot allodynia (Dowdall et al., 2005). Ongoing or spontaneous pain behaviors are more difficult to analyze in rodents than man, however several studies have monitored this outcome, with CCI producing the greatest signs of spontaneous pain in comparison to other models (Kim et al., 1997; Dowdall et al., 2005).

The CCI model produces a range of pain behaviors that model many of the elements of ongoing or stimulus evoked pain in human neuropathies, which may even include the emotional components (Hu et al., 2009). While these attributes have made the CCI model a popular choice for the investigation of the molecular mechanisms of pain, and the testing of experimental therapeutic interventions, the model does have some problems, which is one of the reasons alternative models have been established. As the CCI model requires an exact ligation tension on the sciatic nerve, implementation of the model requires an experienced experimenter. While the complexity of the CCI surgery may not be as great as the SNL model, which requires careful dissection of the transverse spinal processes overlying the spinal nerves (Martin et al., 2003), it does require practice and optimization to perfect the exact tension required for sciatic ligation. In comparison with alternative models, CCI consistently produces the greatest variability in pain behaviors, which may be its greatest pitfall (Kim et al., 1997; Dowdall et al., 2005). In contrast, nerve transection, as in the SNI and CST models, or tight nerve ligation as in the SNL and PSL models are more reproducible, with less variability in pain behaviors. The aim of initial validation of the CCI model was thus to optimize and establish the model with sufficiently low variability in stimulus evoked pain behavior in order to probe the role of the CB2 receptor in neuropathic pain, an aim that has been sufficiently fulfilled in this instance.
2.5.2 Gliosis

In order to assess central inflammation at the spinal level of the pain pathway, immunohistochemistry was performed using antibodies targeted to GFAP and Iba1, to qualitatively assess levels of astrocytic and microglial activation respectively, 10 days post surgery. Strong labeling was observed in the grey matter for both markers. Labeling for Iba1 was particularly strong, ipsilateral to injury in CCI but not sham treated animals, indicating a strong gliotic response. The pattern of labeling in both cases was predominantly in the dorsal horn ipsilateral to the side of injury, although a population of microglia was also present in the ventral horn. This distinctive unilateral pattern of microglial activation is well characterized in peripheral nerve injury models (Eriksson et al., 1993; Svensson et al., 1993), including the CCI model (Zhang et al., 2003; Mika et al., 2009). Similar patterns of labeling have been documented for astrocytic activation in the CCI model (Garrison et al., 1991; Colburn et al., 1997). Microgliosis appears to precede astrogliosis, as markers of microglial activation appear within hours to days following nerve injury, and return almost to baseline levels after a couple of weeks, whereas astrocyte activation first appears several days post injury, with a sustained activation for weeks afterward (Tanga et al., 2004; Cavaliere et al., 2007). The induction of micro- and astrogliosis 10 days post CCI in the current study is thus consistent with previous research.

It is thought that this gliosis is part of a process of neuroinflammation, which has a role in the development and maintenance of neuropathic pain behaviors, particularly allodynia. An early study identified that non-specific suppression of glial activation substantially attenuated evoked pain behavior when delivered during the development of allodynia, and was also moderately effective when delivered at a later stage of allodynic development in the SNT model (Sweitzer et al., 2001). Further studies implicated microglia as key players in the spinal generation of neuropathic pain, as pretreatment with systemic minocycline, a tetracycline antibiotic and inhibitor of microglial
activation, was able to suppress the development of tactile allodynia following peripheral nerve injury (Raghavendra et al., 2003; Guasti et al., 2009). Furthermore, intrathecal delivery of minocycline prior to nerve manipulation is able to inhibit the development of mechanical allodynia following peripheral nerve inflammation (Ledeboer et al., 2005) or injury (Narita et al., 2006), indicating that spinal microglia play a key role in central sensitisation following peripheral nerve injury. Despite a strong research focus on microglial contribution to central sensitization, it has also been reported that astrocyte inhibition is able to block the development of neuropathic pain, though only at a later stage than the effective period for microglial blockade (Wang et al., 2009), corresponding to the known later peak in astrocytic activation (Tanga et al., 2004; Cavaliere et al., 2007).

While the mechanisms of mirror image allodynia are not well understood, there is strong evidence that spinal glia may be also responsible for the development of this phenomenon. In an experimental model of acute inflammatory neuropathic pain, the SIN model, it was discovered that unilateral nerve inflammation could induce bilateral allodynia independent of circulating immune effectors (Chacur et al., 2001), confirming earlier suggestions that mirror image pain was centrally mediated (Koltzenburg et al., 1999). Further studies in the same model found that spinally delivered inhibitors of glial activation reversed bilateral allodynia (Milligan et al., 2003), implicating spinal glial activation as a key element in mirror image pain. Functional studies have identified that both pro-inflammatory cytokine release (Milligan et al., 2003) and glial connectivity via gap junctions (Spataro et al., 2004) are responsible for the spread of glial excitation to the contralateral spinal cord (Grace et al., 2010), and contribute to the mirror image phenomenon.

An interesting observation in this study was that activated microglia ipsilateral to CCI appeared to engulf the resident alpha motor neurons in lamina IX, forming baskets or mantles (Brownjohn et al., 2012a). This has been seen, but not described, previously in both the CCI (Zhang et al., 2003) and CST (Kobbert et al., 2000; Hu et al., 2007) models of sciatic nerve lesion. This is very probably a result
of the lesioning of efferent motor fibers in the sciatic nerve, as this phenomenon has been described following ventral root lesioning alone (Colburn et al., 1999; Kobbert et al., 2000), and corresponds to a reduction in motor neuron synaptic covering and activity following nerve lesion (Zanon et al., 2006; Barbizan et al., 2010). The functional significance of the relationship between reactive microglia and the encapsulated degenerating motor neurons is so far unknown, although it has been postulated that microglia may provide neurotrophic support via NGF and bFGF in order to improve neuronal survival (Kobbert et al., 2000).

2.5.3 Adverse events

Over the course of the study, several of the characteristic adverse effects of the CCI model were observed. Red tears, or chromodacryorrhea, is a sign of inescapable stress in the rodent, and has been shown to occur following limb restraint (Harkness et al., 1980). Much like Bennett and Xie (1988), who reported “a few instances shortly after surgery” out of 148 CCI treated rats, this study produced 9 cases of chromodacryorrhea out of 173 CCI treated animals (an incidence of 5%), and 1 case out of 44 sham treated animals (an incidence of 2%), in addition to 4 cases of nasal chromodacryorrhea in CCI treated animals. Autotomy of the hindpaw has previously been reported with a 70% (Bennett et al., 1988) and 10% (Kim et al., 1997) incidence in CCI treated rats. The 10% incidence rate in the current study is consistent with that reported by Kim et al. (1997), but considerably lower than that reported by Bennett and Xie (1988), however the severity of the mutilation is a key factor to consider. Autotomy in the current study was only recorded when bleeding was observed, however in the Bennett and Xie (1988) study, animals that gnawed their claw tips beyond usual limits were considered to be displaying autotomy. Describing severe autotomy, they report that out of 148 animals, “a few animals injured the distal phalanges of one or
more toes” and two animals amputated more than two toes (Bennett et al., 1988). Similarly in this study severe autotomy consisted of damage to distal phalanges.

In addition to the previously reported and characterized adverse events, ipsilateral paw edema and wound mutilation were seen in several cases, and it was noted that changes in surgical technique and housing influenced the occurrence of these events. Animal bedding, provided by the Taieri-Hercus Resource Unit at the University of Otago, was originally pine shavings, however was switched during the study to heat treated corn cobs for economical reasons. During this period, the incidence of edema in the ipsilateral hindpaw increased markedly (from 9 to 45%). It was noted that with the new bedding, which was much coarser than pine shavings, animals kept their ipsilateral paw in a more guarded position, rarely touching the ground. It is theorized, therefore, that reduced muscle movement in the ipsilateral paw resulted in lymph pooling, and swelling in the affected foot. To avoid this confound, pine bedding was used for the remainder of the study. In addition, wound mutilation was seen in isolated cases, and once again was affected by external factors. It was observed that self mutilation at the wound site increased from 2 – 50% when the gloves used during surgery were changed from synthetic nitrile to powdered latex, and returned to baseline levels upon switching glove type back. It is theorized in this case that latex, being a natural product, induced a local allergen-mediated inflammatory response or irritation of the wound, and thus caused an increase in mutilation. The optimal nitrile glove was employed for the remainder of the study.

2.5.4 Conclusions

This study has established and validated the CCI model. Following CCI but not sham surgery, bilateral mechanical allodynia developed over the course of several days, and was stable 10 days following nerve lesion. This corresponded with extensive microgliosis and mild astrogliosis in the dorsal horn.
of the spinal cord at this time point, which are known to be involved in the development phase of alldynia, and the development and maintenance of bilateral alldynia reported in this study and others. Furthermore, ventral microglia were seen to encapsulate alpha motor neurons in lamina IX of the ipsilateral ventral horn of CCI treated spinal cords, in line with previous reports of sciatic and spinal nerve injury. Therefore, this model was validated to a degree sufficient to allow the assessment of the efficacy of CB2 selective ligands on mechanical alldynia, and thus the investigation of the role of the CB2 receptor in the spinal pain pathway in a stable, reproducible and relevant model of human neuropathic pain.
3 The effect CB2 receptor agonism on allodynia in the CCI model

3.1 Introduction

Delivered systemically, CB2 selective agonists have shown antinociceptive effects in animal models of acute, inflammatory and neuropathic pain (see Chapter 1), yet the mechanism of action remains unclear. Recent studies have implicated spinal CB2 receptors, however, as intrathecal administration of microgram doses of CB2 agonists to the spinal cord has been reported to produce antinociception in a variety of rodent pain models (Romero-Sandoval et al., 2007; Romero-Sandoval et al., 2008; Yamamoto et al., 2008; Curto-Reyes et al., 2010; Curto-Reyes et al., 2011; Gu et al., 2011; Hsieh et al., 2011; Paszczuk et al., 2011). To date, this phenomenon has not been investigated using the CCI model, so the aim of this study was to assess the efficacy of CB2 receptor agonism in this inflammatory neuropathic pain model, using both systemic and spinal administration paradigms.

The CB2 receptor selective partial agonist GW405833 was selected for this study, as it has been shown to be anti-hyperalgesic and anti-allodynic in the surgical model of acute pain (LaBuda et al., 2005) the CFA model of inflammatory pain (Valenzano et al., 2005; Whiteside et al., 2005), and the PSL (Valenzano et al., 2005; Whiteside et al., 2005), SNL (Beltramo et al., 2006) and CCI (Hu et al., 2009) models of neuropathic pain. Further to this, partial agonists such as GW405833 produce less receptor desensitization and internalization than full agonists, in turn tending to induce less tolerance to repeated administration, making them ideal drug candidates for chronic conditions such as neuropathic pain (Clark et al., 1999).
3.2 Experimental aims

This study aimed to assess the efficacy of CB2 selective cannabinoid agonists on mechanical allodynia induced by CCI of the sciatic nerve. In initial experiments, the effects of the CB2 selective agonist GW405833 on CCI induced mechanical allodynia were assessed following systemic administration in a dose response. Assuming this compound was anti-allodynic, as has been previously reported in models of neuropathic pain, subsequent experiments aimed to elucidate a possible spinal location of action of CB2 selective agonists. This was performed by assessing the anti-allodynic effects of CB2 selective and CB1/CB2 non-selective cannabinoid agonists following spinal intrathecal delivery.

3.3 Methods

3.3.1 Experimental design

CCI surgery of the sciatic nerve was performed, and animals were assessed for mechanical allodynia 3 and 10 days post surgery. Following confirmation of alldynia at 10 days, drug or vehicle was administered to animals via systemic intraperitoneal (i.p.) injection, or spinal intrathecal (i.t.) injection, and mechanical allodynia assessed over a range of time points for 5 hours after administration. A timeline of these events is outlined below, in Figure 3.1.
Figure 3.1 Timeline of drug intervention and behavioural testing in CCI treated rats. Mechanical allodynia was assessed at baseline (BL), before CCI surgery, and again 3 and 10 days post surgery. After confirmation of mechanical allodynia at 10 days, drug or vehicle was injected, and allodynia assessed 1, 3 and 5 hours following systemic injection, and 30 min, 1, 3 and 5 hours following intrathecal injection.

Furthermore, for systemic studies, percentage reversal of allodynia for each rat was calculated using the following equation (Equation 1) from Valenzano et al. (2005):

\[
\text{Equation 1:} \quad \% \text{ reversal} = \frac{\text{Postdose threshold} - \text{predose threshold}}{\text{Baseline threshold} - \text{predose threshold}} \times 100
\]

3.3.2 Drugs

The CB2 selective cannabinoid agonists GW405833 and JWH-133, and the CB1/CB2 non-selective cannabinoid agonist WIN 55,212-2, were obtained from Tocris Bioscience (UK). HU910 was
synthesized by Lital Magrid, and was a generous gift from Professor Raphael Mechoulam of the Hebrew University of Jerusalem. For systemic intervention, GW405833 was administered i.p. in 25% hydroxypropyl-β-cyclodextrin dissolved in 0.9% saline, at a concentration of 1, 2.5, or 7.5 mg/mL, for a 1, 10 or 30 mg/kg dose, respectively, as described in Valenzano et al. (2005). The solution was mildly acidified with HCl to increase solubility of the otherwise lipophilic GW405833, before balancing the solution back to pH 5.3 - 7.4 for injection. I.p. dosing volumes were 1 mL/kg for a 1 mg/kg dose, and 4 mL/kg for 10 and 30 mg/kg doses.

For initial intrathecal studies, GW405833 was dissolved to 0.67 mg/mL in 8.3% ethanol in sterilized dH_{2}O, and was delivered at a dose of 15 µl per rat. Due to doubts about solubility with the ethanol vehicle, in later studies GW405833, JWH133, HU910, and WIN 55,212-2 were dissolved in 5% dimethyl sulfoxide (DMSO), 5% Cremophor® EL and 5% ethanol in 0.9% saline (DCE vehicle), at final concentrations ranging between 0.67 and 5mg/mL. The dosing volume for later studies was 15 -20 µl per rat, depending on drug and dose required.

### 3.3.3 Intrathecal drug administration

Acute intrathecal delivery required the construction and implementation of guide cannulae and catheters, as described previously (Milligan et al., 2005). For guide cannulae, plastic hubs were removed from sterile 18 gauge needles, and the bevel side of the needle marked at the hub end. For catheters, sterile polyethylene PE10 tubing, cut to 40 cm lengths, was marked 7.7-7.8 cm from one end, and the opposite end threaded over a sterile 30 gauge needle, attached to a 50 µl Hamilton syringe. Cannulae and catheters were further sterilized with UV light for 10 min, and stored in dry, sterile containers until use.
Ten days following surgery, animals were induced and maintained under 2.5% halothane, and anaesthetic depth confirmed by lack of pedal withdrawal reflex. The dorsal pelvic region was shaved, and swabbed with 70% ethanol. A guide cannula, with the bevel facing up, was inserted between the L5 and L6 vertebrae, at a 45 degree angle. The cannula angle was reduced to 30 degrees, and the cannula advanced through to the subarachnoid space, until it could progress no further. At this point, a characteristic tail flick, and efflux of a drop of clear or pink CSF was often observed at the protruding cannula tip. The pre-loaded catheter was at this stage advanced through the guide cannula, all the while eliciting tail and hind leg twitches, until the 7.7-7.8 cm mark was aligned with the top of the cannula, confirming placement of the catheter at the L4-L6 lumbar enlargement. The drug was administered at a rate of 1 µl/s, before rapid removal of catheter and cannula, and recovery of the animal from anaesthesia. The procedure lasted between 2-4 minutes, and animals had fully recovered from anaesthesia within 10-15 min. No motor or neurological impairments were seen in any animals following the recovery period. Placement of the catheter was verified in naïve test animals several times prior to initiation of, and several times following, the study with injection of India ink, followed by rapid sacrifice and laminectomy, confirming catheter placement and administration of compounds at the level of the L4-L6 lumbar enlargement.

3.3.4 Statistical analysis

Behavioural results were transformed as outlined in Chapter 2, and analyzed parametrically with 2-way repeated measures ANOVA, with time as one factor and drug as the other, with Bonferroni post hoc tests in Prism, Version 5.01 (GraphPad, CA, USA).
3.3.5 Power analysis

To assess the level of power required to detect a significant difference in the intrathecal intervention section, a power analysis was conducted. Using the variance of ipsilateral values obtained from the first 3 animals in each treatment group, a 2 factored analysis in nQuery Advisor® (Statistical Solutions, MA, USA) was performed, with time as a repeated measure, and drug as a second factor. It was estimated that with n = 5 rats per group, the power (β) to detect a difference was 0.89 with alpha set at 0.05.
3.4 Results

3.4.1 Systemic intervention

Systemic administration of the CB2 selective partial agonist GW405833 significantly attenuated mechanical allodynia in the ipsilateral but not contralateral hindpaws of CCI treated rats in a dose dependent manner (Figure 3.2). A 2-way repeated measures ANOVA of paw withdrawal threshold showed that, ipsilateral to injury, the interaction between drug and time was significant (F = 2.80 (15, 110); p = 0.001). Conversion of ipsilateral withdrawal thresholds into percentage reversal of allodynia using Equation 1 revealed that at a dose of 30 mg/kg, GW405833 significantly attenuated mechanical allodynia 3 and 5 hours post administration compared to vehicle as analyzed by Bonferroni post hoc tests (p < 0.05 and p < 0.01, respectively), by 66 and 59%, respectively (Figure 3.2C). Two-way repeated measures ANOVA analysis of contralateral thresholds revealed that the interaction between drug and time was not significant for raw withdrawal threshold values, or percentage reversal of alldynia values, however this interaction tended towards significance in both cases (F = 1.56 (15, 110); p = 0.096 and F = 2.25 (6, 40); p = 0.056, respectively).
Figure 3.2 Effect of systemically (i.p.) administered CB2 selective agonist GW405833 on CCI-induced mechanical allodynia in the rat. GW405833 (GW) significantly attenuated allodynia in the ipsilateral (A, C), but not contralateral (B, D) hindpaws. (C) In the ipsilateral hindpaw, GW405833 significantly reversed mechanical allodynia 3 and 5 hours post administration (* = p <0.05 vs. vehicle). Paw withdrawal thresholds are shown in raw values (A, B), and converted to percentage reversal of allodynia (C, D). Drug was administered at 1, 10 and 30 mg/kg. Data expressed as mean ± SEM. Vehicle = 25% hydroxypropyl-β-cyclodextrin in 0.9% saline. n = 8 for vehicle; n = 6 for GW (1 mg/kg); n = 7 for GW (10 mg/kg); n = 8 for GW (30 mg/kg).

3.4.2 Intrathecal intervention

3.4.2.1 Initial study

GW405833, dissolved in 8.3% EtOH in dH₂O at a dose of 10µg, had no significant effect on CCI induced mechanical allodynia. Analyzed with a 2-way repeated measures ANOVA, there was no
significant interaction between drug and time in the ipsilateral or contralateral hindpaw 3 hours following intrathecal administration ($F = 0.44 \ (3, 39); \ p = 0.73$ and $F = 0.11 \ (3, 39); \ p = 0.95$) (Figure 3.3). This negative result warranted a more detailed investigation involving a variety of drugs, doses, and time points (below).

3.4.2.2 Detailed studies

Intrathecal administration of the CB2 partial agonist GW405833, at doses of 10 and 30 µg, and the CB2 full agonists JWH 133 and HU 910, at doses of 30 and 100 µg, respectively, had no effect on CCI-induced mechanical allodynia in ipsilateral or contralateral hindpaws. Two-way repeated measures ANOVA revealed no significant interaction between drug and time in ipsilateral or contralateral hindpaws ($F = 0.83 \ (24, 174); \ p = 0.70$ and $F = 0.81 \ (24, 174); \ p = 0.73$, respectively) (Figure 3.4).
Figure 3.4 Delivered i.t., the CB2 selective agonists GW405833, JWH-133 and HU910 have no significant effect on mechanical allodynia in CCI treated rats. Data expressed as mean 50% withdrawal threshold ± SEM. GW = GW405833; JWH = JWH-133; HU = HU910. Vehicle was 5% DMSO, 5% Cremophor® EL, 5% ethanol in 0.9% saline. n = 5 for GW (10 µg); n = 5 for GW (30 µg); n = 5 for JWH; n = 6 for HU; n = 13 for vehicle.
In contrast, the non-selective CB1/CB2 cannabinoid agonist WIN 55,212-2, when delivered i.t. at a dose of 30 µg, significantly attenuated mechanical allodynia in the ipsilateral and contralateral hindpaws of animals, 10 days following CCI (Figure 3.5). There was a significant interaction between drug and time following drug administration in ipsilateral and contralateral hindpaws, as determined by 2-way repeated measures ANOVA ($F = 9.00$ (4, 68); $p < 0.001$ and $F = 4.25$ (4, 68); $p = 0.003$, respectively). Furthermore, post hoc analysis revealed a significant difference between WIN 55,212-2 and vehicle in the ipsilateral paw at the 30 min time point ($p < 0.001$). Reversal peaked 30 minutes post administration in both paws, and withdrawal thresholds had returned to baseline levels within 3 hours.

**Figure 3.5** I.t. administration of WIN 55,212-2 ameliorates CCI-induced mechanical allodynia in the ipsilateral (A) and contralateral (B) hindpaws of the rat. (** = $p < 0.001$ WIN vs. vehicle). Data expressed as mean ± SEM. WIN = WIN 55,212-2. Vehicle was 5% DMSO, 5% Cremophor® EL, 5% ethanol in 0.9% saline. $n = 5$ for WIN; $n = 13$ for vehicle.
3.5 Discussion

Systemic delivery of the CB2 selective partial agonist GW405833 significantly attenuated CCI induced mechanical allodynia in a dose dependent manner. When administered spinally via intrathecal injection, however, GW405833 and several structurally unrelated CB2 selective agonists had no significant effect on mechanical allodynia in this model. In contrast, the non-selective CB1/CB2 agonist WIN 55,212-2, when administered intrathecally, was able to significantly reverse mechanical alldynia in the same paradigm.

3.5.1 CB2 selective agonists as a treatment for neuropathic pain

Previous studies have shown that GW405833 is anti-hyperalgesic and anti-allodynic in several animal pain models. In the carrageenan model of inflammatory pain in the rat, GW405833 (i.p.) at 1 mg/kg and above moderately attenuated paw edema and hypersensitivity, respectively, via a CB2 dependent mechanism (Clayton et al., 2002). Using a model of neuropathic pain, the PSL model, Valenzano et al. (2005) found that GW405833 (i.p.) at doses of 1 mg/kg and upwards could partially reverse mechanical hyperalgesia without producing nociception in naïve rats, suggesting a CB1 independent mechanism. This reversal was roughly 50% with a 1 mg/kg dose, and peaked at 64% with a 10 mg/kg dose (Valenzano et al., 2005). In contrast, however, another study by the same investigators revealed that 30 mg/kg was the minimum dose required for anti-allodynic effects using the PSL model in mice (Whiteside et al., 2005). Similar results were obtained in the rat by LaBuda et al. (2005) and Beltramo et al. (2006), who found that 30 mg/kg was the minimum effective dose required for a significant alleviation of mechanical allodynia in a post surgical model of acute pain, and the SNL model of neuropathic pain, respectively (LaBuda et al., 2005; Beltramo et al., 2006).
These results are consistent with the findings of the current study, which suggest that 30 mg/kg is the minimum dose of GW405833 required for anti-allodynic effects in the CCI model of neuropathic pain in the rat.

In contrast to the demonstrated efficacy of systemically delivered GW405833 in neuropathic pain, the results of this study demonstrate that administered intrathecally, a range of structurally unrelated CB2 agonists had no significant effect on CCI-induced mechanical allodynia. While intrathecal administration of GW405833 had not been previously been examined in pain models, efficacy has been demonstrated with several other CB2 selective ligands, including JWH-015, AM1241, and JWH-133, a compound employed in this study. Administered intrathecally in microgram quantities, JWH-015 reduced mechanical allodynia induced by surgery (Romero-Sandoval et al., 2007), and SNT (Romero-Sandoval et al., 2008) in the rat, and bone cancer (Gu et al., 2011) and BPA (Paszczuk et al., 2011) in the mouse. Similarly, in the mouse, intrathecal JWH-133 had an anti-allodynic effect in the PSL model of neuropathic pain (Yamamoto et al., 2008) and the CFA model of inflammatory pain (Curto-Reyes et al., 2011), and AM1241 was efficacious in a bone cancer model (Curto-Reyes et al., 2010) and the CFA model (Curto-Reyes et al., 2011). Where tested for receptor subtype specificity, the effects of JWH-015 were abolished by co-administration with the CB2 antagonist AM630 (Romero-Sandoval et al., 2007; Romero-Sandoval et al., 2008; Gu et al., 2011), but not the CB1 antagonist AM281 (Romero-Sandoval et al., 2007; Romero-Sandoval et al., 2008). Furthermore, the effects of JWH-133 were suppressed when co-administered with the CB2 receptor antagonist SR144528 (Curto-Reyes et al., 2011) and absent in CB2 knockout mice (Yamamoto et al., 2008), and the effects of AM1241 were abolished by co-administration of SR144528, but not the CB1 receptor antagonist AM251 (Curto-Reyes et al., 2010), prompting the investigators in these studies to suggest that these agonists are acting via a CB2, and not CB1, dependent mechanism, at the spinal level. The discrepancies between the results of the current study and those of previously published reports will be addressed in detail below.
In contrast to the negative results obtained in this study with CB2 selective agonists, the non-selective cannabinoid agonist WIN55,212-2 was efficacious in the CCI model when delivered intrathecally, consistent with prior reports in rat neuropathic pain models. Previous studies have shown that intrathecal WIN55,212-2 significantly attenuated mechanical hyperalgesia in the CCI model (Hama et al., 2004), and mechanical allodynia induced by bone cancer (Cui et al., 2011), SCI (Hama et al., 2011), and CCI (Lim et al., 2003). Reversal of allodynia in previous studies peaked approximately 30 min following administration, and where tested, the effect was blocked by co or pre-administration with the CB1 receptor antagonists AM251 (Cui et al., 2011), AM281 (Lim et al., 2003) and SR141716 (Hama et al., 2011), but only weakly blocked with a high dose of the CB2 selective antagonist AM630 (Cui et al., 2011), indicating a strong CB1 receptor dependency. While complete reversal of hyperalgesic effects was reported at 10 µg (Hama et al., 2004), anti-allodynic effects were not usually reported until doses approached 30 µg (Lim et al., 2003; Hama et al., 2011), justifying the dose chosen in the current work. Similarly, the highly potent non-selective CB1/CB2 agonist CP55,940 was efficacious against mechanical allodynia when delivered intrathecally in a surgical pain (Romero-Sandoval et al., 2007) and SNT model (Romero-Sandoval et al., 2008), an effect that was more dependent on CB1 than CB2 receptors (Romero-Sandoval et al., 2007).

3.5.2 Systemic administration of subtype selective cannabinoids

3.5.2.1 Affinity for CB1

An important consideration in the administration of receptor subtype selective ligands is dose, and subsequent concentration at receptors. With unrestrained dose escalation, one cannot be certain that a CB2 selective ligand is binding solely to the CB2 receptor subtype. Indeed it has been shown
with GW405833 that a dose of 100 mg/kg i.p., or as low as 10 mg/kg i.v., results in classical CB1
mediated CNS effects (Valenzano et al., 2005; Beltramo et al., 2006). Given that an effective anti-
allodynic dose in peripheral nerve injury models is reported to be 30 mg/kg, there is a potential for
some overlap between anti-allodynic and CB1 activating doses. In fact, Valenzano et al. (2005)
reported that plasma levels of GW405833 reached 2 µM following a 30 mg/kg dose, and given a high
brain:plasma ration of >5, this equates to potential concentrations of 10 µM in CNS tissues. This is
many orders of magnitude greater than the 3.6 nM Ki for this compound at rat CB2, and importantly,
is well in excess of the 273 nM Ki and 916 nM EC50 for GW405833 at rat CB1 (Valenzano
et al., 2005; Yao et al., 2008b). Although Valenzano et al. (2005) also showed that the anti-allodynic properties of
GW405833 were absent in CB2 receptor knockout mice in the CFA model (Valenzano et al., 2005), it
must be noted that this was specific for a peripheral inflammatory model of pain, and thus far, the
potential action of GW405833 on CB1 receptors has not been disproven in neuropathic models
which involve central sensitization.

In CB2 agonist studies where hyperalgesia and alldynia have been assessed concurrently, it is
apparent that the doses required for alleviation of hyperalgesia are often lower than that required
for reversal of alldynia (Ibrahim et al., 2003; Yao et al., 2008a; Yao et al., 2008b). Hyperalgesia is
gen generally a function of peripheral sensitization, and alldynia one of central sensitization (Chapter
1). It could therefore be hypothesized that the efficacy of low dose CB2 agonists on hyperalgesia is
due to CB2 receptor activation in the periphery, perhaps modulating immune function, yet the
required anti-allodynic doses of these same compounds are acting centrally, either on a small
population of central CB2 receptors, or possibly spilling over to the centrally ubiquitous CB1
receprors. This theory is supported by the work of Yao et al. (2008a), who report that anti-
hyperalgesic doses of the CB2 agonist A-836339 had no effect on locomotion, yet anti-allodynic
doses of the same compound produced deficits in locomotor activity, which were blocked by co-
administration of the CB1 antagonist SR1141716 (Yao et al., 2008a). A key feature of this study was
the use of horizontal activity and exploratory behavior to measure locomotor deficits, rather than the more commonly employed rotorod assay. At a dose of A-836339 causing a CB1 dependent 73% reduction in horizontal motor activity, the Yao group found no effect on rotorod performance (Yao et al., 2008a), questioning the sensitivity of the rotorod assay in determining subtle CB1 receptor activation.

The idea of residual CB1 activation mediating the efficacy of CB2 receptor agonists in pain models has been proposed before. In two recently published letters, a team from Merck Research synthesized an array of structurally similar CB2 selective ligands, differing in their selectivity for CB2 over CB1, and tested them in vivo (Manley et al., 2011; Trotter et al., 2011). Two compounds with 100 – 300 fold selectivity for rat CB2 over CB1, but with some residual CB1 affinity in the micromolar range, were efficacious at high doses (≥ 10 mg/kg) against mechanical hyperalgesia in the CFA model of inflammatory pain. In the same model, however, two structurally related compounds with similar CB2 receptor affinities, but no measurable CB1 affinity were not efficacious, despite higher tested doses (100 mg/kg), and measured levels in the blood, brain and CSF well exceeding CB2 receptor IC50s (Manley et al., 2011; Trotter et al., 2011). The authors point to an elegant study by Gifford et al. (1999) that estimated CB1 receptor reserves in the CNS, and their implications for agonist binding. In that particular study, the investigators calculated that only around 0.1% CB1 receptor occupation was necessary for a 50% inhibition of neurotransmitter release in the CNS, indicating that a low affinity agonist at CB1 has the potential to elicit full agonist effects (Gifford et al., 1999). As most CB2 receptor selective agonists used in pain studies maintain some affinity for CB1 receptors, the implication is that these agonists could be binding CB1 receptors at low affinity and occupancy, and acting via CB1 receptor activation to elicit their anti-nociceptive effects. This is in agreement with a comprehensive in vivo study by Sain et al. (2009), in which the anti-nociceptive effects of the CB1/CB2 non-selective agonist CP55,940 were absent in CB1, but not CB2, knockout mice in both inflammatory and neuropathic pain models (Sain et al., 2009).
Further to CB1 receptor activation at sufficient doses, it has been reported that at least one commonly used CB2 agonist has opposing effects depending on receptor activation state, a phenomenon known as protean agonism. AM1241 reportedly displays 82 fold selectivity for CB2 over CB1 in rodent receptor assays \textit{in vitro} (Ibrahim \textit{et al.}, 2003). Perhaps the most studied CB2 receptor agonist in the pain field, AM1241 has been employed intrathecally by the Curto-Reyes group (Curto-Reyes \textit{et al.}, 2010; Curto-Reyes \textit{et al.}, 2011) and the Hsieh group (Hsieh \textit{et al.}, 2011), and systemically by a host of groups in a multitude of pain models (Malan \textit{et al.}, 2001; Ibrahim \textit{et al.}, 2003; Ibrahim \textit{et al.}, 2005; LaBuda \textit{et al.}, 2005; Beltramo \textit{et al.}, 2006; Rahn \textit{et al.}, 2007; Guerrero \textit{et al.}, 2008; Rahn \textit{et al.}, 2008; Lozano-Ondoua \textit{et al.}, 2010; Rahn \textit{et al.}, 2010; Gutierrez \textit{et al.}, 2011; Saghafi \textit{et al.}, 2011). AM1241 is known to act as a protean agonist, and can have opposing agonist and antagonist effects at the CB2 receptor depending on levels of constitutive receptor activity (Mancini \textit{et al.}, 2009), and assay conditions (Yamamoto \textit{et al.}, 2008), making inference about mechanisms difficult, especially when employing \textit{in vivo} animal models where conditions are less strictly controlled than \textit{in vitro} cell based assays.

Furthermore, while displaying a high selectivity for CB2 receptors over CB1 receptors, AM1241 also has significant affinities for non-cannabinoid targets at micromolar doses, including the kappa opioid receptor, and serotonin, muscarinic, dopamine, histamine and benzodiazepine receptors (Yao \textit{et al.}, 2008a). Similarly, JWH-015, another CB2 agonist used extensively in studies of CB2 agonism and pain, has off target effects at micromolar doses. While this compound has 27 fold selectivity for CB2 over CB1 (Showalter \textit{et al.}, 1996), it is also able to increase intracellular calcium in DRG neurons via binding to the putative cannabinoid receptor GPR55 at low micromolar concentrations (Lauckner \textit{et al.}, 2008). Furthermore, the CB1 receptor antagonists SR141716, AM251 and AM281 have also exhibited binding affinities at GPR55, although the results for these compounds have been mixed.
(for a comprehensive review, see Sharir et al., 2010). The exact role of GPR55 in pain modulation has not yet been elucidated, but it is of note that it is reportedly abundantly expressed on large sensory neurons of the DRG (Lauckner et al., 2008), and peripheral C fibers (Schuelert et al., 2011), and appears to play an essential role in the development of hyperalgesia in inflammatory and neuropathic pain models (Staton et al., 2008). While these off target effects do not necessarily apply to all cannabinoid receptor ligands, it highlights possibilities that have not been studied in enough detail to draw definitive conclusions about selective CB2 receptor activation in pain states.

3.5.3 Intrathecal administration of cannabinoids

While the systemic results of this study are consistent with other reports, the intrathecal results presented here are in stark contrast to previously published research documenting the efficacy of intrathecally delivered CB2 agonists in various pain models. The disparity between these reports requires careful consideration.

3.5.3.1 Validation of results

As i.t. administration in this case was acute, and did not employ an indwelling catheter, it was not possible to verify injection placement in each animal that received an injection. It is therefore plausible that incorrect injection placement could account for the negative results obtained with intrathecal intervention. However, several lines of evidence suggest that cannula placement was correct. First, injection placement was verified in several test animals prior to the intervention study by injecting India ink, before confirming correct placement by sacrifice and laminectomy. This
procedure was repeated at the completion of study, and a 100% success rate of lumbar enlargement catheter placement is reported in these test animals. Furthermore, certain indications during the injection procedure suggest that placement was correct. Observations noted during the procedure matched those of a previously published report of cannula and catheter insertion, that described in detail the characteristic tail flick, hind limb twitch, and CSF efflux during insertion of a correctly placed cannula (Milligan et al., 1999).

The most definitive positive control from the intrathecal set of experiments, however, was the observation that the CB1/CB2 non-selective ligand WIN 55,212-2, at a dose of 30 µg i.t., was able to significantly attenuate CCI induced mechanical alldynia. If it is hypothesized that catheter placement was incorrect, and the drug was inadvertently injected into the systemic circulation, this would become a 30 µg systemic dose in an approximately 300g rat. This would equate to a systemic dose in the region of 0.1 mg/kg, which is below an efficacious dose for any significant effect on mechanical alldynia in peripheral nerve injury models (Bridges et al., 2001; Fox et al., 2001), including CCI (Costa et al., 2004). Moreover, this result confirms that the Von Frey hair paradigm employed to assess mechanical alldynia is sensitive enough to detect a significant amelioration of mechanical alldynia.

With any study using animal behavior as a measure, which can be highly variable, it is essential to ensure blinding of observers to treatment, in addition to randomization of treatment allocation. It has been shown in animal research that studies that do not employ blinding are up to 3 times more likely to report a positive result than those that do, and those that do not employ blinding or randomization are 5 times more likely to report a positive result (Bebarta et al., 2003). In this study, drugs were coded by a third party before administration, in order to blind the administrator and behavioural observer to drug treatment. An additional benefit of third party blinding in this case was randomization of drug treatment, as the third party did not have access to behavioural assessment data. As discussed previously, it was not practically possible to blind the behavioural observer to
surgery and laterality of injury, as the effects of the CCI surgery are so pronounced on the ipsilateral hindpaw, and would be easy to pick even for a fully blinded observer. This is not seen as a limitation in this study, however, as comparisons were not made between hindpaws, and sham treated animals were not employed for this part of the study.

3.5.3.2 Considerations of dose with intrathecal administration

As with peripheral administration, it is important to consider the doses of receptor subtype selective agonists and antagonists administered centrally, and whether these doses correlate with implied receptor subtype selectivity. In these experiments a low, 10 µg, dose of the CB2 selective partial agonist GW405833 was assessed in two alternative vehicles. After determining no significant efficacy, the dose was increased to 30 µg, before switching to the full agonist JWH-133 at 30 µg, and finally a high, 100 µg dose of the highly selective, highly potent agonist HU910. It has previously been shown that intrathecal administration of JWH-133 above 100 µg in adult rats causes motor dysfunction (Yoon et al., 2003), a clear indication of CB1 receptor activation and loss of receptor subtype specificity at this dose. As JWH-133 has an approximately 200 fold selectivity for the CB2 over the CB1 receptor (Huffman et al., 1999), it can assumed that the dose of 30 µg employed in our studies was well in excess of that required for CB2 receptor activation. Furthermore, HU910, a CB2 agonist with a similar CB2 selectivity to JWH-133 (228 fold over CB1), and a high potency at human CB2 receptors (Horvath et al., 2011) was administered at 100 µg, also with no significant effect. Despite delivery in doses approaching CB1 receptor activation, these drugs were unable to elicit an anti-allodynic effect when delivered to the spinal cord in CCI animals.

Issues of subtype selectivity also apply to intrathecally delivered antagonists. For example, in Romero-Sandoval (2007), intrathecal pre-administration of AM630 was able to ameliorate the anti-
allodynic effects of JWH-015 in a surgical model, suggesting a CB2-dependent mechanism (Romero-Sandoval et al., 2007). However, in the same study, the same dose of AM630 was able to inhibit CP55,940 induced catalepsy, a CB1 mediated phenomenon. This result suggests some cross antagonism of CB1 by AM630 that precludes sweeping generalizations about receptor subtype specificity in this model, and questions the conclusions reached by others employing the same antagonists and administration paradigms to infer CB2 involvement in amelioration of allodynia. This is a particularly salient issue in this field, as all but one of the intrathecal CB2 papers has used pre or co-administration of antagonists to ascertain receptor subtype involvement.

3.5.3.3 Intrathecal catheter placement

Inferring a spinal site of action from experiments using intrathecal administration of microgram doses of drug requires careful injection placement. In these studies, if one were to infer any spinal site of action for administered drugs, it was essential for the catheter to sit directly above the L4-L6 lumbar spinal cord, as sciatic nerve pathways synapse through this point. It is important at this stage to understand a fundamental aspect of spinal anatomy. In the rat, the spinal cord terminates around the L3-L4 vertebrae, becoming the cauda equina (Gelder et al., 1977). Therefore, all spinal roots exiting the spinal canal caudal to this termination must travel some distance from their spinal origin to exit the vertebral canal at the appropriate intervertebral foramen. Moving caudally from the L1 lumbar region of the spinal cord, there is an increasing disparity between the origin of nerve roots in the spinal cord, and their intervertebral exit to the periphery. Of note for this study, in the rat, the L4-L6 spinal roots of the sciatic nerve, which originate and terminate in the L4-L6 region of the lumbar spinal cord, must travel 2.7 cm (L4) to 4.5 cm (L6) caudally to exit through the L4-L6 intervertebral foramina (Gelder et al., 1977). It is for this reason in the present study that catheters
were inserted in addition to guide cannulae. Taking into account dead space in the cannula, the catheter, when inserted to the 7.7-7.8 cm mark, is able to extend approximately 3.3 cm from the L5/L6 foramen, thus allowing drug administration directly on to the L4-L6 cord. The anatomical situation is similar in the mouse, in which the L3 – L4 spinal roots that comprise the sciatic nerve (Rigaud et al., 2008) are located 1.8 – 2.1 cm from the L5/L6 foramen (Wu et al., 2004).

Aside from Romero-Sandoval and Eisenach (2007), and Hsieh et al. (2011), in which indwelling catheters were employed, all previous reports of intrathecal CB2 agonist administration have used the Hylden and Wilcox method of intrathecal delivery (Romero-Sandoval et al., 2008; Yamamoto et al., 2008; Curto-Reyes et al., 2010; Curto-Reyes et al., 2011; Gu et al., 2011; Paszcuk et al., 2011), which involves the direct injection of drugs into the intrathecal space at the L5/L6 intervertebral foramen, without the use of a catheter (Hylden et al., 1980). A comparison of the popular Hylden and Wilcox method and the catheter method employed here is illustrated in Figure 3.6. Inferring a spinal site of action in studies employing the Hylden and Wilcox method assumes a wide distribution of cannabinoids throughout the CSF to reach the presumed spinal targets, which is not the case. Using radiolabeled THC, Smith and Martin (1992) showed that rostral spread of intrathecally delivered THC was limited, with considerable amounts of the drug accumulating close to the site of administration (Smith et al., 1992), which is also the case with the more lipophilic opioids (Rathmell et al., 2005). With a limited rostral spread, the action of Hylden and Wilcox delivered CB2 agonists at the proximally located dorsal root, or associated DRG, nestled within the adjacent intervertebral foramina, rather than the more distal lumbar spinal cord, is therefore worth considering.
Figure 3.6 Comparison between methods of intrathecal delivery. (A) The popular Hylden and Wilcox method involves direct administration of drug from the cannula (black) between the L5 and L6 vertebrae (dark grey) to the spinal cord (light grey) adjacent to the L4 – L6 DRGs (red). (B) The catheter method is an adaptation of the Hylden and Wilcox method, employing a catheter (dashed black line) to apply drugs more rostrally, to the L4 – L6 lumbar enlargement adjacent to the L1 vertebrae.

The dural sheath that encases the spinal cord is continuous, to a degree, with the DRGs, and as such the entire dorsal root and proximal half of ganglia are immersed in CSF (Brierley, 1950). The transfer of CSF between the subarachnoid and the subdural spaces would permit the exchange of small
molecular weight drugs such as cannabinoids, allowing them access to the DRG directly from the CSF.

Supporting a DRG site of action, Hsieh et al. (2011) compared the efficacy of CB2 agonist administration via intrathecal and intra-DRG catheterization, the only group to have done so. Using the SNL model, the authors showed that intra-DRG delivery of 30 µg A-836339 significantly reduced CFA induced thermal hyperalgesia by 65% and SNL induced allodynia by 45%, however intrathecal administration of the same dose was unable to attenuate hyperalgesia, and only partially reduced allodynia by 33% (Hsieh et al., 2011). Similarly, when delivered intra-DRG, 50 µg AM1241 attenuated SNL induced mechanical allodynia by 69%, compared to 42% for the same dose delivered via intrathecal catheterization (Hsieh et al., 2011), suggesting that the true site of action may be the DRG. This would explain why administration directly onto the ganglion in this case was more efficacious than delivery to the L4-L6 spinal cord, where, assuming a DRG site of action, the drug would have had to diffuse caudally over a considerable distance. CB2 receptor functionality in the DRG is supported by a set of in vitro experiments conducted by Sagar et al. (2005), in which JWH-133 was able to attenuate capsaicin induced elevations in intracellular calcium in cultured DRG neurons from neuropathic rats (Sagar et al., 2005).

This theory could perhaps explain the lack of efficacy observed in the current study. Had drugs been administered to the L5/L6 intervertebral foramen without the use of a catheter using the Hylden and Wilcox method, or alternatively, administered directly onto the DRGs, a different response profile may have been observed.
3.5.4 Conclusions

There is a large body of evidence supporting the efficacy of CB2 selective agonists in animal models of neuropathic pain, specifically peripheral nerve injury. It has been proposed that CB2 receptors, specifically within the spinal pain pathway may mediate the anti-allodynic effects of these compounds. However after evaluation of the current results, and previous literature, it could be argued that:

1) The efficacy of systemically delivered CB2 selective agonists may be due to cross activation of the CB1 receptor, as common CB2 selective ligands retain residual affinity for the CB1 receptor, and high doses of these compounds are required for anti-allodynic efficacy in neuropathic pain models.

2) The efficacy of intrathecally delivered CB2 agonists may be due to action on cannabinoid receptors in DRGs, as these structures are more proximal to the delivery site when using the Hylden and Wilcox method of intrathecal injection than the lumbar spinal cord, where sensory afferents of the sciatic nerve terminate.

The results of the current work are not consistent with a significant role for the CB2 receptor in the pain pathway at the spinal level in the CCI model of neuropathic pain. This does not, however, rule out CB2 receptor expression in the spinal pain pathway, or possible upregulation subsequent to peripheral nerve injury, which is assessed in the following chapters.
4 CB2 receptor protein expression in the CCI model

4.1 Introduction

The CB2 receptor has traditionally been referred to as the peripheral or immune cannabinoid receptor owing to its conspicuous absence from the CNS in contrast to the ubiquitous neuronal CB1 receptor. Sean Munro and colleagues, detailing the original discovery of CB2 in the rat, reported an absence of CB2 mRNA in the rat brain, using both a homogenate and in situ autoradiographic approach (Munro et al., 1993). Similarly, Galiegue et al. (1995) described the presence of CB2 mRNA transcripts in peripheral, but not central tissues in humans (Galiegue et al., 1995). This is consistent with the anatomical studies of Ibrahim et al. (2003), who reported no cannabinoid receptor binding in the brain and spinal cord of CB1 knockout mice using the high affinity CB1/CB2 agonist CP55,940, also suggesting an absence of CB2 receptors in the CNS (Ibrahim et al., 2003).

More recently, however, it has been reported that CB2 receptors are expressed in activated microglia of the CNS following certain neuropathologies (Benito et al., 2003; Benito et al., 2005; Ashton et al., 2007b; Benito et al., 2007). This phenomenon has also been described in the spinal cord of neuropathic animals. It has been reported that CB2 mRNA transcripts are upregulated ipsilaterally in the spinal cord following CCI and are regionally codistributed with a marker for activated microglia and macrophages (Zhang et al., 2003). In line with this observation, ipsilateral upregulation of CB2 receptor protein has also been described in microglia following SNT injury (Romero-Sandoval et al., 2008). In contrast, Wotherspoon et al. (2005) describe the ipsilateral upregulation of CB2 receptor protein not in microglia, but in primary afferent terminals, following SNL (Wotherspoon et al., 2005). Conditional upregulation of CB2 receptors in immune cells or nerve
terminals in the spinal cord may provide a target for the treatment of neuropathic pain syndromes. The expression patterns and cell types involved must therefore be elucidated and characterized across a range of neuropathies to allow for the optimization of treatment strategies; an aim not yet fulfilled in the CCI model beyond mRNA expression.

Many of the above studies describing central CB2 receptor expression rely on immunohistochemistry or Western blotting with CB2 directed antibodies. The same antibodies have been utilized recently to describe, in contrast to early reports measuring mRNA transcripts, widespread CB2 receptor protein expression in neurons of the healthy CNS (Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006), a finding not without controversy (Atwood et al., 2010). The discrepancies of these studies with earlier reports may be for a number of reasons; however it has been suggested that the CB2 directed antibodies employed may not be specific for the CB2 receptor. It is imperative, therefore, to definitively determine the validity of any antibody used to describe CB2 receptor protein expression.

4.2 Experimental aims

The aim of this chapter was to investigate CB2 receptor protein expression changes and cellular location in the lumbar spinal cord of rats following CCI surgery and intrathecal CB2 agonist administration. Immunolabeling experiments were performed to determine regional protein expression and cell phenotypes, and Western blot studies with mass spectrometric analysis aimed to quantify changes in protein expression. A secondary aim was to confirm the specificity of the antibody employed in this study and other published reports describing CB2 receptor expression in the CNS (Walczak et al., 2005; Wotherspoon et al., 2005; Gong et al., 2006; Walczak et al., 2006; Paszczuk et al., 2011), which has previously been putatively validated (Wotherspoon et al., 2005).
4.3 Methods

4.3.1 Experimental design

CCI and sham surgery was performed on rats, and behavioural testing performed 3 and 10 days post surgery. All animals were sacrificed following 10 day behavioural testing for molecular analysis, except one cohort, which received drug or vehicle via spinal intrathecal injection before sacrifice 3 hours post injection. The presence and regulation of CB2 receptor protein in the spinal cord and DRGs of rats following CCI or sham surgery was assessed. Regional and cellular distribution studies, in addition to quantitative analyses were performed using immunohistochemistry, while Western blot was employed to quantitatively assess protein changes with higher resolution, and to examine protein size. Coupled with Western blot, mass spectrometry was used to identify detected protein species. The specificity of the antibody employed in this study and others was then tested in vitro with immunocytochemistry, and ex vivo with immunohistochemistry of knockout control tissue.

4.3.2 Animals

Animals used for paraffin immunohistochemistry were male Sprague-Dawley rats weighing 330-370 g, and housed at the University of New Mexico. Animals in this cohort also received intrathecal GW405833 or vehicle. Animals used for fresh frozen immunohistochemistry, Western blot, and proteomics were male Wistar rats, weighing 240-340g and housed at the University of Otago. For validation of the N terminus CB2 antibody used in this study, lumbar spinal tissue was obtained from male N terminus CB2 receptor knockout mice (The Jackson Laboratory, ME, USA) and wild type mice.
of the same C57BL/6 strain. This tissue was generously gifted by Professor Ken Mackie, of the Gill Centre and Department of Psychological and Brain Sciences at Indiana University.

4.3.3 CB2 receptor antibodies

The primary antibody employed for quantitative analysis in this study was a rabbit derived polyclonal antibody raised against amino acids 20-33 of the N terminus of the human CB2 receptor (#101550, Cayman Chemical, MI, USA), which has previously been putatively validated (Wotherspoon et al., 2005). Testing of primary antibody-dependent labeling was performed with corresponding immunizing peptide from the same company (#301550, Cayman Chemical, MI, USA).

In a comparative Western blot, two alternative CB2 primary antibodies were tested. These were goat derived polyclonal antibodies raised against an unspecified N terminus sequence of the human CB2 receptor (#10071, Santa Cruz Biotechnology Inc., CA, USA) and an unspecified C terminus sequence of the mouse CB2 receptor (#10076, Santa Cruz Biotechnology Inc., CA, USA). Sources of non-CB2 antibodies are listed in the appropriate methods sections.

4.3.4 Cell culture

Chinese hamster ovary (CHO) cells, over expressing the rat CB2 receptor (EZCells™, Applied Cell Sciences Inc., MD, USA) were grown in Ham’s F12, supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 400 µg/mL G418, in 95% O₂, 5% CO₂ at 37°C. These cells were harvested for immunocytochemistry and Western blot.
For a negative control, human embryonic kidney 293 (HEK 293) cells (American Type Tissue Collection, VA, USA) were grown on coverslips in Dulbecco’s modified Eagle medium, supplemented with 10% FBS, 2 mM L-glutamine, 0.25 µg/mL amphotericin B, 100 U/mL penicillin and 100 µg/mL streptomycin in 95% O₂, 5% CO₂ at 37°C. HEK293 cells were provided by Brie Sorrenson of the Biochemistry department, University of Otago. These cells were used exclusively for immunocytochemistry.

### 4.3.5 Immunohistochemistry

#### 4.3.5.1 Cardiac perfusion

Immediately following behavioural testing at the 10 day time point, animals were anaesthetised with sodium pentobarbital, and upon confirmation of sufficient anaesthetic depth, the chest cavity opened to expose the heart. An incision was made into the left ventricle and right atria, and a catheter inserted into the ascending aorta. Phosphate buffered saline (PBS) containing heparin sodium (500 µL/L) and procaine (1 g/L) warmed to 37°C was run through the catheter, followed by the same solution at room temperature, 4% paraformaldehyde (PFA) at room temperature, and finally 4% PFA at 4°C, until the animal was deemed sufficiently perfused and fixed. Tissues of interest were excised, namely L4-L6 spinal cord sections, ipsilateral and contralateral L4-6 DRGs, and spleens, and stored in ice cold 4% PFA. After 24 hours, tissues were transferred to ice cold PBS for a further 24 hours.
4.3.5.2  Paraffin embedding/Sectioning

Tissues were processed through a graded series of ethanol (70%, 2X 95%, 2X 100%) and xylene, before immersion in 65 °C paraffin, then paraffin under vacuum to remove air bubbles and aid with tissue infiltration. Processed tissue was set into paraffin blocks, before sectioning on a microtome at 3 µm, and mounting on HistoBond® slides (Histology Service Unit, University of Otago), permanently loaded with positive charge to ensure section adhesion. Slides were dried at 60°C for 1 hour, and stored at room temperature until use.

4.3.5.3  CB2 immunolabeling

Sections from the L5 spinal section in each animal were used for analysis, along with DRG sections, and sections of spleen from sham/vehicle treated animals as positive controls for CB2. Sections were dewaxed by immersion in 2 changes of xylene for 5 mins each, before rehydration in 100 and 95% ethanol for 3 mins and 1 min respectively, then dH2O for 1 min. Sections underwent an optimized process of epitope retrieval; sections were immersed in IHC-Tek™ epitope retrieval solution (IHC World, USA), heated in a temperature controlled steamer (95-98 °C) for 30 min, then cooled to room temperature before 3 X 3 min washes in 10 mM PBS. Sections were quenched in 3% H2O2 in PBS, and washed for 3 X 3 min in PBS. Endogenous avidin and biotin binding sites were blocked by incubating sections in dilute egg white (1 egg white/100 mL dH2O) then 5% skim milk powder in PBS for 15 mins each, separated by a single 2 min wash in PBS1. Following blocking with 1.5% normal goat serum in

---

1 Epitope retrieval performed on paraffin embedded sections can unmask endogenous avidin/biotin binding sites which may interfere with an avidin/biotin amplification protocol (Bussolati et al., 1997). Egg whites and
PBS for 30 min at room temperature to reduce non-specific background binding, sections were incubated for 18-20 hours with a polyclonal rabbit α human CB2 antibody (#101550, Cayman Chemical, MI, USA) at a concentration of 1:200 in primary antibody diluent (1 % bovine serum albumin (BSA) and 0.05% sodium azide in PBS) at 4°C. To confirm immunolabeling was primary antibody specific, adjacent sections were incubated with primary antibody occluded, or primary antibody pre-incubated with .025 mg/ml of the corresponding CB2 immunising blocking peptide (#301550, Cayman Chemical, MI, USA), for 1 hour at room temperature.

Visualization of immunolabeling was performed using a Vectastain ABC elite kit (Vector laboratories, CA, USA). Sections were washed 3 times in PBS containing 0.1% Triton-X100 for 3 min each, before incubation with 0.5% goat anti-rabbit biotinylated IgG for 30 min. Sections were then washed thrice in PBS alone for 3 min each, before a 1 hour incubation with an avidin-biotin complex (ABC) horseradish peroxidase made up at half the manufacturer’s recommended concentration. Diaminobenzidine Nickel (DABNi) was prepared immediately prior to use, as per manufacturer’s instructions. Following ABC incubation, sections were washed for a final 3 times in PBS for 3 min each, before application of the DABNi substrate. Development times for DABNi vary depending on antibody and section type, and as such were optimized in pilot studies. The optimized development time of 1 min 50 sec was adhered to for all paraffin embedded spinal sections treated with the Cayman Chemical CB2 polyclonal antibody. To terminate the DABNi development reaction, sections were immersed in dH2O for 2 min.

After termination of the DABNi development reaction, sections were dehydrated in ascending concentrations of ethanol (50, 70, 90, 100%) for 2 min, before clearing in xylene for 2 min. Sections were left to air dry before mounting with di-n-butyl phthalate in xylene (DPX) mountant (BDH, UK).

skim milk have been shown to provide a reliable source of avidin and biotin, respectively, with similar efficacies to commercially available substrates when used in IHC (Miller et al., 1997; Miller et al., 1999).
4.3.5.4  **Double label immunohistochemistry**

Sections underwent identical treatment as for single labeling with CB2, however following DABNi development of CB2 immunolabeling, were sequentially labeled for GFAP, Iba1, or Neuronal Nuclei (NeuN). Following termination of DABNi development, sections were washed 3 times in PBS for 3 min each, before re-quenching in H$_2$O$_2$, and re-blocking of avidin/biotin as outlined above. Sections were blocked for non-specific background staining by incubation at room temperature with normal horse or goat serum (1.5% in PBS) for 30 min at room temperature, before incubation with either mouse anti-rat GFAP (1:1000; Abcam, MA, USA), goat anti-rat Iba1 (1:100; Abcam, MA, USA), or mouse anti-rat NeuN (1:300; Chemicon, CA, USA) for 18-20 hours at 4 °C. Sections underwent identical secondary antibody incubation and ABC amplification step as for single CB2 labeling, except for the use of horse anti-mouse biotinylated IgG for GFAP and NeuN labeling, and rabbit anti-goat biotinylated IgG for Iba1 labeling. Cell markers were visualized using a NovaRED substrate kit (Vector laboratories, CA, USA), made up as per manufacturer’s instructions. Development time was determined in pilot studies, and ranged from 1-3 min, after which development was terminated by immersion in dH$_2$O. Sections were dehydrated and mounted as described above.

4.3.5.5  **Fresh frozen tissue**

In addition to the paraffin embedded tissue discussed above, fresh frozen tissue from a separate cohort of CCI and sham treated animals was analyzed to account for possible differences in CB2 receptor protein antigenicity between paraffin embedded and fresh frozen tissue. Lumbar spinal tissue was removed from animals sacrificed 10 days post CCI or sham surgery, then chilled with powdered dry ice and stored at -80°C until use. Tissue was sliced into 20 µm sections at -20°C on a
Leica CM 1850 cryostat (Meyer Instruments Inc., TX, USA), and thaw mounted on to Histobond® slides, dried, then reanimated in PBS for 5 min. Sections were fixed in 4% PFA, washed 3 X in PBS, then quenched with 0.3% H2O2 in 50:50 methanol:PBS for 10 min. From this point on, sections were treated identically to the paraffin sections as outlined above, with the exception of a lower primary antibody concentration (1:250), and longer development time (4 min, 30 sec). Images were captured, and an analysis of staining density in superficial laminae of the dorsal horn was carried out in ImageJ® by a blinded observer, as described previously. Fresh frozen tissue was not employed for colocalization studies as morphology was substantially reduced compared to paraffin embedded tissue.

4.3.5.6 Image capture

Images were captured using a Zeiss Axioplan microscope, an Axiocam HRc digital camera and Axiovision 3.1 software (Carl Zeiss, Germany). Images were captured at 5 – 100X objective, and saved as Tagged Image File (TIF) images as representative images or for quantitative analysis of immunolabeling. Images for quantitative analysis were captured by an experimenter blind to treatment groups.

4.3.5.7 Analysis of CB2 immunolabeling

Quantitative densitometry of CB2 receptor immunolabeling was performed on images captured of the spinal cord from paraffin embedded and fresh frozen tissue, while cell counting of immunolabeled cells was additionally performed on images from paraffin embedded tissue. All
analysis was performed by an experimenter blinded to treatment. TIF images were converted to 8 bit gray scale in NIH ImageJ®, Version 1.40g, and overlaid with a stereotaxic template of the L5 section of the rat spinal cord, in order to delineate lamina boundaries (Paxinos et al., 1986).

Densitometry was performed on images of paraffin embedded and fresh frozen tissue using ImageJ®. Briefly, optical density was measured in the superficial dorsal horn (laminae I and II), and was normalized to density in adjacent white matter (*Fasciculus cuneatus* and *Fasciculus gracilis*) of the same spinal section, which was unchanged with treatment (data not shown). For additional analysis of immunolabeling in paraffin embedded tissues only, cells in the superficial dorsal horn immunolabeled with DABNi were manually counted. Analysis of paraffin embedded IHC was performed in duplicate, and analysis of fresh frozen IHC was performed singularly.

4.3.6 **Antibody specificity**

4.3.6.1 **Immunocytochemistry**

Cells were fixed for 10 min in 4% PFA, blocked with 1% normal donkey serum and 1% BSA in PBS, and incubated overnight (18-24 hours) at 4°C with a rabbit polyclonal antibody raised against the N terminus of the human CB2 receptor (#101550, Cayman Chemical, MI, USA), at a concentration of 1:200 in antibody diluent (0.1% BSA in PBS). Cells were washed in PBS containing 0.1% Triton-X100, and incubated for 1 hour with donkey anti-rabbit Alexa-Fluor 488 IgG (Invitrogen, CA, USA) at a concentration of 1:1000 in antibody diluent. Nuclei were labeled with Hoechst 33342 (Sigma, MO, USA), and images captured with fluorescence microscopy using a Zeiss Axioplan microscope, an Axiocam HRc digital camera and Axiovision 3.1 software (Carl Zeiss, Germany). Adjacent sections
were incubated with the CB2 antibody preadsorbed for 1 hour with 5 times excess corresponding immunizing peptide (#301550, Cayman Chemical, MI, USA).

4.3.6.2 CB2 receptor knockout mouse tissue

Mice were deeply anaesthetised with sodium pentobarbital, and transcardially perfused with 0.9% saline and 4% PFA. The L4-L6 spinal cord and spleen were excised and cryoprotected in 30% sucrose. Spinal cords were embedded in optimum cutting compound (OCT), and stored at -80°C. Twenty micron sections were cut at -20°C on a Leica CM 1850 cryostat (Meyer Instruments Inc., TX, USA), and thaw mounted on to chrome alum gelatin coated slides.

Sections were permiabilized with 0.2% Triton-X100 in PBS for 15 min, and endogenous peroxidases quenched by incubation for 30 min in 0.3% H$_2$O$_2$ in 50:50 methanol:PBS at 37°C. Following quenching, all procedures, including image capture, and reagents were identical to those used for CB2 immunolabelling in fresh frozen rat tissue, except for an increase in primary antibody concentration from 1:250 to 1:100.

4.3.7 Western blot

4.3.7.1 Tissue preparation

Lumbar spinal cords from L4-L6 of CCI and sham treated animals were rapidly removed by laminectomy following brief CO$_2$ anaesthesia and decapitation, and separated into ipsilateral and contralateral halves. Tissue was homogenized and sonicated in 50 mM Tris-HCl (pH 7.6) with a
complete protease inhibitor cocktail (Roche, IN, USA) to lyse cells, before centrifugation at 12,000 X G at 4°C for 10 min. The supernatant was decanted, and a Bradford protein assay (Bradford, 1976) conducted to standardize protein concentrations. Briefly, Bradford reagent (Biorad) was added to BSA standards at known protein concentrations, as well as unstandardized samples, in a 96 well plate, and absorbance read by the software Microplate Manager®, version 5.2.1 (Biorad, NZ), at 595 nm on a Benchmark Plus™ microplate spectrophotometer (Biorad, NZ). A graph was generated of the absorbance of the known protein standards, and the graph used to determine protein concentrations of the unknown samples, before standardizing the samples to 2 mg/ml in Tris buffer with complete protease inhibitors (Roche, IN, USA). Samples were denatured by means of boiling for 5 min with sample loading buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 2 mM EDTA, and 0.025% bromophenol blue), at a concentration ratio of 1:1, to generate final protein concentrations of 1 mg/ml. Standardized samples were stored at -20 °C until use.

Spleen tissue from naïve Wistar rats, and CB2 over expressing CHO cells were used as positive controls. Spleen tissue was mechanically homogenized before sonication, but otherwise prepared in an identical manner to spinal tissue, and protein standardized to 2.5 mg/ml. CHO cells were sonicated, and the supernatant after 12,000 X g centrifugation collected and re-centrifuged at 50,000 X g to increase protein content, before re-suspension of the pellet and protein standardization to 0.75 mg/ml.

4.3.7.2 SDS-PAGE

Samples were loaded in a 4.75% SDS bis acrylamide-Tris stacking gel, before separation of proteins based on molecular weight in a 7.5% SDS bis acrylamide-Tris resolving gel. Briefly, 0.75 mm glass
spacer plates (Biorad, NZ) were cleaned with 70% ethanol, before placement into a gel casting frame (Biorad, NZ). The catalytic reagents ammonium persulfate (APS), and N,N,N’,N’-tetrakisethylene diamine (TEMED) were added to resolving gel reagents (7.5% bis acrylamide, 0.4% SDS, 4.75% glycerol in 360 mM Tris, pH 8.8), and the resulting mix pipetted between the spacer plates. Methanol was added to smooth the top of the resolving gel, and the gel left to set for 10-20 min. Excess methanol was blotted away, before the addition of APS and TEMED to the stacking gel reagents (4.8% bis acrylamide, 1% SDS in 120 mM Tris, pH 6.8). The stacking gel mix was pipetted over the resolving gel, and the gel left to set for 10-20 min with an embedded well comb. Following gel setting, combs were removed to form the protein loading wells.

Gels were immersed in running buffer (1.4% glycine, 0.1% SDS in 25 mM Tris, pH 8.3), and connected to a Biorad power pack. Sample was loaded into each well, with the exception of the first well of each gel that was loaded with pre-stained kaleidoscope protein standard (Biorad, NZ). Gels were then run at 60 V until the dye front of the samples had passed from the stacking gel to the resolving gel, at which point voltage was increased to 120 V until the dye front had run to the end of the resolving gel, and the kaleidoscope protein standard had separated out.

Gels were separated from glass spacers, and left to soak for 2 X 15 min washes in transfer buffer (1.4% glycine, 20% methanol in 25 mM Tris, pH 8.3). Gels were sandwiched with PVDF membrane, pre-soaked for 1 min in methanol and 15 min in transfer buffer, in between 2 sheets of 3 mm filter paper, and placed inside a Semidry transfer unit (Biorad, NZ). Proteins were transferred from gels to the PVDF membrane at 20 V for 25 min.
Membranes were washed 3 X for 5 min in 0.1% Triton-X100 in tris buffered saline (TTBS), pH 7.4, before blocking with 0.1% BSA and 10% skim milk powder in TTBS at room temperature for 4 hours. Membranes were incubated overnight at 4°C in 1:500 polyclonal rabbit α human CB2 primary antibody (#101550, Cayman Chemical, MI, USA) in 0.1% BSA in TBS. To confirm specificity of this primary antibody, adjacent membrane blots were incubated overnight instead with 1:500 primary antibody pre-adsorbed with a 1:375 concentration of corresponding immunising peptide (#301550, Cayman Chemical, MI, USA). Membranes were then washed 3 X for 5 min with TTBS, and incubated for 2 hours with horse radish peroxidase (HRP) conjugated sheep α rabbit IgG (Chemicon, CA, USA) at 1:1000 in TTBS at room temperature. Following a 5 min wash in TTBS, and two subsequent 5 min washes in TBS, membranes were exposed to freshly prepared electrochemiluminesence (ECL) reagents (GE Healthcare, UK) for 1 min, before exposure to ECL hyperfilm (GE Healthcare, UK) for 2 min. Films were developed by immersion in developer (Kodak, NZ) for 30 sec to 2 min, stopper (Kodak, NZ) for 30 sec, fixer (Kodak, NZ) for 4 min, and running tap water for 4 min, before air drying.

In order to compare band intensity and staining patterns, a range of primary antibodies targeting the CB2 receptor protein were tested on adjacent membranes2. The protocol was identical to that outlined above, except for the use of differing primary antibodies: 1:200 goat α mouse C-terminus M-15 (Santa Cruz, CA, USA) and 1:200 goat α human N-terminus S-16 (Santa Cruz, CA, USA); and differing secondary antibodies: 1:2000 HRP conjugated rabbit α goat (Biorad, NZ) for both goat derived Santa Cruz primaries.

2 Densitometric analysis of bands was only undertaken on films developed after probing with the Cayman Chemical CB2 primary antibody. Analysis of blots probed with alternative antibodies was merely for molecular weight comparison.
Following probing and development of membranes for CB2 protein using the Cayman Chemicals primary antibody, membranes were re-probed for β actin as a loading control. Briefly, following film exposure for CB2, membranes were washed 3 X for 5 min in TTBS, and incubated overnight at 4°C in 1:5000 monoclonal mouse α rat β actin in 0.1% BSA in TBS. Membranes were then washed 3 X for 5 min with TTBS, and incubated for 1 hour with horse radish peroxidase conjugated chicken α mouse secondary antibody at 1:2000 in TTBS at room temperature. Following this step, membranes were treated identically as described above to generate a film of β actin density in addition to CB2 films for each gel.

4.3.7.4 Western Blot densitometry

Molecular weights were determined by the creation of a standard curve of $\log_{10}$ molecular weights of proteins in the kaleidoscope protein standard, versus distance travelled by each protein. Molecular weights of sample protein bands were thus able to be calculated from their distance travelled, by using the rearranged standard curve equation. Densitometric analysis was performed using a digital scanner (GS-710) (Biorad, NZ), and optical density (OD) of each band determined by the image processing software Quantity One®, version 4.6.7 (Biorad, NZ). Background OD was subtracted for each band, and the resulting value divided by the OD of β actin for the corresponding sample to create an OD ratio of CB2/β actin. To confirm that bands detected and measured for densitometric analysis were within the linear dynamic range of detectability, pilot experiments were performed in which exposure time relative to band density was plotted, and optimal exposure conditions obtained. A saturation curve of density relative to exposure time in pilot experiments, and relation to values obtained in the full densitometric analysis can be seen in Figure 9.1 in Appendix 2. Western blots were performed in duplicate.
4.3.8 Mass spectrometric analysis of the CB2 receptor

4.3.8.1 Tissue preparation

Lumbar spinal cord and spleen tissue from naïve animals was harvested after brief CO₂ anaesthesia and decapitation, and rat CB2 over expressing CHO cells were harvested after 72 hours of growth. All samples were sonicated in 50 mM Tris-HCl, pH 7.6, with complete protease inhibitor (Roche, IN, USA), before centrifugation at 1,000 X G at 4°C for 10 min. Resulting supernatant was spun at 50,000 X G for 10 min, and the membrane-rich pellets resuspended in an equal volume of 6 X Laemmli solubilization buffer (10% SDS, 9.3% dithiothreitol (DTT) in 300 mM Tris-HCl, pH 6.8) containing complete protease inhibitors. Samples were combined 1:1 with Laemmli loading buffer (10% SDS, 9.3% DTT, 30% glycerol, 0.025% bromophenol blue in 300 mM Tris-HCl, pH 6.8), and boiled for 5 min.

4.3.8.2 SDS-PAGE and gel digestion

Samples were run on a 7.5% bis acrylamide SDS-PAGE gel, as described above, with 6 identical samples run on each gel, divided into 2 sets of 3. Following this, one half of the gel, containing 3 samples, was processed via Western blot with the Cayman Chemical antibody as described above, while the other half, containing the remaining 3 samples, was fixed in a solution of 10% methanol and 7% acetic acid in dH₂O for mass spectrometry. The fixed gel was soaked overnight in Coomassie blue solution, and then overlaid on to the film from the Western blot processed half. Regions of the fixed gel corresponding to bands on the film were excised with a razor blade.

3 Spleen tissue required additional mechanical homogenization.
Gel fragments were destained with alternating washes in 70% acetonitrile in dH$_2$O and 100 mM ammonium bicarbonate, before dehydration in 70% acetonitrile in dH$_2$O. Samples were incubated in reducing buffer (10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate) for 1 hour at 45 °C, before dehydration in 70% acetonitrile. Next, samples were alkylated in iodacetamide for 15 min in the dark, before successive washes in 50 mM ammonium bicarbonate and 70% acetonitrile. Tryptic digest was performed on samples overnight with a 1:10 concentration of trypsin:protein in 50 mM ammonium bicarbonate at 37 °C. Digested protein was eluted by incubation with 70% acetonitrile in 0.1% formic acid for 30 min, and the supernatant containing the protein digest was lyophilized and saved for analysis.

4.3.8.3 Mass spectrometry

The following mass spectrometry protocols were designed and written by Dr Torsten Kleffman, of the Centre for Protein Research, in the Biochemistry department at the University of Otago.

All mass spectrometric techniques were performed with the following initial procedure, before either untargeted or targeted protein identification: Samples were re-solubilized in 5% [v/v] acetonitrile, 0.2% [v/v] formic acid in water and injected onto an Ultimate 3000 nano-flow uHPLC-System (Dionex Co, CA) that was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter-tip column (75 um ID PicoTip fused silica tubing (New Objectives, Woburn, MA) packed with C-18 material on a length of 8-9 cm) by a gradient developed from 5% [v/v] acetonitrile, 0.2% [v/v] formic acid to 80% [v/v] acetonitrile, 0.2% [v/v] formic acid in water.
4.3.8.3.1 Untargeted protein identification

Full mass spectrometry (MS) in a mass range between m/z 300-2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an AGC target of 5e5. Preview mode for FTMS master scan was used to generate precursor mass lists. The strongest 5 signals were selected for collision induced dissociation (CID) -MS/MS in the LTQ ion trap at a normalized collision energy of 35% using an AGC target of 2e4 and one microscan. Dynamic exclusion was enabled with 2 repeat counts during 30 sec and an exclusion period of 180 sec. Exclusion mass width was set to 0.01. MS/MS data were searched against the SWISS-PROT amino acid sequence database (downloaded in May 2010) using the Mascot search engine (http://www.matrixscience.com). The search was set up for full tryptic peptides with a maximum of 3 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, pyro glutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 10 ppm and the max. Fragment mass error was set to 0.8 Da.

4.3.8.3.2 Targeted protein identification

Peptides were separated by a gradient from 5% to 20% solvent B over 10 min, followed by 20 to 45% solvent B over 7 min and 45 to 90% solvent B over 3 min. Flow rate was set at 800 nl/min. Targeted peptide analysis was performed by selected reaction monitoring (SRM) in the LTQ ion trap using two transitions per precursor ion. Suitable transitions were selected based on the fragmentation pattern from the full CID-MS/MS spectra of peptides positively identified in the untargeted analysis of CB2 over expressing CHO cells.
The transitions used had the following parameters:

(i) Gradient = 5 - 20% B; precursor = 590.29; transition 1 = 676.21 (y6); transition 2 = 906.30 (y8).

(ii) Gradient = 20 – 45% B; precursor = 752.40; transition 1 = 460.15 (y4); transition 2 = 561.19 (y5).

Targeted protein identification data was analyzed manually using the Xcalibur software (Thermo Scientific, San Jose, CA).

4.3.9 Statistical analysis

Densitometry and cell counting data from IHC of paraffin embedded tissues was analyzed by 3 factor ANOVA as part of a general linear model in Minitab 15 (Minitab Inc., PA, USA), while densitometry from IHC of fresh frozen tissues was analyzed by 2-Way ANOVA in Prism, version 5.01 (GraphPad, CA, USA). Western blots were analyzed by 2-way repeated measures ANOVA in Prism, and expressed as mean ± SEM.
4.4 Results

4.4.1 CB2 receptor immunohistochemistry

Using the Cayman Chemical primary antibody, CB2 receptor immunolabeling was detected in the superficial dorsal horn of the lumbar spinal cord of CCI and sham treated rats (Figure 4.1A-D). A similar pattern was detected in fresh frozen tissue (Figure 4.1E), indicating no loss of antigenicity with paraffin embedding. Labeling was situated on cellular membranes (Figure 4.1G), and was reduced with preadsorption of the primary antibody with corresponding immunizing peptide (Figure 4.1F). Furthermore, immunolabeling was detected in fibers of the DRG (Figure 4.1H). Immunolabeling was also detected in spleen tissue, which is commonly used as a positive control for CB2 receptors, and was also reduced with preadsorption of the primary antibody (Figure 4.1I, J).

Quantitative analysis with densitometry (Figure 4.2A) and cell counting (Figure 4.2B) revealed no differences in CB2 immunolabeling between surgical group or between sides, and no modulation of immunolabeling with drug intervention in paraffin embedded tissues. Results were analyzed with a 3 factor ANOVA in a general linear model, with surgery, drug and laterality as factors. Analysis with densitometry revealed no significant differences between surgery ($F = 0.23$ (1, 21); $p = 0.63$), drug ($F = 0.09$ (1, 21); $p = 0.77$) or side ($F = 1.00$ (1, 21); $p = 0.33$), as did analysis of the same factors with cell counting ($F = 0.16$ (1, 21); $p = 0.69$ for surgery; $F = 1.11$ (1, 21); $p = 0.30$ for drug and $F = 0.01$ (1, 21); $p = 0.93$ for side). Additionally, no factor interactions were significant when analyzed with densitometry or cell counting; the most important of which were the interactions between surgery and laterality ($F = 0.02$ (1, 21); $p = 0.88$ and $F = 2.84$ (1, 21); $p = 0.11$ for densitometry and cell counting, respectively) and surgery and drug ($F = 0.45$ (1, 21); $p = 0.51$ and $F = 1.52$ (1, 21); $p = 0.23$ for densitometry and cell counting, respectively). Results were similar for densitometric analysis of fresh frozen tissues (Figure 4.2C). Analyzed with a two-way ANOVA, there was no significant
difference in immunolabeling between the factors: surgery (F = 0.02 (1, 8); p = 0.89), laterality (F =
0.75 (1, 8); p = 0.41) or the interaction between the two (F = 0.19 (1, 8); p = 0.68).

Figure 4.1 CB2 receptor immunolabeling with the Cayman Chemical CB2 antibody. Immunolabeling
was detected in the superficial dorsal horns of the lumbar spinal cord of rats 10 days post sham (A, B)
and CCI (C, D) surgery, following i.t. administration of vehicle (A, C) or GW405833 (B, D). Scale bars
equal to 400 µm. The same pattern was present in fresh frozen tissue (E), and was reduced with pre-
adsorption of the primary antibody with corresponding immunising peptide (F). Scale bars equal to
200 µm. In the dorsal horn, immunolabeling was localized to cellular membrane and neuropil (G).
DRG fibers were also positive for CB2 (H). Scale bars equal to 15 µm. Cellular staining for CB2 was
detected in spleen tissue (I), and was similarly reduced with preadsorption of the primary antibody
with corresponding immunising peptide (J). Scale bars equal to 100 µm.
Figure 4.2 Quantitative analysis of CB2 receptor immunolabeling in the superficial dorsal horn of sham and CCI treated rats. No statistically significant changes in CB2 receptor immunolabeling were detected using quantitative densitometry (A) or cell counting (B) on paraffin embedded tissue, or using quantitative densitometry in fresh frozen tissue (C). $n = 3$ for sham groups and $n = 4$ for CCI groups in paraffin cohort; $n = 4$ for sham and $n = 6$ for CCI in fresh frozen cohort.
Using cell type markers, it was possible to phenotype cells labeled with the Cayman Chemical CB2 antibody. CB2 immunolabeling partially co-localized with Iba1, a marker for microglia and macrophages, and NeuN, a marker for mature neurons (Figure 4.3). CB2 immunolabeling in Iba1 and NeuN positive cells was present on the cellular membrane, consistent with a membrane bound receptor such as CB2. No co-localization was seen with GFAP, a marker of activated astrocytes (not shown).

Figure 4.3 Phenotyping of CB2 immunolabeled cells. CB2 immunolabeling (black) partially co-localized with Iba1 (brown), a marker for activated microglia and macrophages (A), and NeuN (brown), a marker for mature neurons (B). Insets indicate that CB2 labeling was present in the membrane of both cell types. Scale bars equal to 15 µm.
4.4.2 CB2 receptor Western blot

Using the CB2 directed primary antibody from Cayman Chemical in combination with Western blotting, a protein band of approximately 44 kD was detected in the lumbar spinal cord of both sham and CCI treated rats, both ipsilateral and contralateral to induced nerve injury (Figure 4.4A). A band of this weight was detected in spleen tissue, along with 2 additional protein bands of approximately 37 and 59 kD. In CB2 over expressing CHO cells, a single band of approximately 37 kD was detected. All of these bands were abolished following pre-adsorption of the primary antibody with its corresponding immunising peptide (Figure 4.4B). Density of the 44 kD band detected in spinal cord tissue was analyzed by 2-way repeated measures ANOVA, and it was revealed that neither surgery nor laterality were significant factors (F = 0.42 (1, 9); p = 0.53 and F = 1.25 (1, 9); p = 0.29, respectively), nor was the interaction between the two (F = 0.34 (1, 9); p = 0.57) (Figure 4.4C). A similar banding pattern was seen in the same tissues with the use of two alternative primary antibodies, directed to the N terminus and C terminus of the CB2 receptor from Santa Cruz Biotechnology, with a band in spinal tissue detected around 48 kD with both antibodies (Figure 4.4D, E).
Figure 4.4 Western blot analysis of CB2 receptor labeling with the Cayman Chemical antibody. Using the Cayman Chemical CB2 antibody, bands of 37, 44 and 59 kD were detected in rat spleen, while a single band of 37 kD was detected in CB2 over expressing CHO cells and a single band of 44 kD was detected in spinal tissue (A). All bands were abolished with pre-adsorption of the primary antibody with corresponding immunising peptide (B). Two-way ANOVA analysis of the 44 kD band in spinal tissue revealed no significant differences between laterality or surgical group (C); n = 5 for sham, n = 6 for CCI. A similar band pattern to the Cayman Chemical CB2 antibody was seen in the same tissues using an alternative N terminus CB2 antibody (D), and a C terminus CB2 antibody (E), both from Santa Cruz Biotechnology.
4.4.3  Specificity of the Cayman Chemical CB2 antibody

4.4.3.1  Specificity of immunolabeling in vitro

Using the Cayman Chemical primary antibody, immunolabeling was detected in CB2 over expressing CHO cells (Figure 4.5A), and was reduced upon preadsorption of the antibody with its corresponding immunising peptide (Figure 4.5B). No immunolabeling was detected in CB2 negative naïve HEK293 cells (Figure 4.5C). Immunolabeling in CHO cells was present in the membrane and cytosol (Figure 4.5A-C) consistent with immunohistochemistry in spinal sections using the same primary, as well as the nature of membrane bound receptors such as CB2.

Figure 4.5 CB2 receptor immunocytochemistry with the Cayman Chemical CB2 antibody. Immunolabeling was detected in CB2 over expressing CHO cells (A), and was reduced following preadsorption of the primary antibody with corresponding immunising peptide (B). No labeling was detected in CB2 negative HEK293 cells (C). Scale bars equal to 100 µm. CB2 immunolabeling in CHO cells was present in the membrane and cytosol, and did not overlap with Hoechst nuclear stain (D – F). Scale bars equal to 40 µm.
Using the Cayman Chemical CB2 antibody, immunolabeling was detected in cellular membranes in the spinal cord dorsal horn in wildtype mouse spinal tissue (Figure 4.6A, and inset). Immunolabeling was abolished following preadsorption or omission of the primary antibody (Figure 4.6B, C). Similarly, in spinal tissue from CB2 knockout mice, immunolabeling was detected in the cellular membranes of cells in the spinal cord dorsal horn (Figure 4.6D, and inset). This immunolabeling was again abolished by preadsorption or omission of the primary antibody (Figure 4.6E, F).

Figure 4.6 CB2 receptor immunolabeling in wildtype and CB2 knockout mouse tissue with the Cayman Chemical CB2 antibody. Cellular staining was present in the superficial dorsal horn of lumbar spinal tissue from wildtype (A) and CB2 knockout mice (D). Labeling was reduced following primary antibody preadsorption with immunising peptide (B, E), and absent upon primary antibody omission (C, F). Insets show membrane nature of immunolabeling (A, D). Scale bars equal to 15 µm.
4.4.4  CB2 receptor mass spectrometry

4.4.4.1  The effects of membrane enrichment on CB2 labeling

For mass spectrometry, tissue homogenates were prepared as for Western blotting, with an additional ‘membrane enrichment’ step. This procedure entailed concentration of the membrane fraction, and, in the case of spleen and spinal tissue, exclusion of the cytosolic fraction of homogenates which was present in earlier Western blot sample preparations. Preparing samples in this manner resulted in a different Western blot band pattern to that obtained using the cytosolic procedure in earlier Western blot studies (Figure 4.7). The 37 kD band previously detected in CHO cells and spleen was enhanced by membrane enrichment in spleen tissue, and a band appeared at this weight in spinal tissue at a high sample protein concentration. In contrast, the 59 kD band previously detected in spleen, and the 44 kD band previously detected in spleen and spinal tissue were abolished by membrane enrichment indicating that these bands were most likely cytosolic proteins.

\[4\] In the original Western blots, CHO cell preparations, unlike spleen and spinal sample preparations, were partially membrane enriched, and the cytosol already excluded.
Figure 4.7 Differential preparation of Western blot samples for mass spectrometry alters the banding pattern detected by the Cayman Chemical CB2 antibody. Compared to standard preparations, membrane enrichment (+) of spleen and spinal tissue for mass spectrometry resulted in an enhancement of 37 kD band density in these tissues, and abolishment of 44 and 59 kD bands.

4.4.4.1. Mass spectrometry

Using an untargeted approach, the 37 kD band detected by the Cayman Chemical CB2 antibody in CB2 over expressing CHO cells was analyzed by LC-MS/MS following tryptic digest. Comparing sequences from the digested sample to the in silico digested SWISS-PROT database, seven peptides were identified that corresponded to 28% coverage, and thus a positive identification, of the rat CB2 receptor (Figure 4.8). Utilising the same approach on the 37 kD band detected in spleen and spinal tissue yielded none of these sequences, possibly due to relatively low sensitivity of this untargeted procedure.

Using the known elution times and mass to charge ratios of the peptide sequences detected in CHO cells, a targeted approach, employing SIM and SRM techniques was performed on the 37 kD band in spleen and spinal tissue. The expected CB2 receptor protein fragments were not identified with this
approach in either sample at this weight, either, despite positive protein controls indicating sensitivity in the range of 100 attomolar to 1 femtomolar.

Figure 4.8 Mass spectrometric analysis of the CB2 receptor protein in rat CB2 over expressing CHO cells. (A) Representative liquid chromatograph of the 37 kD band detected in CB2 over expressing CHO cell preparations by the Cayman Chemical CB2 antibody. (B) Identification of fragments representing 28% sequence coverage of the rat CB2 receptor protein, using tandem MS and the SWISS-PROT amino acid sequence data base.

<table>
<thead>
<tr>
<th>Position</th>
<th>Observed</th>
<th>Mr (expt)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>67 – 103</td>
<td>1026.2837</td>
<td>1021.1057</td>
<td>R.KPSYLFGSLAGADFLASVIFCHVFIFNQFVDRH</td>
</tr>
<tr>
<td>110 – 131</td>
<td>752.4036</td>
<td>754.1891</td>
<td>R.KPSYLFGSLAGADFLASVIFCHVFIFNQFVDRH</td>
</tr>
<tr>
<td>110 – 131</td>
<td>1126.1023</td>
<td>1125.1900</td>
<td>R.KPSYLFGSLAGADFLASVIFCHVFIFNQFVDRH</td>
</tr>
<tr>
<td>230 – 236</td>
<td>370.7241</td>
<td>739.4336</td>
<td>R.QVPQIA.R</td>
</tr>
<tr>
<td>308 – 318</td>
<td>629.8037</td>
<td>1257.5928</td>
<td>R.SAQQHCLTNWK</td>
</tr>
<tr>
<td>320 – 329</td>
<td>526.2747</td>
<td>1050.5348</td>
<td>R.KYQLQGLGEKE.R</td>
</tr>
<tr>
<td>320 – 334</td>
<td>533.9417</td>
<td>1604.8033</td>
<td>R.KYQLQGLGEKE.R</td>
</tr>
<tr>
<td>335 – 345</td>
<td>590.2907</td>
<td>1178.5669</td>
<td>R.KSYVTVETEAVK.T</td>
</tr>
</tbody>
</table>

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P.
4.5 Discussion

Using immunohistochemistry with a polyclonal rabbit-derived primary antibody for CB2 from Cayman Chemical, a primary antibody-specific signal in cells of the superficial dorsal horn of the lumbar spinal cord as well as DRG neurons in sham and CCI treated rats was detected. This signal was present in spleen tissue, and absent following pre-incubation of the primary antibody with the corresponding immunising peptide. Immunolabeled cell numbers and staining density in the dorsal horn did not change after surgery, or following drug treatment with GW405833. Moreover, no difference was detected between ipsilateral and contralateral sides of the spinal cord. This result was replicated in a separate cohort of fresh frozen tissue, indicating that paraffin embedding and subsequent antigen retrieval did not substantially change immunolabeling. IHC colocalization studies revealed that CB2 labeling partially colocalized with Iba1, a marker of activated microglia or infiltrating macrophages, which was hypothesized, however the pattern of CB2 immunolabeling did not match the regional distribution of microglia at this stage of the neuropathy. Furthermore, there was a partial overlap of CB2 immunolabeling with NeuN, a neuronal marker. The size and morphology of these CB2/NeuN double labeled cells is consistent with interneurons, which play a role in the transmission of sensory information from the periphery to central pain pathways.

Western blot analysis with the same primary antibody produced a band of 44 kD in lumbar spinal tissue. This same band was observed in spleen tissue, which also produced a 37 and 59 kD band. The 37 kD band is consistent with the expected weight of the unmodified rat CB2 receptor, and was the only band present in a preparation of rat CB2 over expressing CHO cells. Quantitative analysis of the sole spinal band mirrored that of the immunohistochemistry studies, indicating no change in CB2 receptor labeling across surgery treatment or laterality in the lumbar spinal cord. A similar banding profile was observed using an alternative human targeted N terminus CB2 antibody, and a mouse targeted C terminus CB2 antibody.
Studies of antibody specificity revealed that while the Cayman Chemical CB2 antibody was sensitive and specific when used in vitro, it was not specific when employed in situ, and detected a signal in tissue from both wildtype and CB2 knockout mice. Preparation of tissue for a combined Western blot/mass spectrometric approach, involving membrane enrichment and cytosol exclusion, revealed a loss of the 44 kD band in rat spinal tissue, and the appearance of a faint 37 kD band, in line with a band detected in cells and spleen with this preparation method. These findings suggest that the 44 kD spinal band detected by this antibody is a non-specific cytosolic protein, and that the 37 kD band may correspond to the actual membrane integral CB2 receptor. However, while the CB2 receptor protein was identified at this weight in CB2 over expressing CHO cells using mass spectrometry, it was not identified in spleen or spinal tissue, despite the use of a targeted approach with high sensitivity.

4.5.1 CB2 receptor expression in the spinal cord

Many studies have suggested that while not present in the healthy spinal cord, the CB2 receptor may be upregulated in the spinal cord following peripheral nerve injury in animal models of neuropathic pain, including the CCI model. Zhang et al. (2003) produced a seminal study that described, for the first time, the upregulation of CB2 mRNA in the spinal cord ipsilateral to nerve injury in the CCI model. It was reported in this case that CB2 mRNA was regionally co-distributed with a marker for activated microglia/macrophages, and it was proposed that microglia or macrophages may selectively express the CB2 receptor under neuropathic conditions (Zhang et al., 2003). Upregulation of CB2 receptor mRNA has also been reported in the ipsilateral spinal cord in the SNL model (Beltramo et al., 2006; Hsieh et al., 2011), although cell type was not determined in these cases. The greatest volume of evidence proposing CB2 receptor expression in the spinal cord,
however, is from immunohistochemical and Western blot studies, which rely on CB2 directed primary antibodies, of which the Cayman Chemical CB2 antibody used in this study is one.

Using Western blot in combination with the Cayman Chemical antibody, Walczak et al. (2005, 2006) described an ipsilateral upregulation of CB2 labeling in the SPL and CCS models of neuropathic pain (Walczak et al., 2005; Walczak et al., 2006). With an alternative antibody, Romero-Sandoval et al. (2008) described an upregulation of CB2 receptor immunolabeling following spinal nerve transection, which colocalized with markers for activated and perivascular microglia (Romero-Sandoval et al., 2008), in line with the microglial expression of CB2 proposed by Zhang et al. (2003). Employing immunohistochemistry, again with the Cayman Chemical CB2 antibody, Wotherspoon et al. (2005) reported an upregulation of CB2 receptor immunolabeling in the ipsilateral superficial dorsal horns of the spinal cord following SNL, but not sham surgery. A novel finding in this study was that CB2 receptor immunolabeling was limited to terminating primary sensory afferents, not immune cells (Wotherspoon et al., 2005), in contrast to the microglial expression proposed by others (Zhang et al., 2003; Romero-Sandoval et al., 2008). CB2 immunolabeling in primary afferent terminations of the superficial dorsal horn has also recently been reported in another model of neuropathic pain, brachial plexus avulsion (Paszcuk et al., 2011). It is of interest to note that this proposed upregulation of spinal CB2 is specific to neuropathic pain models, and has not been reported in models of peripheral inflammation, which lack central sensitization elements (Zhang et al., 2003; Curto-Reyes et al., 2010; Curto-Reyes et al., 2011; Hsieh et al., 2011).

In summary, previous studies have thus suggested that: (i) CB2 receptors are upregulated in the spinal cord ipsilateral to injury in animal models of neuropathic pain; and (ii) CB2 receptors are selectively expressed by microglia/macrophages, or terminating primary afferents. In contrast to these results, using the Cayman Chemical CB2 antibody, and the CCI model of neuropathic pain, the current study revealed that CB2 labeling was (i) not upregulated by CCI injury; and (ii) not regionally codistributed with activated microglia, although some colocalization was seen with a marker for...
activated microglia. Given the disparity of these labeling results with those of previous studies, and the finding that CB2 labeling was detected in sham tissue, which was hypothesized to be absent of CB2 receptor expression, the sensitivity and specificity of the Cayman Chemical antibody was tested both in vitro and in situ, where it was found to lack sufficient specificity for the CB2 receptor. This finding casts doubt on the protein labeling results of this study, and the results of many previous studies, which relied on this non-specific antibody. This finding, however, does not explain why previous studies have reported an upregulation of immunolabeling in the spinal cord ipsilateral to peripheral nerve injury with the same primary antibody (Wotherspoon et al., 2005; Paszcuk et al., 2011). In this regard, methodological differences in quantitative analysis may explain the disparity between the results of previous studies and those of the current study. While Wotherspoon et al. (2005) report an upregulation in neuropathic spinal cords, this difference was not quantified with densitometry, and furthermore, no sham tissue is provided for comparison. Conversely, while Paszcuk et al. (2011) did perform densitometry, it should be noted that this was performed on hematoxylin counter-stained tissue, and may therefore have been more reflective of unspecified changes in cell density, rather than changes in immunolabeling.

4.5.2 Primary antibody specificity

The Cayman Chemical CB2 antibody employed in this study has been used extensively to describe and characterize CB2 receptors, in both the spinal cord in models of neuropathic pain (Walczak et al., 2005; Wotherspoon et al., 2005; Walczak et al., 2006; Paszcuk et al., 2011), and the healthy brain (Gong et al., 2006). In these studies, the antibody was validated with numerous positive and negative controls, including the gold standard of knockout tissue by Wotherspoon et al. (2005), thus providing a rational basis for use of this antibody in the current study. The unexpected CB2 labeling
results of this study, however, justified a more extensive validation of this widely employed antibody.

4.5.2.1 Why antibody validation is essential

As primary antibodies are raised against short peptide sequences which can be partially homologous with many other proteins in a given species, there is always a chance of cross reactivity between the primary antibody and a related yet non-specific antigen, which could potentially generate a false positive signal. Primary antibody specificity testing is therefore a necessity for the reliable interpretation of antibody-based methods, and is all the more essential for polyclonal antibodies, where the chance of non-specific binding is far greater than for monoclonal antibodies (Rhodes et al., 2006). This is of further importance for CB2 directed antibodies, as all of the commercially available antibodies are polyclonal, and therefore require thorough validation before interpretation of immunolabeling or Western blot.

The specificity of antibodies is often tested with the use of immunizing or blocking peptides, which involves pre-incubation of the primary antibody with the peptide sequence the antibody was raised against. While many authors interpret a negative immunizing peptide signal as primary antibody specificity, it must be made clear that this only means the signal is dependent on the primary antibody binding to something within a sample with high affinity; a similar negative control would be to omit the primary antibody, which once again says nothing for specificity of the antibody for the intended binding target. Knockout mice, in which the coding sequence for the protein of interest has been genetically deleted, are the gold standard in primary antibody validation; if specific, the antibody will detect a signal in tissue from wildtype but not knockout mice (Lorincz et al., 2008).
Two CB2 receptor knockout mice lines have been developed for experimental use, and while instrumental in the elucidation of the functional role of the CB2 receptor, they will be discussed here in relation to their role in specificity testing of CB2 directed primary antibodies. The first was created by Buckley and colleagues, and involves a partial deletion of the C terminus (Buckley et al., 2000), while the second, developed by Deltagen Inc. (CA, USA) and distributed by The Jackson Laboratory (ME, USA), involves a partial deletion of the N terminus. In the Buckley knockout, the coding sequence for amino acids 217 – 347 of the C terminus have been deleted, yet the coding sequence for the N terminus and first 5 transmembrane domains remains intact. The loss of CP55,940 binding in knockout spleen certainly points to a loss of CB2 receptor function in these mutants, although the potential translation of the remaining N terminus sequence has not been studied (Buckley et al., 2000). In the Deltagen mutant, the sequence encoding amino acids 26 – 140 has been excised, leading to a loss of the first 3 transmembrane domains. The coding sequence for amino acids 1 – 25 of the extreme N terminus remains, however, and it is unknown if this sequence is translated (Monory et al., 2009).

Although the majority of studies that have tested CB2 receptor antibody specificity have employed the less desirable immunizing peptide approach, some of the more commonly used CB2 antibodies have been validated with knockout controls. C terminus antibodies have been validated from a variety of sources, including one produced by Alpha diagnostics (Van Sickle et al., 2005), one produced by Santa Cruz (Gong et al., 2006), and another produced by Ken Mackie (Suarez et al., 2009). The Cayman Chemical N terminus antibody employed in our studies had also been knockout validated in immunolabeling studies by Wotherspoon et al. (2005) who report a lack of CB2 immunolabeling in CB2 receptor knockout but not wildtype mice (Wotherspoon et al., 2005), arguing for primary antibody specificity and providing a rational basis for use of the same antibody in the
present study. In direct contrast to the results of Wotherspoon et al. (2005), however, the results of this study show that the Cayman Chemical CB2 receptor antibody detected an identical signal in both wildtype and knockout mice, thus failing the specificity test.

One possibility that must be considered is that the Cayman antibody could be detecting a truncated product of the knocked out receptor in the current study. As discussed earlier, the coding sequence for the first 25 amino acids of the N terminus of the CB2 receptor remain in the Deltagen knockout employed in our studies. As the Cayman antibody is raised against amino acids 20 – 33 of the CB2 receptor, there is a 5 amino acid overlap between the antibody epitope and any potential truncated N terminus product, meaning the antibody could be binding to a truncated N terminus fragment retaining a partial binding domain. Arguing against this hypothesis, the subcellular localization of the detected signal - on the plasma membrane - was identical in both wildtype and knockout tissue. If a truncated product were transcribed and translated, it would be unlikely that this product would undergo identical cellular trafficking to the full length receptor protein, and therefore the more plausible explanation is that this antibody was not specific for the detection of CB2 in tissue preparations. Failing the knockout test, despite passing tests for specificity involving in vitro cell preparations and the corresponding immunizing peptide, highlight the insufficiency of relying on these tests for specificity, and the essential role of the knockout animal in antibody validation.

Another explanation for this discrepancy in validation could be the possibility that previous reports, including Wotherspoon et al. (2005), have produced falsely negative staining profiles in knockout tissue. As the antigenic section of the protein of interest is present in wildtype tissue, and should be completely absent from knockout tissue, the immunolabeling profile between the tissues should differ by distribution, not simply intensity. Employing image processing software, it is possible to normalize immunolabeling intensity and contrast post hoc in order to analyze changes in labeling pattern alone, which is the more salient parameter in this case. This approach has been detailed by Ashton in his analysis of Suarez et al. (2009), who validated CB1 and CB2 immunolabeling (using the
Ken Mackie C terminus) with knockout controls, and demonstrated a reduced staining intensity in knockout compared to wildtype tissue for both receptors (Suarez et al., 2009). The output levels of the knockout images, when normalized to the output levels of the corresponding wildtype images, revealed that the staining pattern differed between CB1 immunolabeling in wildtype and knockout tissue, as expected, while the staining pattern for CB2 immunolabeling in wildtype and knockout tissue was strikingly similar, indicating a lack of CB2 antibody specificity (Ashton, 2011b). Applying this method to the images published in Wotherspoon et al. (2005) validating the Cayman Chemical antibody generates similar results.

As presented in the paper (Wotherspoon et al., 2005), CB2 knockout images appear to show a reduction in staining intensity compared to wildtype controls (Figure 4.9, A and D). However, when the output levels are normalized for mean and range, the resulting wildtype and CB2 knockout images are almost indistinguishable from each other (Figure 4.9, B and E), and furthermore, the distribution of labeling in both tissues is almost identical, despite a reduction in staining intensity (Figure 4.9, C and F). Combined with the results of the current study, these findings cast considerable doubt on the specificity of the Cayman Chemical CB2 antibody, and results obtained by other groups using this antibody, and suggest that this antibody may be detecting a non-specific protein target instead of, or in addition to, the CB2 receptor. Unfortunately, this technique could not be employed for the post hoc analysis of knockout validation studies of CB2 receptor antibodies in other publications, as the quality of the published images was not of sufficient standard (Van Sickle et al., 2005; Gong et al., 2006).
4.5.3 Proteomic analysis of the CB2 receptor

4.5.3.1 Molecular weight of the CB2 receptor

Using the Cayman Chemical antibody, a band of 44 kD was detected in spinal tissue using Western blot. This is in accordance with the Cayman Chemical data sheet, and the molecular weight reported by other groups employing the same antibody in the rat brain (Gong et al., 2006; Cox et al., 2007) and spinal cord (Walczak et al., 2005; Merriam et al., 2008). A band of the same molecular weight was detected in spleen tissue, in addition to a 37 and 59 kD band, the smaller of which was also the sole band detected in rat CB2 over expressing CHO cells. Spleen bands at these weights have previously been detected with this antibody, and are hypothesized to be glycosolated variants of the
CB2 receptor (Matias et al., 2002; Gong et al., 2006). A similar banding pattern was detected in the current study with two alternative primary antibodies from Santa Cruz, including one directed at the C terminus of the CB2 receptor, seemingly adding further validity to the results obtained. Interestingly, the pattern of detected bands changed with the use of an alternative sample preparation.

The use of differential centrifugal forces during sample preparation allows the separation of cellular fractions based on weight. In crude homogenates of mammalian tissue, nuclei and whole cells pellet at 600 – 1000 X g, leaving a post nuclear supernatant (PNS) that contains all remaining cellular fragments as well as the cytosol. Mitochondria pellet out of the PNS at 10,000 – 20,000 X g, while microsomes, including plasma membrane fragments, pellet only upon ultrafugation of the PNS at high centrifugal forces of up to 100,000 X g, and leave only cytosolic proteins in the remaining supernatant at this centrifugal force (Castle, 2003). For convenience, Western blot analysis of membrane bound proteins, including CB2, is often performed on the crude PNS after pelleting out the mitochondrial fraction, and thus contains not only the microsomal fraction of interest where the membrane bound CB2 presumably resides, but also all soluble proteins in the cytosol. Purification of the membrane fraction for mass spectrometry requires ultrafugation of the PNS to generate a highly concentrated microsomal pellet. Re-suspension of this pellet in a reduced volume of buffer generates a concentrated, purified and enriched membrane fraction that lacks soluble cytosolic proteins, and also contains a higher concentration of membrane bound receptors. When this enriched fraction was created from spleen tissue, all bands bar the 37 kD band disappeared, and in spinal cord, the 44 kD band disappeared, and was replaced by a faint band at 37 kD, but which was only present at protein concentrations >10 times that of previous cytosolic preparations.

These results indicate that the 59 kD band in spleen tissue, and the 44 kD band in spleen tissue and spinal cord are likely unspecified cytosolic proteins, and the actual membrane integral CB2 receptor signal may be at 37 kD in spleen, and even spinal tissue, in this case, albeit at very low levels. This
finding casts doubt on one of the more influential studies in the field, that of Gong et al. (2006), in which CB2 receptor protein was reported at 50 kD with the Cayman Chemical antibody in naïve brain samples, which were prepared using the cytosolic method, and thus will have been contaminated with cytosolic proteins (Gong et al., 2006). Unfortunately, membrane enrichment with an SDS containing buffer disrupts the Bradford procedure for protein standardization. It would therefore be necessary employ an alternative standardization protocol if one were to quantify any changes in 37 kD band density following CCI surgery.

In this study, the detection of what may be low levels of CB2 protein in naïve spinal cord may have a reasonable explanation. Animals in this case were not perfused during sacrifice, and thus blood remains in vessels throughout bodily tissues, including the CNS, even following removal for analysis. Errant blood cells, trapped within the vasculature of the spinal cord, are therefore incorporated into Western blot tissue preparations. As peripheral blood leucocytes express the CB2 receptor in high levels (Munro et al., 1993; Galiegue et al., 1995), this could explain the positive result for CB2 in this study, albeit at background levels. This could also explain the results of other groups, who use unperfused tissues for Western blot analysis of the CB2 receptor protein (Walczak et al., 2005; Cox et al., 2007; Curto-Reyes et al., 2010; Curto-Reyes et al., 2011). While these studies, detect a band in excess of 40 kD, in contrast to the 37 kD band detected in the current work, despite using membrane enrichment, it is a possibility that these heavier bands represent glycosolated variants of the CB2 receptor, as have been discussed elsewhere (Matias et al., 2002; Gong et al., 2006). This highlights the point that protein weight alone cannot be used to definitively identify the CB2 receptor, which requires more sensitive proteomic analysis.
Mass spectrometry was used to determine the identity of the protein detected by the Cayman antibody at 37 kD in CB2 over expressing CHO cells, spleen, and spinal tissue. Using a non-targeted approach on the 37 kD band in CHO cells, the CB2 receptor was identified by comparing protein fragments obtained by tryptic digest with a theoretical in silico digest of a sequence data base (SWISS-PROT). This confirmed the identity of the CB2 receptor with 28% sequence coverage. Use of the same approach failed to detect CB2 in either spleen or spinal cord tissue at this molecular weight. In order to improve sensitivity, the fingerprints of the protein fragments generated by the tryptic digest of the CHO cells were used in a targeted approach to again search for CB2 in the spleen and spinal cord. Despite high sensitivity confirmed with positive controls, the CB2 receptor was not identified at 37 kD in the spinal cord, or even the spleen, which is often used as a positive control for the CB2 receptor.

The partial sequence coverage of the rat CB2 receptor in over expressing CHO cells in this study is the first time that this receptor has been characterized to any extent by mass spectrometry. The human CB2 receptor, however, has previously been thoroughly characterized by this technique, with almost 100% sequence coverage of the receptor obtained when expressed in Sf-21 cells using a Baculovirus expression system (Zvonok et al., 2007). Although greater than the 28% coverage of the rat CB2 receptor obtained in the current studies, it should be noted that Makriyannis and colleagues detail a long in vitro optimization procedure with less successful attempts achieving as little as 50% coverage of the human receptor sequence (Filippula et al., 2004; Zvonok et al., 2007). The optimized procedure involves purification of the protein by fusion of a FLAG tag to obtain an acceptable protein yield, as well as an in-solution, rather than in-gel, digest with detergents in order to avoid the aggregation and precipitation of the highly hydrophobic, 7 transmembrane domain, CB2.
receptor (Zvonok et al., 2007), which were procedures not possible using the CHO cells at hand, or in vivo tissue.

Sequencing of the rat CB2 receptor was not the aim of the current work, however, which instead aimed to utilize the highly specific and sensitive mass spectrometry approach to determine if the bands identified by the Cayman Chemical CB2 antibody at 37 kD were in fact the CB2 receptor. Although the CB2 receptor was identified using this approach in CB2 over expressing CHO cells, but not in spleen or spinal samples, this is likely due to inherent issues of both low yield when analyzing in vivo tissue, and high hydrophobicity of the CB2 receptor; not necessarily its absence from these tissues. In fact, given that (i) the Cayman Chemical antibody is specific for the CB2 receptor in CB2 over expressing CHO cells when tested in vitro; (ii) a single band of 37 kD was detected by this antibody in Western blot analysis CB2 positive CHO cells; (iii) the CB2 receptor was positively identified in this 37 kD band by mass spectrometry, and (iv) a band at this weight is also detected by this antibody in spleen and spinal tissue after membrane enrichment; there is strong circumstantial evidence that the true, membrane integral CB2 receptor may be present in spleen and unperfused naive spinal tissue at 37 kD, despite a negative result with mass spectrometry.
4.6 Conclusions

Using an N terminus CB2 receptor antibody from Cayman Chemical, labeling was detected in the superficial dorsal horns of the rat lumbar spinal cord using immunohistochemistry and Western blot. As previous studies had not found the CB2 receptor in healthy or sham spinal cord tissue, and as labeling was not modulated following CCI in the current study, as had been expected, the specificity of the Cayman Chemical antibody was tested, and it was found that:

1) While specific \textit{in vitro}, the antibody lacks sufficient specificity \textit{ex vivo}, in tissue.

2) In addition to CB2 protein, the Cayman Chemical antibody detects at least one unspecified cytosolic protein in spinal cord and spleen tissue, which is of similar weight to that expected of a CB2 receptor protein isoform (44 kD).

Further experimentation with alternative tissue preparations, and mass spectrometric analysis found that:

1) If tissue is membrane enriched, and lacks the cytosolic fraction, the Cayman Chemical antibody detects a single band in Western blot at 37 kD, in line with the expected weight of unglycosylated CB2 protein, in CB2 over expressing CHO cells and spleen, and at very low levels in spinal tissue.

2) The CB2 receptor protein was identified in this 37 kD band in CB2 over expressing CHO cells.

Circumstantial evidence, therefore, points to the likely presence of the actual membrane integral CB2 receptor protein at 37 kD in spleen and spinal tissue. While the membrane enrichment procedure precludes use of the Bradford assay in order to quantify changes in this protein band following CCI, it is hypothesized that the low level of expression in unperfused naïve spinal tissue may be accounted for by errant leucocytes trapped in the spinal vasculature. If this is the case, any changes in protein band density following CCI surgery may not reflect changes in spinal CB2 receptor
protein, but instead changes in circulating immune cell CB2. In order to circumvent issues with
antibody specificity, it was decided to test for CB2 receptor upregulation using a functional assay.
5 CB2 receptor function in the CCI model

5.1 Introduction

Reports that have described the presence of the CB2 receptor in CNS tissues have most commonly employed molecular techniques based on the detection of either (i) mRNA transcripts (Zhang et al., 2003; Van Sickle et al., 2005; Beltramo et al., 2006; Gong et al., 2006; Onaivi et al., 2006; Liu et al., 2009; Curto-Reyes et al., 2011; Hsieh et al., 2011; Lanciego et al., 2011; Paszcuk et al., 2011), or (ii) receptor protein (Benito et al., 2003; Benito et al., 2005; Van Sickle et al., 2005; Wotherspoon et al., 2005; Ashton et al., 2006; Gong et al., 2006; Ashton et al., 2007b; Benito et al., 2007; Baek et al., 2008; Romero-Sandoval et al., 2008; Curto-Reyes et al., 2010; Curto-Reyes et al., 2011; Paszcuk et al., 2011). There are a number of problems with both approaches: while the detection of CB2 mRNA transcripts is considered relatively specific, translation to functional protein is not a passive process and is regulated by additional factors (Kelleher et al., 2004); on the other hand, the sensitivity and specificity of CB2 targeted antibodies have so far been highly questionable (Atwood et al., 2010; Ashton, 2011a; Ashton, 2011b). A more powerful method would ideally measure receptor function, rather than merely presence.

An assay that can potentially fulfill this role is the \(^{35}\text{S}\)GTP\(_\gamma\text{S}\) assay, which allows the measurement of basal and ligand stimulated activity of GPCR receptors, of which the CB2 receptor is one. The assay involves the application of excess radiolabeled GTP, in the form of \(^{35}\text{S}\)GTP\(_\gamma\text{S}\), to tissue, which is then incorporated into activated GPCRs in place of endogenous GTP. Unlike endogenous GTP, however, \(^{35}\text{S}\)GTP\(_\gamma\text{S}\) is non-hydrolysable, and thus the radiolabeled analogue accumulates in cellular
membranes relative to GPCR activity, where it can be quantified (Strange, 2010). The principle underlying this assay is outlined in Figure 5.1, below.

![Diagram](image)

Figure 5.1 The $[^{35}\text{S}]$GTPγS assay. (A) Under physiological conditions, activation of a GPCR causes the exchange of GDP for GTP, which activates Gα, and allows the Gα and βγ subunits to dissociate, and regulate signaling proteins. Hydrolysis of GTP to GDP by GTPase results in inactivation of the Gα complex, and re-association of the βγ subunits to reform the inactive G protein complex. (B) In the presence of $[^{35}\text{S}]$ labeled GTP, the exchange of $[^{35}\text{S}]$GTP for GDP occurs, however the Gα($[^{35}\text{S}]$GTPγS) complex is not hydrolysable by GTPase, and thus accumulates in cellular membranes, allowing a direct measurement of GPCR activity. Adapted from Harrison and Traynor (2003).

5.2 Experimental aims

The aim of this experiment was to assess the presence of functional CB2 receptors in the lumbar spinal cord of rats using the $[^{35}\text{S}]$GTPγS assay, and to quantify any changes in receptor function following the induction of neuropathic pain with the CCI model. Membrane enriched homogenates of spinal tissue were assayed to quantify changes in CB2 receptor function, while the same assay was performed in situ on spinal sections to assess regional changes.
5.3 Methods

5.3.1 Experimental design

CCI or sham surgery was performed on animals, which were then assessed for mechanical allodynia 3 and 10 days post surgery. Following behavioural assessment at 10 days, and confirmation of allodynia in CCI rats, animals were sacrificed, and lumbar spinal tissue harvested for functional receptor assays. Spinal tissue was homogenized and resulting homogenates were membrane enriched with ultrafugation, then assayed with $[^{35}S]$GTPγS for gross quantification of CB2 receptor function and any changes following CCI. To assess the regional distribution of functional CB2 receptors in the lumbar spinal cord, and any changes following peripheral nerve injury, $[^{35}S]$GTPγS autoradiography was performed in situ on spinal tissue slices.

5.3.2 Membrane homogenates

5.3.2.1 Membrane preparation

Immediately following assessment of allodynia ten days after CCI or sham surgery, animals were sacrificed by rapid decapitation following brief CO$_2$ anaesthesia. The L4-L6 lumbar spinal cord was rapidly dissected and the tissue homogenized in ice cold homogenization buffer (3 mM MgCl$_2$, 1 mM EGTA, 100 mM NaCl in 50 mM Tris Buffer, pH 7.4). To generate an enriched membrane fraction, homogenized tissue was centrifuged at 48,000 X g for 10 min at 4°C, and the tissue pellet re-suspended in assay buffer (3 mM MgCl$_2$, 0.2 mM EGTA, 100 mM NaCl in 50 mM Tris Buffer, pH 7.4)
with an EDTA-free protease inhibitor (Roche Applied Science, IN, USA). Samples were incubated on ice for 10 min, again centrifuged at 48,000 X g, and the pellet re-suspended in assay buffer. Protein concentration was determined using the method described by Bradford (Bradford, 1976). Protein concentrations were standardized to 1 mg/ml, and stored in aliquots at -80°C until use.

5.3.2.2 \[^{[35]}S\]GTPγS binding

Membrane preparations (10 µg protein/well) were incubated with 50 pM \[^{[35]}S\]GTPγS (Perkin-Elmer, MA, USA), 30 µM guanosine-5'-diphosphate disodium salt (GDP) (Sigma-Aldrich, MO, USA), and 1 µM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma-Aldrich, MO, USA) in assay buffer, in the presence of either 30 µM (R)-(+)-methanadamide (methanandamide), 10 µM [D-Ala^2, D-Leu^5]-Enkephalin (DADLE) (Sigma-Aldrich, MO, USA), or 100 pM to 10 µM JWH-133 for 2 hours at 30°C. Basal binding was determined in the absence of agonist, and non-specific binding determined in the presence of 10 µM unlabelled GTPγS (Roche Diagnostics, IN, USA). Incubation was terminated by rapid filtration through Whatman® GF/B glass fiber filters, and three consecutive washes with ice cold 50 mM Tris buffer. Bound radioactivity was measured in duplicate by liquid scintillation spectrophotometry using a TopCount™ microplate scintillation and luminescence counter (Packard, CT, USA), following overnight extraction with Ecolite(+)™ scintillation fluid (MP Biomedicals, OH, USA). Non-specific binding was subtracted from all values, and stimulated values were normalized to basal binding values. JWH-133 concentration curve experiments were performed in triplicate.
5.3.3  Autoradiography

5.3.3.1  Tissue preparation

Ten days following CCI or sham surgery and subsequent assessment of mechanical allodynia, animals were sacrificed by decapitation following brief CO\textsubscript{2} anaesthesia. The L4 – L6 lumbar enlargement was rapidly removed, embedded in OCT on powdered dry ice, and stored at -80 °C until use. Transverse 20 µm sections of spinal cord were cut at -20 °C, and thaw mounted on to chrome alum gelatin coated slides. Cut sections were again stored at -80°C until use, but were used within 2 weeks to ensure minimal loss of G protein activity.

5.3.3.2  \textsuperscript{[35]S}GTP\textsubscript{gamma}S autoradiography

Sections were reanimated in assay buffer (3 mM MgCl\textsubscript{2}, 0.2 mM EGTA, 100 mM NaCl in 50 mM Tris-HCl, pH 7.4) containing 0.1 mM GDP for 15 min at 25 °C. Sections were then incubated in assay buffer containing 0.1 mM GDP, 50 pM \textsuperscript{[35]S}GTP\textsubscript{gamma}S (Perkin-Elmer, MA, USA) and 1 µM DPCPX, with either 10 µM WIN 55,212-2 or 1 µM JWH-133 for 90 min. Basal binding was determined in the absence of agonist. Incubation was terminated by washing sections twice in ice cold 50 mM Tris-HCl for 5 min, and once in ice cold dH\textsubscript{2}O for 5 min. Sections were dried for 30 min under moving air, then desiccated overnight at -20°C before exposure to ECL hyperfilm (GE Healthcare, UK) for 120 hours at room temperature. Films were subsequently developed by sequential immersion in ice cold developer, stopper then fixative (Kodak, NZ), before a final wash in tap water then air drying.
5.3.3.3 Densitometric analysis of autoradiography

Films were scanned for densitometric analysis using a GS-710 scanner (Biorad, NZ). A stereotaxic template of the lumbar spinal cord (Paxinos et al., 1986) was overlaid on to images, which were then converted to 8 bit gray scale. Using ImageJ®, Version 1.40g, density was measured in the ipsilateral and contralateral dorsal laminae (I-VI), and normalized to adjacent background film density. Template overlay and densitometry were performed by an observer blinded to treatment. To confirm that \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding detected and measured for densitometric analysis was within the linear dynamic range of detectability, pilot experiments were performed in which exposure time relative to \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding density was plotted, and optimal exposure conditions obtained. A saturation curve of binding density relative to exposure time in pilot experiments, and relation to values obtained in the full densitometric analysis can be seen in Figure 9.2 in Appendix 2

5.3.4 Statistical analysis

Membrane homogenate data was analyzed with a two-way repeated measures ANOVA with Bonferroni post hoc comparisons, using Prism® version 5.01 (GraphPad, CA, USA). Autoradiography data was analyzed with a 3 factor ANOVA, using a general linear model with Bonferroni post hoc comparisons in Minitab®, Version 15 (Minitab Inc., PA, USA).
5.4 Results

Both the μ/δ opioid receptor agonist DADLE (Toll et al., 1998), and the CB1 selective agonist methanadamide (Abadji et al., 1994) caused a significant increase in G protein coupled receptor activity, as measured by stimulated $[^{35}\text{S}]\text{GTPyS}$ binding in spinal cord membranes (Figure 5.2A). A 1-way repeated measures ANOVA with a Dunnett post hoc comparison revealed a significant drug effect ($F = 50.5$ (2, 6); $p = 0.0002$), with both DADLE and methanadamide significantly increasing binding levels when analyzed with a Dunnett post hoc comparison (42 and 37%, respectively) ($p < 0.001$ vs. basal). Autoradiography of $[^{35}\text{S}]\text{GTPyS}$ binding in spinal cord sections revealed that basal $[^{35}\text{S}]\text{GTPyS}$ binding was primarily localized to the superficial dorsal horns of the spinal grey matter (Figure 5.2B), and moreover, incubation with DADLE increased binding intensity not only within the superficial dorsal horns, but throughout the deeper dorsal and ventral laminae (Figure 5.2C).

Figure 5.2 Ligand stimulated $[^{35}\text{S}]\text{GTPyS}$ binding in healthy spinal tissue. (A) In membrane homogenates, $[^{35}\text{S}]\text{GTPyS}$ binding is significantly upregulated following incubation with DADLE (10 µM) and methanadamide (30 µM) compared to basal levels ($*** = p < 0.001$ vs. basal) $n = 4$. In spinal cord sections, $[^{35}\text{S}]\text{GTPyS}$ binding is limited to the superficial dorsal horns under basal conditions (B), and is upregulated through the deeper grey matter following incubation with DADLE (10 µM) (C). Scale bars equal to 800 µm.
Analyzed by $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, treatment of membrane homogenates with increasing concentrations of JWH-133 produced a biphasic dose response (Figure 5.3A). $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in sham and CCI spinal membrane homogenates was reduced from basal levels in the presence of 1 nM JWH-133 (45 and 57%, respectively), but was stimulated above basal levels in the presence of 10 µM JWH-133 (67 and 43%, respectively). Analysis with a 2-way repeated measures ANOVA revealed that concentration was a significant factor in binding ($F = 5.82 \ (6, \ 48); \ p < 0.0001$), however neither surgery ($F = 0.15 \ (1, \ 48); \ p = 0.71$), nor the interaction between surgery and concentration ($F = 1.44 \ (6, \ 48); \ p = 0.22$) were significant factors.

The stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in spinal membrane homogenates induced by the highest dose of JWH-133 (10 µM) was reduced to below basal levels when JWH-133 was co-administered with either the CB1 selective antagonist AM251, or the CB2 selective antagonist AM630, both at a concentration of 750 nM (Figure 5.3B). Analysis with a 1-way repeated measures ANOVA revealed that this attenuation was tending towards statistical significance ($F = 3.15 \ (2, \ 6); \ p = 0.11$).
Figure 5.3 JWH-133 stimulated [35S]GTPγS binding in spinal membrane homogenates from CCI and sham treated rats. (A) JWH-133 stimulated binding in spinal cord homogenates from sham and CCI treated rats, as assessed by 2-way repeated measures ANOVA (p < 0.0001 for concentration as a factor), however surgery was not significant, and neither was the interaction between surgery and concentration (p > 0.05), n = 5/group. (B) JWH-133 (10 µM) stimulated binding was attenuated, though not significantly, by co-treatment with the CB1 selective antagonist AM251 or the CB2 selective antagonist AM630, n = 4/group.
Autoradiography of \[^{35}\text{S}]\text{GTPyS}\) binding was performed \textit{in situ} on frozen spinal sections. This technique has a greater resolution than the homogenate approach, and allows mapping of any regional changes in receptor function. Basal binding in sham and CCI treated rat spinal cords was restricted to the superficial dorsal horns (Figure 5.4A, D), and was unchanged following incubation with JWH-133 (Figure 5.4B, E). Incubation with WIN55,212-2, however, induced a strong increase in binding around the superficial dorsal horns of both sham and CCI treated rat spinal cords, extending through the deep dorsal horns to lamina X surrounding the central canal (Figure 5.4C, F arrows).

\textbf{Figure 5.4 \[^{35}\text{S}]\text{GTPyS}\) autoradiography in spinal cord sections from sham and CCI treated rats.} Under basal conditions (A, D), binding is concentrated to the superficial dorsal horns. Binding appears unchanged following incubation with JWH-133 (1 \textmu M) (B, E), however following incubation with WIN55,212-2 (10 \textmu M) (C, F), binding is upregulated around the dorsal horns, and extends deep through the grey matter to lamina X and the central canal (arrows). Binding appears similar between sham and CCI treated spinal cord sections. Scale bars equal to 800 \textmu m.
Densitometric analysis of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ autoradiography with a 3 factor ANOVA in a general linear model revealed that ligand stimulation was a significant factor in determining $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding ($F = 5.23$ (2, 49); $p = 0.009$). Bonferroni post hoc comparisons confirmed that WIN55,212-2, but not JWH-133, increased binding above basal levels in the dorsal horns of both sham and CCI treated rat spinal cords ($p = 0.012$ and $p > 0.05$, respectively) (Figure 5.5A, B). No significant factor interactions were revealed between surgery and ligand ($F = 0.01$ (2, 49); $p = 0.99$) or laterality and ligand ($F = 0.06$ (2, 49); $p = 0.95$), indicating no changes in cannabinoid stimulated binding activity between sides, or following CCI surgery. Bonferroni post hoc analysis revealed that WIN55,212-2 significantly increased binding above basal levels in the ipsilateral and contralateral dorsal horns of sham spinal cords (17 and 27%, $p < 0.05$ and $p < 0.001$, respectively) (Figure 5.5C). Similarly, in CCI treated spinal cords, WIN55,212-2 stimulated binding above basal levels significantly in the ipsilateral dorsal horns (23%, $p < 0.05$), and not significantly in the contralateral dorsal horns (18%, $p > 0.05$)(Figure 5.5 D).
Figure 5.5 Stimulated \({}^{[35]} \text{S} \text{GTP} \gamma \text{S}\) autoradiography in the spinal cord dorsal horn of sham and CCI treated rats. Incubation with JWH-133 (1µM) did not change binding (A), whereas WIN55,212-2 (10 µM) significantly increased binding (B). Binding was increased significantly in the ipsilateral and contralateral dorsal horns of sham treated rats (C), and the ipsilateral dorsal horns of CCI treated rats (D) with WIN55,212-2, but not JWH-133. (* = p < 0.05; ** = p < 0.001 versus basal). n = 5/group for WIN55,212-2 treated and sham JWH-133 treated, and n = 4 for CCI JWH-133 treated.
5.5 Discussion

This investigation into spinal cannabinoid and opioid receptor functionality using the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ assay following the induction of neuropathic pain showed that unstimulated basal GPCR activity is primarily localized to the superficial dorsal horns of the spinal grey matter in naïve, sham, and neuropathic spinal cords. Stimulation with the µ/δ opioid receptor agonist DADLE and the CB1 selective agonist methanandamide increased $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding significantly over basal levels, with DADLE stimulation increasing binding specifically throughout the deeper dorsal and ventral laminae. The high functional levels of opioid and CB1 cannabinoid receptors in the spinal cord described here are in line with previous research (Goodman et al., 1980; Drew et al., 2000), and thus these findings provide a positive control on this assay for its use in the detection and assessment of spinal CB2 receptor functionality.

While stimulation of spinal cord membrane homogenates with the CB2 selective agonist JWH-133 did produce a concentration dependent change in GPCR activity, this took the form of a biphasic dose response curve. At very low concentrations, JWH-133 induced a mild decrease in GPCR activity, while it only induced an increase in binding at the highest concentration tested (10 µM). There was no significant effect of surgery, or surgery interacting with concentration, on GPCR activity, and furthermore there were no significant differences in GPCR activity between sham and neuropathic spinal cords at any concentration. The stimulation of GPCR activity induced by the highest concentration of JWH-133 appeared to be blocked by co-administration with either the CB1 selective antagonist AM251, or the CB2 selective antagonist AM630, but these results were not statistically significant. In line with the results of the homogenate binding studies, an in situ autoradiographic approach on spinal cord sections determined that while the CB1/CB2 non-selective cannabinoid agonist WIN55,212-2 was able to induce significant binding localized to the dorsal horns, JWH-133 had no effect on GPCR activity. Binding stimulated by WIN55,212-2 was not significantly different
between treatment groups or sides. Taken together, these results indicate a strong presence of δ opioid and CB1 receptors, but not CB2 receptors, in both sham and neuropathic spinal cords, in areas considered integral to primary pain processing.

5.5.1 Use of the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ assay to assess CB2 receptor function

In light of the sensitivity and specificity issues surrounding the use of PCR and antibody based approaches to assay the CB2 receptor, the current study aimed to circumvent these problems with the use of a functional receptor assay. The CB2 receptor is a GPCR, and thus the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ assay was rational choice for the measurement of CB2 receptor activation. GPCRs, as their namesake suggests, are coupled with G proteins. Upon agonist binding to the receptor, G protein bound GDP is exchanged for GTP, which activates the G protein complex, resulting in specific downstream effects (Strange, 2010). It had long been a thought that the measurement of the exchange of GTP for GDP would produce a direct measure of agonist induced activation of GPCRs within a given system. It was the development of the non-hydrolysable analogues of GTP that provided a potential method of measuring the exchange, and it was specifically $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ that fulfilled the analogue requirements for stability and thus the measurement of accumulation in biological systems (Sovago et al., 2001).

Initially, agonist stimulated GPCR activity based on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ accumulation was performed in controlled and enriched in vitro preparations, with the purification and transfection of GPCRs and G proteins in to phospholipid vesicles (Asano et al., 1984a; Asano et al., 1984b). While these initial studies provided the proof of concept required for the assay, employment of the assay in intact membranes derived from native tissue was first described several years later. In this context, it was first used to measure the activity of carbachol stimulated muscarinic acetylcholine receptors in preparations of atrial membranes (Hilf et al., 1989), then agonist stimulated adenosine A1 receptor...
activity in bovine CNS membranes (Lorenzen et al., 1993). The application of the $[^{35}S]$GTPγS assay was extended markedly by the efforts of Sim and colleagues, who first described the use of the assay in situ on brain slices, measuring DAMGO ([(D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin)) stimulated opioid receptor activity in the rat brain with an autoradiographical approach (Sim et al., 1995). Since then, the assay has been used to study the activity of various GPCRs, including α2-adrenoreceptors (Newman-Tancredi et al., 2000), serotonin 5-HT(1A) (Newman-Tancredi et al., 2003), and the cannabinoid CB1 receptor (Breivogel et al., 1998; Smith et al., 2007). Furthermore, the assay has been extended for use in spinal tissues, with Drew et al (2000) demonstrating robust stimulation of CB1 receptors in the rat spinal cord with WIN55,212-2 (Drew et al., 2000).

With the traditional radioligand binding approach, ligands specific for a receptor are radiolabeled, often with tritium, before their application to prepared membrane fractions or tissue sections, in order to visualize or measure high affinity binding sites. This approach has been used successfully in the mapping of CB1 receptors in the healthy rat (Herkenham et al., 1990), human (Glass et al., 1997) and mouse (Ibrahim et al., 2003) brain, with tritiated CP55,940 producing clear, crisp autoradiograms. Although CP55,940 is not selective for cannabinoid receptor subtype, the lack of CP55,940 binding in CB1 receptor knockout mice (Ibrahim et al., 2003), and the accepted lack of CB2 receptors in the healthy brain, meant that CP55,940 radiolabeling was inferred as CB1 receptor binding. However, in the case of the present study, where it had been hypothesized that CB2 receptors may be upregulated in the spinal cord following neuropathic pain, it was not appropriate to use non-specific radiolabeled agonists to determine receptor subtype functionality. The unavailability of CB2 selective radiolabeled ligands at the time of testing thus precluded the use of this ‘direct’ autoradiography approach. Of course, while it may have been possible to perform an ‘indirect’ version of this assay, in which non-labeled CB1 and CB2 selective ligands are tested for their ability to outcompete a radiolabeled non-selective ligand such as CP55,940, this approach would lack the functionality aspect that is fulfilled by the $[^{35}S]$GTPγS assay. While the images
produced with [$^{35}$S]GTPγS autoradiography are perhaps not as sharp as those produced with radiolabeled autoradiography, the versatility and simplicity of the assay, in combination with its ability to assess receptor functionality, are driving factors in its popularity and use among researchers (Sovago et al., 2001), and its inclusion in the current investigation.

5.5.2  **Opioid receptor function in the spinal cord**

In both spinal cord membrane homogenates and sections, the µ/δ opioid receptor agonist DADLE was able to strongly increase [$^{35}$S]GTPγS binding, specifically within the grey matter. Consistent with these findings, previous work with radiolabeled autoradiography has indicated a strong density of both µ and δ opioid receptor subtypes in the superficial dorsal horns, and a moderate density throughout the remaining grey matter (Goodman et al., 1980), which correlates with the known role of µ and δ opioid receptors in opioid-induced antinociception at the spinal level (Yaksh, 1983). The ability of this assay to detect a level of spinal opioid receptor function consistent with the known density of opioid receptors in the spinal cord demonstrates the sensitivity of this assay, and its appropriateness for assaying cannabinoid receptor function.

5.5.3  **CB1 receptor function in the spinal cord**

In spinal membrane homogenates, the CB1 selective agonist methanandamide increased binding by 37% above basal levels. Similarly, the CB1/CB2 non-selective agonist WIN55,212-2 increased binding in the dorsal horns of both sham and CCI treated rats, by 17 – 27%. This compares with a previous study by Drew and colleagues (2000), who describe a roughly 25% increase in [$^{35}$S]GTPγS binding in
the dorsal horns of the spinal cord induced by the non-selective cannabinoid agonist HU210, which did not change following the induction of inflammation by carrageenan (Drew et al., 2000). The pattern of cannabinoid stimulated \( ^{35}S \) GTP\( \gamma \)S binding in both cases matches the pattern of CP55,940 binding observed in the spinal cord in radioligand binding studies (Herkenham et al., 1990; Ibrahim et al., 2003) – a pattern which was completely absent in mice lacking the CB1 receptor (Ibrahim et al., 2003). Immunohistochemical studies with CB1 targeted antibodies have also generated a similar pattern (Farquhar-Smith et al., 2000; Lim et al., 2003). Taken together, this strongly suggests that the WIN55,212-2 induced increase in binding in the current study is CB1 receptor dependent.

In contrast to the findings in this study which indicate no change in spinal CB1 receptor functionality following CCI, it has previously been reported that CB1 receptor protein is significantly upregulated in the ipsilateral dorsal horn following CCI injury (Lim et al., 2003). It may be that while able to detect CB1 receptor functionality, the \( ^{35}S \) GTP\( \gamma \)S autoradiography employed in the current work lacks sufficient specificity to detect subtle changes in activity between treatment groups. An alternative explanation may be the methodology employed: the upregulation of CB1 protein described by Lim et al (2003) relies on immunohistochemistry with an unvalidated CB1 directed antibody. CB1 antibodies suffer from similar specificity issues to CB2 antibodies, and must be tested with strict controls to ensure sensitivity and specificity (Grimsey et al., 2008), a point not addressed by the authors in Lim et al (2003).

### 5.5.4 CB2 receptor function in the spinal cord

Despite the ability of the \( ^{35}S \) GTP\( \gamma \)S assay to detect opioid and cannabinoid CB1 receptor function in the spinal cord, these experiments did not find any evidence for functional CB2 receptors in the spinal cords of sham or neuropathic rats. While membrane binding studies indicated that
concentration of the CB2 selective JWH-133 was a significant factor in binding levels, this was largely due to a biphasic dose response, with a reduction in activity in the low nanomolar range, and an increase in activity in the high micromolar range. As JWH-133 is a full agonist at the human CB2 receptor with a Ki of 3.4 nM (Huffman et al., 1999), it would be expected that binding would be stimulated in the low nanomolar range if functional CB2 receptors were present. This study in fact demonstrates the opposite, with a slight reduction in binding in this concentration range, perhaps suggesting inverse agonism by JWH-133. While it is known that the CB2 agonist AM1241 can conversely act as both an agonist and an inverse agonist depending on assay conditions (Yamamoto et al., 2008; Mancini et al., 2009), this is not known to be the case for JWH-133 (Yamamoto et al., 2008), and therefore inverse agonism in the nanomolar range in the current assay, while an intriguing possibility, is unlikely. At a 10 µM concentration, however, JWH-133 did induce a moderate but not significant increase in binding. As the Ki of JWH-133 for the human CB1 receptor is 677 nM (Huffman et al., 1999), a concentration of 10 µM is well in excess of that required for significant CB1 receptor activation. As demonstrated here and in previous studies, the abundance of CB1 receptors in the spinal cord makes it a reasonable hypothesis that the increase in binding seen at this concentration in this study is due to JWH-133 activation of CB1 receptors.

Attempts to block the stimulation of homogenates with selective antagonists produced mixed results with both AM251 and AM630 appearing to attenuate the JWH-133 induced increase in binding in membrane homogenates. These results were not statistically significant, however, which may be reflective of the low statistical power in this experiment. In the binding curve experiments, 10 µM JWH-133 stimulated [35S]GTPγS binding in spinal cord homogenates an average of 55% over basal levels, and it was hypothesized that at least one of the antagonists, at the biorational dose chosen, would reduce JWH-133 stimulated binding to basal levels, i.e. a reduction in the region of 55%. Performing a post hoc power analysis of this experiment in Minitab, it was determined that with an n of 4 per group, this experiment had 80% power to detect a difference of 42%. That 10 µM
JWH-133 unexpectedly induced a binding increase of only 9% in this experiment greatly reduced the statistical power of detecting a significant inhibition of binding by either antagonist, and further highlighted the high variability of this assay on membrane preparations.

Notwithstanding the low statistical power of the antagonist component of this study, it still appeared that both AM251 and AM630 attenuated JWH-133 induced stimulation of \[^{35}\text{S}]\text{GTPyS} binding to a similar degree. AM251 is a CB1 selective cannabinoid antagonist (Gatley et al., 1996), while AM630 is a CB2 selective cannabinoid antagonist (Pertwee et al., 1995). While the binding affinity of AM630 for cannabinoid receptor subtypes in the rat has not been established, *in vitro* assays with CHO cells transfected with human receptors produced Ki values of 31.2 nM and 5.2 µM for CB2 and CB1, respectively, giving AM630 a 165-fold selectivity for CB2 (Ross et al., 1999). On the basis of this, a concentration of 750 nM AM630 should provide selective antagonism of CB2, without cross-antagonizing CB1. However, investigation by the creators of AM630 discovered that the compound can in fact act as an inverse agonist at human CB1 with an unexpectedly low EC50 of 900 nM (Landsman et al., 1998), a value close to the concentration chosen here. It is therefore possible that the apparent attenuation of JWH-133-induced binding by AM630 was in fact due to inverse agonism of AM630 at the CB1 receptor. More importantly, taken together, these results indicate that AM630 may lack sufficient specificity for the CB2 receptor in functional assays.

Due to the high variability of the \[^{35}\text{S}]\text{GTPyS} assay in membrane homogenates, and seemingly low expression of the CB2 receptor in the spinal cord, it is possible that any stimulation of CB2 receptors was masked by a low signal to noise ratio. For this reason, the \[^{35}\text{S}]\text{GTPyS} assay was performed *in situ* on spinal cord slices using an autoradiography approach, to generate greater resolution for the detection of any possible small populations of CB2 receptors. Once again, however, it was demonstrated that while the non-selective agonist WIN55,212-2 was able to significantly stimulate binding, the CB2 selective agonist JWH-133 was not. WIN55,212-2 has an approximately 10-fold lower affinity for the CB2 receptor than JWH-133, with an estimated Kd at the rat CB2 receptor of
35.6 nM (McPartland et al., 2007) compared to a Ki of 3.4 nM for JWH-133 at the human CB2 receptor (Huffman et al., 1999). In contrast, the affinity of WIN55,212-2 for the CB1 receptor is much greater than JWH-133, with a Kd of only 2.4 nM for WIN55,212-2 (McPartland et al., 2007) compared to a Ki of 677 nM for JWH-133 (Huffman et al., 1999). By incubating spinal sections with either JWH-133, or a 10 fold greater concentration of WIN55,212-2, the relative binding affinities for the CB2 receptor were normalized, and the only difference was a markedly greater affinity for CB1 in the case of WIN55,212-2. Therefore, it is reasonable to assume that any stimulation following WIN55,212-2 incubation not seen with JWH-133 was due to CB1 and not CB2 receptor activation.

The absence of any binding stimulation by CB2 selective concentrations of JWH-133 in membrane homogenates and the more sensitive autoradiographical approach in spinal sections strongly argues for an absence of functional spinal CB2, in spite of extensive immunohistochemical and PCR evidence suggesting otherwise. An analogous situation has been reported with the putative cannabinoid receptor and GPCR, GPR55. While initial PCR studies suggested a strong presence of GPR55 mRNA transcripts in various brain regions (Sawzdargo et al., 1999), further studies with the \[^{35}S\]GTP\(\gamma\)S assay found no evidence of functional GPR55 in any tested brain region (Rojo et al., 2010). The results of the current study and those of Rojo and Fowler (2010) suggest either that the \[^{35}S\]GTP\(\gamma\)S assay lacks sufficient sensitivity for some molecular targets, or that the highly sensitive PCR and antibody based approaches are not sufficient evidence to demonstrate receptor functionality.
5.6 Conclusions

This study of functional opioid and cannabinoid receptor activity in sham and CCI treated spinal cord has shown that while CB1 cannabinoid and opioid receptors exist in high densities within regions essential for pain processing, functional CB2 receptors do not, even following the induction of neuropathic pain. These results correlate with those of the Chapter 3, in which spinally delivered CB2 selective agonists were not effective in the reduction of allodynia following nerve injury. Taken together, these results do not support a role for the CB2 receptor in the pain pathway at the spinal level following CCI of the sciatic nerve.
6 Discussion

6.1 Summary of findings

6.1.1 Establishment and validation of the CCI model of neuropathic pain

This study documents the first implementation of the CCI model of neuropathic pain in the rat at the University of Otago. Mechanical allodynia, a common measure of neuropathic pain, developed in the ipsilateral and contralateral hindpaws of CCI, but not sham treated rats over the course of several days, and was fully established 10 days following surgery. Markers for activated astrocytes and microglia were strongly upregulated in the spinal cord ipsilateral to injury 10 days following CCI surgery, indicative of spinal gliosis in response to peripheral nerve injury. The robust establishment of mechanical allodynia, pattern and intensity of spinal gliosis, and the incidence rate of adverse effects following surgery were all consistent with previous reports describing CCI. The establishment of this model, coupled with its well characterized pathology, justified its use in this investigation into the role of the CB2 receptor in neuropathic pain.

6.1.2 The effect of CB2 selective agonists on neuropathic pain

When delivered systemically, the CB2 selective partial agonist GW405833 partially attenuated mechanical allodynia in the ipsilateral hindpaw of the rat induced by chronic constriction injury of the sciatic nerve. The effect was dose dependent, with 30 mg/kg the minimum effective dose...
required for significant alleviation of mechanical allodynia. When delivered intrathecally, however, the same compound, and two other structurally unrelated CB2 selective agonists had no significant effect on mechanical allodynia, even when delivered in doses approaching those thought to be required for these drugs to activate the CB1 receptor. In contrast, intrathecal delivery of the CB1/CB2 non-selective cannabinoid agonist WIN55,212-2 significantly reversed CCI-induced mechanical allodynia, both ipsilateral and contralateral to injury.

6.1.3 CB2 receptor protein expression in the neuropathic spinal cord

When a commonly employed polyclonal primary antibody targeted to the human CB2 receptor, developed by Cayman Chemical, was used to determine CB2 protein expression, immunolabeling was detected in cells in the spleen, and in the superficial dorsal horn of the spinal cord. The labeling was restricted to cell membranes, and did not change following CCI surgery or after treatment with a CB2 agonist. The labeled cells were phenotyped as neurons and microglia/macrophages. Western blot analysis of spinal tissues with the same antibody revealed a single band of 44 kD, corresponding to the weight of a known isoform of CB2. The density of this band did not differ between ipsilateral and contralateral sides, and was unchanged following CCI surgery. Assaying of spleen tissue, commonly used as a positive control for CB2, produced bands at 37 and 44 kD, whereas CB2 over-expressing CHO cells produced a single band at 37 kD. The 37 kD band corresponds to the weight of another known isoform of the CB2 receptor. Analysis with alternative primary antibodies directed to CB2 revealed similar band patterns across positive control and sample tissue, providing an additional positive control for the Cayman Chemical antibody.

Attempts at validation of the antibody with controls produced mixed results, however. The commonly employed negative control of pre-incubation of the primary antibody with its
corresponding immunizing peptide produced a clear negative result when employed in immunohistochemistry and Western blotting. Similarly, the antibody was sufficiently specific in vitro to detect a signal in CB2 over expressing CHO cells, but not in CB2 negative HEK293 cells. However, when employed ex vivo in tissue sections, immunolabeling was detected in the spinal cord of both wildtype and CB2 knockout mice. These results cast considerable doubt on the reliability of results obtained with the Cayman Chemical antibody, both in this study, and in many other published reports describing CB2 receptor expression.

Enriching the cellular membrane fraction and excluding the cytosolic fraction of homogenized Western blot samples, the 44 kD band detected by the Cayman Chemical antibody in naïve spinal cord and spleen disappeared. At the same time, a faint band at 37 kD appeared in the spinal cord, in line with the band detected in spleen and CB2 over-expressing CHO cells at this weight. This suggests that the Cayman Chemical antibody detects a non-specified cytosolic protein at 44 kD in spleen and spinal tissue, and the actual membrane integral CB2 receptor may be present at 37 kD. Using mass spectrometry, the CB2 receptor was identified at 37 kD in CB2 over expressing CHO cells, however was not identified in preparations of spleen or spinal tissue at this weight despite the use of a highly sensitive targeted approach. Failure to detect CB2 at 37 kD in spleen and spinal samples was likely due to the limitations in analyzing ex vivo tissues with this technique, and not necessarily lack of CB2 receptor protein in these samples. The possible presence of very low levels of CB2 protein in spinal cord at 37 kD may be explained by peripheral leukocyte contamination of spinal tissue samples.

6.1.4 CB2 receptor function in the neuropathic spinal cord

Partially in order to circumvent issues of antibody specificity, a functional approach to CB2 expression was employed using a receptor assay based on a non-hydrolysable, radiolabeled GTP
analogue, the $[^{35}\text{S}]$GTPγS assay. The principle of the assay is that the basal and stimulated activity of a GPCR, such as CB2, can be quantified by measurement of the incorporation of non-hydrolysable, radiolabeled $[^{35}\text{S}]$GTPγS into membrane bound GPCRs, using either a homogenate or in situ autoradiographic approach. The use of receptor subtype selective ligands, such as CB2 agonists, allows the measurement of specific receptor subtype GPCR activity. It was determined that while opioid and CB1 cannabinoid agonists stimulated activity in spinal cord homogenates, the CB2 selective agonist JWH-133 did not stimulate activity at concentrations selective for CB2. Similarly, using an autoradiographic approach, it was determined that while non-selective CB1/CB2 cannabinoid agonism could stimulate binding in key pain processing areas of the spinal cord of sham and CCI treated rats, selective CB2 receptor agonism could not. These results indicate that while functional cannabinoid CB1 receptors are present in the spinal cord, in line with their known role in the pain pathway, functional CB2 receptors are either absent from the spinal cord, or present in levels below the limits of detection for this assay. These findings complement the results of the drug intervention experiments in Chapter 3, which indicate a limited role for CB2 at the spinal level in this model of pain.

6.1.5 Conclusions

This study has determined that CB2 receptors have a limited role in the pain pathway at the spinal level following CCI injury of the sciatic nerve, and found no behavioural or functional evidence supporting the existence of the CB2 receptor in the spinal cord following either sham or CCI surgery. Furthermore, this study has determined that an antibody commonly used to assess CB2 receptor protein is not specific for the CB2 receptor, casting serious doubt on previous work employing this antibody.
6.2 **CB2 selective agonists for the treatment of pain**

Despite the results of this investigation suggesting that selective targeting of the CB2 cannabinoid receptor is unlikely to be a fruitful strategy in the treatment of neuropathic pain, much preclinical evidence has suggested otherwise. Many studies have demonstrated that CB2 selective agonists are efficacious in the treatment of painful conditions, including neuropathic pain.

### 6.2.1 Preclinical evidence for CB2 selective agonists

CB2 receptor agonists been shown to be antinociceptive in a wide variety of animal pain models (Chapter 1, Table 2). Based on its designation as a peripheral immune cell receptor, cannabinoids selective for CB2 were initially tested, and found to be efficacious, in models of inflammation; specifically in the attenuation hyperalgesia and inflammation associated with intra-paw formalin (Hanus et al., 1999) carrageenan (Clayton et al., 2002; Nackley et al., 2003; Quartilho et al., 2003), and CFA injection (Valenzano et al., 2005; Whiteside et al., 2005), models of acute inflammatory pain. Furthermore, it was somewhat unexpectedly discovered that these compounds were also antinociceptive in models of neuropathic pain. Of particular interest was the attenuation of allodynia following peripheral nerve injury in the CCI (Yao et al., 2008a; Yao et al., 2008b; Hu et al., 2009; Hsieh et al., 2011), SNL (Ibrahim et al., 2003; Beltramo et al., 2006; Hsieh et al., 2011) and PSL (Valenzano et al., 2005; Whiteside et al., 2005) models.

It was initially proposed that CB2 agonists were acting via peripheral immune cells to suppress inflammation and thereby indirectly influence the transmission of pain signals (Hanus et al., 1999; Clayton et al., 2002; Quartilho et al., 2003). It was also suggested that peripheral sensory afferents and their cell bodies in the DRG may express functional CB2 receptors, and that this could also
explain the anti-hyperalgesic action of CB2 agonists. The discovery that local delivery of a CB2 agonist produced antinociception in the dorsal hindpaw of naïve rats via a CB2 dependent mechanism provided circumstantial evidence that peripheral nociceptors may contain CB2 receptors (Malan et al., 2001), in keeping with previous work that had suggested a CB2-like receptor in peripheral nerves (Griffin et al., 1997). CB2 receptor protein has since been described in human sensory afferents (Stander et al., 2005). Functional evidence for the presence of CB2 receptors on peripheral nociceptive afferents was reported in an electrophysiological study by Elmes et al. (2004).

In naïve, carrageenan inflamed and SNL rats, local intraplantar administration of JWH-133 inhibited innocuous and noxious stimulus-induced firing of WDR neurons in the spinal dorsal horn, in a CB2 dependent manner (Elmes et al., 2004). A similar finding was reported by Beltramo et al. (2006), who reported a CB2 dependent reduction in calcitonin gene-related peptide (CGRP) neurotransmitter release from primary afferents terminating in the spinal cord following CB2 agonist administration in naïve rat spinal sections (Beltramo et al., 2006).

However these theories do not appear to provide a coherent explanation for the efficacy of these drugs in neuropathic pain models. In particular, CB2 agonists are regularly cited as efficacious in the reversal of allodynia, which develops over the course of days, with a particular reliance on processes in the CNS where the CB2 receptor is reportedly absent (Munro et al., 1993; Galiegue et al., 1995; Buckley et al., 2000; Ibrahim et al., 2003). However, a seminal study by Zhang et al. (2003) reporting spinal CB2 mRNA upregulation in response to nerve injury changed the dogma of the CB2 receptor as a peripheral receptor, proposing the possibility that CB2 selective drugs were acting on CB2 receptors in the CNS. Since then, a raft of research has purportedly identified functional CB2 receptors in spinal (Romero-Sandoval et al., 2007; Romero-Sandoval et al., 2008; Yamamoto et al., 2008; Hsieh et al., 2011; Paszcuk et al., 2011) and supraspinal (Jhaveri et al., 2008) centers in pain models, which could account for the efficacy of CB2 selective agonists under neuropathic conditions.
The current study aimed to build on previous work in this field, and assess the mechanisms by which CB2 selective agonists may be exerting their anti-allodynic effects following peripheral nerve injury. This study successfully replicated the results of previous studies which have demonstrated the anti-allodynic effects of the CB2 selective compound GW405833 when delivered systemically in the PSL (Valenzano et al., 2005; Whiteside et al., 2005) and CCI (Hu et al., 2009) models of neuropathic pain. Furthermore, while previous studies have assessed the anti-allodynic effects of GW405833 weeks following nerve lesion, the current experiment has shown for the first time that GW405833 is also efficacious only 10 days following nerve injury. However, in contrast to previous publications suggesting a spinal site of action, this study failed to show efficacy of CB2 selective agonists when delivered to the spinal cord, and did not find any evidence supporting the expression of the CB2 receptor in the spinal cord of naïve or CCI treated rats. These findings suggest that targeting of the CB2 receptor in the spinal cord is likely to be an ineffective strategy for the treatment of pain following peripheral neuropathy. To explain the apparent discrepancies between the systemic and intrathecal efficacy of CB2 agonists, and the results of this study with others employing intrathecal delivery of CB2 agonists, the following hypotheses are presented:

Hypothesis 1: Including the results of this study, the anti-allodynic activity of systemically administered CB2 agonists in neuropathic pain models may be explained by the high doses employed significantly interacting with CB1 receptors, or other unidentified targets; and

Hypothesis 2: Reports of anti-allodynic activity following intrathecal administration of CB2 agonists in neuropathic pain models may be explained by subtle differences in cannula placement, with previous reports likely to be targeting receptors in the DRGs, part of the peripheral, not central, nervous system.

As discussed in Chapter 3, the doses at which systemically administered CB2 agonists are effective at reversing allodynia potentially exceed the activation threshold for CB1 (Valenzano et al., 2005),
providing the rationale for hypothesis 1. This could easily be tested in future studies employing rodent models of neuropathic pain through the use of CB1 and CB2 receptor knockout mice, or to avoid the issues with changes in binding affinities across species, silencing mRNA against CB1 delivered to regions of interest within the pain pathway in rats. Regarding hypothesis 2, although an initial study reported that CB2 mRNA is absent from naïve DRGs (Hohmann et al., 1999a), studies have since suggested that CB2 mRNA (Hsieh et al., 2011) and receptor function (Sagar et al., 2005) are markedly increased following peripheral nerve lesion, providing a target for intrathecally administered cannabinoids distinct from the spinal cord or CNS. This idea is supported by the results of a recent study in the SNL model, which indicated that intra-DRG administration of CB2 selective agonists is more effective at attenuating allostynia than intrathecal administration (Hsieh et al., 2011). A comparison of the efficacy between intrathecal versus intra-DRG administration of CB2 agonists would go some way to answering this question in the current CCI model, although this procedure is not well established in the rodent, and presents with a number of technical challenges (Fischer et al., 2011).

If either of these hypotheses is correct, then it is unlikely that CB2 agonists as a class are a good candidate for future neuropathic pain research. If their action in these conditions is mediated via CB1, there is already an arsenal of clinically available cannabinoids that act at CB1. If their action is modulated via a small subset of receptors in the DRG that are only activated by local drug administration, then selective targeting of DRGs would be invasive and not clinically desirable. It is perhaps worthwhile at this point examining some of the current clinical evidence for CB2 selective agonists.
6.2.2 CB2 selective agonists in the clinic

Despite the strong interest in the development and testing of CB2 selective ligands in recent times, only limited clinical data are available. One CB2 agonist that has been tested in the clinic is GW842166, developed by GlaxoSmithKline. Preclinical testing indicated that this compound had a moderate affinity and very high selectivity for the human CB2 receptor (>475X selective over CB1), and was efficacious at low doses in the CFA model of inflammatory pain in the rat (Giblin et al., 2007). While initially promising, the compound failed to change pain scale scores when given as a single dose in a phase 2 RCT of dental pain, and was not as efficacious as the positive control, ibuprofen (Ostenfeld et al., 2011). The authors discuss the possibility that plasma levels were suboptimal, but acknowledge that plasma levels obtained were greater than those in preclinical trials demonstrating efficacy in the rat, and thus are difficult to explain. It is more than likely, however, that a model of acute dental pain was in fact not the best application for a drug class thought to be most efficacious in chronic inflammatory conditions. Phase 2 clinical testing for osteoarthritis has also been completed, although at the time of writing, the results of these trials are not available (Ostenfeld et al., 2011).

Another CB2 selective compound PRS-211,375 (cannabinor), developed by Pharmos Corporation, has been tested in clinical trials, and while the results have not been published, the company has issued a press release detailing the outcomes (Pharmos, 2011). While promising in preclinical trials of painful conditions, clinical results were not as positive as hoped. In initial phase 2 trials, the compound was effective at reducing pressure and heat induced pain, but not capsaicin induced pain in healthy volunteers. Results were similarly mixed in the second round of phase 2 trials, in which cannabinor was tested in acute dental pain; cannabinor was found to be efficacious, however only at low doses. Due to these mixed outcomes, cannabinor is no longer under investigation for painful conditions, and the company is directing its efforts elsewhere (Pharmos, 2011).
CB2 agonists have thus far followed a similar pattern to a variety of other drugs. That is, many compounds with efficacy in animal models of pain do not exhibit the same effect in the clinic. This may be for a number of reasons, including the methods chosen for preclinical drug assessment in animal pain models.
6.3 Preclinical pain testing

An intriguing finding in this study was the encapsulation of motor neurons in the ventral horn of the spinal cord following CCI (Brownjohn et al., 2012a). This phenomenon has been previously reported following sciatic nerve lesion (Kobbert et al., 2000; Zhang et al., 2003; Hu et al., 2007) and is likely the result of lesioning of motor nerve fibers themselves (Colburn et al., 1999; Kobbert et al., 2000). While it is not known if this encapsulation represents a protective or detrimental response, it is a clear indication that motor fiber pathways are damaged by injury to “mixed” peripheral nerves, which contain both sensory and motor fibers, as is the case with CCI of the sciatic nerve.

Preclinical animal pain research often involves testing paw withdrawal reflexes to thermal or mechanical stimuli to measure allodynia and hyperalgesia, which are then assessed as outcomes of treatment efficacy. It is therefore worth considering the contribution of damaged motor fiber pathways to this measured outcome, and whether in fact this feature of whole nerve lesion is a confounding factor in neuropathic pain research. Given the assumed importance of gliosis in the dorsal horn in the development and maintenance of neuropathic pain behaviors (Milligan et al., 2009), there is a very real possibility that gliosis in the ventral horn associated with motor neurons may also have an effect on reflexive pain testing.

While it is not known at this stage whether or not this is a confounding factor, there are two ways of dealing with this issue in future pain research if this is indeed found to be the case, both of which are currently being explored. Neuropathic pain models that involve lesioning of purely sensory nerves could avoid any confounding effects of motor nerve damage, and indeed both a partial tight ligation (Walczak et al., 2005), and an adapted CCI (Gunduz et al., 2011) of the saphenous nerve have already been developed. Lesioning of this sensory nerve led to the development of pain behaviors very similar to those induced in mixed nerve models, which were sensitive to gold standard pharmacological treatments, including Gabapentin (Walczak et al., 2005; Gunduz et al., 2011).
Alternatively, the use of pain tests that are independent of limb reflexes would circumvent motor confounds, and is arguably a more accurate representation of the human condition. Although reflexive testing is the most widely used category of pain testing in animals (Mogil et al., 2004), it has long been argued that reflexes do not accurately reflect pain sensation in humans, and may only provide an indirect measure of pain that is also sensitive to changes in motor processing (Chapman et al., 1985). This is relevant to therapeutic intervention, as drugs that alter motor processing may be falsely interpreted as anti-nociceptive based on their ability to influence paw withdrawal reflexes. It has instead been proposed that methods based on operant behavioural testing, which measures learned behavior in response to positive or negative reinforcement, or measurement of spontaneous pain, will avoid confounding changes in motor processing, while at the same producing more clinically relevant data (Mogil, 2009).
6.4 CB2 receptor expression in the CNS

If CB2 agonists were acting via a CB2 dependent mechanism at the level of the spinal cord to attenuate allodynia in neuropathic pain, as has been suggested, this would require the expression of functional CB2 receptors in the spinal pain pathway. Given that early studies failed time and again to find evidence for the expression of CB2 in the healthy CNS, including the spinal cord, the hypothesis of inducible CB2 receptor expression in the spinal pain pathway in response to peripheral nerve damage is an attractive one to explain the efficacy of spinally delivered CB2 agonists. While the current study did not produce evidence to support the functional expression of CB2 receptors in the spinal pain pathway in healthy or neuropathic rats, many studies have reported otherwise. It is essential to determine the site and mechanism of action of CB2 agonists for reasons of drug optimization, and therefore the evidence for CB2 receptors in the spinal cord will be considered in the following sections. Evidence for CB2 receptor expression in the brain will also be considered in parallel, as much of the previous work on central CB2 expression has been conducted in supraspinal, rather than spinal centers.

6.4.1 CB2 receptor mRNA expression in the CNS

Early studies that investigated CB2 mRNA expression in the CNS used Northern blotting, in which total RNA is separated by weight and hybridized with a targeted probe, and in situ hybridization, in which a similar procedure is performed directly on tissue sections. In the first description of the CB2 receptor, Munro et al. (1993) used both techniques to identify the presence of CB2 mRNA in the marginal zone of the spleen, comprised largely of macrophages, and an absence of the same transcripts in the rat brain (Munro et al., 1993). These results agree with those obtained by Galiegue
et al. (1995) for human and Schatz et al. (1997) for rat. Both of these studies characterized the expression of CB2 transcripts in peripheral immune cells, and confirmed an absence of CB2 transcripts in central nervous tissues (Galiegue et al., 1995; Schatz et al., 1997). The same results were reported by Griffin et al. (1999), who did not detect any CB2 mRNA in the healthy rat CNS, including the spinal cord, using Northern blotting (Griffin et al., 1999).

While initial evidence pointed to an absence of CB2 mRNA in the CNS, this was challenged when Zhang et al (2003) produced results that provided some of the first evidence of CB2 upregulation in the spinal cord following peripheral nerve injury. The authors reported that CCI of the sciatic nerve or tight ligation of spinal nerves resulted in an upregulation of CB2 gene expression, regionally codistributed with an upregulation of OX-42, a marker for microglia/macrophages. While sense controls confirming specificity of the riboprobes were performed, representative images are not provided, precluding assessment of these vital controls. Furthermore, an independent group of researchers could not replicate these results in a more recent study, and did not detect CB2 mRNA in the spinal cords of SNL treated rats with in situ hybridization using a validated probe, despite strong microglial activation (Elphick et al., 2008). This report casts doubt on the reproducibility of the key findings in Zhang et al. (2003).

A more sensitive and quantifiable method of detecting mRNA transcripts is reverse transcription polymerase chain reaction (RT-PCR). This technique involves the amplification of mRNA transcripts within a sample, using probes directed towards the target. The amplification process allows the detection of minute quantities of mRNA, which can be quantified following positive identification with Northern blotting. Matching earlier works that used Northern blotting alone or in situ hybridization, many reports using RT-PCR have failed to identify CB2 transcripts in naive central nervous tissues, including whole human (Galiegue et al., 1995) and mouse (McCoy et al., 1999) brain, and rat brainstem (Derbenev et al., 2004) and cortex (Beltramo et al., 2006). In more recent times, however, the expression of CB2 mRNA has been reported in healthy central tissues, in striking
contrast to earlier works. In 2005, Van Sickle and coworkers reported the presence of CB2 transcripts in the rat brainstem, in addition to weak expression in the cortex (Van Sickle et al., 2005). Since this discovery, there have been many more, confirming the presence of low levels of CB2 transcripts in the brainstem and hypothalamus of the rat (Gong et al., 2006; Onaivi et al., 2006; Viscomi et al., 2009), brainstem, striatum, mid brain, hippocampus, and prefrontal cortex of the mouse (Liu et al., 2009), globus pallidus of the crab-eating macaque (Lanciego et al., 2011), and amygdala, caudate, putamen, nucleus accumbens, cortex, cerebellum and hippocampus of man (Liu et al., 2009).

These results have been corroborated by a recent study that used a sensitive method of in situ hybridization to examine CB2 expression in the brain of the crab-eating macaque (see above) (Lanciego et al., 2011). This paper provides convincing evidence for CB2 mRNA in a small population of cells in CA1 of the hippocampus, the cerebral cortex and the globus pallidus of Macaca fascicularis. Importantly, these results were validated by the lack of staining produced by sense control probes. Cell phenotyping was not performed on CA1 or cortical cells, although the morphology appears, and was interpreted by the authors as neuronal. A possible explanation for this is suggested by the finding that in culture, neuroprogenitor cells, precursors to neurons, express CB2 mRNA before their differentiation into mature neurons, and subsequent loss of CB2 expression (Palazuelos et al., 2006). Neurogenesis, or the differentiation of neuroprogenitor cells into mature neurons, occurs in the adult mammalian brain in the hippocampus (van Praag et al., 2002), including CA1 (Becq et al., 2005), as well as in the subventricular zone (Lois et al., 1994), and potentially the cortex (Gould, 2007). It is perhaps feasible that neuroprogenitor cells in CA1 of the hippocampus as well as the cerebral cortex are the source of the low levels of CB2 mRNA expression detected by Lanciego et al. (2011) in the adult crab-eating macaque. This hypothesis, however, does not explain why CB2 positive cells, later identified as mature neurons, were also found in the globus pallidus, for which there is no evidence of neurogenesis in mature animals.
RT-PCR is frequently used in the CB2 field due to its superior specificity and sensitivity over both early RNA techniques such as in situ hybridization and Northern blotting, as well as over antibody based approaches such as immunohistochemistry. However this technique also has several limitations and a number of important considerations must be made when interpreting data of this kind. First, while superior to in situ hybridization in terms of sensitivity, RT-PCR on whole tissue sections is not compatible with cell phenotyping, owing to tissue homogenization creating a heterogeneous cell population. For this reason, while it may be possible to quantify the level of a transcript within a sample, it is not possible to identify the source – a clear limitation if one is to determine the relevance of a target within a given physiological process. The use of single cell RT-PCR would circumvent this issue (Phillips et al., 2000), however this approach has not, to this author’s knowledge, been applied in this field. Alternatively, in vitro culture of CNS cells allows the purification of mRNA from homogenous populations of neural or glial cells. Using such a method, a recent study using this technique was able to show that CB2 mRNA was absent in mature cortical neurons derived from the mouse (Palazuelos et al., 2006).

A second issue is that the sensitivity of the RT-PCR technique may be a double edged sword. Successive replications of transcripts using PCR can amplify even infinitesimal transcript quantities up to observable and measureable levels. In fact, the technique of single cell RT-PCR demonstrates that enough material necessary to positively identify a given mRNA transcript can be obtained from a lone cell (Phillips et al., 2000). While this may appear to be a positive attribute, and is in certain applications, the results of RT-PCR studies of CB2 which involve homogenization of tissue sections must be interpreted with caution in this respect. Unless perfused sufficiently, central nervous tissues will contain residual blood cells in the vasculature, which will become part of the homogenate analyzed by RT-PCR. As peripheral leukocytes unequivocally express CB2 receptor mRNA (Galiegue et al., 1995), any errant leukocytes trapped within the vasculature could lead to a CB2 positive RT-PCR result, which may falsely be interpreted as expression of the CB2 receptor within the CNS itself.
While perfusion has been detailed in at least one of the RT-PCR studies above (Van Sickle et al., 2005), almost all of the remaining studies make no mention of perfusion of any kind, making it likely blood cells were contaminating the samples. Moreover, the spinal cord is considerably less vascular than the whole brain, and it has proven difficult in this laboratory and others to completely and satisfactorily purge the spinal cord of all blood cells using perfusion so as to avoid potential contamination.

6.4.2 CB2 receptor binding in the CNS

While there is some evidence for CB2 receptor mRNA expression at background levels in the healthy CNS, three very informative radioligand binding studies do not support a hypothesis of appreciable levels of CB2 receptor binding in the healthy CNS. It has been conclusively demonstrated that genetic disruption, or knockout, of the CB1 receptor gene abolishes all cannabinoid receptor binding in the brain, as measured by radioligand binding with tritiated CP55,940 (Zimmer et al., 1999; Ibrahim et al., 2003). CB1 receptor knockout also abolishes all cannabinoid binding in the spinal cord (Ibrahim et al., 2003), but does not alter binding in the spleen, where the greatest density of CB2 receptors is located (Zimmer et al., 1999). Conversely, CP55,940 binding in the brain is unaltered by CB2 receptor knockout, whereas cannabinoid binding in the spleen is completely abolished in this case (Buckley et al., 2000). These complementing studies argue strongly against appreciable CB2 receptor expression in the healthy CNS, suggesting that PCR studies in healthy brain tissue are measuring either irrelevant background levels of CB2 expression, or contamination from white blood cells.
6.4.3 Evidence for neuronal CB2 expression

6.4.3.1 In vivo CB2 expression

There are a handful of controversial studies that describe widespread neuronal expression of the CB2 receptor in the CNS. A team originating from the United States National Institutes of Health (NIH) has published work describing CB2 receptor immunolabeling in neurons from a number of brain regions in naïve rodents, including, among others, the cerebellum, hippocampus, olfactory bulb, cerebral cortex, striatum, thalamus and amygdala (Gong et al., 2006; Onaivi et al., 2006; Onaivi et al., 2008). There are two key reasons to question the widespread distribution of CB2 immunolabeling reported in these studies. First, aside from the plethora of previous work describing the absence of CB2 receptor mRNA above background levels in the naïve CNS, RT-PCR studies published alongside the immunolabeling work also failed to identify CB2 mRNA transcripts in key brain regions in which CB2 immunolabeling was described, aside from some trace levels in the brainstem and hypothalamus (Gong et al., 2006; Onaivi et al., 2006). These results are an apparent contradiction given the necessity of mRNA in subsequent protein translation and expression.

Second, and perhaps more important, is the reliance of these studies on controversial immunolabeling techniques. As discussed in detail earlier, validation of antibodies with appropriate knockout controls is an essential part of immunohistochemistry and Western blotting, especially in the CB2 field in which a reliable monoclonal CB2 directed antibody has yet to be produced. The immunolabeling work of the NIH team lacks the appropriate negative controls, and is almost entirely reliant on the Cayman Chemical antibody (Gong et al., 2006; Onaivi et al., 2006; Onaivi et al., 2008); that the current study shows is not specific for the CB2 receptor in vivo. While Gong et al. (2006) does show an antibody knockout control image, firstly the image is not of critical brain regions, and
secondly, the image is not a validation of the Cayman Chemical antibody which is used for the entirety of the remaining study, and is instead validation of an alternative CB2 primary antibody. Similarly, the work of Wotherspoon et al. (2005) describing CB2 expression in primary sensory neurons synapsing in the dorsal horn is difficult to interpret, as that study also employed the same Cayman Chemical antibody. Although this group did run appropriate knockout controls, the results of this validation are not conclusive, as discussed in detail in Chapter 3.

As the results of these immunolabeling studies are not commensurate with those from most mRNA studies which indicate absence or very low expression of CB2 mRNA transcripts, and rely on a problematic antibody, they do not provide convincing evidence for neuronal CB2 expression on their own.

6.4.3.2  In vivo CB2 function in the healthy CNS

Many authors have postulated a potential role for the CB2 receptor in the CNS, and several studies have proposed to show evidence of a functional role for neuronal CB2 receptors in physiological central processes. While this approach bypasses the issues with the molecular approaches discussed above, studies of this kind often have their own distinct issues which may hamper interpretation e.g. ligand selectivity.

6.4.3.2.1  Functional characterization of brainstem CB2 receptors (Van Sickle et al. (2005))

In 2005, Van Sickle and colleagues described the first such evidence for functional CB2 in the naive CNS, in the brainstem of the ferret, in a study which changed the dogma surrounding CB2 in the CNS
(Van Sickle et al., 2005). Following their description of CB2 receptor immunolabeling in brainstem nuclei involved in emesis, or vomiting, the authors wished to test the functional significance of these receptors. As cannabinoids are antiemetic in humans, it was hypothesized by Van Sickle and colleagues that the CB2 receptors in the brainstem which they described with immunolabeling may have a role in this process. They therefore tested the effects of the non-selective endocannabinoids anandamide and 2-AG on induced emesis, in the presence and absence of antagonists in order to determine receptor subtype involvement. The experiments show that both endocannabinoids were antiemetic, and furthermore, that while the effect of anandamide was only blocked by the CB1 selective antagonist AM251, the effect of 2-AG was blocked by either AM251 or the CB2 selective antagonist AM630. The authors concluded that while the antiemetic effects of anandamide are mediated by CB1, the effects of 2-AG are mediated by both CB1 and CB2. While this paper is frequently cited as some of the most concrete evidence of CB2 receptor involvement in central processes, there are several fundamental problems involved in interpretation of the results and the experimental design itself that cast doubt on this assertion.

In the first experiment, in which the endocannabinoids were administered in the absence of antagonists, anandamide completely abolished all episodes of emesis induced by the emetic agent morphine-6-glucuronide (MG6), whereas 2-AG only reduced the number of episodes, albeit significantly, from ~7.5 to ~2.5 over the observation period. The first issue is that the complete abolition of emetic episodes by anandamide also removes the variance from this group, violating the assumptions of the ANOVA used to analyze the results. Secondly, and more saliently for later assumptions, the antiemetic effect of anandamide is essentially constrained, and there is no way to accurately quantify the true difference in efficacy between the two endocannabinoids. In another set of experiments, the more efficacious anandamide was antagonized only by AM251, while 2-AG was antagonized by both AM251 and AM630, a result which is interpreted as anandamide acting via CB1, and 2-AG acting via CB1 and/or CB2. However, it could be more parsimoniously explained that
both are acting via CB1; anandamide is a more selective agonist for CB1 than 2-AG (McPartland et al., 2007), fitting with the apparent increased antiemetic effect observed in the initial set of experiments, and the reversal of this effect with AM251 alone. On the other hand, 2-AG, being a weaker CB1 agonist, could well be more easily blocked by partial antagonism of CB1 by AM630 at the relatively high dose employed in the study (5 mg/kg). It should be noted that the binding affinities and potencies of cannabinoid agonists and antagonists at ferret cannabinoid receptors are wholly untested. Given the great interspecies variability in ligand selectivity for cannabinoid receptors (Ashton et al., 2008b), this creates a potential issue with the use of subtype selective ligands in this study, which are only selective for subtypes within a specific concentration range, as discussed previously.

Aside from the debatable evidence arguing for a CB2 receptor role in emesis, there are two strong pieces of evidence in this study arguing against a role for CB2 in this process. Firstly, it is described that AM251, but not AM630, was able to potentiate the emetic effect of the emetic agent MG6. This indicates that the endogenous cannabinoid tone involved in emesis is mediated solely via CB1, with no evidence for CB2 involvement. However, perhaps the strongest evidence arguing against a CB2 mediated anti-emetic effect is this statement in the discussion section, describing a follow up set of experiments: “These results led us to investigate whether selective CB2 receptor agonists reduced emesis. We observed no statistically significant reductions in emesis in animals given the CB2 receptor agonists AM1241 (1 or 2 mg/kg) or JWH-133 (1 or 5 mg/kg)” (Van Sickle et al., 2005). Plainly stated, two structurally distinct CB2 receptor agonists had no effect on emesis when delivered in comparable doses to the efficacious non-selective cannabinoids anandamide and 2-AG. These results are in stark contrast to those expected if the CB2 receptor is involved in emesis, as the authors of this paper have suggested, and instead strongly argue against a role for the CB2 receptor in this process.
Several investigations by Onaivi and colleagues have described a role for CB2 in locomotion and alcohol consumption in mice. In the locomotor studies, it was discovered that systemic administration of the CB2 selective agonist JWH-015 was able to dose dependently reduce locomotor activity in several strains of mouse; a result interpreted by the authors as clear evidence of a role for central CB2 receptors in locomotion (Onaivi et al., 2006; Onaivi et al., 2008). Although the doses employed (5 – 20 mg/kg) are perhaps lower than might be expected to produce central CB1 mediated motor deficits (Valenzano et al., 2005), it should be noted firstly that JWH-015 is far less selective for CB2 over CB1 than most of the other commonly employed CB2 selective agonists (Ashton et al., 2008b), and secondly that the horizontal locomotor tests employed by Onaivi et al. (2006) are thought to be more sensitive tests of motor function than the more commonly used rotorod tests of motor coordination (Yao et al., 2008a). In this case, both studies may be in fact be detecting CB1 not CB2 mediated effects in this dose range. Similarly, another study by the same group found that chronic administration of JWH-015 exacerbated stress-induced increases in alcohol consumption in mice, once again only high doses (20 mg/kg) (Ishiguro et al., 2007). In the same study, the CB2 selective antagonist AM630 had no significant effect on stress-induced alcohol consumption, perhaps suggestive of a lack of cannabinoid tone mediated via CB2 in this process. There was no attempt to block the effects of JWH-015 with receptor knockout or antagonism in any of the three studies, making it difficult to ascertain receptor subtype involvement in this phenomenon.

The involvement of the CB2 receptor in anxiety and depression has also been investigated by this group. Researchers found that chronic dosing of JWH-015 caused an increase in sucrose consumption (a measure of hedonism) and that acute administration of JWH-015 attenuated stress-
induced anxiety (Onaivi et al., 2008). Again, however, the dose was high (20 mg/kg), and the effects were not tested by receptor knockout or antagonism, casting doubt on the true target of the administered compound. An intriguing approach in the same study assessed the effects of i.c.v. administration of an antisense oligonucleotide for the CB2 receptor protein on anxiety, using the elevated plus maze paradigm. Chronic administration of the antisense oligonucleotide to the cerebral ventricles via twice daily microinjection over 3 days reduced anxiety, as determined by an increase in time spent in the open arms of the maze (Onaivi et al., 2008). While this appears to be convincing evidence for a role for CB2 in anxiety, the experiment is not well described, and the natures of the probe, or the controls used to compare the response, are ambiguous and not clear. Without the full data set, or even the sample sizes disclosed, it is difficult to draw definitive conclusions about this study.

6.4.3.2.3  Functional CB2 type cannabinoid receptors at CNS synapses (Morgan et al. (2009))

The only published ex vivo electrophysiology study that purportedly provides any evidence of neuronal CB2 receptor activity analyzed the effect of various cannabinoids on inhibitory post synaptic currents (IPSCs) from entorhinal cortical/hippocampal sections taken from the healthy rat brain (Morgan et al., 2009). Experiments show that the inhibitory actions of both the non-selective endocannabinoid 2-AG and the CB2 selective cannabinoid JWH-133 on IPSC amplitude are impeded by the CB2 selective antagonist/inverse agonist AM630, but not the CB1 selective inverse agonist LY320135. While these experiments are interpreted as clear evidence for neuronal CB2 receptor activity in this brain region, the experimental design does not allow unambiguous conclusions to be drawn. In a traditional experimental design that employs antagonists to discern receptor subtype involvement in an agonist-induced response, the agonist response is tested in the presence of
subtype selective antagonists. In the study by Morgan et al. (2009), however, this design is reversed, and the responses induced by cannabinoid receptor subtype-selective inverse agonists are tested before and after the addition of of selective or non-selective cannabinoid agonists.

For example, in one set of experiments, the CB1 selective inverse agonist LY320135 increases IPSC amplitude; an increase that is returned to baseline levels by the non-selective endocannabinoid 2-AG and the CB2 selective agonist JWH-133. This is interpreted as evidence that CB1 inverse agonism was unable to attenuate 2-AG or JWH-133 induced inhibition of spike amplitude, despite no evidence in this study that either 2-AG or JWH-133 modulate IPSC amplitude independently, and instead the distinct possibility that both agonists are simply outcompeting the inverse agonist. It should be acknowledged that it may be difficult if not impossible to ascertain any electrophysiological responses from the CB2 receptor in such a pathway without first inhibiting what is likely to be a high level of constitutive CB1 receptor tone. Taken together, however, the results of Morgan et al (2009) do not provide unambiguous evidence for functional neuronal CB2 receptors.

6.4.3.2.4 Brain CB2 receptors modulate cocaine’s actions in mice (Xi et al. (2011))

A recent study by Xi and colleagues (2011) provides perhaps the strongest evidence of a role for CB2 in a central process. The authors report that the CB2 selective agonist JWH-133 is able to modulate cocaine addiction and self-administration in mice, and furthermore, that this is due to CB2 receptor activation in the nucleus accumbens (NAc), implying expression of the CB2 receptor in this nucleus. Upon scrutinizing the experimental design and data interpretation in this paper, however, it becomes clear that these claims are not unambiguously supported by the data.

In one set of experiments using intra-NAc delivery, the authors show that JWH-133 reduced cocaine self-administration in wildtype mice, and that this effect was reversed by AM630, and absent in CB2 -
/- mice. Firstly it should be noted that this result obtained with AM630, while presented on the same graph, was not part of the same matched JWH-133 experiment, and therefore comparison across an unmatched design in this case does not confirm a CB2 dependent effect. Secondly, although asserted that any response in WT mice is abolished in CB2 -/- mice, it is clear to see that JWH-133 appears to modulates cocaine self administration similarly in WT and CB2 -/- mice, causing a drop of 40 and 30%, respectively, in both sets of animals, although only in WT mice does this difference reach significance (p < 0.05). It is of note then that while the WT mice in this comparison number 9, with 4 dose levels, the CB2 -/- mice only number 6, with 2 dose levels. With a P value of 0.15 in the CB2 -/- group, it is a distinct possibility that the comparison is underpowered, and thus insufficient to claim CB2 dependent effects on cocaine modulation.

The mechanism behind the authors’ claims of CB2 modulation of cocaine self administration was hypothesized to be via modulation of dopamine release, and further experiments are proposed to show evidence that JWH-133 is able to modulate dopamine release specifically via the CB2 receptor in the NAc. In one set of experiments, dopamine release in the NAc is directly measured in response to systemic and central JWH-133 administration. It is demonstrated that JWH-133, when given intranasally or via NAc cannulation, reduces dopamine release in wildtype and CB1 -/-, but not CB2 -/- mice, and furthermore, that inhibition of dopamine release by systemic JWH-133 is attenuated by intra-NAc AM630. While the authors contend that this is clear evidence for central CB2 involvement in this process, they fail to explain or discuss the greatest source of variation in this experiment: the interaction between receptor knockout and drug treatment. It is clearly shown that centrally administered JWH-133 actually increases dopamine release in CB2 -/- mice, and conversely, that centrally administered AM630 could not attenuate inhibition of dopamine release induced by systemic JWH-133 in CB1 -/- mice. These two findings implicate a distinct CB1/CB2 independent target for JWH-133 in the CNS, questioning the specificity of the ligand, which is delivered directly to the CNS at unknown concentrations at the site of action. The pharmacokinetics of intra-NAc
delivered compounds are as yet unknown, and given the crucial importance of correct dose administration in guaranteeing receptor subtype selectivity with cannabinoid ligands, the interpretation of results of experiments involving this method of cannabinoid drug delivery are fraught with difficulty.

Perhaps a further concession in this study is the observation that cocaine administration is not modulated by CB2 receptor deletion, but is markedly reduced following CB1 receptor deletion. This suggests that CB2, unlike CB1, is not involved in the regulation of endogenous tone in the reward pathway. Coupled with the potentially underpowered CB2 -/- self administration experiments, and the dopamine release experiments, which implicate a CB1/CB2 independent target for JWH-133, assertions by Xi et al. (2011) that central CB2 receptors modulate dopamine release or cocaine self administration in naïve mice remain inconclusive.

6.4.3.3  
In vivo CB2 function in the neuropathic CNS

While little concrete evidence exists supporting a role for functional CB2 receptors in CNS neurons in naïve conditions, there is a suggestion that functional CB2 in spinal or supraspinal structures may be upregulated under conditions of neuropathic pain, and that these receptors may a direct role in neuronal function. This section discusses the current evidence for such a hypothesis.
Some of the only functional evidence for the upregulation of CB2 receptors in the spinal pain pathway comes from the work of Sagar et al. (2005), who performed in vivo electrophysiology on dorsal horn neurons in anaesthetized sham and SNL treated rats. Delivered directly on to the lumbar spinal cord following laminectomy, the authors reported that JWH-133 inhibited mechanically stimulated responses of dorsal horn neurons in SNL, but not sham treated rats. Furthermore, the authors reported that the CB2 selective antagonist SR144528, but not the CB1 selective antagonist SR141716, was able to attenuate the effects of JWH-133. The authors concluded from these results that functional CB2 receptors were upregulated in the lumbar spinal cord following spinal nerve lesion, and that targeting of these receptors with CB2 agonists alters nociceptive processing at the spinal level (Sagar et al., 2005).

It was hypothesized by the authors that primary sensory afferents, terminating in the dorsal horn, presynaptically express functional CB2 receptors; a theory supported by the finding, in the same study, that functional CB2 receptors are present in the sensory afferent cell bodies from DRGs of sham-operated and SNL rats, with greater function in neuropathic rats (Sagar et al., 2005). This correlates well with functional data obtained from the studies of Beltramo et al. (2006), who determined that CB2 selective agonists could inhibit neurotransmitter release from first order neurons in ex vivo preparations of spinal cord sections from healthy rats (Beltramo et al., 2006). In the study of Sagar et al. (2005), it is also entirely possible that microglia are expressing functional CB2, and suppression of microglia by JWH-133 is altering neuronal firing indirectly, a hypothesis that could not be dismissed by the authors (Sagar et al., 2005).
At least one study has investigated CB2 function in pain at the supraspinal level. The thalamus is a key supraspinal modulator in the discriminative nature of pain, and despite a relatively low density of cannabinoid receptors (Herkenham et al., 1990) it had been implicated in non-selective cannabinoid-induced analgesia in naïve rats, presumably via CB1 receptor activation (Martin et al., 1999). Jhaveri et al. (2008) however, investigated a possible role for CB2 receptors in cannabinoid-induced analgesia in the thalamus, in naïve and neuropathic rats, using JWH-133. While their results clearly show that JWH-133, when delivered into the ventral posterior nucleus, appears to reduce both the spontaneous and evoked firing rates of VPL neurons in neuropathic but not sham treated rats, their conclusion that this is due to neuronal CB2 receptor activation is not necessarily supported by the data.

In the first experiment it was demonstrated that intra-VPL administration of JWH-133 inhibits spontaneous firing of VPL neurons in SNL but not sham treated rats, an effect attenuated by co-administration with SR144528, thus implying CB2 receptor involvement. It should be noted that intra-VPL cannulation involves the administration of compounds to a very small brain region, and consequently the pharmacokinetics of drugs administered via this route are largely unknown. This is a major consideration for the interpretation of results involving the administration of compounds only selective for receptor subtypes to a certain degree, such as CB2 selective JWH-133 and SR144528, which have affinities for rat CB1 receptors in the nanomolar range (Rinaldi-Carmona et al., 1998; Huffman et al., 1999). In this study, JWH-133 and SR144528 were administered at 192 and 201 µM, respectively - concentrations well in excess of those required for CB1 receptor binding, making interpretation of the results extremely difficult. As a CB1 selective antagonist was not utilized in the first experiment as a comparator, it therefore cannot be concluded with certainty that
the observed reduction in spontaneous VPL neuron firing induced by JWH-133 was mediated via CB2 receptors.

In further experiments it was determined that increased VPL neuron firing evoked by both innocuous and noxious touch was also attenuated by intra-VPL JWH-133 administration in neuropathic but not sham rats. Co-administration with subtype selective antagonists, however, did not produce a clear description of receptor subtype involvement in this phenomenon. While attenuation of increased firing induced by innocuous touch was inhibited by JWH-133, this was not significantly attenuated by either CB1 or CB2 antagonism, potentially implicating an unknown central target for JWH-133. Conversely, JWH-133 mediated attenuation of increased VPL firing evoked by noxious touch was fully attenuated by CB2 receptor antagonism, and partially attenuated by CB1 receptor antagonism, perhaps indicating cross antagonism of cannabinoid receptor subtypes in this instance. Interestingly in this final experiment, while SR144528 had no effect on VPL firing rate when given alone, it dramatically enhanced the noxious touch-induced increase in firing rate when co-administered with JWH-133, a seemingly inexplicable result that appears to account for the greatest degree of variation in this experiment, and one that is not discussed by the authors.

As these effects are only reported for neuropathic rats, another intriguing explanation could be that attenuation of VPL firing rate by JWH-133 is mediated via CB2 receptors resident on thalamic microglia. This theory is supported by a recent paper, which found that intra-VPL microinjection of minocycline, an inhibitor of microglial activation, reduced thermal hyperalgesia in neuropathic rats (LeBlanc et al., 2011). An indirect effect of JWH-133 on VPL neurons via microglial suppression could explain the results of Jhaveri et al. (2008) independent of functional CB2 receptor expression in VPL neurons.
Taken together, the results of this study, while indeed demonstrating a central action of JWH-133 in neuropathic pain, and producing some intriguing results, do not convincingly demonstrate central CB2 receptor functionality in neurons, as argued by the authors.

6.4.4 Evidence for microglial CB2

While the unequivocal expression of CB2 in the CNS, particularly in neurons, has not been determined, there is a large body of evidence suggesting that microglia may express CB2 receptors, particularly in certain pathological states. This section discusses some of the anatomical and functional evidence for microglial CB2 expression.

6.4.4.1 In vitro expression

The first convincing evidence for CB2 expression in microglia came from a study by Carlisle et al. (2002) utilizing RT-PCR. The investigators report that while CB2 mRNA was absent from rat cortex, in line with previous research, it was present in primary glial cultures from the neonatal rat cortex. Furthermore, while minimally expressed in mixed glial cultures, expression was greater in homogenous microglial cultures, and inducible following microglial priming with interferon gamma (INFγ) but down regulated following microglial activation with lipopolysaccharide (LPS) (Carlisle et al., 2002). These results suggested that CB2 levels are low in the naïve CNS, are most likely expressed in microglia, not astrocytes, and that CB2 levels are dependent on microglial activation states. The presence of CB2 mRNA in primary cultured rat microglia has been verified by others (Carrier et al., 2004; Beltramo et al., 2006), and has also been described in primary cultured mouse (Walter et al.,
2003; Ehrhart et al., 2005) and human (Klegeris et al., 2003) microglia. The effect of activation state on CB2 receptor expression was also investigated by Maresz et al. (2005), who found that priming primary microglial cultures with INFγ and/or granulocyte macrophage-colony stimulating factor (GM-CSF) dramatically induced CB2 mRNA expression.

6.4.4.2 Microglial CB2 function in vitro

There have been many in vitro studies indicating a functional role for the CB2 receptor in microglial processes. Some of the first work in this area was conducted by Walter et al. (2003) in primary cultures of mouse microglia, who reported that microglial migration was increased by 2-AG. This effect was inhibited by pre-incubation of cells with the CB2 selective antagonists SR144528 and cannabiol, and the abn-CBD antagonists cannabidiol and O-1918, but not the CB1 selective agonist SR141716A. Furthermore, the effect of 2-AG on microglial migration was not mimicked by the CB1 selective agonist methanandamide at CB1 selective concentrations (Walter et al., 2003). Franklin and Stella (2003) reported similar findings for CB2 function in BV-2 microglia cell lines. The cannabinoid agonist arachidonylcyclopropylamide (ACPA) induced cell migration in mouse BV-2 cells; an effect that was blocked by CB2 or abn-CBD antagonism but not CB1 antagonism, an unexpected result given that ACPA was considered at the time to be a putative CB1 receptor selective agonist (Franklin et al., 2003). Taken together, these results suggest that CB2 receptors mediate a pro-migrational effect of cannabinoids, a suggestion further enhanced by the finding that CB2 receptors were found to be strongly expressed in the leading edges of mobile activated microglia (Walter et al., 2003). Carrier et al. (2004) also reported that 2-AG and the CB2 selective agonist JWH-133 induced proliferation in primed primary rat microglia. This effect was inhibited by CB2 but not CB1 selective antagonism (Carrier et al., 2004).
Selective agonism of CB2 receptors also inhibits microglial activation and subsequent pro-inflammatory cytokine release. Klegeris et al. (2003) report that the CB2 selective agonist JWH-015 inhibited the release of IL-1β and TNFα from LPS/INFγ activated human microglia, which were positive for CB2 mRNA but negative for CB1 mRNA (Klegeris et al., 2003). Similarly, Ehrhart et al. (2005) reported that JWH-015 inhibited INFγ induced TNFα and NO release from microglial culture, an effect ameliorated by co-incubation with small interfering RNA (siRNA) targeted against the CB2 receptor. Microglial activation was also inhibited by non-selective cannabinoid agonism in the same study (Ehrhart et al., 2005). Another CB2 selective compound, JWH-133, has also been shown to inhibit β amyloid induced TNFα release from neonatal rat cortical microglia, via a CB2 dependent mechanism (Ramirez et al., 2005). Similar results have been reported in cultured BV-2 cells, in which WIN55,212-2 reduced iNOS protein levels, and inhibited production of NO via CB2 receptors, and anandamide delayed the release of IL-6, another pro-inflammatory mediator (Eljaschewitsch et al., 2006).

Given that microglia and neurons share a close relationship in vivo, and microglia are known to play a key role in neuroinflammation and neurotoxicity processes, several studies have now used models of neurotoxicity to assess whether cannabinoid induced changes in microglial activation state and cytokine release directly affect these processes. Activated microglia cause a decrease in the viability of neurons when both are cultured together in vitro, an effect reversed when the co-culture is treated with the CB2 selective agonists JWH-015 (Klegeris et al., 2003) and JWH-133 (Ramirez et al., 2005). Furthermore, Eljaschewitsch et al. (2006) used an ex vivo model of excitotoxic damage, the organotypic hippocampal slice culture model, in which excitotoxicity is induced by NMDA, and BV-2 microglia are applied to the slices. Addition of BV-2 microglia increased hippocampal neurotoxicity, with CB2, but not CB1 receptor antagonism further exacerbating the damage (Eljaschewitsch et al., 2006).
6.4.4.3 Expression of CB2 in microglia in vivo

A group led by Julián Romero, of the Francisco de Vitoria University in Madrid, has described the upregulation of CB2 immunolabeling in central microglia in human Alzheimer’s disease (Benito et al., 2003), MS (Benito et al., 2007) and Down’s syndrome (Nunez et al., 2008) patients, as well as in simian HIV-infected Macaques (Benito et al., 2005). Microglial CB2 receptor immunolabeling has been corroborated by independent groups in the brains of Alzheimer’s patients (Ramirez et al., 2005) and HIV encephalitis patients (Cosenza-Nashat et al., 2011), and in the spinal cords of MS and ALS affected patients (Yiangou et al., 2006). Suggesting a role for microglial CB2 in neuroinflammation, CB2 labeling has been described in activated microglia following ischemic brain injury in the rat (Ashton et al., 2007b). Microglial CB2 immunolabeling has also been described in the healthy human (Nunez et al., 2004) and rat (Ashton et al., 2006) cerebellum, where labeling was localized to perivascular microglia. As Ashton et al. (2007) only used cell morphology to phenotype CB2 positive cells, and the cell markers used by Nunez et al. (2004) cannot distinguish between microglia and macrophages, it is, as noted above, possible that these CB2 labeled cells are in fact macrophages infiltrating from the periphery. In neuropathic pain, CB2 mRNA was shown to be codistributed with OX-42 in the spinal cord of CCI and SNL treated rats (Zhang et al., 2003), and an immunolabeling study has described CB2 immunolabeling colocalizing with Iba1 following spinal nerve transection (Romero-Sandoval et al., 2008).

All but one of these studies describing CB2 expression in microglia in vivo, relied upon immunohistochemistry. As has been shown in this study and others, and discussed at length earlier,

---

5 Incidentally, none of these studies reports CB2 immunolabeling in surrounding neurons, further questioning the results of studies describing widespread neuronal CB2 labeling (Gong et al., 2006; Onaivi et al., 2006; Onaivi et al., 2008).
this method is particularly controversial in the cannabinoid field, given the unreliability of currently available CB2 directed antibodies, particularly in fixed tissue (Brownjohn et al., 2012b). When assessing mRNA in vivo, in situ hybridization has issues of sensitivity, while RT-PCR is generally restricted to use in heterogeneous tissues where cell phenotype is not able to be determined. Perhaps one of the most elegant studies to circumvent these issues was that by Maresz et al. (2005), which investigated CB2 receptor regulation following the induction of experimental autoimmune encephalomyelitis (EAE) in mice, a model of MS. Ten days following EAE induction, brain tissue was perfused to remove errant blood cells, before homogenization, then sorting of resident immune cells, using a range of cell markers, into T cells, macrophages and resting and activated microglia. It was on these homogenous cell populations that the highly sensitive RT-PCR technique was performed, and the level of CB2 receptor mRNA quantified. It was discovered that CB2 mRNA was upregulated 10 fold in activated microglia and macrophages over T cells and resting microglia (Maresz et al., 2005).

6.4.4.4 Function of microglia CB2 in vivo

Thus there seems to be reasonably good evidence that microglia express CB2, at least under some conditions. Both in vitro and in vivo evidence suggest that in certain states, microglia upregulate CB2 expression, which may then regulate cellular proliferation, activation, and migration. It has been argued that targeting microglial CB2 receptors may be a viable therapeutic strategy in a variety of central neuropathies (Ashton et al., 2007a; Cabral et al., 2008; Rivers et al., 2010), however the functional relevance of these receptors in specific neuropathies is yet to be fully elucidated.

Aside from efficacy in neuropathic pain states as discussed extensively above, CB2 selective agonism has also improved outcomes following cerebral ischemia (Zhang et al., 2007), and in animal models
of MS (Arevalo-Martin et al., 2003; Ni et al., 2004), Huntington’s disease (Palazuelos et al., 2009; Sagredo et al., 2009), Parkinson’s disease (Price et al., 2009), with authors in many cases attributing the effects to CB2 receptors on microglia. In support of a role for CB2 in some of these processes, receptor knockout studies have indicated that loss of the CB2 receptor worsens disease outcomes in a number of central neuropathies (Palazuelos et al., 2009; Sagredo et al., 2009; Garcia et al., 2011), including neuropathic pain (Racz et al., 2008). It should be noted however, that in all of these studies using CB2 selective agonism to treat central neuropathies (with the notable exception of some neuropathic pain studies), the route of administration was systemic, and therefore an indirect effect of CB2 agonists on circulating CB2+ immune cells cannot be ruled out. In fact, decreases in peripheral leukocyte rolling and adhesion (Ni et al., 2004; Zhang et al., 2007), and infiltration into the CNS (Arevalo-Martin et al., 2003) reported in some of these studies provides an alternative explanation for the reduction in neuroinflammation seen following systemic CB2 agonist administration.

6.4.5 Evidence for CB2 receptor expression in the CNS

A review of the published literature, and the results of this study, allow the following conclusions to be drawn on the presence of CB2 receptors in the healthy and pathological CNS:

(1) There is no strong evidence for the widespread expression of CB2 receptors in the healthy CNS.

(2) There is no compelling evidence for the expression of functional CB2 receptors in CNS neurons, either in naïve or pathological states.
(3) Some evidence exists for CB2 receptor expression in peripheral DRGs and primary sensory afferents, which terminate in the dorsal horn of the spinal cord. There is also some evidence for increased function following neuropathic pain, but this was not apparent in the current study.

(4) There is strong evidence for microglial expression of CB2 receptors in vitro, and in at least some pathological conditions in vivo, notably EAE. It is possible that CB2 receptors are expressed by microglia in the early stages of neuropathic pain development, but this hypothesis is not supported by the current study.

6.4.6 Implications for future CB2 research

Clearly many of the current methodologies that have been employed to investigate the presence and function of CB2 receptors in the CNS under naïve or inducible conditions leave a lot of questions unanswered. It is most likely that any central expression of CB2 in the healthy CNS is limited, and that any upregulation may be condition and time dependent in neuropathologies, and therefore requires sensitive, yet robust methodologies if one wishes to accurately describe and quantify it.

One of the main issues in the current published work on CB2 receptor expression, as discussed above, is that assays are often performed on homogenized CNS tissues, producing two serious issues; contamination from peripheral blood cells, and loss of the ability to identify potential CB2 positive cell types. The first of these issues can be addressed by adequate perfusion of tissue to ensure minimal contamination, which could easily be assessed by confirming a lack of red blood cell markers in the extracted perfused tissue for example. The second, however, requires more consideration of the experimental design employed. The elegant study by Maresz et al., (2005) employed fluorescent assisted cell sorting (FACS) to sort homogenized brain and spinal tissue
samples from EAE mice into distinct immune cell populations, based on the expression profile of specific cellular markers, in order to determine the specific cell type within the CNS expressing CB2 receptor mRNA (Maresz et al., 2005). A similar approach with FACS and/or the related magnetic assisted cell sorting (MACS) would be of great value in studies exploring CB2 expression and upregulation in other models of neuropathology, including neuropathic pain models. Studies delving further than immune cell populations, and instead purifying distinct neuronal populations, could answer burning questions in the field, for instance: are the CB2 mRNA positive hippocampal neurons in the crab-eating macaque described by Lanciego et al. (2011) neural progenitors or mature neurons?; do CGRP expressing primary afferents terminating in the spinal cord dorsal horn also express the CB2 receptor, as the functional evidence from Sagar et al. (2005) and Beltramo et al. (2006) suggests they are?

Another classical method yet to be employed in the field is the use of radioligand binding directly targeting the CB2 receptor, of which an autoradiographic approach would generate crisp, clear images to analyze. While there is a distinct lack of radiolabeled cannabinoid ligands selective for the CB2 receptor, it is entirely possible to perform this assay in an indirect manner, by attempting to outcompete a bound radiolabeled ligand such as \([^3H]\)CP55,940 with unlabelled ligands selective for the CB2 receptor. A thorough study of this type in healthy or diseased animals, examining the entire CNS, would allow the identification of any small pockets of CB2 binding sites, clarifying whether any of the CB2 receptor mRNA identified in previous studies translates to functional binding domains. This was a method considered for the current study in place of the \([^{35}S]\)GTPyS assays, although it notably lacks the functionality and versatility associated with the assay format settled upon.

It is clear that no one technique is sufficient to rely upon in the characterization of this molecular target, and in a recent insightful review from the laboratory of Professor Ken Mackie, the pros and pitfalls of the various techniques used to describe CB2 receptor expression and function in the CNS are discussed in depth (Atwood et al., 2010). The review divides techniques in to functional,
biochemical, and anatomical techniques, and the authors state that “the most convincing studies will incorporate a combination of these techniques” (Atwood et al., 2010).
6.5 Suppression of microglia as a treatment strategy for neuropathic pain

If suppression of microglial activation via CB2 agonism could treat neuropathic pain, then other drugs that reduce microglial activation should have a similar effect. Minocycline is a tetracycline antibiotic that has immunosuppressive actions via suppression of microglial activation, attenuating iNOS upregulation (Yrjanheikki et al., 1998) and TNFα release (Lee et al., 2003), and suppressing the enzyme caspase 1, the protease responsible for cleavage of the inactive pro-IL-1β to the active pro-inflammatory IL-1β (Yrjanheikki et al., 1998). These intriguing, antibiotic-independent effects of minocycline have been assessed as a treatment strategy for neurological disease (Stirling et al., 2005; Plane et al., 2010), and provide a unique opportunity to study the role of microglia in the development and maintenance of central neuropathies, including neuropathic pain.

It was first reported by Raghavendra et al. (2003) that systemic minocycline, when given preemptively and then daily, could attenuate SNL-induced mechanical allodynia, in line with a significant reduction in IL-1β and TNFα levels in the lumbar spinal cord. It was also reported, however, that delay of minocycline treatment until after the development of allodynia (5 days) nullified any beneficial effects, despite similar reductions in pro-inflammatory cytokines (Raghavendra et al., 2003). Ledeboer et al. (2005) extended these findings, using the zymosan SIN model of inflammatory neuropathic pain. They reported that intrathecal administration of minocycline prevented the onset of, or partially reversed existing, acute SIN-induced allodynia, but could only delay the onset of chronic SIN-induced allodynia, and, consistent with the findings of Raghavendra et al. (2003) for the SNL model, had no effect on established chronic neuropathy (Ledeboer et al., 2005). Therefore, minocycline, presumably acting via suppression of microglia, appears to be solely preventative, and has no effect on established allodynia, a finding replicated again in the SNL model (Zhang et al., 2011b), and also reported in the CCI model (Padi et al., 2008), and an animal model of PHN (Zhang et al., 2011a).
While minocycline has also performed favorably in alternative models of neuropathy, inhibiting the development of allodynia induced by HIV (Wallace et al., 2007b) paclitaxel (Liu et al., 2010), burns (Chang et al., 2010), SCI (Cho et al., 2011) and diabetes (Pabreja et al., 2011), it should be noted that in these studies, minocycline was always administered preemptively, and was not tested on established allodynia. It appears that minocycline, an inhibitor of microglial activation, has no effect on established neuropathic pain, consistent with the hypothesis that microglia may be important for the development of neuropathic pain, while astrocytes may have a greater role in the long term maintenance of pain behaviors in the chronic state (Hald et al., 2009; Gosselin et al., 2010). If this is the case, then perhaps targeting of microglia with CB2 agonists or other suppressing agents is not a biorational strategy in the treatment of existing neuropathic pain conditions, as the window for therapeutic intervention may be prohibitively small. It is worth noting, however, that while minocycline is often considered a selective microglial inhibitor, it has also recently been shown to have direct effects on neuronal firing in the spinal cord and DRGs (Cho et al., 2006; Kim et al., 2011), which would have obvious implications in the study of the development and maintenance of pain behaviors, and the presumed role of microglia in these processes.

It is worth discussing at this point a study by Leichsenring et al. (2009), which investigated the effect of chronic low dose GW405833, on mechanical allodynia and spinal glial activation in the SNL model of neuropathic pain. Starting 2 days post SNL surgery, daily 0.1 mg/kg i.p. GW405833 attenuated the development of mechanical allodynia, coincident with a reduction in spinal microglial activation. Termination of GW405833 treatment resulted in a resurgence of mechanical allodynia and glial activation. While this study does not provide direct evidence for functional CB2 receptors in spinal microglia in neuropathic pain (suppression of peripheral immune response to nerve lesion by GW405833 is also possible), it does provide additional evidence that glial activation and neuropathic pain behaviors are linked, and that direct or indirect suppression of glial activation may result in transient attenuation of allodynia, but only in the development phase.
6.6 Final conclusions

This study has extensively evaluated both the efficacy of CB2 selective cannabinoid ligands, and the expression and functionality of the CB2 cannabinoid receptor in a well established animal model of neuropathic pain, the chronic constriction injury (CCI) model. It was found that only at high, potentially CB1 activating, doses, a CB2 selective agonist was effective in the alleviation of mechanical allodynia induced by CCI. Furthermore, no pharmacological or functional evidence for the CB2 receptor was found at the spinal level of the pain pathway in this model of neuropathy, also failing to support the hypothesis that spinal CB2 receptors are the target of drug action in this case. Moreover, a commonly employed CB2 receptor directed antibody was found to be non-specific for the CB2 receptor, questioning the integrity of results obtained in other studies using this tool.

The CB2 receptor is an important component in the endocannabinoid system, and clearly has a function in immunity, inflammation and in some cases, pain. It is essential to elucidate the true role of CB2 in pain, particularly of neuropathic origin, as new therapeutic strategies are essential in a condition which remains intractable to many current therapies. While the current study has produced negative results in relation to CB2 expression in the CNS, the use of a range of techniques and methodologies has strengthened the conclusions that may be drawn from it regarding the role of the CB2 receptor in neuropathic pain.
7 References


IASP (1979) Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. Pain 6(3): 249.


McHugh, D, Page, J, Dunn, E, Bradshaw, HB (2011) Delta(9) -THC and N-arachidonyl glycine are full agonists at GPR18 and cause migration in the human endometrial cell line, HEC-1B. *Br J Pharmacol*: In Press.


8 Appendix 1 – Publications arising from this thesis

Peer reviewed publications


Book chapters

Appendix 2 – Densitometry saturation plots

Figure 9.1 Densitometry saturation curve for Western blot experiments. (A) A pilot experiment of exposure time versus pixel density was performed to determine optimal exposure conditions. Western blot was performed as described on sham spinal cord samples with the Cayman Chemical CB2 receptor antibody, in the presence or absence of immunizing peptide, and the membranes exposed to ECL film for varying lengths of time. (B) A plot of raw densitometry values from all bands in the actual Western blot experiment, without immunizing peptide, performed with 1 min exposure to ECL film, confirms that bands generated and measured in this experiment were within the detectable linear range.
Figure 9.2 Densitometry saturation curve for \[^{35}\text{S}]\text{GTP}\gamma\text{S} experiments. (A) A pilot experiment of exposure time versus pixel density was performed to determine optimal exposure conditions. \[^{35}\text{S}]\text{GTP}\gamma\text{S} assay without agonists was performed as described on sham spinal cord sections, with standard or low GDP levels, and the membranes exposed to ECL film for varying lengths of time. (B) A plot of raw basal densitometry values from all sections in the actual \[^{35}\text{S}]\text{GTP}\gamma\text{S} experiment, performed in the presence of standard levels of GDP with 120 hours exposure to ECL film, confirms that the density of basal \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding measured in this experiment was within the detectable linear range.
Cannabinoids are lipophilic compounds that require solubilization in solvents, emulsifiers, or carrier molecules for parenteral administration. Combinations of solvents and emulsifiers are regularly used to dissolve cannabinoids, often followed by dilution in physiological saline or buffered solutions for drug administration. As solvents and emulsifiers contain potentially bioactive compounds, it is essential to assess the effects of vehicle combinations on the paradigms to be tested. While this study used a combination of 5% DMSO; 5% Cremophor® EL; 5% ethanol in saline (DCE vehicle) to dissolve cannabinoids for intrathecal injection without issue, it was discovered that a similar combination, 5% Cremophor® EL; 5% ethanol in saline (CE vehicle), intended as a systemic vehicle, was active when injected systemically. Systemic CE vehicle significantly reversed CCI-induced mechanical allodynia 1 hour post administration when delivered alone, compared to intrathecal DCE (80% vs. 5% reversal, p < 0.01) (Figure 10.1). The antinociceptive effects of ethanol and Cremophor® EL appeared to be limited to systemic delivery, as both DCE vehicle and moderate dose ethanol (8.3%) had no effect on CCI-induced allodynia when delivered intrathecally (p > 0.05) (Figure 10.1). While DCE therefore appeared to be an acceptable vehicle for intrathecal delivery, the antinociceptive effects of systemic CE precluded use of this vehicle for the systemic administration of cannabinoids in this case. Instead a carrier molecule, hydroxypropyl-β-cyclodextrin, was employed as the vehicle for systemic administration of cannabinoids, which had no significant effect on allodynia when delivered alone (p > 0.05) (Figure 10.1).
Figure 10.1. The effect of vehicle and administration paradigms on the reversal of CCI-induced mechanical allodynia. Data expressed as mean % reversal of allodynia ± SEM. Cyclodextrin = 25% hydroxypropyl-β-cyclodextrin dissolved in 0.9% saline; CE = 5% Cremophor® EL, 5% ethanol in 0.9% saline; DCE = 5% DMSO, 5% Cremophor® EL, 5% ethanol in 0.9% saline; E = 8.3% ethanol in dH₂O. i.p. = intraperitoneal; i.t. = intrathecal. ** = p < 0.01; CE (i.p.) vs. DCE (i.t.)Nb. Data for the E (i.t.) group was only available for the 3 hour time point. n = 6 for cyclodextrin; n = 4 for CE; n = 13 for DCE; n = 6 for E.

The finding that low dose ethanol and cremophor are anti-nociceptive when delivered systemically has far reaching implications for the cannabinoid and pain fields, as these compounds are commonly used as vehicles for lipophilic drug solubilization. It has previously been shown that systemic ethanol is analgesic in mice, increasing latency in the hotplate test via an NMDA-dependent mechanism (Mogil et al., 1993). This effect was seen with 2.5 ml/kg, which is far greater than the 50 µl/kg used in the current experiment, although lower doses were not tested by Mogil et al. (1993). Cremophor® EL is made by reacting castor oil with ethylene oxide, and contains a heterogeneous mixture of glycol based compounds. It has been shown that some compounds within cremophor are biologically active, and can activate complement cascades within peripheral immune cells in order to illicit a hypersensitivity reaction in some humans (Szebeni et al., 1998). While this does not provide a mechanism for possible cremophor-induced antinociception, it does highlight the uncharacterized nature of this potentially active vehicle.
It is essential to determine the safety of drugs destined for clinical application. As CB2 agonists are a relatively new class of compounds, with minimal clinical use thus far, it was deemed necessary to determine any possible toxicological effects of chronic CB2 agonist administration. The effects of daily systemic administration of low dose GW405833 over 8 days on weight, water intake, liver enzyme activity and wet organ weight were determined in parallel to the pharmacological intervention studies outlined in Chapter 3. GW405833 was dissolved to 1 mg/mL in 25% hydroxypropyl-b-cyclodextrin in 0.9% saline following acidification and rebalancing to pH 5.3 – 7.4, and administered i.p. at 1 ml/kg. Naïve Wistar rats were randomly assigned to receive either drug or vehicle, and were dosed between 1100 and 1300 hours each day. Body weight and water consumption were recorded daily prior to drug injection. Twenty four hours post injection on day 8, animals were sacrificed via decapitation following brief CO\textsubscript{2} anaesthesia. Blood was collected from the carotid arteries, and spun at 3000 rpm for 5 min to remove blood cells, before storage of resultant serum at -20 °C for alanine aminotransferase (ALT) measurement – a determinant of liver enzyme function. Furthermore, primary organs (heart, liver, lungs, spleen, kidneys and testes) were weighed and weights converted to percentage of body weight. For the ALT assay, 100 µl of serum from each animal was combined with 1 mL ALT (GPT) reagent (Thermo Scientific, VA, USA), and absorbance read at 340 nm in a SmartSpec™ Plus spectrophotometer (Biorad, USA) at baseline, and again after 3 min incubation at RT. Samples were run in duplicate, and ALT activity determined by calculating the rate of absorbance change per minute at 340 nm.
Two-way ANOVA analysis of body weight and water consumption revealed the effect of GW405833 on these factors was not significant compared to vehicle alone (p = 0.5912 and p = 0.7851, respectively). There was no statistically significant difference in ALT activity of serum from vehicle and drug treated animals when analyzed with unpaired two tailed t test (37.82 ± 2.335, and 32.41 ± 2.406 U/L, respectively, T = 1.611, p = 0.1382), indicating no liver toxicity. Wet organ weights at sacrifice of drug and vehicle treated animals were analyzed with unpaired two tailed t tests, and are outlined in Table 4. While there were no significant differences in liver, lung or testes weights, there was a significantly greater mean heart and spleen weight in the GW405833 versus vehicle group (+14.65% ± 4.12, p = 0.005; and +15.93% ± 5.84, p = 0.022, respectively). Furthermore, drug treated animals, on average, had modestly larger kidneys at sacrifice than vehicle treated animals, however this difference was not significant (+7.65% ± 3.62, p = 0.061).

Table 4. Organ weights of Wistar rats following daily administration of GW405833 or vehicle. GW405833 was administered at a dose of 1 mg/kg, and drug and injection volumes were 1 ml/kg. Vehicle was 25% hydroxypropyl-β-cyclodextrin in 0.9% saline. Organ weights were normalized to total body weight. Unpaired, 2-tailed T-tests were used to compare normalized values, and p values less than 0.05 were considered significant (bold).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Vehicle</th>
<th>GW405833</th>
<th>% Difference</th>
<th>T value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.310 ± 0.012</td>
<td>0.355 ± 0.005</td>
<td>+14.65 ± 4.12</td>
<td>3.556</td>
<td>0.005</td>
</tr>
<tr>
<td>Liver</td>
<td>3.266 ± 0.097</td>
<td>3.308 ± 0.060</td>
<td>+1.28 ± 3.48</td>
<td>0.369</td>
<td>0.720</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.529 ± 0.068</td>
<td>0.501 ± 0.026</td>
<td>-5.43 ± 13.74</td>
<td>0.395</td>
<td>0.701</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.206 ± 0.011</td>
<td>0.239 ± 0.006</td>
<td>+15.93 ± 5.84</td>
<td>2.723</td>
<td>0.022</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.692 ± 0.015</td>
<td>0.745 ± 0.020</td>
<td>+7.65 ± 3.62</td>
<td>2.114</td>
<td>0.061</td>
</tr>
<tr>
<td>Testes</td>
<td>1.058 ± 0.113</td>
<td>1.088 ± 0.032</td>
<td>+2.81 ± 11.08</td>
<td>0.254</td>
<td>0.805</td>
</tr>
</tbody>
</table>
The finding that chronic GW405833 administration caused a statistically significant increase in spleen and heart weight was unexpected, and is not readily explained. These results may be specific for GW405833, although additional unpublished observations from the Ashton laboratory have indicated that another CB2 agonist, JWH-133, may be cardioactive. In the presence and absence of noradrenalin, JWH-133 significantly altered the rate of contraction in isolated rat atria. These findings, in addition to those of the current toxicological results, must be explored in more detail if CB2 agonists are to be widely employed in the clinic.