Attenuation of morphine tolerance, reward, and spinal gliosis in neuropathic pain by ultra-low dose alpha2-adrenergic antagonists

By

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Abstract

Introduction: Opioid use to treat chronic pain is limited by the development of tolerance and increased risk of side effects as dosages are increased to compensate for loss of analgesia. Ultra-low dose (ULD) α₂-adrenergic receptor (AR) antagonists appear to attenuate tolerance, though have only been tested in pain naïve rats, never in chronic pain models, and effects on the affective components of pain have never been assessed.

Aims: To determine the effects of ULD α₂-AR antagonists on: i) sensory responses in morphine tolerant and nerve injured rats, ii) chronic morphine and nerve-injury induced spinal gliosis and neuronal activation, iii) morphine conditioned place preference (CPP), a paradigm that assesses the affective or emotional component of pain processing.

Methods & Results: In a model of opioid tolerance, ULD atipamezole attenuated the loss of morphine antinociception in pain naïve rats, consistent with the literature. Next, a model of neuropathic pain (chronic constriction injury (CCI)) was employed and changes in responses to mechanical and thermal nociceptive stimuli were tracked over time. This was the first study to show positive effects of ULD α₂-AR antagonists in alleviating pain hypersensitivity associated with nerve injury and attenuation of morphine tolerance in neuropathic animals. Using immunohistochemistry, tissue collected from all the animals was labeled to determine if molecular changes correlated with the behavior induced by ULD atipamezole. Morphine and CCI-induced gliosis in the spinal dorsal horn were attenuated in animals chronically administered ULD atipamezole. Neuronal activity inferred by c-Fos cell counts was likewise attenuated in neuropathic animals. Finally, through the use of the CPP paradigm, it was shown ULD atipamezole is neither rewarding nor aversive on its own, but disrupts the development of morphine CPP in CCI animals, but not the sham or pain naïve animals. Currently, experimental
evidence suggests a reduction in opioid reward in the neuropathic rats.

**Conclusion** ULD $\alpha_2$-AR antagonist atipamezole inhibits morphine tolerance and enhances opioid analgesia in pain naïve and chronic pain states, alleviates the mechanical hypersensitivity following nerve injury, inhibits chronic morphine and nerve injury-induced glial and neuronal activation in the spinal dorsal horn, and disrupts opioid reward in chronic pain states.
Co-Authorship

The research upon which this thesis is based was conducted by Patrick Grenier under the supervision of Dr. Catherine M. Cahill and Dr. Mary C Olmstead. Ms. Lihua Xue aided with perfusion of some animals.
ACKNOWLEDGEMENTS

This road has sometimes been a long and painful one, but having finally reached its end (or at least the next fork), much credit is due to the many people who have helped me along the way. Some of the help has been of a professional nature, providing lamplight and signposts for me to find my way. Some of it has been financial; without provisions and shelter this journey would not have been possible. And finally, the hardest to quantify but undoubtedly the most valuable, has been the emotional support and inspiration of people I know personally, but also those I don’t. Many Long Dark Teatimes of the Soul were overcome by sympathetic ears when I had people to talk to and by my love for film and literature when I did not.

Thank you to Cathy and Cella for allowing me the opportunity to perform this work and for helping give it some direction. My best memories will be the conferences I was lucky enough to go to and I know not every graduate student gets those experiences. Being given the opportunity to see Italy and Greece and to present this work to researchers I have a great deal of respect for would not have been possible otherwise. After Cathy left and I no longer had a home, Cella invited me into her own, for which I am very grateful. I also need to acknowledge Dr. Jhamandas and Dr. Milne for always providing valuable insight on these and other topics, both from a basic science and a clinical perspective, Dr. Racz for giving me plenty of opportunities to TA over the years, Dr. Reynolds for being part of my committee and giving his own perspective on this work, Dr. Winn who has helped me navigate administrative issues, and the rest of the Departments of Pharmacology & Toxicology and Psychology. Thank you also to Dr. Stone who kindly agreed to be an external examiner on quite short notice.

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To Mom, Dad and Erica, thank you for being patient with me for so long. Now I can actually get a “real job”. I don’t have the space here to highlight everything you have done for me, but please know I wouldn’t have gotten where I am without you and your sacrifices have meant a lot to me.

Kathryn, Kate, Alain, Liam, and Mike: even though I don’t get to see you as much I would like, know that you have been some of the most important people in my life and I wish you all the best. Maybe I will finally have the time and means to pay more visits in the future.

Finally, the ambitions of many great writers and filmmakers have provided much of the motivation for me to create work I am proud of and that will survive after I am gone. During this final year, especially, when stress levels were high and moods were low, I have spent a disproportionate amount of time watching old movies and reading things that have nothing to do with this thesis work. Re-reading Gravity’s Rainbow and Infinite Jest, and having marathons of old Tarkovksy films might not seem like a wise use of time, but often they were the things that kept me centered and motivated.

This sentence is the end of the road less traveled.
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<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>α₁-AR</td>
<td>alpha-1 adrenergic receptor</td>
</tr>
<tr>
<td>α₂-AR</td>
<td>alpha-2 adrenergic receptor</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
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<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
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<td>Amy.</td>
<td>amygdala</td>
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<td>analysis of variance</td>
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<td>area of interest</td>
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<tr>
<td>AR</td>
<td>adrenergic receptor</td>
</tr>
<tr>
<td>Ati</td>
<td>Atipamezole</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>β₁-AR</td>
<td>beta-1 adrenergic receptor</td>
</tr>
<tr>
<td>β₂-AR</td>
<td>beta-2 adrenergic receptor</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
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<tr>
<td>BOLD</td>
<td>blood-oxygen level dependent</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Ca²⁺</td>
<td>calcium ion</td>
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<td>calcium/calmodulin-dependent protein kinase</td>
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<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CB</td>
<td>cannabinoid</td>
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<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
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<td>calcitonin gene-related peptide receptor</td>
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<td>cisplatin-induced peripheral neuropathy</td>
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<td>Cl⁻</td>
<td>chloride ion</td>
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<td>caudate nucleus</td>
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<td>conditioned place aversion</td>
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<td>CPP</td>
<td>conditioned place preference</td>
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<td>CPS</td>
<td>Canadian Pain Society</td>
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<td>DAG</td>
<td>diacyl glycerol</td>
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<td>DAMGO</td>
<td>[D-Ala², N-MePhe⁴, Gly-ol⁵]-enkephalin</td>
</tr>
<tr>
<td>δ</td>
<td>delta</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MCOPPB</td>
<td>1-[1-(1-methylcyclooctyl)-4-piperidinyl]-2-(3R)-3-piperidinyl-1H-benzimidazole</td>
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<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>min.</td>
<td>minutes</td>
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<td>μ</td>
<td>mu</td>
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<tr>
<td>MOR</td>
<td>mu-opioid receptor</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>morphine sulfate</td>
</tr>
<tr>
<td>NA</td>
<td>nucleus accumbens</td>
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<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>NC</td>
<td>neocortex</td>
</tr>
<tr>
<td>NeuPSIG</td>
<td>Neuropathic Pain Special Interest Group</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NK1</td>
<td>neurokinin 1</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NNT</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>neuropathic</td>
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<tr>
<td>NRM</td>
<td>nucleus raphe magnus</td>
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<td>OIH</td>
<td>opioid-induced hyperalgesia</td>
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<tr>
<td>OR</td>
<td>opioid receptor</td>
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<tr>
<td>ORL-1</td>
<td>opioid receptor-like-1</td>
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<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>pERK</td>
<td>phosphorylated extracellular signal-regulated kinase</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PF</td>
<td>propentofylline (3-methyl-1-(5-oxohexyl)-7-propyl-3,7-dihydro-1H-purine-2,6-dione)</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PFC</td>
<td>prefrontal cortex</td>
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<tr>
<td>PHN</td>
<td>post-herpetic neuralgia</td>
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<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PJ34</td>
<td>N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride</td>
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<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>PSNL</td>
<td>partial sciatic nerve ligation</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>RVM</td>
<td>rostral ventromedial medulla</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>SI</td>
<td>primary somatosensory cortex</td>
</tr>
<tr>
<td>SII</td>
<td>secondary somatosensory cortex</td>
</tr>
<tr>
<td>Sal</td>
<td>saline</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>SCN</td>
<td>sciatic cryoneurolysis</td>
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<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIN</td>
<td>sciatic inflammatory neuritis</td>
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<tr>
<td>SN</td>
<td>substantia nigra</td>
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<td>SNC80</td>
<td>4-[(R)-<a href="3-methoxyphenyl">(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl</a>methyl]-N,N-diethylbenzamide</td>
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<td>spared nerve injury</td>
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<tr>
<td>SNL</td>
<td>spinal nerve ligation</td>
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<tr>
<td>SNRI</td>
<td>serotonin norepinephrine reuptake inhibitor</td>
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<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
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<tr>
<td>STZ</td>
<td>streptazotocin</td>
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<td>TB</td>
<td>tris buffer</td>
</tr>
<tr>
<td>TBC</td>
<td>tibial bone cancer</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TCA</td>
<td>tricyclic antidepressant</td>
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<tr>
<td>THC</td>
<td>delta-9-tetrahydrocannabinol</td>
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<tr>
<td>TIPN</td>
<td>Taxol-induced peripheral neuropathy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Trk</td>
<td>tyrosine receptor kinase</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>ULD</td>
<td>ultra-low dose</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage-dependent calcium channel</td>
</tr>
<tr>
<td>VIPN</td>
<td>vincristine-induced peripheral neuropathy</td>
</tr>
<tr>
<td>VP</td>
<td>ventral pallidum</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>WDR</td>
<td>wide dynamic range</td>
</tr>
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</table>
CHAPTER 1:
GENERAL INTRODUCTION

1.1 Pain

In animals and humans, pain plays an important protective role in survival by warning the body of injury and driving the body away from the source of damage. Afferent signals relay sensory information from the periphery to the spinal cord, then up to the brain stem and higher brain regions where the nociceptive signal is interpreted as painful. Efferent motor signals are then relayed back down the spinal cord and then to the periphery allowing the organism to move away from danger.

1.1.1 Primary sensory afferents

Primary sensory afferents respond to external stimuli including touch, temperature, nociception, and proprioception (the body’s position in space). The dorsal root ganglia (DRG) contain the cell bodies of these primary afferent neurons, which project from the periphery to the spinal cord. The diameter of the neuron and whether or not it is coated in a myelin sheath dictate its conduction velocity. The speed of nerve impulse propagation and the temperature thresholds vary somewhat among mammalian species (Miyamoto et al., 1990).

Different types of primary sensory afferents respond to different stimuli. Aδ and C fibers are primarily involved in the propagation of a nerve impulse following nociceptive stimulation. Some studies, however, have shown C fibers can be engaged by light touch (Olausson et al., 2002). Most Aβ fibers, which primarily respond to light touch and play a role in proprioception, do not respond to nociceptive stimuli, but help maintain the tonic inhibitory tone of other nociceptive fibers, and appear to be important in the development of allodynia (a painful
response to a normally innocuous stimulus) following peripheral nerve injuries (Zimmerman, 2001). There is some often overlooked evidence that is not present in textbooks and most of the literature, however, that small populations of mammalian Aβ fibers do, in fact, have nociceptive activity of their own (Djouhri and Lawson, 2004).

In general, Aβ fibers have the largest diameter cell body of the three groups of sensory afferents and are coated in a myelin sheath, giving them very fast conduction velocity (Le Pichon and Chesler, 2014). Aδ fibers are also myelinated, but their smaller diameter results in somewhat slower conduction velocity. C fibers are unmyelinated and have a very small diameter, making them the slowest at conducting a nerve impulse by a large margin. Because of the different conduction velocities, myelinated Aδ fibers are responsible for the first sensation of pain, which is generally sharp and transient, while the unmyelinated C fibers fire more slowly and are responsible for the more diffuse, longer lasting sensation of pain that occurs following injury, often described as a burning or aching type of pain. Unmyelinated C fibers primarily respond to nociceptive stimulation of all types, including chemical, mechanical, and thermal, while Aδ fibers respond primarily to nociceptive stimuli of thermal and mechanical origin, and also respond to non-nociceptive heat and cold. In humans, cutaneous temperatures higher than 43°C are perceived as painful (LaMotte and Campbell, 1978), and these temperatures correspond to firing of afferent nerve fibers (Raja et al., 1999) and activation of capsaicin-sensitive transient receptor-potential vanilloid 1 (TRPV1) receptors (Caterina et al., 1997). Chemical sensitive primary afferents synapse on secondary interneurons in laminae I and II of the spinal dorsal horn (Janscó and Király, 1980). Primary sensory afferent fibers can be classified as peptidergic or non-peptidergic based on the neurotransmitters they produce and varying responses to nociceptive and non-nociceptive stimuli depend on the receptors the fibers express. Peptidergic fibers
produce and release peptides such as substance P and calcitonin gene-related peptide (CGRP). Aδ and C fibers consist of both peptidergic and non-peptidergic populations that project to distinct areas of the spinal dorsal horn, while Aβ fibers are primarily non-peptidergic.

Regardless of the type of stimuli that activates a peripheral nociceptor, a nerve impulse is propagated along the length of the axon toward the nerve terminal allowing communication between two cells through synaptic neurotransmission. Figure 1.1 shows the projection of the different types of primary sensory afferents involved in nociceptive signaling and where they synapse on neurons within the spinal dorsal horn. Table 1.1 summarizes the properties of each type of sensory afferent.
Figure 1.1 Primary sensory afferent projections to the spinal cord. The spinal cord is divided into ten distinct laminae. The spinal dorsal horn is comprised of laminae I-VI. Large diameter, myelinated Aβ fibers, medium diameter, myelinated Aδ fibers, and small diameter, unmyelinated C fibers project to distinct laminae within the spinal dorsal horn.
<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Sheathing</th>
<th>Relative Conduction Velocity</th>
<th>Relative Size</th>
<th>Sensory Responses</th>
<th>Projects to</th>
<th>Neurotransmitters/Neuropeptides and Receptors expressed</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>Myelinated</td>
<td>Fast</td>
<td>Large</td>
<td>-Vibration</td>
<td>-Laminae III, IV, V (mainly non-peptidergic)</td>
<td>-Most are non-peptidergic but some may show low expression of substance P, CGRP.</td>
<td>-Tonic inhibition of nociceptive inputs</td>
</tr>
<tr>
<td>Aδ</td>
<td>Myelinated</td>
<td>Medium</td>
<td>Medium</td>
<td>-Thermal (noxious heat and cold)</td>
<td>-Laminae I, II V (peptidergic and non-peptidergic)</td>
<td>-Glutamate -Type II: TRPV1 expressing (low thermal threshold, capsaicin sensitive) -Type I: TRPV2 expressing (higher thermal threshold, lower mechanical threshold, capsaicin insensitive)</td>
<td>-“first pain”</td>
</tr>
<tr>
<td>C</td>
<td>Unmyelinated</td>
<td>Slow</td>
<td>Small</td>
<td>-Thermal</td>
<td>-Laminae I, II V (peptidergic and non-peptidergic)</td>
<td>-Glutamate -Substance P, CGRP (peptidergic) -trkA neurotrophin receptor, TRPV1 (peptidergic) -IB4 (non-peptidergic)</td>
<td>-“second pain”, itch</td>
</tr>
</tbody>
</table>

Table 1.1: Properties of primary sensory afferents. The table denotes the sheathing, diameter, conduction velocity, and projections to the spinal dorsal horn of primary sensory afferents involved in nociceptive transmission. The neurotransmitters and neuropeptides produced by each cell type define the sensory information the cell will respond to and relay to the spinal cord. CGRP = calcitonin gene-related peptide, IB4 = isolectin B4, trk = tyrosine receptor kinase, TRPV = transient receptor potential vanilloid. Adapted from Djouhri and Lawson, 2004.
1.1.2 Neurotransmission

The resting membrane potential of a neuron is maintained by sodium- and potassium-gated ion channels that regulate membrane polarity. Thermal, mechanical, or chemical stimuli cause activation of peripheral nociceptors, initiating depolarization of the primary sensory afferents, thus causing propagation of an action potential along the axonal length of the cell toward the dorsal horn of the spinal cord where it synapses on a secondary projection neuron.

Depolarization causes an influx of calcium ions at the nerve terminal, resulting in release of neurotransmitters and neuropeptides into the synaptic cleft. Neurotransmitter molecules released into the synapse can bind to respective post-synaptic receptor sites, leading to a wide range of actions depending on the receptor activated. Glutamate, the primary excitatory neurotransmitter in the nervous system binds α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors post-synaptically, causing depolarization of the post-synaptic neuron and continued propagation of the nerve impulse (Mayer et al., 1999).

1.1.3 Ascending systems involved in nociceptive transmission

Second order neurons in the spinal cord project to the brain stem and higher brain areas and are modulated by descending inhibition. These second order neurons are classified according to the types of afferents they receive input from. Wide dynamic range (WDR) neurons receive inputs from both nociceptive and non-nociceptive afferents, while others receive input only from nociceptive Aδ or C fibers.

Three main tracts project to the brain: the spinothalamic tract, the spinoreticular tract, and the spinoparabrachial tract. The spinothalamic tract consists of second order neurons originating in laminae I, IV, V, and VI of the spinal dorsal horn, while the spinoreticular tract consists of
second order neurons originating from laminae VII and VIII. Second order neurons in the lateral spinothalamic tract project to the thalamus and synapse on third order neurons that project to the primary and secondary somatosensory cortices, which are primarily responsible for the sensory component of the stimulus interpretation. Second order neurons in the medial spinoreticular tract also project to the thalamus, but the third order neurons synapse with projections to the insula and the anterior cingulate cortex (ACC), limbic structures responsible for the affective or emotional components of pain processing (how one feels about the pain), and memory of past painful events. Second order neurons in the medial spinoreticular tract can also synapse with other neurons in the brain stem, primarily within the periaqueductal grey (PAG) and the nucleus raphe magnus (NRM), areas involved in modulation of pain through descending inhibition by noradrenergic and serotonergic systems. The spinoparabrachial tract projects from the superficial dorsal horn to the parabrachial nuclei between the pons and midbrain, then projects to the nucleus accumbens (NA) and amygdala as well (Bushnell et al., 2013). These higher brain regions are crucial for turning a simple nociceptive stimulus into a more complex interpretation of pain, including its location, intensity and unpleasantness.

1.1.4 Processing of pain in the brain

While many blood-oxygen level dependent (BOLD) functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies in humans have attempted to elucidate a “pain matrix” (Bushnell and Apkarian, 2005; Peyron et al., 2000), it has become increasingly apparent that the interpretation of imaging data must be considered carefully (Legrain et al., 2011). The same areas of the brain that appeared to show activation to nociceptive stimuli, including the secondary somatosensory cortex, the insula, and the ACC, have now been shown to respond to many types of sensory inputs, noxious or not. Visual and
auditory (Mouraux et al., 2011), and tactile (Liu et al., 2008) stimuli all cause similar activation patterns in the brain compared to a nociceptive stimulus, confirming that the network is not entirely pain-specific (Mouraux and Iannetti, 2009) and appears to be a more generalized system of salience detection (Legrain et al., 2011).

Despite the fact that a specific “pain matrix” may not exist, it is accepted that many different brain regions are at least important in pain processing. The amygdala, which is within the temporal lobe, plays a role in fear response and modulates pain in stress situations. It has projections to the brain stem, the thalamus and higher cortical regions (Veinante et al., 2013). The nucleus accumbens and ventral tegmental area (VTA) make up part of the mesolimbic dopaminergic reward system (Fields et al., 2007), dysfunction of which occurs during chronic pain leading to negative affective or emotional states associated with anhedonia and depressive mood disorders (Nicholson and Verma, 2004; Elman et al., 2013). Other limbic structures involved in affective pain processing include the septum, ventral pallidum, and hippocampus (Cahill et al., 2014b). Nociceptive or sensory discriminative processing in the brain, which interprets information about the location and intensity of a pain stimulus, involves the somatosensory cortices, PAG and the thalamus. The thalamus, which acts as an intermediary “relay” station that receives ascending inputs from the spinal cord, projects to cortical regions while also modulating affective and emotional responses to pain as well (Aziz and Ahmad, 2006). Changes in functional connections between the limbic systems involved in affective pain processing and cortical regions involved in sensory discrimination predict development of chronic pain states (Baliki et al., 2012). The frontal cortex becomes hyperactivated in chronic pain (Apkarian et al., 2001) and a loss of grey matter density is observed over time within prefrontal and thalamic regions of the brain (Apkarian et al., 2004).
1.2  **Nociception and pain are not equivalent**

Pain can occur without a nociceptive stimulus, for example in phantom limb pain (Subedi and Grossberg, 2011), and a nociceptive stimulus is not always interpreted as “painful” (Lee et al., 2009), and non-noxious stimuli may become noxious if stimulation occurs repeatedly over time through temporal summation or wind-up, or when applied to a large area, thus affecting a larger population of sensory afferents (spatial summation).

In humans, repeated heat (Nielsen and Arendt-Nielsen, 1998), cold (Mauderli et al., 2003), ultrasonic (Wright et al., 2002), electrical (Arendt-Nielsen et al., 2000) and mechanical (Nie et al., 2005) stimuli cause tissue sensitization leading to pain. Spatial summation of mechanical (Defrin et al., 2003; Greenspan et al., 1997) and thermal (Defrin and Urca, 1996) stimuli leads to lower nociceptive thresholds and sensitization. Both spatial and temporal summation appear to be important in chronic widespread pain syndromes (Nie et al., 2009).

1.3  **Central sensitization and chronic pain**

Pain serves an important biological function by warning the body of harm, but injury or illness can cause these processes to become maladaptive and persistent, and chronic pain is considered a disease state. Central sensitization is defined as an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy as well as reduced inhibition, and is a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation, and neural injury (Latremoliere and Woolf, 2009). Central sensitization occurs both within the spinal cord and in regions of the brain including the limbic system and cortical areas leading to maladaptive sensory and emotional processing of pain (Costigan et al., 2009).
Under normal physiological conditions, the post-synaptic N-methyl-D-aspartate (NMDA) receptor is inactive due to the presence of a magnesium ion plug, however repeated stimulation causes removal of the plug, allowing activation of the receptors by excitatory glutamate (Riley and Boulis, 2006). NMDA receptors in the spinal cord and higher brain regions including the amygdala, the hippocampus and medial septum play an important role in long-term potentiation (LTP), both in learning and memory (Izquierdo, 1994; Rockstroh et al., 1996; Rowland et al., 2005), and also in central sensitization following nerve injury. While most of the literature describes post-synaptic NMDA receptors in the brain and spinal cord, it has been shown that they are also expressed in the periphery on small diameter DRG neurons that propagate nociceptive signals (Hummel et al., 2008b).

Postsynaptic NMDA receptor activation and activation of the neurokinin-1 (NK1) receptor by substance P causes an influx of calcium ions, leading to activation of the enzyme nitric oxide (NO) synthase (NOS), which converts the amino acid arginine to citrulline with the release of NO. NO diffuses from the post-synaptic neuron and is taken up presynaptically to act in a retrograde fashion on soluble guanylyl cyclase, leading to an increase in intracellular cyclic guanosine monophosphate (cGMP). Increased cGMP enhances release of excitatory neurotransmitters and substance P. NO also increases post-synaptic excitability and activates microglia and astrocytes, leading to a shift from quiescent to an “activated” or pro-inflammatory state (Latremoliere and Woolf, 2009; Mayer et al., 1999). Genetic deletion or knock-down of NOS with pharmacological agents has been shown to inhibit the development of mechanical allodynia in mice following nerve injury (Guan et al., 2007), confirming its importance in chronic pain development. Fig. 1.2 shows a diagram of changes in signaling in chronic pain.

10
Fig. 1.2 Alterations in neurotransmission in chronic pain states.

1. Activation of thermal, chemical or mechanical nociceptors causes depolarization and propagation of an action potential along the primary sensory afferent to the axon terminal.

2. Depolarization causes voltage-gated calcium channels (VGCC) to open and Ca\(^{2+}\) to enter the cell.

3. Calcium influx triggers extravasation of vesicles containing excitatory neurotransmitters and peptides including glutamate, substance P, and calcitonin-gene related peptide (CGRP) into the synapse.

4. Substance P binds post-synaptic neurokinin-1 (NK1) receptors, CGRP binds CGRP-R, and glutamate binds metabotropic glutamate receptors (mGluR) (6), all of which are G-protein-coupled receptors (GPCRs).

5. CGRP-R activation activates adenylyl cyclase (AC), which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA).

6. NK1-R activation also leads to the production of PKA through the AC signaling pathway, but also activates phospholipase C (PLC), which cleaves membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into diacyl glycerol (DAG) and inositol triphosphate (IP\(_3\)). mGluR activation by glutamate activates the PLC pathway as well.

7. DAG activates protein kinase C (PKC) which, along with PKA increase sodium influx through AMPA receptors that are normally activated by excitatory glutamate.

8. In acute nociceptive pain, the NMDA receptor is inactive due to the presence of a magnesium ion plug, but repeated stimulation leads to PKC removing the plug and the NMDA receptor becomes active, responding to excitatory glutamate to allow post-synaptic calcium influx.

9. Increased intracellular calcium leads to activation of nitric oxide (NO) synthase, which catalyzes the conversion of arginine to citrulline with the release of NO.

10. NO diffuses to act on the post-synaptic cell to increase neuronal excitability, and pre-synaptically increases excitatory neurotransmitter release.

11. Under normal physiological conditions, glutamate transporter 1 (GLT-1) and glutamate-aspartate transporter (GLAST) are expressed on astrocytes and remove glutamate from the synaptic cleft, but activation of astrocytes by inflammatory mediators causes down-regulation of the glutamate transporters and build-up of excess glutamate in the synapse. This sensitizes the neurons and further recruits and activates microglia and astrocytes.
1.4 Modulation of pain

Modulation of pain occurs through several endogenous systems including the opioid, cannabinoid, and adrenergic receptor systems, all of which involve different classes of G-protein-coupled receptors (GPCRs). An overview of GPCR signaling and function will be followed by in-depth looks at the noradrenergic systems involved in descending inhibiton pathways, the endogenous opioid system and the problems with tolerance and hyperalgesia that limit opioid use in the treatment of chronic pain, and finally the ways these two systems interact and modulate each other.

1.4.1 Overview of GPCRs

GPCRs are serpentine receptors with seven helical transmembrane domains with both a C (cytosolic)- and N-terminus (facing the extracellular space), and are coupled to G-proteins that consist of trimeric alpha, beta, and gamma subunits (Venkatakrishnan et al., 2013). They regulate two main signaling pathways: adenylyl cyclase/cyclic adenosine monophosphate (cAMP) signaling, and phosphatidylinositol pathways. Extracellular ligand binding to a GPCR induces a conformational change at the intracellular side, leading to G-protein activation. In the case of stimulatory G-proteins (G_S), guanosine diphosphate (GDP) bound to the alpha subunit of the G-protein is displaced by guanosine triphosphate (GTP), causing the alpha subunit of the G-protein to dissociate from the beta-gamma dimer (Hamm and Gilchrist, 1996). The alpha subunit moves to activate the membrane-bound enzyme adenylyl cyclase (AC), which catalyzes cAMP formation. cAMP activates protein kinase A (PKA) that further phosphorylates intracellular proteins (Gainetdinov et al., 2004). The proteins activated by PKA are diverse but they all contain a common serine or threonine phosphorylation site. The dissociated beta/gamma
dimer plays an important role in recycling of the receptor complex by recruiting G protein-coupled receptor kinases (GRKs) that phosphorylate the C-terminus of the receptor leading to β-arrestin binding, dissociation of the beta/gamma dimer from the receptor, and internalization of the receptor through endocytosis (Gainetdinov et al., 2004). β-arrestin binding initiates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways. Once internalized, β-arrestin dissociates and the receptor can be recycled to the membrane (Ferguson et al., 1996, 1998; Zhang et al., 1997). β-arrestin binding and receptor internalization was believed to be the main mechanism through which tolerance develops, which will be discussed in more detail later. cAMP is eventually broken down by phosphodiesterases and Gs is inactivated by hydrolysis of the bound GTP back to GDP. The affinity of the ligand for the receptor varies depending on whether GTP or GDP is bound to the G-protein. In its inactive state (GDP-bound), ligand affinity is highest, but once it is GTP-bound, the affinity of the ligand for the receptor is low, causing dissociation. Recycling of the receptor takes time and prolonged stimulation can cause desensitization that will result in decreased response to the ligand or activation of alternate signaling pathways due to lack of functional receptors at the membrane (Böhm et al., 1997).

Depending on chemical structure and concentration, ligands can exert differential effects on multiple receptors systems and the effects of ligand-binding through GPCR signaling can be tissue specific. For example, norepinephrine is an agonist for β1 and β2-adrenergic receptors, which signal though Gs-mediated pathways, but it also activates α2-adrenergic receptors, which signal through Gi-mediated pathways, and α1-adrenergic receptors which signal through Gq.

Inhibitory GCPRs including opioid and α2-adrenergic receptors (which will be discussed in more detail later) are normally coupled to inhibitory G-proteins (Gi/o), and their activation leads to inhibition of adenylyl cyclase and cAMP signaling pathways. The Gβγ subunit released
from the inhibitory G-proteins following opioid receptor activation hyperpolarizes the neuron by inhibiting voltage-dependent calcium channels (VDCCs; Saegusa et al., 2000) and by activating inwardly-rectifying potassium channels (GIRKs; Ikeda et al., 2000). Thus, activation of pre-synaptic opioid receptors, for example, by agonists inhibits the release of excitatory amino acids. Post-synaptic opioid receptor activation hyperpolarizes the cell, making it harder for a nerve impulse to be propagated.

$G_q$ signaling, which occurs following $\alpha_1$-adrenergic receptor activation, for example, occurs through an alternate signaling pathway. Ligand binding causes a conformational change and exchange of GDP for GTP on the intracellular G-protein and dissociation of the alpha subunit from the beta/gamma dimer, as is the case for stimulatory $G_S$; however instead of activating adenylyl cyclase, membrane-bound phospholipase C (PLC) is activated, causing cleavage of phosphatidylinositol 4,5 bisphosphate (PIP$_2$) to diacylglycerol (DAG), which remains membrane-bound, and inositol triphosphate (IP$_3$), which is released into the cytosol. The cytosolic IP$_3$ attaches to a binding pocket on calcium channels on the endoplasmic reticulum (ER), causing them to open and release calcium ions into the cytosol. These calcium ions, along with the membrane-bound DAG, activate protein kinase C (PKC) leading to phosphorylation of multiple substrates through a kinase signaling cascade. High intracellular calcium ion concentration also leads to the activation of calmodulin, which is a structural component of another group of kinase enzymes, the calcium/calmodulin-dependent protein (CaM) kinases. Intracellular calcium and CaM kinases affect a plethora of different proteins depending on the tissue, including nitric oxide synthase, regulation of adenylyl cyclase and cAMP activity, control of the opening of certain ion channels, and facilitation of contraction of muscle fibers, among other functions.
Agonist-selective signaling can result in the activation of different signaling pathways through the same receptor, and can alter processes involved in receptor desensitization, recycling or degradation (Arttamangkul et al., 2008; Virk and Williams, 2008).

Whereas GPCRs are related on a structural level and sub-classes share common signaling pathways, there appears to be a great deal of variation in the genetic sequences coding for different GPCRs, and even related receptor types can share little homology. Among the adrenergic receptors, for example, less than half of the genetic sequence is common to both β-adrenergic sub-types, and the α-adrenoreceptors have even less in common. Despite these differences, adrenoreceptors share common endogenous ligands, though the affinity of specific ligands varies by receptor type. Post-translational modifications, coiling of the helices and folding into the tertiary structure, trafficking from intracellular sites, and insertion into the plasma membrane are all as important as the DNA sequences that code the amino acids that make up the receptors. Thus, despite a lack of sequence homology between receptors and families, GPCRs are able to maintain a similar general structure and signaling mechanisms with varying affinities for specific ligands. Biased ligands can result in differential G-protein coupling leading to altered and unexpected functional responses, including undesirable side effects, and polymorphisms in the genetic coding region for these receptors could lead to individual differences in drug responses (Venkatakrishnan et al., 2013).
1.4.2 Adrenergic receptor distribution and function

Adrenergic receptors (ARs) are GPCRs that are expressed throughout various tissues in the central nervous system (CNS) and peripheral nervous system (PNS) (particularly in the sympathetic autonomic nervous system and along primary sensory afferents). They consist of two main classes: the β-ARs of which there are three sub-types (β₁, β₂, and β₃, all coupled to Gₛ), and the α-ARs, α₁ (coupled to G_q) and α₂ (coupled to G_i/o). The diversity of G-proteins to which the sub-types are coupled means they have distinct effects on the tissues in which they are expressed.

β₁-ARs are primarily localized in cardiac muscle and their stimulation leads to increased heart rate and contractility (Yoo et al., 2009). Activation of β₁ receptors in adipocytes leads to lipolysis (Robidoux et al., 2004), while activation in kidney cells increases renin release (Osborn et al., 1981). β₂-ARs are expressed in smooth muscle tissues and their activation triggers relaxation and dilation of blood vessels (Tanaka et al., 2005). β₂ receptors are expressed in the pancreas where activation induces insulin secretion (Philipson, 2002), in the liver where they initiate the breakdown of glycogen stores and increase glucose synthesis (Erraji-Benchekroun et al., 2005), in the kidney where they increase renin secretion (Gaál et al., 1979), in the lungs where they lead to bronchodilation (Bai, 1992), and on mast cells where they inhibit histamine (Wang and Lau, 2006) and tumor necrosis factor-alpha (TNF-α) release (Bissonnette and Befus, 1997), thus exerting anti-inflammatory effects. Unlike β₁ and β₂-ARs, less is known about β₃, though it appears to be expressed in adipocytes and muscle tissue and its activation triggers the breakdown of fat stores and increases thermogenesis through PKA and MAPK-mediated mechanisms (Collins and Surwit, 2001). More recent studies have shown β₃ receptors in smooth muscle of the urinary and gastrointestinal (GI) tract as well (Tanaka et al., 2005).
On primary sensory afferents, α-AR expression is low under normal physiological conditions, but following nerve injury adrenergic receptor expression is increased leading to greater noradrenergic sensitivity (Lee et al., 1999; Sato and Perl, 1991). In rats, following peripheral axotomy, $\alpha_{2A}$ and $\alpha_{2C}$ were found in motor areas of the ventral horn of the spinal cord, $\alpha_{2A}$ was found in all layers of the dorsal horn, but especially superficially in laminae I and II, and deeper in lamina V, while $\alpha_{2C}$ was also found in all layers as well but was mostly in the motor neurons of the ventral horn (Shi et al., 1999). They didn’t see a change in expression, however, following axotomy. On DRG neurons in humans, mRNA (messenger ribonucleic acid) quantification studies show generally low expression but the sub-types that are predominantly expressed are $\alpha_{2B}$ and $\alpha_{2C}$, while in the spinal cord, $\alpha_{2A}$ and $\alpha_{2B}$ are predominant (Ongioco et al., 2000). Spinal analgesia and opioid synergy are mediated by $\alpha_{2A}$ (Stone et al., 1997) and $\alpha_{2C}$ (Fairbanks et al., 2002). Peripherally the $\alpha_{2B}$ subtype appears to be involved in development of hyperalgesia to norepinephrine (Khasar et al., 1995). Hyperalgesia will be discussed in more detail in the section on opioid receptors.

Within the brain and brain stem, distribution varies somewhat for the $\alpha_2$ sub-types (Saunders and Limbird, 1999). Immunohistochemical studies show distribution of $\alpha_{2C}$ within several areas of the brain involved with pain processing, motivation and reward including the amygdala, the VTA, septum, the substantia nigra (SN), and the ventral pallidum, as well as certain cortical regions, and sparser labeling in the striatum (Rosin et al., 1996). For $\alpha_{2A}$, mRNA expression was detected in the amygdala, hippocampus, hypothalamus, and cortical regions, and various other areas of the midbrain and brainstem (Wang et al., 1996). $\alpha_{2B}$ mRNA has mainly been found within parts of the hippocampus and thalamus and within the olfactory system (Weinshank et al., 1990).
On sympathetic nerve fibers, α₂-ARs are primarily expressed on pre-synaptic nerve terminals and control release of norepinephrine through negative feedback inhibition. High levels of norepinephrine in the synaptic cleft bind to pre-synaptic receptors, inhibiting further release. Pre-synaptic α₂-ARs modulate glutamate release into the synaptic cleft within the superficial dorsal horn of the spinal cord (Kawasaki et al., 2003), which can be blocked by BRL44408 (Li and Eisenach, 2001), an α₂A-selective antagonist, further confirming the importance of that subtype in modulating excitatory neurotransmission. Despite this classic view of α₂-ARs, there is evidence showing that they are also expressed on postsynaptic neurons (Zhang et al., 2009), and multiple types of adrenergic receptors are also directly expressed on glial cells in vitro (Ebersolt et al., 1981; Tanaka et al., 2002). Cultured astrocytes express low levels of α₂-ARs which appear to be coupled to multiple second messenger pathways, and signaling changes are observed following glial activation (Enkvist et al., 1996). In cultured microglia, α₁-, α₂-, β₁-, and β₂-AR mRNA have all been observed (Mori et al., 2002). In vivo, glial cells express adrenergic receptors in many mammalian species including humans as demonstrated in autoradiographic and immunohistochemical studies (Mantyh et al., 1995), and the upregulation of GFAP on astrocytes associated with chronic morphine-induced gliosis is attenuated by yohimbine, an α₂-AR antagonist (Garrido et al., 2005). Whether this is due to direct activity at α₂-ARs expressed on astrocytes or an indirect result of reduced excitatory neurotransmission due to action on neuronal ARs is not known, but suggests a complicated interplay between opioid and noradrenergic receptor systems and their ability to modulate interactions between neurons and glia. The role of glia in opioid tolerance and chronic pain states and how glial cells are modulated by opioid and noradrenergic receptor systems will become more apparent in Chapters 2 and 4.
Table 1.2 shows a summary of the distribution of the various classes of adrenergic receptors, the types of G-proteins and second messengers pathways to which they are coupled, and the role they play in various tissue types.
<table>
<thead>
<tr>
<th>Adrenergic Receptor</th>
<th>Known sub-types</th>
<th>Distribution and function</th>
<th>Predominant Signalling Pathway</th>
<th>Endogenous Ligands</th>
<th>Agonists</th>
<th>Antagonists</th>
</tr>
</thead>
</table>
| α₁                 | A, B, D         | Mostly expressed **post-synaptically** on effector tissue.  
- **Smooth muscle of blood vessels** (activation leads to vasoconstriction and contraction)  
  - eg: Urogenital (contraction) and gastrointestinal tracts, skin (decreased blood flow), lungs  
- **Cardiac muscle** (may increase or decrease contraction, increased blood pressure and peripheral resistance)  
- **Liver** (increase glucose production from energy stores)  
- **Kidneys** (involved in sodium reabsorption)  
- Salivary and sweat glands (secretion)  
- **Olfactory system** (inhibitor)  
- **Neurons** (activation inhibits neuronal excitability).  
  Found in brain and periphery. | -G<sub>Q</sub>-mediated.  
  - Activation of PLC, increase in IP₃, DAG and Ca<sup>2+</sup> | Epinephrine, norepinephrine | - Clinically useful for treating low blood pressure or nasal congestion.  
  - Phenylephrine  
  - Methoxamine | - Clinically useful for treating hypertension or benign prostate hyperplasia  
  - Prazosin  
  - Doxazosin |
| α₂                 | A, B, C         | Mostly expressed **pre-synaptically on neurons** (negative feedback), but may also be found post- and extra-synaptically.  
- Expression in DRGs, spinal cord and brain regions.  
- **Smooth muscle of blood vessels**  
  - eg: Gastrointestinal tract (activation inhibits motility and secretions)  
- **Platelets** (activation increases clotting)  
- **Pancreas** (activation decreases release of insulin and increases glucagon release → increase in blood glucose)  
  Mostly found in **cardiac muscle** (activation increases heart rate and blood pressure)  
  - In adipose tissue, activation leads to lipolysis.  
  - In kidney, activation leads to renin release. | -G<sub>I</sub>-mediated.  
  - Inhibition of AC, decrease in cAMP | Epinephrine, norepinephrine | - Clonidine (also binds imidazoline receptors)  
  - Dexmedetomidine,  
  - Xylazine | - Yohimbine (non-selective)  
  - Atipamezole (selective for α<sub>2</sub> in general but to any subtype)  
  - Efaroxan,  
  - BRL44408 (α<sub>2A</sub>-selective) |
| β₁                 |                 | Mostly found in **cardiac muscle** (activation increases heart rate and blood pressure)  
- In adipose tissue, activation leads to lipolysis.  
- In kidney, activation leads to renin release. | -G<sub>S</sub>-mediated.  
  - Activation of AC, increase in cAMP | Epinephrine, norepinephrine | - Denopamine  
  - Xamoterol (partial agonist)  
  - Isoprenaline (non-selective) | - Atenolol  
  - Esmolol  
  - Propranolol |
| β₂                 |                 | - Activation is **smooth muscle** leads to relaxation.  
- Activation in **blood vessels** leads to dilation.  
  - Activation of AC, increase in cAMP | Epinephrine, norepinephrine | - Salbutamol (short duration of action)  
  - Salmeterol (long duration of action) | - Butoxamine (selective) |
| β₃                 |                 | Mostly found in **muscle tissue** (smooth and skeletal) and **adipose tissue**, but also in **bladder** and **gallbladder**.  
- Activation leads to breakdown of adipose tissue, prevents urination. | -G<sub>S</sub>-mediated.  
  - Activation of AC, increase in cAMP | Epinephrine, norepinephrine | - SR-58,611A  
  - GW-427,353 | SR59230A (also acts at α<sub>1</sub>) |

**Table 1.2: Adrenergic receptor sub-types, distribution, and function.** AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, DAG = diacyl glycerol, IP₃ = inositol triphosphate, PLC = phospholipase C
1.4.3 Opioid receptor systems

As mentioned above, opioid receptors are inhibitory GPCRs located both pre- and post-synaptically throughout the peripheral and central nervous system. Pre-synaptically opioid receptor activation inhibits excitatory neurotransmitter release into the synapse and post-synaptically, their activation hyperpolarizes the cell, making it more difficult for a nerve impulse to propagate.

To date, four main classes of opioid and opioid-like receptors have been identified: the mu-opioid receptor (MOR), the delta-opioid receptor (DOR), the kappa opioid receptor (KOR) and the opioid-like receptor-1 (ORL-1). Each of the main types of OR consists of several sub-classes.

Scherrer et al. (2009) have suggested that despite nociceptors being polymodal, certain nociceptor subtypes respond more selectively to heat or mechanical stimulation and these neurons express different opioid receptor populations. For example, they have shown through immunohistochemistry using green fluorescent protein (GFP)-tagged DORs and antibodies for MORs that DORs are expressed in myelinated and non-myelinated non-peptidergic neurons projecting from the skin that respond primarily to mechanical stimulation, with small molecule DOR agonist SNC80 having no effect on thermal response latencies following destruction of TRPV1-containing neurons by capsaicin. They did not observe any GFP-DOR in lamina I of the spinal cord, only in inner lamina II. Conversely they proposed MORs are expressed in peptidergic (containing substance P and CGRP), heat-sensitive primary afferents in both the skin and viscera, that levels of substance P do not regulate DOR trafficking, and despite what dozens of previous studies have suggested, that DOR is trafficked to the plasma membrane following synthesis to become functionally competent independent of certain reported triggers (for
example, chronic morphine or chronic pain which will be discussed in more detail in the next section). This study used GFP-tagged DORs due to growing concerns about the non-specificity of the available DOR antibodies, but the presence of the large, bulky GFP molecule itself could, in fact, alter normal DOR signalling and receptor trafficking. The study also used fluorescent images and merged channels to look for areas of co-localization, but this is problematic since the images are two-dimensional cross-sections in a single plane and better techniques including 3-dimensional reconstructions, higher resolution electron microscopy, co-immunoprecipitation or fluorescence resonance energy transfer (FRET) imaging were not performed, so it remains unclear whether what was previously assumed about opioid receptor distribution, trafficking, dimerization, and function should be called into question.

MORs are distributed throughout the CNS. They are expressed within the superficial layers of the spinal dorsal horn (Kemp et al., 1996), within the brain stem (including regions involved in control of respiration), the cerebellum, and areas of the brain associated with pain and reward processing including cortical localization (primary and secondary somatosensory cortices, ACC, prefrontal cortex), thalamus, VTA and NA, amygdala, hippocampus and the striatum among others. DORs are expressed in some of the same regions (Mansour et al., 1993; Peng et al., 2012) such as the amygdala, the hippocampus, striatum and NA, as well as the superficial spinal dorsal horn (Arvidsson et al., 1995a), and all opioid receptor sub-types are found on peripheral DRGs.

Because of the wide distribution of opioid receptors throughout the PNS and CNS, side effects such as CNS and respiratory depression, gastrointestinal disturbances, and nausea may limit the use of opioids in some patients. Table 1.3 summarizes in greater detail the distribution of opioid receptors in various tissues, and the result of receptor activation in those tissues.
### Opioid Receptor Subtypes and Function

<table>
<thead>
<tr>
<th>Opioid Receptor</th>
<th>Distribution</th>
<th>Function</th>
<th>Endogenous Ligands</th>
<th>Examples of Agonists</th>
<th>Examples of Antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MOR</strong> (µ&lt;sub&gt;1&lt;/sub&gt;, µ&lt;sub&gt;2&lt;/sub&gt;, µ&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>On pre- and post-synaptic excitatory neurons throughout the CNS and PNS.</td>
<td>-Activation is analgesic due to receptors’ inhibitory effects on excitatory neurons&lt;sup&gt;4&lt;/sup&gt;.</td>
<td>Endorphins</td>
<td>-Codeine (pro-drug)</td>
<td>-Naloxone</td>
</tr>
<tr>
<td></td>
<td>-CNS:</td>
<td>-Involved in opioid reward due to distribution throughout non-dopaminergic and dopaminergic reward pathways&lt;sup&gt;5&lt;/sup&gt;.</td>
<td></td>
<td>-Morphine</td>
<td>-Naltrexone</td>
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<td></td>
<td>-Brain&lt;sup&gt;1&lt;/sup&gt; (eg: thalamus, ACC, PFC, S&lt;sub&gt;1&lt;/sub&gt;, S&lt;sub&gt;2&lt;/sub&gt;, amy., insula, NA, VTA, PAG, RVM, LC, striatum, CN, hipp., cerebellum)</td>
<td>-Respiratory depression due to inhibitory effects on respiratory centers in the brain stem and cortical regions that modulate them&lt;sup&gt;6&lt;/sup&gt;.</td>
<td></td>
<td>-Oxycodone</td>
<td></td>
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<td></td>
<td>-Spinal cord (largely in the superficial dorsal horn)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>-Activation of MORs in GI tract inhibits motility&lt;sup&gt;7&lt;/sup&gt;.</td>
<td></td>
<td>-Deltorphin (natural peptide)</td>
<td></td>
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<td></td>
<td>-Periphery:</td>
<td></td>
<td></td>
<td>-Meperidine (synthetic, has serotonergic effects)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-DRGs&lt;sup&gt;3&lt;/sup&gt;, GI tract&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DOR</strong> (δ&lt;sub&gt;1&lt;/sub&gt;, δ&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>-CNS:</td>
<td>-Activation of DORs causes some antinociception&lt;sup&gt;11&lt;/sup&gt;. Analgesic effects are enhanced in chronic pain states&lt;sup&gt;12&lt;/sup&gt;. -Role in neuroprotection following ischemic injury by regulating ionic homeostasis&lt;sup&gt;13&lt;/sup&gt;. -Anti-depressant-like&lt;sup&gt;14&lt;/sup&gt; and anxiolytic&lt;sup&gt;15&lt;/sup&gt; effects.</td>
<td>Dynorphins</td>
<td>-Bremazocine (highly selective)</td>
<td>-Norbinaltorphimine</td>
</tr>
<tr>
<td></td>
<td>-Brain and brain stem (eg: NC, NA, amy., PFC, VTA, VP, hipp.)&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>-Nalfurafine (highly selective, approved for use in humans)</td>
<td>-JDTic (delayed onset, extremely long duration of action)</td>
</tr>
<tr>
<td></td>
<td>-Spinal cord (largely in the superficial dorsal horn, motor nuclei of ventral horn, areas involved in autonomic control&lt;sup&gt;10&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>-Periphery:</td>
<td></td>
<td></td>
<td>-J-113,397 (selective)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-DRGs&lt;sup&gt;9&lt;/sup&gt;, pancreas, adrenal gland, small intestine&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>-Difelikefalin (peripheral only)</td>
<td></td>
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<tr>
<td><strong>KOR</strong> (κ&lt;sub&gt;1&lt;/sub&gt;, κ&lt;sub&gt;2&lt;/sub&gt;, κ&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>-CNS:</td>
<td>-Some analgesia through modulation of stress pathways&lt;sup&gt;16&lt;/sup&gt;. -Hallucinogenic&lt;sup&gt;17&lt;/sup&gt; and catatonic effects&lt;sup&gt;18&lt;/sup&gt;.</td>
<td>Dynorphins</td>
<td>-Buprenorphine (partial agonist, also has action at MOR)</td>
<td>-JTC801 (selective)</td>
</tr>
<tr>
<td></td>
<td>-Brain (eg: NA, BNST, amy., PFC, VTA, VP, hipp.)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-Activation induces aversive responses&lt;sup&gt;19&lt;/sup&gt;. Mediates negative emotional states/dysphoria&lt;sup&gt;20&lt;/sup&gt;.</td>
<td></td>
<td>-MCOPPB (full agonist. Low to moderate affinity to other opioid receptor sub-types).</td>
<td>-J-113,397 (selective)</td>
</tr>
<tr>
<td></td>
<td>-Spinal cord&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-Conflicting evidence of anxiogenic effects&lt;sup&gt;21,22&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td>-Periphery:</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>-DRGs&lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td><strong>ORL-1</strong></td>
<td>-CNS:</td>
<td>-Activation inhibits MOR- and stress-induced analgesia&lt;sup&gt;24&lt;/sup&gt;.</td>
<td>Orphanin/Nociceptin</td>
<td></td>
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<tr>
<td></td>
<td>-Brain (eg: cortex, thalamus, limbic structures, PAG, SN)&lt;sup&gt;15&lt;/sup&gt;</td>
<td>-Roles in anxiety and stress, reward, tolerance, dependence, learning and memory&lt;sup&gt;22&lt;/sup&gt;.</td>
<td></td>
<td>-Buprenorphine (partial agonist, also has action at MOR)</td>
<td>-JTC801 (selective)</td>
</tr>
<tr>
<td></td>
<td>-Spinal cord&lt;sup&gt;23&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>-Periphery:</td>
<td></td>
<td></td>
<td>-MCOPPB (full agonist. Low to moderate affinity to other opioid receptor sub-types).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-DRGs, smooth muscle, immune cells&lt;sup&gt;23&lt;/sup&gt;</td>
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</tbody>
</table>

Table 1.3: Opioid receptor sub-types, distribution and function. ACC = anterior cingulate cortex; amy. = amygdala, BNST = bed nucleus of the stria terminalis, CN = caudate nucleus, CNS = central nervous system; DAMGO = (D-Ala<sup>4</sup>, N-MePhe<sup>5</sup>)-enkephalin; DOR = δ-opioid receptor; DRG = dorsal root ganglia; GI = gastrointestinal; hipp. = hippocampus; KOR = κ-opioid receptor; LC = locus coeruleus, MOR = µ-opioid receptor receptor NA = nucleus accumbens; NC = neocortex, ORL-1 = opioid receptor-like; PAG = periaqueductal grey; PFC = prefrontal cortex, PNS = peripheral nervous system; RVM = rostral ventromedial medulla; S<sub>1</sub> = primary somatosensory cortex, S<sub>2</sub> = secondary somatosensory cortex, SN = substantia nigra, VP = ventral pallidum, VTA = ventral tegmental area..

<sup>1</sup>Cahill et al., 2014b, <sup>2</sup>Kemp et al., 1996, <sup>3</sup>Coggleshall et al., 1997, <sup>4</sup>Ostermeier et al., 2000, <sup>5</sup>Laviolette et al., 2004, <sup>6</sup>Shook et al., 1990, <sup>7</sup>Sobczak et al., 2014, <sup>8</sup>Mansour et al., 1993, <sup>9</sup>Peng et al., 2012, <sup>10</sup>Arvidsson et al., 1995a, <sup>11</sup>Gavériaux-Ruff and Kieffer, 2011, <sup>12</sup>Cahill et al., 2007, <sup>13</sup>Staples et al., 2013, <sup>14</sup>Jutkiewicz, 2006, <sup>15</sup>Perrine et al., 2006, <sup>16</sup>Tejeda et al., 2013, <sup>17</sup>Chaillet et al., 1983, <sup>18</sup>Laud et al., 2008, <sup>19</sup>Knoll et al., 2008, <sup>20</sup>Kudryavtseva et al., 2004, <sup>21</sup>Mollereau and Mouledous (2000), <sup>22</sup>Xie et al., 2008, <sup>23</sup>Mogil and Pasternak, 2001.
1.4.3.1 Tolerance and changes in MOR functional competence at the membrane

While tolerance can occur with other GPCRs as well (for example it is observed and has been well-studied for the β2-AR), the focus here will be on the opioid receptor system as an example since one of the main purposes of the research carried out in this thesis was to investigate treatment of opioid tolerance. While opioid receptor agonists like morphine are very effective in relieving moderate to severe acute pain, their usefulness in the treatment of chronic pain is limited by the onset of tolerance. Repeated stimulation of opioid receptors results in the progressive loss of analgesic effect, whereby escalating doses are required to maintain the same level of pain relief. This is characterized by a rightward shift in dose-response curves. A lower concentration of opioid receptors expressed on the membrane was thought to partially explain loss of analgesic effect. For GPCRs to be functionally competent, they need to be expressed on the plasma membrane of the cell where they can bind endogenous or exogenous ligands to initiate (in the case of agonists) or block (in the case of antagonists) a signaling cascade and exert their effects.

Most newly synthesized MORs are packaged into vesicles and trafficked to the plasma membrane, while the majority of DORs are believed to remain in vesicles in the cytosol and are trafficked to the plasma membrane under certain scenarios (Wang et al., 2008) such as repeated administration of mu-opioid receptor agonists (Cahill et al., 2001) or under chronic pain conditions (Cahill et al., 2003). Both chronic morphine administration and chronic pain cause MORs to become desensitized or removed from the plasma membrane so the functional competence of mu- and delta-opioid receptors appears to be inversely related (Cahill et al., 2007). The anti-nociceptive effects of DOR agonists are increased following nerve injury in rats, likely due to enhanced functional competence at the plasma membrane (Holdridge and Cahill,
Though the mechanisms are not known, glial activation may play an important role as well (Holdridge et al., 2007).

Early studies had suggested that ligand binding to opioid receptors resulted in recruitment of β-arrestin, which triggered internalization of the receptor and its removal from the membrane, decreasing its functional competence, though newer evidence points not to a single mechanism through which tolerance occurs, but multiple mechanisms at molecular, cellular and circuitry levels (Williams et al., 2013). While receptor downregulation and desensitization have sometimes been used interchangeably and are important components of tolerance development, they are not synonymous. In radioligand binding studies, a downregulation of receptors at the membrane would equate to a smaller $\beta_{\text{max}}$ value (Tsao and von Zastrow, 2000), and the removal of receptors from the membrane or a decrease in *de novo* receptor synthesis or trafficking occurs over a longer timeframe than simple receptor desensitization. Desensitization is a decrease in G-protein coupling to effector molecules and a reduction in subsequent intracellular signaling pathways occurs within minutes of ligand binding (Gainetdinov et al., 2004). Acute tolerance occurs over hours, while chronic tolerance occurs over many days (Williams et al., 2013).

Over time it has become apparent that different ligands can affect GPCR coupling and signaling, phosphorylation, β-arrestin binding, receptor internalization, recycling and degradation pathways in distinct ways, and this sequence and timing of events from ligand binding to receptor recovery can vary a great deal depending on the molecular structure of the agonist binding to the receptor (Williams et al., 2013). Whether this agonist-selective signaling is a result of differential changes in conformation of the receptor induced by the ligand is not definitively known but has been extensively investigated and reviewed (Bailey and Kelly, 2011; Berger and Whistler, 2010; Christie, 2008; Kelly et al., 2008; Koch and Höltt, 2008; Martini and Whistler,
For example, a ligand can cause receptor desensitization without necessarily inducing internalization (Arttamangkul et al., 2006), a receptor can recover without being internalized (Quillnan et al., 2011), and a receptor is not always shut down by arrestin binding but can lead to signaling through alternate pathways (Macey et al., 2006). While the mechanisms of opioid tolerance are not completely understood and appear to be more complicated than originally thought, tolerance and dose escalation remain a significant problem hindering opioid use in chronic pain treatment.

1.4.3.2 Opioid-induced hyperalgesia

Loss of opioid efficacy over time is not merely a result of tolerance but can also be attributed to opioid-induced hyperalgesia (OIH), which can be difficult to differentiate clinically. Opioids predominantly have inhibitory effects on neurons, but at low doses or following repeated administration they can exert pro-nociceptive effects as well due to sensitization of nociceptive pathways. What may look like opioid tolerance behaviorally, may actually be a result of disease progression or increased pain caused by the analgesic itself. While the mechanisms of OIH are not entirely understood, they do appear to be distinct from the neurophysiological adaptations that lead to tolerance, and, at least in animals, may involve increased descending facilitation in the rostral ventromedial medulla (RVM) (Vanderah et al., 2001), increased dynorphin and brain-derived neurotrophic factor (BDNF) expression (Liang et al., 2014), increases in expression of excitatory neuropeptides substance P and CGRP (Ossipov et al., 2005) and TRPV1 in the DRG (Vardanyan et al., 2009), and increased glutamatergic and NMDA receptor expression and function (Mao et al., 1994). The clinical relevance of OIH has been debated (Fishbain et al., 2009; Lee et al., 2011; Tompkins and Campbell, 2011), but human studies have shown increased
pain and morphine requirements following surgery in patients receiving the short-acting opioid remifentanil during surgery (Guignard et al., 2000). Moreover with the increase in opioid prescriptions within the last decade reports of OIH have risen (Arout et al., 2015). Sales of prescription medications have skyrocketed with an increase in retail sales of 1,293% for methadone, 866% for oxycodone, and 525% for fentanyl from 1997-2007 (Manchikanti et al., 2010).

1.4.4 Co-localization and dimerization of opioid and adrenergic receptors

Newer attempts in vivo to generate an interactive brain atlas of MOR and DOR co-localization have used double knock-in mouse models to tag both receptors (Erbs et al., 2014). It appears that MOR and DOR co-localization varies by brain region. In some subcortical areas of the brain, MOR and DOR do, in fact, appear to be expressed in the same neurons, but in other areas of the brain they may not be. Co-immunoprecipitation shows MOR and DOR exist at least in close proximity within the hippocampus, but whether they are close enough to physically dimerize is unclear (Erbs et al., 2014). While the interaction between MOR and DOR is becoming clearer over time, less is known about whether the interactions between MOR and α2-AR occur at a systems level or if there is a more direct interaction at a molecular level between the receptors in vivo. Co-localization of opioid and adrenergic receptors within the same neurons has been observed by immunohistochemistry and Western blotting in rat spinal cord and synaptosome preparations. Riedl et al. (2009) observed high levels of co-localization for DOR and α2A-AR within the superficial dorsal horn of the spinal cord in substance P containing neurons, but observed little co-localization between MOR and α2A-AR, MOR and α2C-AR, and DOR and α2C-AR. This study contradicts Scherrer et al.’s (2009) assertion that DORs exist only
on non-peptidergic neurons and surprisingly Riedl et al. (2009) did not observe co-localization of MOR and substance P either. Immunohistochemical studies are only as good as the antibodies available and the authors admitted that the lack of expected co-localization between the other opioid and adrenergic receptor sub-types could have been due to problems with the antibodies themselves. Alas, co-localization and close physical proximity does not necessarily mean the receptor pairs will form functional heteromers, but does help explain the analgesic synergy between these two systems. Much more work needs to be done to determine which GPCRs are expressed within specific neurons and whether cross-talk occurs at a broader systems level or whether the receptors interact directly to alter each other’s functionality in vivo (Massotte, 2015).

The likelihood of dimerization of two GPCRs increases the more each monomer is expressed in a system and there appears to be some controversy over whether certain GPCRs are expressed in the same neurons in vivo and whether they can form functional dimers. X-ray crystallography of inactive mu- (Manglik et al., 2012), delta- (Granier et al., 2012), kappa- (Wu et al., 2012) and nociception/orphanin FQ- (Thompson et al., 2012) opioid receptors bound to antagonist combined with in silico molecular modeling and bioinformatics (Filizola et al., 2002; Filizola and Weinstein, 2005) has helped predict and identify possible sites of interaction between GPCR monomers, but the conformation of the crystallized receptors may not be reflective of how they actually interact in living tissue (Massotte, 2015) despite efforts to model and simulate a more realistic lipid-water environment (Johnston and Filizola, 2014). Lipid rafts (Pike, 2009; Simons and Toomre, 2000), caveolins (Liu et al., 2001; Smart et al., 1999) and associations of intracellular scaffolding proteins (Garrington and Johnson, 1999; Pawson and Scott, 1999) help control interactions between receptors, structural proteins and enzymes to help regulate microenvironments and facilitate signalling pathways and cross-talk between multiple
systems to coordinate complicated feedback loops within the cell (Burack and Shaw, 2000).

Despite a few studies questioning the co-localization of certain receptors, there is still a
great deal of evidence, however, that suggests opioid receptors do in fact form functional homo
and heterodimers, both among their own receptor types (MOR-KOR: Chakrabarti et al., 2010;
MOR-DOR: George et al., 2000; Gomes et al., 2000; Gomes et al., 2004; DOR-KOR: Jordan and
Devi, 1999; Jordan et al., 2000; Law et al., 2005) and with other GPCRs including, but not
limited to, adrenergic receptors (MOR-α2AR: Jordan et al., 2003; DOR-β2AR: Jordan et al.,
2001), the NK-1 receptor (Pfeiffer et al., 2003), cannabinoid (Rios et al., 2006), and dopamine
receptors. These functional dimers display altered signal transduction activity and affinities for
agonists that would normally bind one or both of the GPCR monomers (Salahpour et al., 2000).
For example, MORs that heterodimerize with DORs have less functional activity (Gomes et al.,
2000), while MOR-α2AAR dimers show increased signaling when an agonist of either receptor is
administered alone, but reduced signaling when agonists for both receptors are administered
concurrently (Jordan et al., 2003). Table 1.4 summarizes much of the work that has been done
showing dimerization between GPCRs, especially focusing on opioid and adrenergic receptor
pairs. It is important to recognize that not every pairing has been demonstrated in vivo; some
studies have seen dimerization when the receptors are over-expressed in culture, but there is a
growing body of evidence suggesting many do exist under normal physiological conditions.

Whether due to heterodimer formation or not, opioid and adrenergic receptors cross-
modulate one another (Hao et al., 2000b; Jordan et al., 2003) through the involvement of N-type
calcium channels (Wei et al., 1996) and intracellular signaling pathways (Vilardaga et al., 2008)
including p38 MAPK and β-arrestin- (Tan et al., 2009), and PKC-mediated signaling (Wei and
Roerig, 1996). Opioid and α2-ARs demonstrate analgesic additivity (Stone and Wilcox, 2004) or
synergy when co-administered (Chabot-Doré et al., 2015; Tajerian et al., 2012; Fairbanks et al., 2002; Fairbanks et al., 2000; Fairbanks and Wilcox, 1999; Ossipov et al., 1999; Ossipov et al., 1997; Przesmycki et al., 1997; Drasner and Fields, 1988). These additive or synergistic effects are potentially opioid-sparing when used clinically, allowing greater analgesia at lower opioid doses (Park et al., 1996) and thus less incidence of side effects, lowered abuse potential and decreased tolerance development.
Table 1.4: Homo-and heterodimerization between opioid, adrenergic and other select GPCRs involved in pain, opioid analgesia and reward.

<table>
<thead>
<tr>
<th>Opioid receptors</th>
<th>Adrenergic receptors</th>
<th>Other receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MOR</strong></td>
<td><strong>DOR</strong></td>
<td><strong>KOR</strong></td>
</tr>
<tr>
<td><strong>ORL-1</strong></td>
<td><strong>α1A-AR</strong></td>
<td><strong>α1D-AR</strong></td>
</tr>
<tr>
<td><strong>α2-AR</strong></td>
<td><strong>β1-AR</strong></td>
<td><strong>β2-AR</strong></td>
</tr>
<tr>
<td><strong>CB1</strong></td>
<td><strong>D1</strong></td>
<td><strong>D2</strong></td>
</tr>
<tr>
<td><strong>NK1</strong></td>
<td><strong>X1</strong></td>
<td><strong>X2</strong></td>
</tr>
<tr>
<td><strong>X3</strong></td>
<td><strong>X4</strong></td>
<td><strong>X5</strong></td>
</tr>
<tr>
<td><strong>X6</strong></td>
<td><strong>X7</strong></td>
<td><strong>X8</strong></td>
</tr>
<tr>
<td><strong>ORL-1</strong></td>
<td><strong>X4</strong></td>
<td><strong>X5</strong></td>
</tr>
<tr>
<td><strong>X6</strong></td>
<td><strong>X7</strong></td>
<td><strong>X8</strong></td>
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<td><strong>X12</strong></td>
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<td><strong>X21</strong></td>
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<td><strong>X24</strong></td>
<td><strong>X25</strong></td>
<td><strong>X26</strong></td>
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<tr>
<td><strong>X27</strong></td>
<td><strong>X28</strong></td>
<td><strong>X29</strong></td>
</tr>
</tbody>
</table>

X indicates dimerization between two receptor sub-types, altering cellular signaling, receptor internalization and recycling or degradation properties. Some studies that have found dimerization between receptors were transfected cell models where the receptors were heterologously over-expressed and thus not every interaction indicated here has been demonstrated in vivo under normal physiological conditions. AR = adrenergic receptor, CBR = cannabinoid receptor, D = dopamine receptor, DOR = delta opioid receptor, KOR = kappa opioid receptor, MOR = mu opioid receptor, NK = neurokinin, ORL-1 = opioid receptor-like. Loosely adapted from Rozenfeld et al., 2010 and Prinster et al., 2005. 1Li-Wei et al., 2002; 2George et al., 2000; 3Wang et al., 2005a; 4Evans et al., 2010; 5Jordan et al., 2003; 6Rios et al., 2004; 7Cvejic and Devi, 1997; 8Stanasila et al., 2003; 9Uberti et al., 2003; 10Uberti et al., 2005; 11Xu et al., 2003; 12Prinster et al., 2006; 13Ramsay et al., 2002; 14Jordan et al., 2001; 15Wager-Miller et al., 2002; 16Kearn et al., 2005; 17Small et al., 2006; 18Lee et al., 2004; 19Lee et al., 2000; 20Hudson et al., 2010; 21Bushlin et al., 2012.
1.5 Types of neuropathic pain and animal models

Various animal models exist of neuropathic pain including those where the peripheral or spinal nerves are damaged mechanically through surgery, genetic knockout (KO) models that make the animal more prone to developing diseases that manifest in neuropathy, or injection of drugs or chemicals that damage the nerve directly. Chronic constriction injury (CCI) is a common model of peripheral nerve injury whereby the sciatic nerve of the hindpaw is exposed and ligated, either by tying four loose ligatures around the nerve with suture thread (Bennett and Xie, 1988) or by wrapping a plastic polyethylene cuff around the nerve (Mosconi and Kruger, 1996), resulting in spontaneous and stimulus-evoked pain in the ipsilateral hindpaw. Over the week or so following the sciatic nerve injury and persisting for a couple months after, rats develop mechanical and cold allodynia and thermal hyperalgesia in the affected limb, and adopt a cupped guarding posture to protect the injured leg. Neurophysiological changes are observed in the periphery (due to Wallerian degeneration, local ischemia and edema) and in the spinal dorsal horn to which the primary sensory afferents project, including the superficial (laminae I, II) and deep (lamina V) areas of the lumbar dorsal horn, as there is an inflammatory component to the CCI model that changes the chemical environment surrounding the nerves. Studies comparing the composition of the suture thread used to ligate the nerve confirm that chromic gut resulted in more pronounced pain behaviours and gait and postural changes than identical surgeries performed with normal gut thread or silk (Maves et al., 1993). Both A- and C- fibers are sensitized following CCI, but more damage occurs to myelinated afferents, leaving unmyelinated C fibers largely intact.

Other models of partial deafferentation include the spinal nerve ligation (SNL) model in which the lumbar dorsal roots around L5-L6 are damaged (Kim and Chung, 1992), the spared
nerve injury (SNI) model in which the tibial and peroneal nerves that branch off the sciatic nerve nearer the paw are lesioned and the sural nerve is left intact (Decosterd and Woolf, 2000), and the partial sciatic nerve ligation model (PSNL) in which only the upper sciatic nerve is ligated (Seltzer et al., 1990; later modified by Lindenlaub and Sommer, 2000).

The sciatic cryoneurolysis (SCN) model (DeLeo et al., 1994) involves damaging the nerve by freezing it with a -60°C cryoprobe, resulting in mechanical allodynia and autotomy that is short-lasting and resolves over the 21 days post-surgery. Interestingly, in the SCN model, the allodynia is not attenuated by administration of α2-adrenergic receptor agonists like clonidine and dexmedetomidine (Willenbring et al., 1995) unlike other models such as the CCI, so this model may not be ideal in testing analgesics that are known to reduce pain in humans, especially those that function through noradrenergic mechanisms.

The sciatic inflammatory neuritis (SIN) model involves injection of zymosan (derived from the cell walls of yeast) around the sciatic nerve to induce immune activation, and like the CCI model, has an inflammatory component (Chacur et al., 2001). The SIN model is somewhat unique in that it induces mechanical allodynia in a dose-dependent manner, with higher doses causing lower mechanical withdrawal thresholds on the contralateral side as well, though thermal hyperalgesia does not appear to manifest. Radiation can also be used to damage the nerve, as is seen in a photochemical-induced sciatic nerve injury model whereby a photosensitive dye is injected around the sciatic nerve and an argon LASER (light amplification by stimulated emission of radiation) is directed at the site to damage the nerve through ischemic injury due to the formation of thrombi in blood vessels surrounding it (Hao et al., 2000a; Kupers et al., 1998). Hao et al. (2000a) observed mechanical allodynia and increased thermal hypersensitivity that was bilateral, with axonal damage and partial demyelination of large myelinated afferents, which
is mostly consistent with Kupers et al. (1998), though the latter also observed an increase in spontaneous pain behaviors. Another similar model does not use a photosensitive dye, but a carbon dioxide LASER is used to irradiate the blood vessels around the nerve directly, reducing blood flow and causing ischemic injury (Myers et al., 1985).

Different models have slightly different behavioural profiles and timelines for those behaviours to develop. Both SNL and PSNL models result in guarding of the hindpaw, thermal hyperalgesia and mechanical hypersensitivity. Unlike the CCI model, however, SNL and PSNL do not appear to manifest cold allodynia and the inflammatory component is less pronounced. The simplicity of the CCI procedure and the speed with which it can be performed, the quick recovery and low impact on the general well-being of the animal compared to other surgical models, its high reproducibility and consistency, and its relatively high predictive validity in terms of translation to neuropathic pain in humans, make it is one of the most widely used surgical models of peripheral nerve injury.

Trigeminal neuralgia, an extremely painful condition resulting from compression or damage of the trigeminal nerve in the face is often described as shocking or paroxysmal-type pain (Nurmikko and Eldridge, 2001). Idiopathic trigeminal neuralgia is modeled in rats by anesthetizing the animals, placing the head in a stereotactic frame to drill a hole through which to insert a cannula and inject agar which will compress the nerve. This results in mechanical allodynia (to air puffs), mechanical hyperalgesia (to pin pricks), and spontaneous scratching behavior (Ahn et al., 2009). Another model involves CCI of the infraorbital nerve (ION) resulting in bilateral heat hyperalgesia that resolves quickly in around 12 days (Imamura et al., 1997). Dynamic mechanical allodynia has also been reported (Alvarez et al., 2009). The CCI-ION is a model of secondary trigeminal neuralgia (Jaggi and Singh, 2009), which are caused in
humans by surgical injuries or other traumas (Nurmikko and Eldridge, 2001).

Not all neuropathic pain in humans is a result of trauma, however, and models of neuropathic pain resulting from a specific disease etiology such as diabetic neuropathy exist as well. A common animal model involves the injection of streptazotocin (STZ) to induce type I diabetes in animals. STZ injection results in destruction of islet β-cells which are responsible for insulin production within the pancreas, thus disrupting the ability to control blood glucose levels (Courteix et al., 1993). Because STZ resembles glucose, structurally, it is selectively taken up by GLUT2 glucose transporters (Hosokawa et al., 2001) and, as an alkyalating agent, damages DNA (Bennett and Pegg, 1981), destroying the β-cells. Elevated intracellular glucose leads to dysfunction of metabolic pathways, changes in proton gradients and electron transport chains, an increase in reactive oxygen species (Dobretsov et al., 2007), and can increase the formation of advanced glycation end products, where glucose covalently binds proteins, thus inducing structural changes in biological molecules, impairing catalytic functions of enzymes, disrupting normal ligand-receptor binding, and causes stiffening of cells making them more prone to damage (Singh et al., 2014). Dysregulation of glucose metabolism ultimately leads to narrowing of blood vessels and hypoxia resulting in ischemic neuronal damage (Singleton et al., 2003). In humans with type I diabetes, damage to neurons causes spontaneous pain often characterized as burning, pricking or tingling, or loss of sensation, usually at the extremities making them more prone to damage, infection and amputation. This loss of sensation seen in humans is partially reflected in the STZ animal model of diabetic neuropathy where there is an increase, not a decrease, in heat thresholds, but there is an increase in mechanical hypersensitivity (Courteix et al., 1993). Genetic mouse models of diabetes have also been developed. In ob/ob (obese mouse) and db/db (diabetic dyslipidemia) mice, genetic deletion of the region coding the leptin hormone
leads to an inability to control food intake and the animals become obese leading to secondary health problems including neuropathies (Drel et al., 2006). This is a model of type II diabetes.

Other drug-induced neuropathy models in animals mimic the effects seen in cancer patients treated with chemotherapeutic agents such as vincristine (Authier et al., 1999), paclitaxel (Authier et al., 2000) or cisplatin (Authier et al., 2003), for example. Injection of these drugs causes axonal damage in animals, a decrease in conduction velocity of the nerve, decreased sensitivity to heat, but increased sensitivity to cold and mechanical stimulation, as well as spontaneous pain, similar to what is observed in humans undergoing chemotherapy. Cancer pain in humans is not only a result of drug treatment but is also caused by nerve compression due to tumor growth and can be modelled in animals by injecting and growing cancer cells in the vicinity of a nerve; for example, the cancer-invasion pain (CIP) model where mammary gland sarcoma cells are introduced around the sciatic nerve results in mechanical allodynia, thermal hyperalgesia, and spontaneous pain behaviors (Shimoyama et al., 2002). As the tumor grows in size and the pressure on the nerve increases, there is a loss of sensation and development of mechanical hypoalgesia. Bone cancer pain is modelled by injecting cancer cells directly into bone structures; for example the tibial bone cancer (TBC) model where carcinoma cells are injected into the tibial bone (Medhurst et al., 2002) or the femur bone cancer model (FBC) where sarcoma cells are injected into the bone marrow of the femur (Schwei et al., 1999).

Drugs used to treat HIV (human immunodeficiency virus) can lead to neuropathic pain as well, as do viral infections themselves including infection with HIV and varicella zoster virus. Varicella zoster virus initially causes chicken pox, but can remain dormant for many years in the peripheral nervous system following infection, leading to shingles when it is re-activated. This re-activation leads to skin lesions and can be followed by post-herpetic neuralgia (PHN):
extreme pain characterized by constant burning or stabbing that can last for months or longer (Garry et al., 2005). Light mechanical stimulation of the affected dermatome is often painful and patients report spontaneous pain as well. PHN is modelled in animals through the injection of virally infected cells into the skin (Sadzot-Delveaux et al., 1995), or through injection of resiniferatoxin, a capsaicin analog and agonist of TRPV1 receptors that desensitizes and destroys capsaicin-sensitive afferents resulting in less response to thermal stimulation but higher mechanical sensitivity (Pan et al., 2003).

Many types of neuropathy can manifest in patients with HIV and AIDS (acquired immune deficiency syndrome) and their classification depends on multiple factors including the stage of infection, whether it is caused by the virus itself, the drugs used to treat it, resultant dysregulation of the patient’s own immune system, opportunistic infections, whether it affects only sensory neurons or motor neurons as well, and whether it causes increased sensitivity or a loss of sensation (Pardo et al., 2001). Thus it is difficult to develop a general model of HIV neuropathy when so many factors contribute to various phenotypes. It has been shown, however, that chemokine receptors CXCR4/CCR5 on glia and neurons bind to the virus’s gp120 envelope glycoprotein which is normally recognized by CD4 receptors on helper T cells to allow the virus to merge with the host cell and infiltrate it (Tamamis and Floudas, 2013). Binding to the chemokine receptors is cytotoxic, causing a cascade of inflammatory and apoptotic events that damage neurons and activate glia (Catani et al., 2000). Thus, a specific model of HIV-induced peripheral neuropathy involves exposing the sciatic nerve of the hindpaw and wrapping it with a soaked cotton gauze matrix containing the HIV gp120 envelope protein (Herzberg and Sagan, 2001).

It is important to recognize the species differences between humans and animal models.
The time spent in pain is much longer in human patients suffering from chronic pain than the animal models typically allow. The validity of the surgical model and the nociceptive test being used are important considerations in designing an animal experiment. Table 1.5 summarizes many of the animal models available of peripheral nerve injury.
<table>
<thead>
<tr>
<th>Affected nerve or tissue</th>
<th>Model</th>
<th>Description</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sciatic nerve</td>
<td>Chronic constriction injury (CCI)</td>
<td>Bennett &amp; Xie (1988) Four loose ligatures tied around nerve (mid-thigh)</td>
<td>Mechanical allodynia, cold allodynia, thermal hyperalgesia, spontaneous pain, autotomy</td>
</tr>
<tr>
<td></td>
<td>Partial sciatic nerve ligation (PSNL)</td>
<td>Seltzer et al., 1990 Lindenlauf and Sommer, 2000 Ligation of sciatic nerve at upper thigh level</td>
<td>Mechanical hyperalgesia, cold allodynia, spontaneous pain, behaviors, chemical hypersensitivity</td>
</tr>
<tr>
<td></td>
<td>Sciatic cryoneurolysis (SCN)</td>
<td>DeLeo et al., 1994 Mosconi and Kruger (1996) Nerve exposed and frozen with a -60°C cryoprobe</td>
<td>Mechanical allodynia, autotomy Short duration (~21 days), reversible</td>
</tr>
<tr>
<td></td>
<td>Laser-induced nerve injury</td>
<td>Hao et al., 2000a Kupers et al., 1998 A photosensitizing dye is injected around the nerve and an argon laser beam is used to damage the nerve by inducing ischemic injury caused by photochemical reaction.</td>
<td>Bilateral mechanical allodynia, heat and cold allodynia, conflicting reports of spontaneous pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myers et al., 1985 Blood vessels around the nerve are irradiated directly with a carbon dioxide laser, damaging the nerve by causing ischemic injury.</td>
<td>Mechanical allodynia, thermal hyperalgesia, spontaneous pain behaviors.</td>
</tr>
<tr>
<td></td>
<td>Sciatic inflammatory neuritis (SIN)</td>
<td>Chacur et al., 2001 Nerve exposed and coated with zymosan to induce inflammation</td>
<td>Unilateral or bilateral allodynia, but no thermal hyperalgesia</td>
</tr>
<tr>
<td></td>
<td>HIV</td>
<td>Herzberg and Sagan, 2001 Cotton gauze soaked in solution containing HIV gp120 envelope protein wrapped around nerve</td>
<td>Allodynia, thermal hyperalgesia, spontaneous pain</td>
</tr>
<tr>
<td>Cancer</td>
<td>Cancer-invasion pain (CIP)</td>
<td>Shimaoyama et al., 2002 Sarcoma cells are injected around the nerve and allowed to proliferate, causing pressure on the nerve.</td>
<td>Mechanical allodynia, thermal hyperalgesia</td>
</tr>
<tr>
<td>Tibial nerve or common peroneal nerve</td>
<td>Spared nerve injury (SNI)</td>
<td>Decosterd and Woolf, 2000 Damage to tibial and peroneal nerves that branch off the sciatic nerve. Sural nerve is left intact.</td>
<td>Mechanical allodynia, thermal hyperalgesia</td>
</tr>
<tr>
<td>Spinal nerves</td>
<td>Spinal nerve ligation (SNL)</td>
<td>Chung model (Kim and Chung, 1992) Ligature tied around L5 or L6 (or both) spinal nerve</td>
<td>Mechanical and cold allodynia, thermal hyperalgesia, spontaneous pain, no autotomy</td>
</tr>
<tr>
<td>Trigeminal nerve</td>
<td>Trigeminal neuralgia models</td>
<td>Model of idiopathic trigeminal neuralgia (Ahn et al., 1997) Cannula inserted through a hole in the skull to inject agar onto the nerve, causing compression.</td>
<td>Mechanical allodynia and hyperalgesia, spontaneous scratching</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Model of secondary trigeminal neuralgia (Imamura et al., 1997) Ligature tied around the infraorbital nerve</td>
<td>Bilateral heat hyperalgesia, short duration, resolves after two weeks. Dynamic mechanical allodynia</td>
</tr>
<tr>
<td>Polyneuropathy</td>
<td>Diabetic neuropathy models</td>
<td>Streptozocin (STZ) model (type I diabetes) (Courteix et al., 1993) STZ injection destroys islet β cells in pancreas resulting in loss of insulin production. This leads to increased blood glucose causing vascular changes and neuronal damage.</td>
<td>Cold and heat allodynia, Thermal and mechanical hyperalgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ob/db or db/db model (type II diabetes) (Drel et al., 2006) Genetic deletion of leptin hormone causing obesity due to overeating.</td>
<td></td>
</tr>
<tr>
<td>Cancer (pain from chemotherapy drugs)</td>
<td>Cisplatin-induced peripheral neuropathy (CIPN)</td>
<td>Authier et al., 2003 Chronic daily injections of cisplatin</td>
<td>Mechanical allodynia, thermal hyperalgesia</td>
</tr>
<tr>
<td></td>
<td>Taxol-induced peripheral neuropathy (TIPN)</td>
<td>Authier et al., 2000 Chronic daily injections of Taxol</td>
<td>Mechanical allodynia, thermal hyperalgesia</td>
</tr>
<tr>
<td></td>
<td>Vincristine-induced peripheral neuropathy (VIPN)</td>
<td>Authier et al., 1999 Chronic daily injections of vincristine</td>
<td>Thermal hypoalgesia, mechanical allodynia and hyperalgesia</td>
</tr>
<tr>
<td>Dermatome</td>
<td>Post-herpetic neuralgia</td>
<td>Varicella zoster-induced neuropathy (Sadzot-Delveaux et al., 1995) Viral infected cells injected into the paw.</td>
<td>Mechanical allodynia, thermal hyperalgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resiniferatoxin-induced neuropathy (Pan et al., 2003) Resiniferatoxin injected which causes destruction of capsaicin-sensitive afferent fibers</td>
<td>Mechanical allodynia, thermal hypoalgesia</td>
</tr>
<tr>
<td>Bone</td>
<td>Cancer (pain from bone cancer)</td>
<td>Tibial bone cancer (TBC) (Medhurst et al., 2002) Carcinoma cells from breast tissue injected directly into tibial bone</td>
<td>Mechanical allodynia, mechanical hyperalgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Femur bone cancer (FBC) (Schwe ii et al., 1999) Sarcoma cells injected into the marrow of the femur</td>
<td>Mechanical allodynia, mechanical hyperalgesia</td>
</tr>
</tbody>
</table>

Table 1.5 Common animal models of peripheral nerve injury. 
- surgical component to model. 
- neuropathy is primarily a result of mechanical injury or pressure. 
- neuropathy is primarily a result of chemical exposure or introduction of a virus or cancer cells. 
- neuropathy is a result of genetic deletions. Adapted from Jaggi and Singh, 2009.
1.6 Nociceptive testing in animals

Many tests exist for assessing nociceptive responses in animals, and selection of appropriate testing modalities is important for assessing sensory pain responses following tissue or nerve injury, or for testing the antinociceptive effects of pharmacological agents.

Most tests of stimulus-evoked pain including the thermal tail flick (in which a beam of radiant light is directed at the 5cm distal portion of the rats’ tail (D’Amour and Smith, 1941)), hot water bath, paw hot plate and Hargreave’s plantar test (in which a beam of light is directed at the paw from underneath a glass floor), and cold allodynia (using a drop of cold acetone applied to the paw) measure the time to withdraw from the aversive stimulus and are not a measure of the threshold to withdraw. Tests of mechanical allodynia using a series of calibrated von Frey filaments applied in an up-down fashion to the plantar portion of the hindpaw do measure withdrawal threshold, with filaments of higher or lower tension applied depending on whether the animal withdraws its paw or not (Chaplan et al., 1994). An alternate means of mechanical sensitivity testing involves applying a single filament of known tension (usually 2g or 12g) repeatedly and measuring the number of times the animal withdraws its paw (Colburn et al., 1999; Raghavendra et al., 2003). The surface upon which the animal is placed can affect von Frey testing results (Pitcher et al., 1999), but the wire mesh is nearly always used. Dynamic allodynia, which is modelled using brush strokes instead of discrete filament applications differs from the static methods that employ the up-down application of von Frey filaments and may be mediated through distinct mechanisms, but is not as often used in most pain studies in animals.

Unlike stimulus-evoked pain, spontaneous pain in animals is much harder to assess and there has been a lot of disagreement about whether assessing certain behaviors is valid. For example, following peripheral nerve injury changes in gait and posture are common as animals
try to protect the injured limb and favor the contralateral side (Mogil and Krager, 2004), so measuring changes in these behaviors was proposed as an appropriate way to infer spontaneous pain in these animals. By placing animals on a narrow elevated Catwalk, cameras track the animal’s gait as it moves from one end to the other and software compares movement to pain naïve animals (Vrinten and Hamers, 2003). Another measure of spontaneous pain has been proposed and used in published studies where the animals are observed for a short time interval and the amount of time the animal spends favoring the contralateral paw, flinching and licking the ipsilateral paw are used to generate a weighted pain score, with the more severe behaviors such as licking the injured foot being given higher weighting. A problem with the weighted pain scoring is that the score is heavily skewed in the nerve injured animals from the start as almost all animals will spend almost the entire time favoring the contralateral side since the ipsilateral paw develops into a guarding cupped posture, and the other behaviors such as flinching and licking are rare. This makes it especially hard to observe whether a drug has an analgesic effect in these animals because even if the drug is providing pain relief, the animals still maintain a cupped paw and thus there is very little difference in the pain scores. A study by Mogil et al. (2010) assessed a number of these spontaneous pain tests and concluded that they were not appropriate measures of neuropathic pain, at least in mice, and cautioned interpreting them as such. In the twenty two mouse strains they tested they saw no correlation between the severity of mechanical allodynia following nerve injury and gait changes on the Catwalk. They also saw that administering analgesics including morphine and gabapentin had no effect on abnormal gait in neuropathic animals, and other behaviors such as lifting and licking the hind paw were too rare to be compared appropriately. Thus, while spontaneous pain may be more analogous to the debilitating pain in human patients than some of the stimulus-evoked pain tests and could have
greater clinical relevancy and may be more predictive of translational success (Backonja and Stacey, 2004), there remains some concern and a lack of consensus about whether the tests are actually measuring spontaneous pain specifically. A newer method called the Rat Grimace Scale uses camera tracking and software to identify facial expression in the rat associated with pain and discomfort, including squinting of the eyes, less bulging of the cheeks, pointing of the ears and pushing the whiskers forward, and has been validated in post-operative pain assays (Sotocinal et al., 2011). It remains to be seen if it will be adopted as a common and reliable measure of spontaneous pain in animal models.

Basic science data is only as good as the models that are used. The next section will discuss current treatments of neuropathic pain in humans and will highlight the fact that different types of neuropathic pain in humans respond differently to different classes of drugs. A negative outcome in treating neuropathic pain of one etiology does not necessarily mean it will be ineffective in another model, and a positive outcome with a drug in treating a certain type does not mean it will be effective in other types. Model selection and validation is thus an extremely important part of designing an experiment to test a drug in animals. Table 1.6 summarizes the characteristics of common nociceptive tests in animals.
<table>
<thead>
<tr>
<th>Type of Pain Assessed</th>
<th>Test</th>
<th>Description</th>
<th>What is Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tests of stimulus-evoked pain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thermal hyperalgesia</strong></td>
<td>Thermal tail flick (D’Amour and Smith, 1941)</td>
<td>A beam of radiant light is directed at the 5cm distal portion of the tail</td>
<td>Time to withdraw</td>
</tr>
<tr>
<td></td>
<td>Hargreaves’ test (Hargreaves et al., 1988)</td>
<td>Beam of radiant light directed at plantar portion of the paw from underneath a glass floor</td>
<td>Time to withdraw. Modified versions are also able to detect threshold.</td>
</tr>
<tr>
<td></td>
<td>Hot plate (Menéndez et al., 2002)</td>
<td>Paw is placed on top of a hot plate with a pre-set temperature</td>
<td>Time to withdraw</td>
</tr>
<tr>
<td></td>
<td>Hot water bath (Bölcskei et al., 2010 modified version)</td>
<td>Animal is wrapped/restrained and tip of tail is lowered into hot water of pre-set temperature</td>
<td>Originally assessed time to withdraw. Modified versions are also able to detect threshold.</td>
</tr>
<tr>
<td><strong>Cold allodynia</strong></td>
<td>Acetone test (Choi et al., 1994)</td>
<td>A drop of acetone (or ethyl chloride) is applied to the paw. As it evaporates, heat is pulled from the skin, cooling it.</td>
<td>Time spent flicking the paw</td>
</tr>
<tr>
<td></td>
<td>Cold plate (Jasmin et al., 1998)</td>
<td>Animal is placed on glass plate and dry ice pellets are applied to the area of the glass under the paw</td>
<td>Number of jumps or paw withdraws, time to withdraw</td>
</tr>
<tr>
<td></td>
<td>Ice bath (Moss et al., 2002)</td>
<td>Animal is wrapped/restrained and tip of tail is lowered into cold water of pre-set temperature</td>
<td>Time to withdraw, time spent out of water</td>
</tr>
<tr>
<td></td>
<td>Escape test (Mauderli et al., 2009)</td>
<td>Two chambers, each with floors of different temperatures. Rat chooses to escape to the more comfortable, less cold side.</td>
<td>Time spent on each side</td>
</tr>
<tr>
<td><strong>Mechanical allodynia</strong></td>
<td>Von Frey testing</td>
<td>Animal is paced on an elevated wire grid and a series of calibrated filaments are applied to plantar portion of paw (Chaplan et al., 1994)</td>
<td>Withdrawal threshold</td>
</tr>
<tr>
<td></td>
<td>Randall and Selitto (1957) paw pressure test</td>
<td>Pressure is continually applied to the paw until the animal starts flinching and trying to escape.</td>
<td>Withdrawal threshold</td>
</tr>
<tr>
<td><strong>Tests of “spontaneous pain”</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tests assessing gait changes</td>
<td>Catwalk (Vrinten and Hamers, 2003)</td>
<td>Camera tracks animals movement and gait as they walk along a narrow beam</td>
</tr>
<tr>
<td></td>
<td>Tests assessing postural changes</td>
<td>Weighted pain score</td>
<td>Animal is observed over a set time frame and the number of instances and duration of paw favoring, flinching and licking are used to generate a weighted pain score</td>
</tr>
<tr>
<td></td>
<td>Tests assessing discomfort</td>
<td>Rat Grimace Scale (Sotocinal et al., 2011)</td>
<td>Camera and software tracks facial movements and recognizes facial expressions associated with pain</td>
</tr>
</tbody>
</table>

Table 1.6. Common nociceptive tests used in rodents to assess severity of injury and the analgesic potential of drugs.
1.7 Mechanisms of action of currently available treatments

While earlier estimates placed the prevalence of chronic neuropathic pain to be approximately 2-3% of the general population (Foley, 2003; Gilron et al., 2006), newer surveys and epidemiological studies suggest the prevalence may actually be two to threefold higher, affecting up to 8% of individuals (Bouhassira et al., 2008; Torrance at al., 2006).

The Canadian Pain Society (CPS), the European Federation of Neurological Societies (EFNS), and the Neuropathic Pain Special Interest Group (NeuPSIG), a division of the International Association for the Study of Pain (IASP), have all proposed evidence-based treatment guidelines for physicians treating patients suffering from neuropathic pain. Because of the difficulty in doing meta-analyses of randomized clinical trials to compare the efficacy of different classes of drugs in treating chronic pain arising from a number of different etiologies, it is common to use numbers needed to treat (NNT) for a drug or drug class in the treatment of a specific type of neuropathic pain. NNT is defined as the number of patients that one must treat in order for a single patient to experience a significant reduction in pain scores. A general consensus is that first-line treatment of neuropathic pain should be anti-depressant agents, specifically tricyclic antidepressants (TCAs) or serotonin/norepinephrine reuptake inhibitors (SNRIs), or anticonvulsant drugs such as gabapentin or pregabalin.

TCAs have been in use for decades in the treatment of mood, stress and anxiety disorders. While they have been replaced to a large degree by newer, more selective classes like the selective serotonin reuptake inhibitors (SSRIs) in the treatment of clinical depression, they have emerged as a first-line treatment for neuropathic pain (Moulin et al., 2014). Because of their effects on multiple systems, the list of unwanted side effects is extensive and pinpointing an exact mechanism of action has proven difficult. TCAs reduce reuptake of neurotransmitters
norepinephrine and serotonin, allowing them to remain in the synapse for longer periods of time to exert their effects (Sindrup et al., 2005), so they may modulate pain at least partially through descending inhibition pathways. While there has been growing skepticism over the years about whether antidepressants actually alter mood pharmacologically or whether their effects are largely due to placebo, there is evidence that TCAs may directly inhibit the excitability of neurons through sodium channel blockade, thus making it more difficult for an action potential to propagate along the cell (Dick et al., 2007). TCAs can also act as NMDA receptor antagonists (Eisenach and Gebhart, 1995), thus reducing central sensitization, an important component in chronic pain development. Delta-opioid receptors, whose functional competence at the neuronal plasma membrane is increased following chronic opioid administration (Cahill et al., 2001; Morinville et al., 2003; Morinville et al., 2004) and in chronic pain states (Cahill et al., 2003; Gendron et al., 2007), appears to be crucial in mediating the antinociceptive effects of amitriptyline following nerve injury (Benbouzid et al., 2008), and radioligand binding studies have shown direct agonist activity of TCAs at the delta-opioid receptor (Onali et al., 2010). These studies confirm that TCAs do, in fact, have pharmacological effects on multiple receptors and cellular signaling systems that cannot be attributed to placebo alone.

While TCAs are first-line treatment for certain types of neuropathic pain, adverse effects may result in discontinuation, even in patients for whom the drugs prove efficacious. Inhibition of L-type calcium channels by TCAs can lead to dangerous cardiac issues (Zahradnik et al., 2008), and unpleasant antimuscarinic side effects are common (Sindrup et al., 2005).

Serotonin and norepinephrine reuptake inhibitors (SNRIs) such as venflaxine and duloxetine are also effective in some cases in the treatment of neuropathic pain. While the side effect profile of SNRIs is less extensive than the TCAs, effects on the adrenergic system can lead
to increases in heart rate and blood pressure, which can be dangerous to those with cardiac
disease. Sexual dysfunction and liver problems can also lead to discontinuation, which can
trigger withdrawal-type symptoms if stopped abruptly (Sindrup et al., 2005).

It is because of the broad side effect profile that TCAs have been phased out for the more
selective SSRIs in the treatment of depression, but in human populations suffering from chronic
pain, SSRIs are not as effective. Randomized clinical trials have shown only modest
improvements over placebo in patients with painful polyneuropathy, with an NNT = 6.8 (Otto et
al., 2008), despite animal studies demonstrating antinociceptive effects of SSRIs in multiple
rodent models including streptozotocin-induced diabetic neuropathy (Anjaneyulu and Chopra,
2004), spinal nerve ligation and persistent pain following formalin injection (Wang et al., 1999).
Thus, it appears that inhibition of norepinephrine reuptake is more important than serotonin for
the relief of neuropathic pain (Mochizucki, 2004), likely due to the fact that serotonin can act to
both inhibit and facilitate pain pathways (Silveira et al., 2010; Wang et al., 2013).

Along with the antidepressants, anti-epileptic/anti-convulsant agents such as
carbamazepine and the gabapentinoids (gabapentin and derivatives like pregabalin) are
recommended as first-line treatment for neuropathic pain. Carbamazepine inhibits nerve impulse
propagation by stabilizing the inactive state of sodium channels, and also potentiates the activity
of GABA (gamma-aminobutyric acid) leading to greater inhibitory effects (Ambrosio et al.,
2002). Carbemazepine is primarily used in the treatment of idiopathic trigeminal neuralgia
(Zakrzewska, 2010), however, and it is recommended only as a fourth-line treatment for other
types of neuropathic pain if other treatments fail. The gabapentinoids have multiple mechanisms
of action as well. At the spinal level they reduce the calcium-induced release of excitatory
neurotransmitters like glutamate into the synapse (Kumar et al., 2010) by binding N-type
VDCCs at the $\alpha_2$-$\delta$ subunit (Field et al., 2006), and reduce excitatory synapse formation by blocking thrombospondin binding to $\alpha_2$-$\delta$ (Eroglu et al., 2009). Autoradiography studies have shown pregabalin binding to the $\alpha_2$-$\delta$ subunit of VDCCs not only in the spinal cord but in brain regions as well, including the cortex, amygdala, and hippocampus (Bian et al., 2006). Supraspinally, gabapentinoids also modulate pain through noradrenergic descending inhibition (Tanabe et al., 2008). Pregabalin does not directly interact with GABA\textsubscript{A} or GABA\textsubscript{B} receptors (Li et al., 2011), has no effect on L-type calcium channels, and does not alter post-synaptic receptors (Joshi and Taylor, 2006).

If antidepressants and anticonvulsants are not effective, tramadol and opioids are recommended as second-line treatment. Tramadol’s mechanisms of action include inhibition of norepinephrine and serotonin reuptake, similar to the TCAs and SNRIs, but it also acts as a weak opioid receptor agonist (Eggers, 1995). Tolerance to opioids and side effects including respiratory and CNS depression, nausea, and gastrointestinal disturbances are problematic and may lead to discontinuation. Long-term endocrine disruptions and hypogonadism may occur with chronic opioid use (Katz and Mazer, 2009). Patients taking medications for depression, a common co-morbidity with chronic pain, may not be able to also take tramadol due to increased risk of adverse drug effects. Also, though it should not be a primary concern in considering opioids for the treatment of chronic non-cancer pain, physicians may be reluctant to prescribe opioids as abuse and misuse has increased over the years in up to 15% of individuals, though these numbers are skewed with a much lower percentage in patients who had no history of illicit drug use prior to the start of chronic opioid therapy to treat chronic pain (0.19%) (Fishbain et al., 2008). This suggests that careful screening and monitoring of patients’ history and ongoing response to drug therapy is important both for the physician’s peace of mind and to ensure their
patients are not deprived of medications that would reduce pain and suffering.

Lidocaine functions primarily as a sodium channel blocker, thus inhibiting nerve impulse propagation, though it can affect potassium and calcium channels as well (Scholz, 2002). Topically it is administered as a cream or patch and is thus most useful for localized peripheral pain. For post-herpetic neuralgia, it is recommended as a second-line treatment, but for other types of neuropathic pain it is not efficacious and is recommended as fourth-line treatment only if other options have failed (Moulin et al., 2014).

Cannabinoids are recommended as third-line treatment of neuropathic pain if antidepressants (TCAs and SNRIs), anticonvulsants, and opioids are not effective. Though historically stigmatised, cannabinoids have slowly gained societal acceptance as recreational substances and the growing body of literature has provided irrefutable evidence of the modulation of pain by endogenous cannabinoids and the efficacy of tetrahydrocannabinol (THC) and cannabidiol in the treatment of neuropathic pain, with an average NNT of 3.4 (Finnerup et al., 2010). Cannabinoid side effects are usually mild. In addition to a reduction in pain scores, patients often report less sleep disturbances and an overall improved quality of life.

Tapentadol is currently recommended by the CPS only as a fourth line treatment, likely due to the fact that it is a relatively new drug and there is much that is currently unknown about it. It is somewhat similar to tramadol in that it has activity at opioid receptors and also modulates noradrenergic activity. Isobolographic analysis has shown synergistic interactions between the two receptor systems in producing analgesia (Schröder et al., 2011). Unlike tramadol, however, tapentadol does not appear to modulate serotonin.

Methadone is a synthetic opioid receptor agonist with activity at the NMDA receptor as an antagonist. It has a long duration of action and a long half-life compared to other opioids, thus
requiring less frequent dosing. Methadone is most often used in maintenance programs for people struggling with opioid addiction. While some patients may find relief from neuropathic pain with methadone, The CPS currently recommends its use only as a fourth-line option if other treatments have failed due to lack of reliable clinical trial data (Moulin et al., 2014). Table 1.7 shows a summary of current treatment hierarchies as recommended by the CPS.

While most of the Society guidelines list treatment hierarchies of single drug classes, combination therapies often prove more useful than one drug alone and more randomized clinical trials are looking at drug combinations for the treatment of chronic pain (Gilron et al., 2009; Gilron and Max, 2005; Romano et al., 2009; Schechtmann et al., 2010). Though clinical trials with drug cocktails are more challenging and could lead to an unexpected adverse effect, combining drug classes may be beneficial in some cases, allowing for multiple modalities through which relief might be attained. Drug additivity or synergy may allow for a greater reduction in pain scores than a single drug could achieve on its own and lower doses of individual drugs used in combination may decrease the likelihood of side effects that one drug alone would have at a higher dose.

One combination therapy that has proven useful in animal models and clinical trials is the use of ultra-low dose (ULD) GPCR antagonists, which will be discussed in more detail in the next section.
Table 1.7 Results of randomized clinical trials of current neuropathic pain treatments. Table shows treatment hierarchy as recommended by the Canadian Pain Society for prescribing medications to treat neuropathic pain based on etiology and the mechanisms through which they appear to be working. The numbers needed to treat (NNT) represent the number of people that require treatment with that class of drug for one person to experience a reduction in pain. CNS = central nervous system, HIV = human immunodeficiency virus; MS = multiple sclerosis; NMDAR = N-methyl-D-aspartate receptor, NNT = numbers needed to treat, PNS = peripheral nervous system, RCT = randomized clinical trial, SNRI = serotonin-norepinephrine reuptake inhibitor, SSRI = selective serotonin reuptake inhibitor, TCA = tricyclic antidepressant. Adapted from Moulin et al., 2014.

<table>
<thead>
<tr>
<th>1st line treatment</th>
<th>Drug class</th>
<th>Most effective for</th>
<th>NNT</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabapentin</td>
<td>Painful polyneuropathy</td>
<td>6.4</td>
<td>Inhibition of excitatory glutamate and substance P release</td>
<td></td>
</tr>
<tr>
<td>Pregabalin (Gabapentinoids)</td>
<td>Diabetic neuropathy</td>
<td>4.5</td>
<td>Inhibition of voltage-gated calcium channels in spinal dorsal horn</td>
<td></td>
</tr>
<tr>
<td>TCAs (amitriptyline, nortriptyline, desipramine)</td>
<td>Diabetic neuropathy</td>
<td>2.1</td>
<td>Inhibition of norepinephrine and serotonin reuptake from synapse</td>
<td></td>
</tr>
<tr>
<td>SNRIs (duloxetine, venlafaxine)</td>
<td>Diabetic neuropathy</td>
<td>5.0</td>
<td>Inhibition of norepinephrine and serotonin reuptake from synapse</td>
<td></td>
</tr>
<tr>
<td>2nd line treatment</td>
<td>Tramadol</td>
<td>Diabetic neuropathy</td>
<td>4.9</td>
<td>Weak agonist at opioid receptors</td>
</tr>
<tr>
<td>Opioid (controlled release)</td>
<td>Painful polyneuropathy</td>
<td>2.6</td>
<td>Opioid receptor activation</td>
<td></td>
</tr>
<tr>
<td>Topical lidocaine</td>
<td>Post-herpetic neuralgia</td>
<td>?</td>
<td>Blockade of sodium channels inhibits nerve impulse propagation</td>
<td></td>
</tr>
<tr>
<td>3rd line treatment</td>
<td>Cannabinoids</td>
<td>HIV neuropathy</td>
<td>3.4</td>
<td>Cannabinoid receptor-mediated analgesia. Cannabinoid receptors are inhibitory GPCRs found throughout the CNS and PNS on both excitatory and inhibitory neurons and thus have a wide spectrum of action dependent on receptor localization.</td>
</tr>
<tr>
<td>Methadone</td>
<td>No reliable RCT data available.</td>
<td>?</td>
<td>-Agonist at opioid receptors</td>
<td></td>
</tr>
<tr>
<td>Anticonvulsants</td>
<td>RCT data are mixed, negative or poor quality.</td>
<td>10-12</td>
<td>Sodium channel blockade.</td>
<td></td>
</tr>
<tr>
<td>Tapentadol</td>
<td>Diabetic neuropathy (new compound, more studies needed)</td>
<td>?</td>
<td>Similar mechanism as tramadol, but without serotonergic effects. Activity as MOR agonist, inhibition of norepinephrine reuptake</td>
<td></td>
</tr>
<tr>
<td>SSRIs</td>
<td>Diabetic neuropathy</td>
<td>6.8</td>
<td>Selective inhibition of serotonin reuptake from synapse</td>
<td></td>
</tr>
<tr>
<td>Topical capsaicin</td>
<td>Postherpetic neuralgia</td>
<td>8-10</td>
<td>Excitatory response, then desensitization of nociceptors through TRPV1 activation</td>
<td></td>
</tr>
<tr>
<td>Topical lidocaine</td>
<td>Other types of peripheral nerve injury except postherpetic neuralgia</td>
<td>?</td>
<td>Blockade of sodium channels inhibits nerve impulse propagation</td>
<td></td>
</tr>
</tbody>
</table>
1.8 Ultra-low dose (ULD) G-protein-coupled receptor (GPCR) antagonists

While opioids are highly efficacious in the treatment of moderate to severe acute pain, their usefulness in the treatment of chronic pain states is limited by the development of analgesic tolerance over repeated administration, necessitating the use of higher doses to maintain the same level of pain relief, which also increases the risk of adverse effects. Paradoxically, the use of ultra-low dose (ULD) GPCR antagonists has been shown to attenuate the development of tolerance to opioids and enhance their analgesic effects.

ULD is defined as a dose several log-units below what would normally result in receptor blockade in the case of antagonists (usually in the pico- to nanogram range). While at high doses opioid antagonists like naloxone and naltrexone block the effects of opioid agonists like morphine and oxycodone, at ULD they enhance their analgesic effects.

1.8.1 ULD opioid receptor antagonists

1.8.1.1 Animal studies

In animal models, ULD naltrexone blocks acute tolerance to spinal morphine (Powell et al., 2002), chronic tolerance to systemic morphine (Tuerke et al., 2011) and prevents thermal hyperalgesia produced from low dose opioids (McNaull et al., 2007). This low dose morphine hyperalgesia is believed to occur through activation of $G_{\alpha S}$, resulting in activation of L-type calcium channels via a protein kinase C (PKC) pathway (Esmaeili-Mahani et al., 2008). Similar behavioural effects have been observed in mice with both oral and intraperitoneal injection of ULD naltrexone (Shen and Crain, 1997). Though there appears to be no difference in the effects of ULD opioid antagonists between male and female rats, the effects are strain-dependent. Both Sprague-Dawley and Long-Evans rats show similar attenuation of opioid tolerance, but the ULD
effects are lost in Lewis and F344 strains at all doses tested (Terner et al., 2006).

ULD administration of highly selective MOR antagonist peptides CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$) and CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$), as well as ULD DOR antagonist naltrindole enhance the acute effects of morphine, and not only prevent the development of acute and chronic opioid tolerance but can reverse it once it is already established (Abul-Husn et al., 2007).

ULD opioid antagonists also appear to reduce the reinforcing effects of opioids in rats, which can be partially dissociated from their analgesic effects (Burns, 2005). In a self-administration model, rats receiving intravenous injections of ULD naltrexone (1pg/kg/infusion) with oxycodone (0.1mg/kg/infusion) administered significantly more injections compared to oxycodone alone, possibly due to lower reward. Following extinction, re-instatement showed drug seeking was significantly lower in the animals co-administering ULD naltrexone with oxycodone after drug-priming, conditioned cues, or a stress response resulting from foot shock (Leri and Burns, 2005). In a progressive ratio paradigm where animals need to work harder and harder to receive each subsequent injection, rats that were co-administered ULD naltrexone did not reach their breakpoint significantly sooner compared to those receiving oxycodone alone (Leri and Burns, 2005). It has been suggested that the progressive ratio paradigm is more sensitive to the rewarding effects of stimulant class drugs like cocaine than it is for the opioid analgesics (Ward et al., 2005), which could explain the discordance seen between the lack of effect in the progressive ratio and the positive effects in the other self-administration and re-instatement models.

In a conditioned place preference paradigm (CPP), co-administration of ULD naltrexone blocks morphine and oxycodone CPP in a dose-dependent manner in pain naïve rats, suggesting
a reduction in the rewarding properties of opioids, and inhibits conditioned place aversion (CPA) caused by the unpleasant withdrawal effects upon abrupt discontinuation of opioids (Olmstead and Burns, 2005). Conversely, Powell et al. (2002) observed an increase in morphine CPP (at 1mg/kg) with systemic ULD naltrexone (16.7, 20.0 and 25.0 ng/kg) in pain naïve rats. Shen and Crain (1997) have also shown in mice that ULD naltrexone inhibits withdrawal behaviours such as jumping upon cessation of morphine.

ULD opioid antagonists are effective in animal models of chronic pain as well. In a SNL model of neuropathic pain in rats, co-administration of ULD naltrexone with oxycodone (either intrathecally through catheters terminating in the lumbar portion of the spinal cord or through oral administration) enhanced the anti-allodynic and anti-hyperalgesic effects compared to oxycodone alone, and attenuated the development of chronic opioid tolerance over time (Largent-Mills et al., 2008).

1.8.1.2 Randomized clinical trials with ULD opioid antagonists

Clinically, the use of ULD opioid antagonists appears useful in human populations as well. In a randomized double-blind trial, a single injection of ULD naloxone prolonged the analgesic effects of fentanyl and lidocaine following cataract surgery by significantly extending the time until rescue analgesic was needed (Ezz and Elkala, 2015). In patients with moderate to severe osteoarthritis pain, Oxytrex (combination oxycodone and ULD naltrexone) provided significantly greater pain relief when administered twice daily compared to oxycodone alone administered four times daily (but in equivalent total daily doses), with no difference in adverse effect scores (Chindalore et al., 2005). Some post-operative clinical trials have found opioid side effects are reduced in patients co-administered ULD naloxone compared to morphine alone.
The antinociceptive effects of buprenorphine are enhanced in healthy volunteers when co-administered with ULD naltrexone (Hay et al., 2011) or ULD naloxone (La Vincente et al., 2008) in a cold pressor pain task, with no difference in the prevalence of adverse effects. While most of the ULD antagonist literature have investigated the effects on tolerance or analgesia, a few studies have tried to determine their effects on dependence, reward, or abuse liability of opioids in humans.

In chronic opioid users, a randomized, placebo-controlled clinical trial showed no difference in abuse liability indices of oxycodone with or without ULD naltrexone (Tompkins et al., 2010). This study used a within-subject cross-over design with placebo and two doses of oxycodone (20mg and 40mg), with or without ULD naltrexone (0.00001mg and 0.001mg, the doses currently available in commercial Oxytrex formulations). Participants were asked to rate their “liking” of the drugs on a subjective visual analog scale and a drug versus money questionnaire was administered whereby the participants rated what they perceived the relative street value would be based on their experience. The higher dose of oxycodone ranked the highest on the visual analog scale and had the highest perceived street value. Co-administration of either dose of ULD naltrexone had no effect on the likability of oxycodone or its perceived money value. The study only recruited participants with a history of opioid abuse, however, not patients taking opioids for chronic pain, it did not assess analgesia at all, it had a small sample size (n=14 total from all groups) with only one female, and there was a disconnect between adverse effects and the “likability” and perceived value of the oxycodone. Despite the fact that there was no difference in the visual analog scale between the oxycodone alone or oxycodone combined with naltrexone, fewer adverse effects were reported in the higher 40mg oxycodone
with naltrexone group than the lower 20mg oxycodone with naltrexone group.

1.8.2 ULD adrenergic receptor antagonists

Most ULD studies have investigated the use of ULD naloxone and naltrexone, but the ULD phenomenon is not restricted to opioid antagonists. In an acute tolerance model in rats, the analgesic effects of intrathecally-administered norepinephrine and the $\alpha_2$-adrenergic agonist clonidine are enhanced by ULD $\alpha_2$-adrenergic receptor antagonist atipamezole (Milne et al., 2011).

Animal models have shown that there is cross-modulation between the opioid and noradrenergic receptor systems. When administered spinally through intrathecal administration in rats, ULD $\alpha_2$-adrenergic receptor antagonists yohimbine (0.02ng, 2ng), atipamezole (0.08ng, 0.8ng), mirtazapine (0.02ng), and idazoxan (0.08ng) prolonged the analgesic effects of acute intrathecally-administered morphine in rats, and inhibited development of acute and chronic tolerance, even after tolerance had been established (reversal) (Milne et al., 2008). Destruction of noradrenergic inputs in the spinal cord by injection of cytotoxic 6-hydroxydopamine does not prevent the attenuation of acute morphine tolerance (Milne et al., 2008). Intrathecal administration of other ULD $\alpha_2$-AR antagonists including efaroxan (Milne et al., 2013) and the $\alpha_{2A}$-AR-selective BRL44408 (Milne et al., 2014) also inhibit spinal morphine tolerance and opioid-induced hyperalgesia.
1.8.3 Proposed mechanisms of action of ULD GPCR antagonists

On their own, ULD opioid antagonists appear to exert biphasic dose-responses. At low
doses they seem to act as partial agonists, possibly at distinct sub-populations of opioid receptors
or at distinct receptor sites, causing analgesia, but become hyperalgesic at higher doses in both
rats (Woolf, 1980) and humans (Levine et al., 1979) as opioid receptor blockade disrupts tonic
inhibitory control.

In slices from rodent brain stem, low dose naloxone increased high potassium-evoked
release of met-enkephalin into the synapse by blocking presynaptic autoinhibitory mechanisms
(Ueda et al., 1986). At higher doses, met-enkephalin release was greatly reduced, and
corresponded to hyperalgesic behaviour in thermal tail flick and writhing tests (acetic acid
injected into the abdominal cavity).

Low dose opioid agonist administration can induce excitatory rather than inhibitory
effects (Shen and Crain, 1989; Crain and Shen, 1990). In cultured DRG neurons, ULD (in the
picomolar range) opioid antagonists selectively block excitatory but not inhibitory effects of low
dose morphine (Crain and Shen, 1995). Similarly, behavioural models in mice show low dose
morphine-induced thermal hyperalgesia is attenuated by ULD naltrexone (Crain and Shen,
2001). Part of the hyperalgesia associated with chronic morphine administration is believed to be
caused by a G-protein coupling shift and consequent changes in effector activation and signaling
pathways. Wang et al. (2005b) observed that in the PAG and spinal cord, MORs couple to G\textsubscript{i} and
G\textsubscript{o}, while in the striatum they couple to G\textsubscript{o} in opioid naïve rats. Chronic morphine administration
induces a coupling shift to stimulatory G\textsubscript{s} and G\textsubscript{βγ} signaling. G\textsubscript{s} activates adenylyl cyclase
pathways leading to hyperalgesia. Adenylyl cyclase activation can also occur through stimulatory
G-protein-associated G\textsubscript{βγ} (Belevych et al., 2001), causing an increase in L-type VDCC function
and changes in potassium ion flow through GIRKs (Robillard et al., 2000), leading to opioid
tolerance (Wang and Gintzler, 1997). The morphine-induced shift to stimulatory G-protein
coupling is attenuated by co-administration with ULD opioid antagonists (Wang et al., 2005).

Similar results have been observed in chronic pain models. In a SNL model of
neuropathic pain in rats, no change in total MOR or \( G_\alpha \) expression was observed within the
dorsal horn of the spinal cord; however, a shift in MOR G-protein coupling was observed in the
dorsal horn, ipsilateral to the site of nerve injury. The neuropathy-induced shift to excitatory \( G_S \)
was increased following chronic oxycodone administration, which was attenuated in the animals
that were co-treated with ULD naltrexone (Largent-Milnes et al., 2008).

The inhibition of spinal morphine tolerance and hyperalgesia by ULD \( \alpha_2 \)-AR antagonist
efaroxan is stereoselective in rats (Milne et al., 2013). Both a racemic mixture and the active (+)
stereoisomer of ULD efaroxan block the development of tolerance and hyperalgesia, while the
inactive (-) stereoisomer has little to no effect, suggesting that direct functional interaction with
the \( \alpha_2 \)-adrenergic receptor is at least partially responsible for the drug’s effects, and is not a direct
result of off-target receptor binding. Intrathecal administration of BRL44408, an \( \alpha_2 \)-AR
antagonist that is highly selective for the \( \alpha_{2A} \) receptor subtype prolonged the antinociceptive
effects of acute morphine and norepinephrine administration and blocked tolerance development,
again suggesting the effects are at least partially mediated directly through the \( \alpha_2 \)-AR and that the
\( \alpha_{2A} \) subtype is important for these effects (Milne et al., 2014).

Like MORs, \( \alpha_{2A} \)-adrenergic receptors are GCPRs coupled to inhibitory \( G_{i/o} \) pathways
(Jordan et al., 2003). Their activation leads to inhibition of adenylyl cyclase activation, inhibition
of calcium channels and increased potassium influx (Morita and North, 1981; Limbird, 1988;
Richman and Regan, 1998). Likewise, the G-protein coupling shift observed with opioid
receptors appears to extend to other GPCRs as well (Wang et al., 2005). Cannabinoid receptors are important inhibitory receptors localized throughout the central and peripheral nervous systems. Like Opioid receptors, they are normally coupled to $G_{i/o}$ and their activation leads to inhibition of the neurons on which they are expressed. As is the case with opioid receptors, certain conditions can induce a shift to stimulatory $G_S$ pathways (Jarrahian et al., 2004; Bonhaus et al., 1998). $\beta_2$-adrenergic receptors, which are typically coupled to $G_S$ can undergo a coupling shift to $G_i$ leading to altered effector activation and signaling pathways (Daaka et al., 1997).

Prevention of the excitatory $G_S$ coupling shift is only one mechanism through which ULD opioid antagonists appear to be exerting their effects. Co-administration of a single injection of spinal morphine with ULD naltrexone produces antinociception, which is blocked by 8-phenyltheophylline, an adenosine receptor antagonist (McNaull et al., 2007). Modulation of glial activation and alterations in cytokine profiles appear to be important as well. Mattioli et al. (2010) have shown a positive correlation between attenuation of chronic morphine tolerance and a reduction in morphine-induced spinal gliosis by ULD naltrexone. Chronic morphine administration did not cause an increase in the total number of glial cells in the spinal cord but did cause an increase in glial cell size and immunolabelling consistent with a change in activation states as microglia and astrocytes shift to a pro-inflammatory phenotype. The morphine-induced increase in astrocyte size was confirmed by 3-dimensional reconstructions of cells within the spinal dorsal horn. Co-administration of ULD naltrexone attenuated the chronic opioid-induced increase in astrocyte volume to levels that were not different from saline controls (Mattioli et al., 2010).

Similar results have been observed further downstream. Both chronic morphine (Hutchinson et al., 2007) and chronic pain (Inoue and Tsuda, 2009) are associated with glial
activation in the spinal dorsal horn as previously discussed. Activated glia have a different metabolic profile than those in a quiescent state and release inflammatory mediators including pro-inflammatory cytokines interleukin 1-beta (IL-1β), interleukin-6 (Il-6), and TNF-α (Watkins and Maier, 2004). A decrease in the anti-inflammatory cytokine interleukin-10 (IL-10) is also observed. The chronic morphine-induced upregulation of IL-1β, Il-6, and TNF-α in the spinal cord is attenuated in rats co-administered ULD naloxone, while expression of the anti-inflammatory IL-10 is increased (Lin et al., 2010). They observed a threefold increase in IL-10 mRNA compared to animals treated with morphine alone, with a fifty percent increase in levels of IL-10 functional protein. The ability of ULD naloxone to attenuate the development of morphine tolerance is reduced in animals injected daily with IL-10 antibodies intrathecally (Lin et al., 2010). ULD naloxone enhances the removal of excitatory glutamate from the synapse by attenuating the downregulation of glutamate transporters GLAST and GLT-1 on astrocytes within laminae I and II of the dorsal horn of the spinal cord following partial sciatic nerve transection in rats (Yang et al., 2011). These studies suggest that at least at the spinal level, glia play an important role in the mechanism of action of ULD opioid antagonists. It is unknown if similar effects on glia occur following chronic ULD adrenergic antagonist administration.
1.8.4 Limitations of current studies

While there is evidence in both the animal literature and in randomized clinical trials that ULD GPCR antagonists augment opioid analgesia and may have some analgesic effects on their own, the mechanisms of action are not entirely understood. It is feasible that the reported change in G-protein coupling is a more universal phenomenon that affects GPCRs in general and is not MOR specific, and it appears that glial modulation may be an important component in ULD GPCR antagonist action, though it is not known if these mechanisms extend to the α2-adrenergic receptor. One must also be cautious not to imply causality to these events. Do ULD opioid antagonists inhibit nerve-injury or chronic morphine-induced glial activation through direct activity at astroglial or microglial opioid receptors or some unknown target, or is their effect on glia indirect and downstream of changes in neurotransmission and sensitization?

Previous studies with ULD adrenergic antagonists have only been performed in rodents and the antagonists have only been administered intrathecally in acute and chronic tolerance models. These studies have only assessed animal behaviour and no molecular techniques have been performed to try to determine if their mechanism of action differs from the opioid antagonists. It is also unknown what effects ULD α2-adrenergic receptor antagonists have in models of chronic pain. While there is some overlap between the opioid tolerance literature and chronic pain, mechanistically the actions may differ. To be more clinically relevant, systemic administration of ULD α2-adrenergic antagonists must be assessed in a model of chronic pain, and both the sensory and affective components of pain should be assessed.

1.9 Statement of purpose and objectives

Due to the limitations of the previous studies, the main global aim of this thesis work was to assess the use of ULD α2-adrenergic antagonists in more clinically relevant models of opioid
tolerance and chronic pain. The first specific aim was to more clearly elucidate the interactions between neuronal and glial activation in chronic pain states and select appropriate methodology moving forward. Thus, the aim of Chapter 2 was to find a reliable way to assess changes in spinal gliosis and neuronal activation and to compare different surgical models of peripheral nerve injury and nociceptive testing paradigms to select the most reliable methods.

The second specific aim was to assess the sensory behavioral effects of ULD α2-AR antagonists in more relevant models of opioid tolerance and neuropathic pain. Thus, Chapter 3 had several goals. Firstly, to replicate previous findings by using systemic rather than spinal administration of the antagonists (atipamezole and efaroxan) in a model of chronic rather than acute opioid tolerance. Secondly, to induce neuropathic pain in animals and assess whether the ULD α2-AR antagonists had any effect on nociception on their own. Third, to assess whether chronic administration of the antagonists following nerve injury altered acute opioid antinociception. The final aim of Chapter 3 was to assess whether co-administration of ULD α2-AR antagonists could alter opioid tolerance in chronic pain states.

The third specific aim was to determine whether any of the behavioral effects seen in Chapter 3 correlated with changes in gliosis, neuronal activation, or MOR expression in the spinal dorsal horn, specifically in the deep and superficial laminae of the L4-L5 lumbar region to which the primary sensory afferents project. Thus, Chapter 4 aimed to use immunohistochemical methods to label specific proteins and to image and quantify their expression in 2- and 3-dimensions.

The final aim of this thesis was to assess the effects of ULD α2-AR antagonists in the conditioned place preference paradigm, a model of the emotional or affective component of pain.

It was hypothesized that chronic systemic administration of ULD atipamezole and efaroxan would attenuate morphine tolerance in pain naïve and nerve-injured animals, inhibit the spinal gliosis associated with those models, and increase time spent in the drug-paired compartment in the CPP paradigm, either due to reward or relief from pain.
CHAPTER 2:
NEUROPATHIC PAIN STATES, NEURONAL ACTIVATION AND GLIOSIS:
SELECTION OF SURGICAL MODEL, BEHAVIOURAL TESTING MODALITIES AND
OPTIMIZATION OF IMMUNOHISTOCHEMISTRY

Abstract

In the present study, using two different surgical models of sciatic nerve injury and two methods of mechanical sensory testing, we sought to determine the role of microglia in the management of neuropathic (NP) pain using a reportedly specific microglial inhibitor. Propentofylline (PF), a microglia and astrocyte inhibitor, and PJ34, a purportedly selective microglia inhibitor, were used to determine the relative contribution of microglia and astrocytes in two animal models of NP pain through the use of immunohistochemical (IHC) and behavioural experiments. Most of the pharmacological tools used to suppress microglial activation have numerous effects on other systems; the antibiotic minocycline also alters ionotropic receptor conductance. PJ34, a poly[ADP-ribose] polymerase (PARP-1) inhibitor has been shown to be neuroprotective in animal models of ischemia, but has not been used previously in model of NP pain. Following chronic constriction injury (CCI) of the sciatic nerve, chronic intrathecal administration of PF attenuated the up-regulation of both glial fibrillary acidic protein (GFAP) and CD11b ipsilateral to the site of nerve injury. Similar effects were seen with PJ34 despite reports in the literature that it was selective to microglia. It is possible that attenuation of astrogliosis with PJ34 was an indirect result of microglial inhibition, however, as microglial activation usually precedes astrogliosis. With fewer “activated” microglia releasing pro-inflammatory cytokines and other inflammatory mediators, astrocyte activation may have
been indirectly impacted.

Using c-Fos as a marker of neuronal activation, we demonstrate that glial inhibition by either PF or PJ34 attenuates the bilateral nerve injury-induced up-regulation of c-Fos in the dorsal horn of the spinal cord. Surprisingly, however, the glial inhibitors either had no effect or only modestly attenuated pain hypersensitivities including mechanical allodynia in two NP pain models. These results suggest a discordance between glial inhibition and pain hypersensitivity associated with nerve injury.

Of the two CCI models compared, the Bennett & Xie model produced more consistent and robust development of mechanical allodynia. Of the two test paradigms for assessing mechanical allodynia that were compared, the number of withdrawals to repeated stimulation by a single 12g filament appeared to be more sensitive to changes in mechanical hypersensitivity than the 50% withdrawal threshold. While the two tests supposedly assess the same thing, on closer inspection the threshold to respond is not the same as the number of responses to a single filament. The latter method may show little response at first but after repeated stimulation the frequency of withdrawals increases as the tissue becomes sensitized, which may involve temporal summation or wind-up. Because the two tests are so similar but at the same time so different, both tests will be used moving forward.
2.1 INTRODUCTION

In recent years, a role for glial cells in the development of NP pain states has been identified by contributing to an increase in neuronal excitability, inflammation and neurotoxicity (Milligan and Watkins, 2009). Studies have identified various mechanisms by which glial cells contribute to the genesis and maintenance of chronic NP pain. For example, activation of microglia and astrocytes following nerve injury results in the release of pro-inflammatory cytokines and other inflammatory mediators such as fractalkine, reactive oxygen species (ROS), ATP, NO, substance P, and glutamate that further recruit and activate glia and increase neuronal sensitization, enhancing nociceptive transmission (Watkins and Maier, 2004). Genetic deletion of the CX3CR1 fractalkine receptor, an important mediator in neuronal-glial interaction causes a reduction in inflammation and neuropathic pain in mice (Old et al., 2014; Staniland et al., 2010). Inhibiting the activation of glia following nerve injury has been studied extensively in recent years as a novel approach to treat NP pain, however most pharmacological agents (minocycline, propentofylline, pentoxifylline, fluorocitrate, and AV411) used to inhibit glial activation are promiscuous in that they alter function of multiple proteins and/or produce various non-specific effects through inhibition of common intracellular signalling cascades. Minocycline is a tetracycline-like antibiotic that also reduces glial activity by suppressing inducible NOS and p38 MAP kinase phosphorylation (Hua et al., 2005) and may affect T-cell/glial contact (Giuliani et al., 2005), but also has the ability to modulate Na\(^+\) and Ca\(^{2+}\) currents (González et al., 2007). In the substantia gelatinosa (lamina II) of the superficial dorsal horn, whole cell patch-clamp recording has shown inhibition of hyperpolarizing cation currents by minocycline that was not affected by sodium channel blockade or antagonism of GABA\(_A\) or glycine receptors (Liu et al., 2015), suggesting that even after so many years the mechanism of action of glial inhibitor drugs
are not completely understood. Fluorocitrate, another glial inhibitor drug, inhibits aconitase (Lauble et al., 1996), blocking the citric acid cycle preferentially in glial cells, but its action is not restricted to such cells. Propentofylline and pentoxifylline are methylxanthine derivatives, that in addition to AV411, inhibit adenosine transporters (Parkinson et al., 1993) and phosphodiesterases (Schubert et al., 1997), resulting in reduced proliferation and activity of both microglia and astrocytes (Gwak et al., 2008; Holdridge et al., 2006; Raghavendra et al., 2003; Sweitzer et al., 2001). However, methylxanthines also have the ability to inhibit such proteins in neurons and thus the ability to alter neuronal excitability.

PJ34 is a compound that inhibits the activity of poly-ADP ribose polymerase 1 (PARP-1), an enzyme involved in regulation of DNA repair mechanisms. PARP-1 regulates the activation of the transcription factor NF-κB, thus impacting the transcription and expression of pro-inflammatory cytokines and other inflammatory mediators that enhance neuronal excitability and increase glial cell activation and recruitment. In reperfusion injury models of ischemia, systemic administration of PJ34 has been shown to only inhibit microglial activation (Chiarugi and Moskowitz, 2003; Kaupinen and Swanson, 2005; Ullrich et al., 2001), is able to reduce inflammation and tissue damage, and may have neuroprotective effects (Crawford et al., 2010; Stone et al., 2009; Kaupinnen et al., 2009). To our knowledge the effects of PJ34 had not been demonstrated in a model of NP pain, although it has several advantages over existing microglial inhibitors for assessing the effects of these immunocompetent cells in the development of pain hypersensitivities associated with peripheral nerve injury. In this study, we aimed to determine what glial cell type was primarily responsible for the occurrence of pain-like behaviours associated with nerve injury by using PJ34 and compared its effects to a non-specific inhibitor of astrocytes and microglia PF.
Previous unpublished work in our lab did not see a significant decrease in GFAP labeling in nerve injured animals treated chronically with intrathecal PJ34, but did see a decrease in CD11b. All the imaging and quantification from those studies, however, was done with a low quality fluorescent microscope and the 2-dimensional images were only captured in a single layer in the z-plane. While GFAP was not significantly attenuated, the p-value was borderline and the trend looked very similar to the CD11b labeling. Thus, we wanted to re-assess GFAP labeling in sham and nerve injured animals using a higher quality confocal microscope capable of capturing not just single layers in the z-plane, but whole stacks than can be collapsed into more representative images.

The second purpose of this study was to compare and contrast the effects of both glial inhibitor drugs in different models of neuropathic pain and to compare different nociceptive testing modalities to select the optimal methodology moving forward in subsequent chapters. Two different models of chronic constriction injury (CCI) of the sciatic nerve were assessed: the Bennett & Xie (1988) model involves the tying of four loose ligatures around the sciatic nerve of the hindpaw, while the Mosconi and Kruger (1996) model involves wrapping polyethylene tubing around the nerve. While both surgical models are commonly used, are relatively easy and quick to perform, and the animals rarely show signs of poor health following sciatic nerve ligation (initial weight loss is usually recovered within 36-48 hours post-surgery), the composition of the chromic gut suture thread used to ligate the nerve in the Bennett & Xie model is derived from ovine or bovine intestine and thus induces an inflammatory component to the nerve injury that is not observed to the same extent in the Mosconi-Kruger model. Because of this, it was hypothesized that the Bennett & Xie model would produce more robust pain behaviors.
In addition to selecting the better surgical model, two similar yet different nociceptive tests involving the use of von Frey filaments were assessed following nerve injury to determine whether one has more value in assessing mechanical allodynia in the rat hindpaw. Most often, 50% withdrawal thresholds are assessed whereby the animal is placed on an elevated mesh grid and a series of calibrated filaments are applied sequentially in an up-down fashion to the plantar portion of the ipsilateral and contralateral hindpaws after surgery (Chaplan et al., 1994). Filament tension is increased or decreased accordingly depending on whether or not the rat withdraws its paw and the response pattern will determine the threshold at which the normally non-noxious stimulus of the filament becomes painful in the injured paw. Another method of assessing mechanical allodynia with von Frey filaments involves simply using a single filament (typically 2g or 12g in rats), but applying it repeatedly to the plantar portion of the hindpaw to see how many times the animal withdraws (usually out of several bins of 10 applications spaced a short time apart so as not to over-stimulate the tissue). The more reliable and consistent method of assessing mechanical allodynia was chosen moving forward.
2.2 METHODS

2.2.1 Animals and Housing

Male Sprague-Dawley rats (225-250g; Charles River, Quebec, Canada) were housed two per cage on a reverse 12/12 h light/dark cycle, and allowed ad libitum access to food and water. Animals were weighed daily as an assessment of general health. All experiments were performed in accordance with Queen’s University Animal Care Committee guidelines established by the Canadian Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues. After arrival within the housing facility, animals were allowed to acclimatize for three to four days and were then habituated to the testing apparatuses.

2.2.2 Surgery

Rats were randomly assigned to either one of two NP groups or a sham control group. Prior to surgery, all animals received 20mg of liquid acetaminophen. Under isoflurane and oxygen anaesthesia (5L/min induction, 2-3L/min maintenance), NP pain was induced by two different models: chronic constriction injury (CCI) of the sciatic nerve as described by Bennett and Xie (1988) by tying four loose ligatures around the nerve using 4-0 chromic gut thread, or as described by Mosconi and Kruger (1996) by wrapping a 2mm polyethylene (PE90) cuff around the nerve. The sham animals received a similar surgery but without manipulation of the nerve. During surgery, all animals received 0.1ml Tribrissen antibiotic (containing 4mg trimethoprim and 20mg sulfadiazine) and 5ml of lactated Ringer’s solution. Immediately following surgery, animals received acetaminophen crushed in gelatine (50mg), which was administered again the following morning.
2.2.3 Drug Administration

CCI and sham rats received either saline vehicle or propentofylline (PF, 10μg) through once daily intrathecal (i.t.) injections (30μl) beginning approximately one hour prior to surgery and continuing for eleven days post-surgery. Two other separate groups of CCI and sham animals received either saline vehicle or PJ34 (15μg). Drug administration was performed blind to treatment and drugs were coded by letters.

2.2.4 Tests for Mechanical Allodynia

Mechanical withdrawal thresholds were assessed prior to surgery (baselines) and four, seven and ten days post-surgery as described by Chaplan et al. (1994) by applying von Frey filaments in an up-down fashion to the plantar surface of the ipsilateral and contralateral hind paws. A second alternative method of assessing changes in mechanical sensitivity was also used whereby the rats were placed on top of the elevated wire mesh and using a single 12g von Frey filament, the number of withdrawals was assessed out of 10 applications (in three bins) on days 0, 4, 7 and 10. While it is not possible to blind oneself to surgery group between sham and CCI animals because the nerve-injured animals adopt a very distinct gait and guarding posture not exhibited in the shams, testing was blind as to which animals received which CCI surgery, as it was not possible to tell those groups apart visually.
2.2.5 Sacrifice, fixation and spinal cord isolation

Eleven days post-surgery, animals were anaesthetized with sodium pentobarbital (75mg/kg i.p; MTC Pharmaceuticals, Cambridge, ON, Canada) and perfused transaortically with 500ml of cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Rats were decapitated, spinal cords were removed and post-fixed for 30 min. in 4% PFA in 0.1M PB on ice. Spinal cords were cryoprotected in 30% sucrose in PB for 48 hours at 4°C. Spinal cords were then snap-frozen in -70°C isopentane over dry ice and stored at -80°C until used. These cords were either submitted to fluorescent IHC to assess changes in glial activation, or to DAB (3,3’-diaminobenzidine) IHC for light microscopy to assess changes in neuronal activation via c-Fos.

2.2.6 Fluorescent Immunohistochemistry

The L4-L5 region of spinal cords was isolated and the contralateral ventral side was notched for later identification of orientation. Using a freezing sledge microtome, cords were cut transversely into 40μm sections and collected in 24-well plates containing 0.1M tris-buffered saline (TBS). Sections were washed for 5 min. in 0.1M TBS, then for 5 min. in 0.1M TBS-triton (TBS-T). Sections were blocked for two hours at room temperature with 10% normal goat serum (NGS) in TBS-T. Sections were incubated overnight at 4°C in primary antibody solution (1% NGS and 1% bovine serum albumin (BSA) in 0.1M TBS-T) to label for astrocytes (1:2500 dilution anti-glial fibrillary acidic protein [GFAP], Z0334, lot: 00045904, Dako, Glostrup, Denmark).

The next day, sections were washed 2 x 5 min. in 0.1M TBS-T, then 2 x 10 min. in 0.1M TBS-T. Sections were incubated for two hours in the dark with secondary antibody conjugated to Alexa fluorophores (anti-rabbit 488nm, Molecular Probes, Invitrogen, ON, CA) at a 1:200
dilution in 5% NGS in 0.1M TBS-T. Sections were washed 3 x 10 min. in TBS-T, then 1 x 10 min. in 0.1M TBS. Sections were mounted on glass slides and coverslipped with Aquamount (Polysciences).

Three dimensional stacks of labelled astrocytes were captured on a Leica confocal microscope (TCS SP2 multi photon) within the superficial dorsal horn, ipsilateral and contralateral to the site of injury. At least three slices, randomly selected, were imaged per animal. Stacks were collapsed in ImageJ (NIH) using the Z Project function and fluorescent intensity of GFAP labeling was quantified as mean pixel intensity. The average of the sections was calculated per animal. Imaging and quantification was performed blind to treatment.

2.2.7 Quantification of neuronal activity via c-Fos

2.2.7.1 3,3’-diaminobenzidine (DAB) IHC

The L4-L5 region of spinal cords was isolated and the contralateral ventral side was notched for later identification of orientation. Using a freezing sledge microtome, cords were cut transversely into 30μm sections and collected in 24-well plates containing 0.1M phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by incubating sections in a 1:100 dilution of 30% hydrogen peroxide (in water) for 10 min. at room temperature. Sections were washed 4 x 5 min. in 0.1M PBS until no bubbles remained, then blocked for two hours at room temperature in 3% NGS & 3% BSA in 0.1M PBS). Sections were then incubated in primary antibody solution for 24 hours to label for c-Fos, a marker of neuronal activation (anti-c-Fos, rabbit polyclonal, ab7963-1, lot: 701686, Abcam, Cambridge, England. 1:2000 dilution in 0.5% NGS & 0.5% BSA in 0.1M PBS).
The next day, sections were washed three times in 0.5% NGS & 0.5% BSA in 0.1M PBS, then incubated for one hour at 4°C with a biotinylated secondary antibody diluted in the same washing solution (goat-anti-rabbit IgG, 1:200, Vector Labs, Burlingame, CA). Sections were then incubated with Vectastain ABC (avidin-biotin complex; Vector, 50μl solution A and 50μl solution B in 0.1M PBS). Sections were washed 2 x 10 min. in 0.1M TBS then 1 x 10 min. in 0.1M TB. Sections were then incubated for 5 min. with 500μl of 3,3’-diaminobenzidine (DAB) solution (1ml of 5mg/ml DAB, 9ml of 0.1M TB, 100μl of 8% nickel chloride). The reaction was initiated by the addition of 5μl hydrogen peroxide solution (500 μl of 30% H₂O₂ in 15ml water). The reaction was timed to ensure all wells developed for the same time and the reaction was terminated by washing with 0.1M TB once a light purple colour was achieved (5 min). Sections were washed 2 x 10 min. in 0.1M TB, then mounted on gelatine-coated slides. Slides were dehydrated using increasing concentrations of ethanol (5 min. 70%, 5 min. 80%, 5 min. 90% 2x 10 min. 100%), then soaked in CitriSolv® (2 x 15 min.). Slides were cover-slipped with Permount® then visualized on a Leica microscope (DM 4000B) and camera (DFC 350X).

2.2.7.2 Light microscopy and c-Fos immunoreactive cell counts

Using light microscopy, the number of immunoreactive cells labelled for c-Fos was counted at 40x magnification within the deep and superficial dorsal horn, ipsilateral and contralateral to the site of injury. At least three sections, randomly selected, were imaged per animal. Open Lab 4.0.2 was used for image capture (Improvision / Quorum Technologies, Guelph, CA). Imaging and counting were performed blind to treatment.
2.2.8 Statistical analysis

Two-way analysis of variance (ANOVA) was used for statistical analysis of IHC data using GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA) with surgery and treatment as between-subject factors. P < 0.05 was considered statistically significant. Tukey-Kramer post-hoc analysis was performed for multiple comparisons. Behavioural responses were analyzed by two-way ANOVA using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA, USA) with time as a within-subject factor and treatment as a between-subject factor to account for repeated measures design. P < 0.05 was considered statistically significant, and Bonferroni post hoc analysis was performed for multiple comparisons. Data are expressed as mean ± S.E.M. The hypothesis tested was considered two-tailed.

2.3 RESULTS

2.3.1 Prolonged treatment with intrathecal PJ34 inhibits spinal astrocytic activation

Previous studies have suggested that PJ34 inhibits microglia without affecting astrocyte activation, so to determine whether this was true, fluorescent IHC was performed using antibodies for GFAP (astrocytes) and high resolution confocal microscopy was used to capture images in three dimensions. 2-way ANOVA showed a significant effect of surgery (F(1,56) = 17.64, P < 0.0001); treatment effect was borderline but not significant (F(1,56) = 3.223, P = 0.0780) and no significant interaction was observed (F(1,56) = 3.36, P = 0.0731). CCI animals showed a significant upregulation of GFAP labeling in the superficial dorsal horn, ipsilateral to the site of injury, compared to sham saline controls (*** P < 0.001). The nerve injury-induced upregulation in GFAP (*** P < 0.001) was attenuated in CCI animals chronically treated with i.t. PJ34 (15µg) compared to CCI saline-treated controls (* P < 0.05) (fig. 2.1).
Chronic intrathecal administration of the glial inhibitor PJ34 attenuates the nerve injury-induced upregulation in GFAP in the spinal dorsal horn ipsilateral to the site of nerve injury.

Fig. 2.1 Representative micrographs of glial fibrillary acidic protein (GFAP) labeling in the superficial dorsal horn in neuropathic (NP, top) and sham animals (bottom) ten days post-surgery on the ipsilateral side. Animals were treated chronically over the ten days post-surgery with intrathecal (i.t.) PJ34 (15µg), a glial modulator (right) or saline vehicle (left). Micrographs are 63X magnification, captured on a confocal microscope under oil immersion.

Quantification of mean pixel intensity in collapsed stacks showed a significant upregulation in GFAP in the NP animals receiving saline compared to the sham animals receiving saline *** P < 0.001. Neuropathic animals treated chronically with intrathecal PJ34 showed significantly attenuated GFAP labeling compared to NP saline controls (*P < 0.05). Data are displayed as mean +/- S.E.M.
2.3.2 Prolonged treatment with both PF and PJ34 attenuates nerve injury-induced c-Fos activation

To confirm previous reports that the glial inhibitors PF and PJ34 had neuroprotective effects, c-Fos was used as a marker of neuronal activation and DAB IHC and light microscopy were employed to count the number of immunoreactive cells. Two-way ANOVA revealed a significant treatment effect ($F_{(1,56)} = 95.13, P < 0.0001$). CCI caused a bilateral increase in c-Fos immunoreactive cell counts compared to sham controls within the superficial dorsal horn (fig. 2.2a). There was no significant difference between the ipsilateral or contralateral sides in either group.

Comparing between groups on the ipsilateral side, one-way ANOVA revealed a significant treatment effect ($F_{(5,96)} = 41.91, P < 0.0001$). The increase in neuronal activation in NP animals was significantly attenuated by chronic treatment with PF compared to saline controls ($p < 0.001$), with the number of c-Fos immunoreactive cells reduced nearly to sham levels (fig. 2.2b). No difference between PF and saline was seen in the sham animals. Similarly, chronic treatment with PJ34 was able to attenuate neuronal activation compared to saline in the CCI animals ($p < 0.001$), but unlike PF, PJ34 could not reduce neuronal activation to sham levels (fig. 2.2b). Again, like PF, PJ34 had no effect on c-Fos cell counts in sham animals when compared to saline.
Chronic Constriction Injury (CCI) Causes A Bilateral Upregulation in c-Fos Expression in the Dorsal Horn Which Can Be Attenuated By Glial Inhibitor Drugs Like PJ34 and Propentofylline (PF)

Fig. 2.2 Micrographs show representative images of c-Fos (a marker of neuronal activity) labeling by DAB IHC in the dorsal horn of the L4-L5 spinal cord in sham (top row) and CCI (bottom row) animals chronically treated intrathecally with saline (left column) or one of two glial inhibitor drugs, PJ34 (15 µg, middle column) or propentofylline (PF, 10µg, right column) over the ten days post-surgery (10X magnification). A) shows immunoreactive counts for c-Fos in sham saline (yellow) and CCI saline (red) animals, ipsilateral and contralateral to the site of injury. CCI causes a bilateral upregulation in c-Fos compared to shams. B) shows immunoreactive cell counts for c-Fos ipsilateral to the site of surgery. The left triad shows sham saline (yellow), CCI saline (red), or CCI PF (green). The right triad shows separate groups of sham saline (purple), CCI saline (red) and PJ34 (blue). The nerve injury induced upregulation in c-Fos is attenuated when glial inhibitor drugs are administered. 3-5 sections were imaged per side per animal, with three animals per group. Bars represent mean +/- S.E.M. **** P < 0.0001, *** P < 0.001. CCI = chronic constriction injury, DAB = 3,3'-diaminobenzidine, IHC = immunohistochemistry, PF = propentofylline.
2.3.3 Prolonged treatment with PJ34 moderately attenuated nerve injury induced mechanical allodynia, while PF had no effect

To determine how changes in glial and neuronal activation correlated with changes in pain responses, mechanical withdrawal thresholds were assessed before surgery and four, seven and ten days post-surgery. In the Bennett & Xie model (fig. 2.3a), two way ANOVA revealed a significant effect of time \( \left( F_{(3,18)} = 13.48, P < 0.0001 \right) \). No significant treatment effect was observed \( \left( F_{(1,6)} = 0.03695, P = 0.8539 \right) \), and there was no significant time x treatment interaction \( \left( F_{(3,18)} = 0.6353, P = 0.6019 \right) \). In the Mosconi-Kruger model (fig. 2.3b), two way ANOVA revealed a significant effect of time \( \left( F_{(3,30)} = 5.452, P < 0.0041 \right) \). No significant treatment effect was observed \( \left( F_{(1,10)} = 1.195, P = 0.300 \right) \), and there was no interaction \( \left( F_{(3,30)} = 1.725, P = 0.1830 \right) \). Rats receiving CCI surgery demonstrated development of mechanical allodynia within the ipsilateral paw. The development of mechanical allodynia was more intense in animals receiving the Bennett & Xie CCI surgeries than those receiving CCI by the Mosconi-Kruger model (fig. 2.3). Compared to saline control, chronic administration of PF did not attenuate the development of mechanical allodynia in either the Bennett & Xie or the Mosconi-Kruger models (fig. 2.3a, 2.3b).

In the Bennett & Xie model (fig. 2.3e), two way ANOVA revealed a significant effect of time \( \left( F_{(3,42)} = 59.11, P < 0.0001 \right) \) and an interaction \( \left( F_{(3,42)} = 3.200, P = 0.0328 \right) \). No significant treatment effects was observed \( \left( F_{(1,14)} = 3.524, P = 0.0815 \right) \). In the Mosconi-Kruger model (fig. 2.3f), two way ANOVA revealed a significant effect of time \( \left( F_{(3,42)} = 7.090, P < 0.0006 \right) \). No significant treatment effects were observed \( \left( F_{(1,14)} = 0.06359, P = 0.8046 \right) \), and there was no interaction \( \left( F_{(3,42)} = 0.4708, P = 0.7042 \right) \). Compared to saline controls, chronic administration of PJ34 significantly attenuated the reduction in 50% withdrawal thresholds on day 10 post-surgery.
in the Bennett & Xie (p < 0.05) (fig. 2.3e), but not the Mosconi-Kruger model (fig. 2.3f).

Because some previous studies describing the efficacy of PF assessed changes in mechanical allodynia not by determining withdrawal thresholds, but by comparing the number of withdrawals in response to application of a single von Frey filament (either a 2g or a 12g filament), behaviour was repeated with separate groups of animals using this approach following both Bennett & Xie CCI vs sham, as well as Mosconi-Kruger CCI vs sham surgeries. In the Bennett & Xie model (fig. 2.3c), two way ANOVA revealed a significant effect of time (F(3,18) = 13.47, P < 0.0001). No significant treatment effect was observed (F(1,6) = 1.115, P = 0.3316), and there was no interaction (F(3,18) = 0.8983, P = 0.4613). In the Mosconi-Kruger model (fig. 2.3d), two way ANOVA revealed a significant effect of time (F(3,30) = 4.394, P = 0.0112). No significant treatment effects were observed (F(1,10) = 1.082, P = 0.3228), and there was no interaction (F(3,30) = 0.9461, P = 0.4308). Again, there was no significant decrease in the number of withdrawals to the 12g von Frey filament following chronic PF treatment compared to saline in either Bennett & Xie (fig. 2.3c) or Mosconi-Kruger (fig. 2.3d) surgical models. Data for the number of withdrawals to the 2g filament was highly variable and no significant differences were observed between PF and saline in either CCI surgical model.

For the PJ34 experiments, two way ANOVA revealed a significant effect of time (F(3,42) = 53.03, P < 0.0001), treatment (F(1,14) = 10.31, P = 0.0063), and an interaction (F(3,42) = 5.126, P = 0.0041) in the Bennett & Xie model (fig. 2.3g), and a significant effect of time (F(3,39) = 12.31, P < 0.0001), treatment (F(1,13) = 5.368, P = 0.0375), and an interaction (F(3,39) = 4.206, P = 0.0114) in the Mosconi-Kruger model (fig. 2.3h). Compared to saline controls, chronic administration of PJ34 significantly reduced the number of withdraws to a 12g von Frey filament 4 and 10 days post-surgery (*** p < 0.001) in the Bennett & Xie model (fig. 2.3g), and 7 and 10 days post-
surgery in the Mosconi-Kruger model (* p<0.05) (fig. 2.3h).
Chronic intrathecal administration of glial inhibitor PJ34 moderately attenuates mechanical allodynia following peripheral nerve injury in a modality-specific manner, while propentofylline has no effect.

**Bennett & Xie**

**PF**

**Mosconi-Kruger**

Fig. 2.3 Comparison of the antinociceptive effects of two glial modulators propentofylline (PF, A-D) and PJ34 (E-H) in two different surgical models of chronic constriction injury (CCI) of the sciatic nerve. The Bennett & Xie model involving the tying of four loose ligatures around the nerve is shown on the left-hand side (A, C, E, G); the Mosconi-Kruger model involving wrapping a polyethylene cuff around the nerve is shown on the right-hand side (B, D, F, H). Mechanical responses were assessed using two different testing modalities with von Frey filaments; the 50% withdrawal threshold which determine the tension at which the pressure becomes painful (A, B, E, F), and a second method involving the repeated application of a single 12g filament to the paw to see how many times the animals withdraw (C, D, G, H). For the 50% withdrawal method, higher values correspond to less pain, while for the 12g method, lower values equate to less pain. Red bars show saline-treated animals, blue bars show PF-treated animals, green bars show PJ34 treated animals.

Chronic intrathecal PF did not reduce mechanical allodynia in either the 50% withdrawal paradigm in either the Bennett & Xie model (A) or the Mosconi-Kruger (B), nor did it alter the number of withdrawal to the 12g filament in either surgical model (C and D) compared to saline treated controls. PJ34, on the other hand, attenuated mechanical allodynia by day 10 in the 50% withdrawal method in Bennett & Xie surgery (E, *P < 0.05), and by day ten had attenuated the mechanical alldynia in the 12g withdrawal method in both surgical groups (G, H). Bars represent mean +/- S.E.M. N=4-6/group. CCI = chronic constriction injury, PF = propentofylline.
2.4 DISCUSSION

Consistent with what other studies have shown (Sweitzer 2001; Sweitzer, 2006; Tawfik, 2007), chronic administration of PF attenuated the activation of both microglia and astrocytes (data not shown as this was done prior to the beginning of this PhD project). Though studies have already demonstrated that the microglial inhibitor minocycline suppresses NP pain hypersensitivity (Marchand et al., 2009), we wanted to find a substitute because it causes liver toxicity, and preliminary experiments using this drug demonstrated that animals had significant weight loss so the experiments had to be terminated. Thus, it became important to find a new compound that could inhibit microglia without affecting astrocytes to address the research objectives. Chronic administration of the PARP-1 inhibitor PJ34 inhibited the activation of microglia and astrocytes, which is contrary to what previous literature showed so it may or may not be a good substitute for minocycline to study the cell-specific contribution of activated glia following nerve injury. Previous studies have shown PJ34 to only inhibit microglia, not astrocytes, but this was done in reperfusion injury models of ischemia (Chiarugi and Moskowitz, 2003; Kauppinen and Swanson, 2005; Ullrich et al., 2001); to our knowledge PJ34 has not been administered in CCI nerve injury models. Previous studies have used the streptozotocin (STZ) diabetes model to assess changes in the levels of high energy blood metabolites and endoneurial blood flow (Li et al., 2004), while another looked at oral administration of PJ34 on mechanical allodynia and hyperalgesia and found no effect (Berti-Materra et al., 2008), but neither assessed the effect on glial activation.

Administration of either PF or PJ34 was able to attenuate the nerve injury-induced upregulation of c-Fos, a marker of neuronal activation, confirming that glial inhibitors have neuroprotective effects. Though there was a significant attenuation of c-Fos upregulation in NP
animals chronically treated with PJ34 compared to saline, PJ34 was unable to reduce c-Fos cell counts to sham levels, while chronic administration of PF did, suggesting that perhaps astrocytes may be playing a more significant role in neuronal activation than microglia this long (11 days) post-surgery. Some arguments may be made against the use of c-Fos as a suitable marker eleven days following nerve injury since its upregulation is so rapid (Harris, 1998), however there were significant differences in the c-Fos cell counts between the sham and NP groups eleven days post-surgery suggesting the nerve injury itself was still affecting c-Fos levels. This was repeated several times and effects on c-Fos were consistent. Of similar importance is the fact that neither PF nor PJ34 had any effect on c-Fos cell counts in sham animals, suggesting that activated glia are contributing to neuronal activation. Validating the use of c-Fos cell counts as a marker of neuronal activity in NP pain models, a study published by Narita et al. (2003) has also shown an up-regulation in c-Fos activity in a sciatic nerve ligation model in rats, using immunoblotting. They observed an increase in c-Fos levels within supraspinal sites within the brain associated with pain processing, namely the frontal cortex, thalamus and periaqueductal grey. Similarly, Frieboes et al. (2010) have demonstrated increased c-Fos expression following chronic nerve compression injury even after two weeks post-surgery, and Shimoyama et al. (2005) have also shown an increase in c-Fos positive cells within both the deep and superficial dorsal horn up to twenty-five days after injury in a NP cancer model, lending support that c-Fos upregulation can be prolonged following nerve injury in a number of different NP pain models.

Surprisingly, despite the fact both PF and PJ34 inhibited c-Fos upregulation, few effects were seen in the behavioural paradigms tested. Because some previous studies that showed positive results used variations on the mechanical allodynia testing, experiments were performed both to determine the withdrawal thresholds as well as the number of withdrawals to a single
filament. Similarly, some previous studies that showed positive outcomes with PF used the Mosconi-Kruger CCI model as opposed to the Bennett & Xie CCI model, which has a significant neuroinflammatory component to nerve injury, so mechanical allodynia was assessed using both models.

It appears that the effects observed are modality-specific, and both the surgical model selected and the behavioural testing procedures impact outcomes. The fact that PJ34 reduced the number of withdrawals to a 12g filament but only affected 50% withdrawal thresholds in the Bennett & Xie model may be important, and investigating the difference between these two supposedly similar testing paradigms could give an indication which neurons are affected by glial inhibition, for example those involved in wind-up.

The mechanism through which these drugs are modulating glial activation is important as well, since PJ34 (a PARP-1 inhibitor) was able to attenuate mechanical allodynia in several paradigms, while PF (a phosphodiesterase and adenosine reuptake inhibitor that modulates both microglia and astrocytes) had no effect in any. It is very possible that these drugs could be having direct effects on neurons as well.

One explanation for the lack of effect seen in the behavioural paradigms could be that an insufficient dose of drug was used, but the positive outcomes in the immunohistochemical labelling for glial and neuronal activation markers suggest that this is not the case. Despite the fact that glial inhibitors such as PF have shown promise at the basic science level for the treatment of a number of neurological disorders, translation into humans has not been successful, with several clinical trials and meta-analyses failing to show effect over placebo in patients suffering from Alzheimer’s disease and vascular dementia (Frampton et al., 2003). A phase II clinical trial investigating the use of PF in the treatment of post-herpetic neuralgia was
carried out by Solace Pharmaceuticals in 2009 in collaboration with Joyce DeLeo, a pioneer in the field of glia and pain, but the trial failed to show efficacy over placebo, the company was shuttered and the data were never published. New clinical trial data with minocycline has also failed to show clinically meaningful outcomes in lumbar radicular neuropathic pain (Vanelderen et al., 2015) or persistent pain after lumbar discectomy (Martinez et al., 2013), but was effective for diabetic neuropathy (Syngle et al., 2014).

The idea that glia exist in a “resting” state is simplistic and flawed, as microglia are constantly moving about and scanning the environment for debris and are highly mobile (Davalos et al., 2005). Upon injury, purinergic receptor activation leads microglia to migrate to the damaged tissue (Domercq et al., 2013). This process may be at least partially controlled by astrocytes (Haynes et al., 2006). Though “activated” glia are thought of as pro-inflammatory and cytotoxic, their functional state is actually dramatically impacted by the conditions that initiated the response. Microglia release anti-inflammatory molecules when cleaning up apoptotic cells and damaged myelin (Magnu et al., 2001). Microglia activated by IL-4 (which plays an important role in adaptive immune responses mediated by T cells) are actually neuroprotective (Bukovsky et al., 2005).

Microglial profiles are not only shaped by a primary stimulus, but by long-term changes in the environment that can result in vastly different metabolic or phenotypic states such as changes in signaling and cytokine production or alterations in phagocytic activity (Fumagalli et al., 2015; Kraft and Harry, 2010; Luo and Chen, 2012). Conductance changes are regulated by chloride channels (Ducharme et al., 2007), allowing the cell to change its shape and size, and over time microglia may become more “excitable”. A “postactivated” microglia may retain some form of memory, whereby a specific phenotype is generated when the cell is initially exposed to
a stimulus, causing distinct upregulation and expression of cell surface markers and production of a specific pattern of cytokines and chemokines, allowing it to remain primed to respond again more quickly in the future (Olson and Miller, 2004). Microglia of mesenchyme origin (Ginhoux et al., 2013) resemble a monocyte (which is a myeloid cell that differentiates into macrophages and dendritic cells). Parenchymal microglia are distinct from those that are macrophage-like (Li et al., 2013). Thus, the location and origin of the microglia in the CNS and the environment in which they are found can affect their metabolic or phenotypic state without necessarily changing their morphology.

Inhibiting microglia, which may reduce the release of pro-inflammatory cytokines and other inflammatory mediators, also inhibits myelin repair by trophic support factors (Kotter et al., 2005) and prevents removal of debris from damaged myelin (Reichert and Rotshenker, 2003). The debris may then be presented to T cells resulting in enhanced immune activation (Hanisch and Kettenmann, 2007).

Microglial recruitment following ischemic injury has been shown to be neuroprotective in some cases, not detrimental. Treatment with gancyclovir (an antiviral) to destroy microglia led to increased neuronal damage following ischemic injury and an increase in neuronal programmed cell death (Lalanchette-Hebert et al., 2007). Injection of microglia following ischemic injury also produces neuroprotective effects (Kitamura et al., 2004). Additionally, lipopolysaccharide activation of microglia increases TNF-a production, but this leads to an autocrine upregulation of the GLT-1 glutamate transporter normally found on quiescent astrocytes and this leads to increased removal of excitatory glutamate from the synaptic cleft, reducing neuronal excitability (Persson et al., 2005). Finally, microglia are involved in a process called synaptic stripping in which aberrantly functioning synapses are
removed by degrading specific dendrites (Cullheim and Thams, 2007). Thus, it is apparent that
glia exist in a spectrum of phenotypes and general inhibition of glia by PF and PJ34 may lead to
not only a reduction in release of pro-inflammatory factors from certain glia, but may also inhibit
those glia that are exhibiting neuroprotective effects, leading to negligible net changes in pain
responses. See Uwe-Karsten and Kettenmann (2007) for review. It is for these many reasons that
glial “inhibition” may not necessarily equate to changes in pain states, as many glial cells are
phenotypically different and inhibiting all glia in general may result in no net change.

2.5 CONCLUSION

Chronic pain states are unequivocally correlated with increased gliosis, but modulation of
glial activation does not necessarily appear to result in less pain. Without discounting the
possibility that activated microglia and astrocytes contribute to the development of pain
hypersensitivity in NP pain states, these data provide little evidence for glia as a driving force
behind the development of mechanical allodynia, though gliosis was positively correlated with
nerve injury and neuronal activity within the spinal dorsal horn, demonstrating a tight interplay
between glia and neurons in the periphery and spinal cord.

More consistent results were obtained when intensity of fluorescent labeling of glial cells
was quantified based on collapsed stacks collected on a confocal microscope, rather than
quantification of a single layer in a 2-dimensional image as was often the case in the past. In
future chapters, confocal imaging will be performed despite the increased amount of time it takes
to collect 3-dimensional stacks. Future chapters will also attempt to quantify cell size through 3-
dimensional reconstructions, as mean pixel intensity says more about the density of cells in an
area, but not much about the actual size of individual cells, both of which are important.
The Bennett & Xie model of peripheral sciatic nerve injury will continue to be used in future chapters as it provided more robust and consistent development of mechanical allodynia compared to the Mosconi-Kruger model.

While hypothetically the 50% withdrawal threshold and the # of withdrawals to a single filament should be measuring the same thing in terms of mechanical allodynia as they both employ the use of von Frey filaments applied in an up-down manner to the plantar portion of the hindpaw, there was some discrepancy between the two models leading us to believe that perhaps different sub-populations of neurons were being engaged by the two mechanical tests; for example, perhaps wind-up plays a role in the responses seen to the 12g method as the same filament tension is applied to the same area repeatedly within a short period of time. Thus, in future chapters, both mechanical testing paradigms will be used as they both provide valuable information.

This chapter only compared mechanical sensory tests. Moving forward, future chapters will also employ a thermal sensory test (the thermal tail flick assay), as different populations of neurons respond to different types of nociceptive stimuli and it will be important to know once we start administering the ULD α₂-adrenergic antagonists in the various models if it has a more selective effect on certain testing modalities or neuronal sub-populations.

In addition to the neuropathic pain model, a well-established model of chronic morphine tolerance will be used as well to see if ULD α₂-adrenergic antagonists have greater (if any) effects in the tolerance or chronic pain models, despite the two causing a lot of overlapping molecular changes in the periphery, spinal cord and higher brain regions.
Chapter 3: Effects of ULD α2-adrenergic antagonist atipamezole on morphine tolerance, opioid analgesia and hypersensitivity following nerve injury

Abstract

Background and purpose: Opioid treatment for chronic pain is limited by the development of analgesic tolerance as increasing the dose exacerbates side effects such as constipation and opioid-induced hyperalgesia. Spinal administration of ultra-low dose (ULD) α2 adrenergic receptor (AR) antagonists paradoxically enhances morphine effectiveness and attenuates analgesic tolerance in pain-naïve conditions. Here, we determined whether systemic ULD α2-AR antagonists would attenuate the development of pain hypersensitivities and would enhance morphine-induced effects in a model of neuropathic pain.

Experimental approach: NP pain was induced by chronic constriction injury of the sciatic nerve in rats. Sham and neuropathic pain animals were treated daily with subcutaneous morphine (5mg/kg), morphine & ULD atipamezole (5ng), atipamezole or vehicle for 10 days beginning one hour prior to surgery (N=5-6 per group). The effects of another α2-AR antagonist efaroxan were tested in separate animals. Thermal and mechanical withdrawal thresholds were assessed prior to and post-nerve injury. Key results: ULD α2-AR antagonist atipamezole did not modify nociceptive thresholds in sham animals, but partially alleviated mechanical allodynia associated with injury. Tolerance to morphine developed more quickly in neuropathic compared to sham animals. Chronic atipamezole improved the acute and chronic effects of morphine in neuropathic pain animals.

Conclusions and implications: Chronic systemic ULD α2-AR antagonists attenuate the
development of morphine tolerance in pain-naïve and improve opioid effectiveness in alleviating pain hypersensitivities in NP pain animals. Moreover, ULD atipamezole partially alleviated mechanical allodynia following peripheral nerve injury without altering nociceptive thresholds in the absence of persistent pain.
3.1 Introduction

While opioids are highly efficacious in the treatment of moderate to severe acute postoperative and traumatic injury-induced pain, their usefulness in the treatment of many chronic and persistent pain states is limited by a decrease in efficacy (Ballantyne and Shin, 2008), decreased potency (Christie, 2008), and the development of opioid-induced hyperalgesia (Mao, 2002). One novel strategy to mitigate analgesic tolerance is through concomitant use of ultra-low dose (ULD) G-protein coupled receptor (GPCR) antagonists. ULD is defined as a concentration several log units below the levels that result in ligand-induced receptor activity. Preclinical studies have shown that chronic administration of ULD opioid antagonists, like naloxone and naltrexone, do not inhibit opioid-induced pharmacological effects, but enhance the antinociceptive effects of morphine (Tsai et al., 2008), suppress the development of opioid analgesic tolerance (McNaull et al., 2007; Powell et al., 2002; Shen and Crain, 1997; Terner et al., 2006; Tuerke et al., 2011), and reduce opioid withdrawal symptoms (Mannelli et al., 2004; Olmstead and Burns, 2005). This phenomenon has been observed clinically as well, with studies demonstrating that ULD naltrexone enhances and prolongs oxycodone analgesia in patients suffering from osteoarthritis (Chindalore et al., 2005), and reduces physical dependence compared to oxycodone alone in chronic low back pain patients (Webster et al., 2006).

This ULD phenomenon is not restricted to opioid receptor antagonists. Recent studies have demonstrated that chronic intrathecal (i.t.) administration of ULD α2-adrenergic receptor antagonist atipamezole enhances clonidine analgesia in rats (Milne et al., 2011). Additionally, structurally diverse α2-adrenergic receptor antagonists are able to augment spinal morphine analgesia and attenuate development of acute and chronic opioid tolerance (Milne et al., 2008). To date, the preclinical studies on ULD opioid and α2-adrenergic receptor antagonists in models
of opioid tolerance have been performed in pain-naïve animals and may not be relevant to translational medicine where opioids are administered to attenuate moderate to severe pain. To our knowledge, no study has determined whether ULD α2-adrenergic receptor antagonists will modulate pain hypersensitivities associated with chronic pain or whether this treatment may improve opioid analgesia in a clinically meaningful model of persistent pain.

In the present study we hypothesize that systemic administration of ULD α2-AR antagonist will attenuate the development of pain hypersensitivities in an animal model of chronic pain. This study aimed to determine whether systemic administration of ULD α2-adrenergic receptor antagonists atipamezole and efaroxan would enhance morphine analgesia in pain-naïve animals and in animals following nerve injury to induce a neuropathic pain state. Thus, we investigated the effects of chronic systemic ULD atipamezole and efaroxan on (i) the development of chronic morphine tolerance in pain-naïve animals, (ii) on the development of mechanical allodynia and thermal hyperalgesia following nerve injury, (iii) on acute opioid analgesia in animals where neuropathic pain was established, (iv) on the development of morphine tolerance in nerve-injured animals.

3.2 Methods

3.2.1 Animals

Male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec, Canada) weighing 250-300g were used for all experiments. Animals were kept on a reverse 12h/12h light/dark cycle with lights off at 0700h and were allowed ad libitum access to food and water. Upon arrival, animals were allowed to habituate to their new surroundings for three days before handling. All experiments were performed in accordance with guidelines set by the Queen’s
University Animal Care Committee and the Canadian Council on Animal Care. All behavioural testing was performed blind to treatment. All experiments were performed during the animal’s active phase before 1400 h. Animals were assigned to treatment groups by ensuring that all animals within a cage received the same treatment. There was no computer assisted randomization of groups.

3.2.2 Surgery

For the neuropathic pain experiments, rats were assigned to either neuropathic or sham surgery groups. Neuropathic pain was induced through chronic constriction (CCI) of the sciatic nerve as described by Bennett and Xie (1988). Rats were anaesthetised under isoflurane anesthesia (5L/ min induction, 2-3L/ min maintenance). All animals received 5ml of Lactated Ringer’s Solution by subcutaneous injection. The left hind limb was shaved and cleaned with alcohol and then Betadine solution. An incision was made on the hind limb and the muscle layers were bluntly dissected with scissors to expose the sciatic nerve. Using 4-0 chromic gut suture thread, four ligatures were tied loosely around the sciatic nerve, and the muscle and skin were sutured with Monocryl 3-0 thread. Sham surgeries were used as controls whereby the animals received the same skin and blunt muscle dissection, but without manipulation of the sciatic nerve. All animals received Tylenol dissolved in Jello cubes (50mg/animal) immediately following surgery and again the following morning.
3.2.3 Drug administration

All drugs were administered through once daily subcutaneous injection. Atipamezole hydrochloride (5ng, Orion Pharma, Espoo, Finland), efaroxan hydrochloride (5ng, Tocris Bioscience, Ellisville, USA) morphine sulfate (MS, 5mg/kg, Sandoz Canada Inc., Boucherville, QC) were dissolved in 0.9% sterile saline. While the ULD antagonists were administered at 5ng doses, relative to body weight this is approximately 17ng/kg.

3.2.4 Behavioural nociceptive testing

3.2.4.1 Thermal Tail Flick Assay

The five cm distal portion of the rat tail was marked with a permanent black marker and a thermal tail flick analgesiometer (IITC Life Science Inc., Woodland Hills, CA) was used to determine tail flick latencies as described previously (D’Amour and Smith, 1941). Baseline thresholds were assessed prior to the start of each experiment on the same day, with the beam intensity adjusted to elicit tail flick latencies between 2-3 seconds. A cut-off was set at three times baseline to avoid tissue damage.

3.2.4.2 Mechanical Paw Withdrawal Thresholds

Using a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL), 50% withdrawal thresholds were assessed in the ipsilateral hindpaw as described by Chaplan et al. (1994). Filaments were applied sequentially in an up-down manner to the plantar portion of the hindpaw and tension was increased or decreased accordingly. An additional method of assessing mechanical responses was also performed on the same animals whereby the number of withdrawals to a single 12g filament (average out of a total of thirty applications in three sets of
ten) was assessed (Colburn et al., 1997). In all animals, 50% mechanical withdrawal threshold was assessed prior to determining the number of withdrawals to the 12g filament.

3.2.5 Experiment 1: effects of chronic systemic ULD alpha2-adrenergic antagonists on development of chronic morphine tolerance in pain-naïve animals

Animals were randomly assigned to one of four groups: morphine (5mg/kg, subcutaneous (s.c.)), morphine & ULD atipamezole (5ng, s.c.), ULD atipamezole alone (5ng/kg, s.c.), or vehicle (saline, 0.1ml/100g, s.c.). Thermal tail flick latencies were assessed daily prior to and following drug injections for seven days. This protocol was also used to assess the effects of efaroxan (5ng, s.c.) in separate groups of animals.

3.2.6 Experiment 2: effects of chronic ULD alpha2-adrenergic antagonists on development of mechanical allodynia and thermal hyperalgesia following nerve injury

Animals were randomly assigned to either a neuropathic pain surgical group receiving CCI of the sciatic nerve or a sham control group. Half the animals in each group received once daily injections of ULD atipamezole (5ng) and the other half received equivalent volumes of vehicle (saline). Drug or vehicle treatment was initiated one hour prior to surgery. Thermal and mechanical behavioural testing was performed on days -1, 4, 7 and 10 post-surgery. To determine if either ULD atipamezole or efaroxan had any acute effects on their own in the absence of opioid administration, thermal and mechanical response thresholds were assessed before and after injection ten days following surgery.
3.2.7 Experiment 3: effects of chronic systemic ULD alpha2-adrenergic antagonists on acute opioid analgesia in nerve injured animals

On day 10 post-surgery, all animals (sham and neuropathic) from experiment 2 received a single subcutaneous injection of morphine (5mg/kg) and thermal and mechanical responses were assessed over a two-hour time course post-injection to determine the effects of treatment on acute opioid analgesia. This experiment was repeated with separate groups of animals to determine the effects of ULD efaroxan (5ng), rather than atipamezole.

3.2.8 Experiment 4: effects of chronic systemic ULD alpha2-adrenergic antagonists on development of chronic morphine tolerance in nerve injured animals

All animals underwent CCI surgery to induce neuropathic pain. Animals received once daily injections of morphine (5mg/kg), morphine plus ULD atipamezole (5ng), or ULD atipamezole (5ng) for ten days post-surgery. Thermal and mechanical behavioural testing was performed on days -1, 4, 7 and 10 post-surgery before and after injection.

3.2.9 Data Analysis

Behavioural responses were analyzed by two-way analysis of variance (ANOVA) using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA, USA) with time as a within-subject factor and treatment as a between-subject factor to account for repeated measures design. P < 0.05 was considered statistically significant, and Bonferroni post hoc analysis was performed for multiple comparisons. Data are expressed as mean ± S.E.M. All data collected was included in the data analysis and no data was omitted. The hypothesis tested was considered two-tailed.
3.3 RESULTS

3.3.1 Experiment 1: Effects of ULD \(\alpha_2\)-adrenergic antagonists on development of morphine tolerance in pain naïve animals

Chronic administration of morphine (5mg/kg, s.c., Q24h) significantly reduced the ability of morphine to elicit thermal antinociception (Figure 3.1), as evidenced by a decrease in tail flick latencies measured 30 minutes after daily morphine injections. Concomitant administration of either ULD alpha-2 adrenergic antagonist (atipamezole or efaroxan) with morphine significantly attenuated the development of analgesic tolerance (Figure 3.1A,B). Statistical analysis by a 2-way ANOVA revealed a significant effect of treatment (\(F_{(3,12)} = 101.7, ***p<0.001\)), time (\(F_{(4,48)} = 35.43, ***p<0.001\)) and an interaction (\(F_{(12,48)} = 11.59, ***p<0.001\)) for the atipamezole experiment and a significant effect of treatment (\(F_{(1.6)} = 8.61, *p<0.05\)), time (\(F_{(5,30)} = 11.67, ***p<0.001\)) and an interaction (\(F_{(5,30)} = 2.78, *p<0.05\)) for the efaroxan experiment. Post hoc analysis revealed a significant effect at day 4 in the atipamezole experiment and days 4-6 in the efaroxan experiment compared to animals receiving morphine alone. Chronic systemic administration of ULD atipamezole alone or saline-vehicle did not change thermal withdrawal latencies over the seven day trial (Figure 3.1A). A two-hour time course of morphine or morphine and \(\alpha_2\) adrenergic antagonist was determined on day 1 and day 7 following daily drug administrations. On the first day of injections there was no difference in thermal tail flick latencies between animals receiving morphine and those receiving morphine co-administered with ULD atipamezole (5ng) (Figure 3.1A center) or morphine co-administered with efaroxan (Figure 3.1B center). On the seventh day of daily drug treatment, a two-hour time course was
performed post-drug injections. Statistical analysis by a 2-way ANOVA revealed a significant effect of treatment ($F_{(3,12)} = 10.83$, ***$p<0.001$), time ($F_{(6,72)} = 6.568$, ***$p<0.001$) and an interaction ($F_{(18,72)} = 3.387$, ***$p<0.001$) for the atipamezole experiment (fig.3.1A right panel) and a significant effect of treatment ($F_{(1.6)} = 6.346$, *$p<0.05$), time ($F_{(6,36)} = 5.914$, ***$p<0.001$) and an interaction ($F_{(6,36)} = 2.50$, *$p<0.05$) for the efaroxan experiment (fig. 3.1B right panel). Animals that had been treated with morphine elicited tail flick latencies not significantly different than their baseline responses prior to any drug treatment, while the animals that had been chronically treated with morphine and atipamezole (Figure 3.1A right) or morphine and efaroxan (Figure 3.1B right) had significantly higher tail flick latencies compared to morphine only groups.

In order to determine whether acute synergistic or additive effects exist between morphine and ULD atipamezole that would not be detected at maximal analgesic doses of morphine, we co-administered ULD atipamezole with submaximal doses of morphine. No additive effects of the combination were evident (data not shown), suggesting that any potential additive effects do not account for the ability of ULD atipamezole to attenuate the development of tolerance.
Fig. 3.1 Chronic co-administration of ULD alpha2-adrenergic receptor antagonists attenuates morphine tolerance in pain naive animals. No difference in opioid analgesia was observed over the two hours after the first injection, with most animals receiving morphine still experiencing analgesia after 120 minutes (A and B middle panels). By day 7, however, following chronic daily injections, the animals receiving morphine alone experienced little to no analgesia following injection, while the animals that had been chronically co-administered ULD atipamezole (5ng) and morphine (5mg/kg) experienced significantly more analgesia even 120 minutes postinjection (A right panel). Co-administration of efaroxan (5ng) with morphine (5mg/kg) resulted in less enhancement of opioid analgesia over the two hour time course on day 7 (B right panel). Peak analgesia occurred around 30 minutes post-injection and tracking this time point over the seven day trial (A and B left panel) demonstrated that the development of chronic morphine tolerance was reduced in the animals co-administered either ULD atipamezole (5ng) or efaroxan (5ng) with morphine compared to morphine alone. No acute or chronic effects were observed for either atipamezole alone or saline (A left panel). MS = morphine, Ati = atipamezole, Efx = efaroxan. Data displayed as mean +/- S.E.M. Group numbers: Panel A: Saline n=4, morphine n=5, morphine and atipamezole n=5, atipamezole n=4. Panel B: morphine n=5, morphine and efaroxan n=5. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to MS (5mg/kg).
3.3.2 Experiment 2: Effects of chronic systemic ULD α2-adrenergic antagonists on development of mechanical allodynia and thermal hyperalgesia following peripheral nerve injury

To determine the effects of chronic administration of ULD α2 adrenergic antagonists on the development of pain hypersensitivities following nerve injury, sham and CCI animals were treated daily with atipamezole (5ng, s.c., Q24h), efaroxan (5ng, s.c., Q24h) or saline for either 7 or 10 days beginning 1h prior to surgery. CCI of the sciatic nerve produced a significant change in mechanical withdrawal thresholds ipsilateral to nerve injury (Figure 3.2B), indicative of the development of mechanical allodynia. However, nerve injury did not alter thermal withdrawal thresholds as determined by tail flick latencies (Figure 3.2A). No changes in thermal or mechanical withdrawal thresholds compared to baseline responses were evident in sham animals (data not shown). To determine whether chronic systemic administration of ULD atipamezole would attenuate the development of mechanical allodynia in the CCI animals, animals were tested for withdrawal thresholds daily prior to the administration of the ULD α2-AR antagonist. Statistical analysis by a 2-way ANOVA revealed a significant effect of treatment ($F_{(1,34)} = 23.31$, ***p<0.001), time ($F_{(3,102)} = 73.22$, ***p<0.001) and an interaction ($F_{(3,102)} = 15.26$, ***p<0.001) for number of withdrawals to 12g filament (fig. 3.2B bottom left), and a significant effect of treatment ($F_{(1,10)} = 4.93$, *p<0.05), time ($F_{(3,30)} = 25.06$, ***p<0.001) but no interaction ($F_{(3,30)} = 0.745$, p=0.544) for 50% withdrawal threshold (fig. 3.2B upper left). Figure 3.2 demonstrates chronic treatment with ULD atipamezole attenuated mechanical allodynia as evidenced by fewer withdrawals to a 12g filament on day 4 (p<0.01), 7 (p<0.01) and 10 (p<0.001) post-surgery compared to animals receiving saline, and attenuated the decrease in mechanical withdrawal thresholds on day 10 post injury (Figure 3.2B, left panels). However, atipamezole had no effect
on thermal withdrawal thresholds compared to saline treated animals (Figure 3.2A). No
differences between saline and efaroxan treatment groups were evident in CCI animals, in either
the thermal tail flick assay or the mechanical withdrawal tests (Figure 3.2, right panels).
Chronic ULD Atipamezole Attenuates Mechanical Allodynia But Has No Effect on Thermal Hypersensitivity Following Nerve Injury

Fig. 3.2 Chronic administration of ULD atipamezole attenuates mechanical allodynia following nerve injury. CCI did not result in the development of thermal hyperalgesia in the tail over ten days post-surgery (A), but did result in the development of mechanical allodynia in the ipsilateral hindpaw (B). Chronic systemic administration of ULD atipamezole (5ng) had no effect on thermal tail flick latencies (A), but did attenuate mechanical allodynia by increasing the withdrawal thresholds (B upper left) and decreasing the number of withdrawals to a single 12g filament (B bottom left) compared to saline controls. Chronic administration of ULD efaroxan (5ng) alone had no effect on thermal tail flick latencies (A top right) or mechanical paw withdrawal thresholds (B right upper and bottom panels) compared to saline over the ten days following nerve injury. Ati = atipamezole, Efx = efaroxan. Data displayed as mean +/- S.E.M. * = p < 0.05, *** = p < 0.001 compared to CCI saline.

Group numbers: Panel A: saline n=18, atipamezole n=18, saline n=4, efaroxan n=4. Panel B upper: saline n=6, atipamezole n=6; saline n=5, efaroxan n=5. Panel B lower: saline n=18, atipamezole n=18; saline n=12, efaroxan n=12.
3.3.3 Experiment 3: Effects of chronic systemic ULD α₂-AR antagonists on acute effects of morphine in nerve injured animals

To determine whether ULD α2 adrenergic antagonists alter acute morphine analgesic effects in neuropathic pain animals, all animals received a single acute injection of morphine (5mg/kg, s.c.) and withdrawal thresholds were assessed over a two hour time course on either day 10 (atipamezole experiment) or day 7 (efaroxan experiment) post nerve injury. Morphine injection significantly increased thermal tail flick latencies in CCI and sham animals (Figure 3.3). In the tail flick test, morphine produced maximal withdrawal thresholds for the first forty-five minutes post-injection in both saline and ULD α₂-AR antagonist groups. In the CCI animals in the atipamezole experiment (fig. 3.3A left), statistical analysis by a 2-way ANOVA revealed a significant effect of treatment ($F(1,10) = 42.00, ***p<0.001$), time ($F(6,60) = 58.48, ***p<0.001$), and an interaction ($F(6,60) = 6.53, ***p<0.001$). Atipamezole significantly increased morphine effects at 60 (p<0.01), 90 (p<0.001), and 120 (p<0.01) minutes post-injection compared to control saline-treated animals (Figure 3.3A left). No significant differences between atipamezole and saline treatment groups were evident in the sham animals (Figure 3.3A right). No differences in acute morphine-induced effects were produced in the tail flick assay in either the CCI or sham animals that had been chronically treated with ULD efaroxan compared to saline (Figure 3.3B left and right, respectively).

The ability of ULD α2 adrenergic antagonists to enhance acute morphine-induced effects in neuropathic pain animals was also assessed in two mechanical tests. Acute morphine administration significantly attenuated mechanical pain hypersensitivities in CCI animals (Figure 3.4). Animals that had been chronically treated with atipamezole (5ng, s.c., Q24h) or efaroxan
(5ng, s.c., Q24h) showed significantly prolonged morphine-induced anti-allodynic effects (Figure 3.4). Statistical analysis by a 2-way ANOVA revealed a significant effect of treatment ($F_{(2,17)} = 5.052$, *p*<0.05), time ($F_{(4,68)} = 31.86$, ***p*<0.001) but not an interaction ($F_{(8,68)} = 2.06$, *p*=0.052) for the 50% withdrawal threshold and a significant effect of treatment ($F_{(2,57)} = 11.15$, ***p*<0.001), time ($F_{(4,228)} = 55.10$, ***p*<0.001) and an interaction ($F_{(8,228)} = 6.498$, ***p*<0.001) for number of responses to application of a 12g filament. *Post hoc* analysis revealed a significant effect of efaroxan treatment at 90 and 120 min post morphine injection as determined by 50% withdrawal threshold and significant effect of atipamezole and efaroxan treatment at 90 and 120 min post morphine injection as determined by the number of responses to a 12g filament compared to saline-treated animals.
Chronic Systemic Administration of ULD Atipamezole Increases the Thermal Analgesic Effects of Acute Morphine Administered Ten days Following Nerve Injury

Fig. 3.3 Chronic systemic administration of ULD atipamezole increases the thermal analgesic effects of acute morphine ten days following nerve injury. On day 10 post-surgery, all animals received a single acute injection of morphine (5mg/kg) and behaviour was assessed over two hours. Neuropathic animals that had been chronically treated with ULD atipamezole displayed significantly prolonged thermal analgesia compared to chronic saline following acute morphine injection (A left panel), while no effect was observed in sham animals (A right panel). Chronic ULD efaroxan (5ng) had no effect on thermal tail flick latencies compared to saline following acute morphine administration ten days after surgery in neuropathic (B left panel) or sham (B right panel) animals.

Ati = atipamezole, Efx = efaroxan. Data displayed as mean +/- S.E.M. ** = p < 0.01, *** = p < 0.001 compared to CCI Saline. Group numbers: Panel A CCI: saline n=6, atipamezole n=6; sham: saline n=6, atipamezole n=6. Panel B CCI: saline n=4, efaroxan n=4; Sham: saline n=4, efaroxan n=4.
Chronic Systemic Administration of ULD Atipamezole Increases the Mechanical Analgesic Effects of Acute Morphine Administered Ten days Following Nerve Injury

Fig. 3.4 Chronic systemic administration of ULD alpha2-adrenergic antagonists increases the mechanical analgesic effects of acute morphine ten days following nerve injury. On day 10 post surgery, all animals received a single acute injection of morphine (5mg/kg) and mechanical behavioural responses were assessed over two hours in neuropathic animals. Animals that had been chronically treated with ULD atipamezole displayed significantly prolonged mechanical analgesia compared to chronic saline following acute morphine injection (B). Chronic ULD efaroxan (5ng) enhanced the acute morphine mechanical analgesia in both testing paradigms (A and B). Saline controls were pooled from separate groups of animals (saline vs efaroxan or saline vs atipamezole groups).

Ati = atipamezole, Efx = efaroxan. Data displayed as mean +/- S.E.M. * = p < 0.05, *** = p < 0.001 compared to CCI saline. N=6/group for atipamezole and efaroxan. N=10 for saline.
3.3.4 Experiment 4: Effects of chronic systemic ULD α2 adrenergic antagonists on development of chronic morphine tolerance in nerve injured animals

To determine whether concomitant treatment of ULD α2 adrenergic antagonists with morphine would attenuate the development of tolerance in a chronic pain model, animals received once daily subcutaneous injections of morphine alone (5mg/kg), morphine plus ULD atipamezole (5ng), or ULD atipamezole (5ng) alone for ten days following nerve injury. Behavioural responses were assessed on days 4, 7, and 10 post surgery thirty minutes after injections (at peak antinociceptive effect, Figure 3.5 left panels).

In the thermal tail flick assay, opioid tolerance developed in the animals treated with morphine alone (Figure 3.5A, left panel). By day ten, the thermal tail flick latency in the morphine treated animals was around five seconds, compared to eleven seconds on day four post-surgery. Over the ten days post-surgery, statistical analysis by 2-way ANOVA revealed a significant effect of treatment (F(1,13) = 8.832, *p<0.05), time (F(2,26) = 5.624, **p<0.01) and an interaction (F(2,26) = 4.412, *p<0.05) for thermal testing (fig. 3.5A left). The development of tolerance was completely abolished in the animals that received daily concomitant ULD atipamezole with morphine over the ten days (Figure 3.5A, left panel). Post hoc analysis revealed that CCI animals co-administered morphine with ULD atipamezole showed significantly higher tail flick latencies on day 10 post-surgery compared to the morphine-only group (Fig. 3.5A, left panel, ** P < 0.01).

To determine whether the time course of morphine analgesia was altered by co-administration of ULD α2-AR antagonists in animals with established neuropathic pain, thermal tail flick latencies were assessed over a two hour time course on day 10 post-surgery. Statistical analysis by a 2-way ANOVA revealed a significant effect of treatment (F(2,18) = 34.64,
***p<0.001), time (F(6,108) = 10.15, ***p<0.001) and an interaction (F(12,108) = 4.462, ***p<0.001) for thermal testing (fig. 3.5A right). In the thermal tail flick assay, pre-injection baselines on day 10 were not different among animals that had been receiving morphine alone, morphine plus ULD atipamezole, or ULD atipamezole alone (Figure 3.5A, right panel). Injection of ULD atipamezole alone had no effect on the thermal tail flick latencies over the two hour time course, suggesting the antagonist has no acute effects on its own, at least over that time frame. When co-administered with morphine, however, opioid analgesia was prolonged and the peak effect was greater than in the animals that had received morphine alone. Animals that received morphine with ULD atipamezole had significantly higher tail flick latencies at 30 (*** P < 0.001), 45 (*** P < 0.001), and 120 (* P < 0.05) minutes post-injection compared to morphine alone. By 120 minutes, morphine alone was no longer analgesic and was not different than ULD atipamezole alone, while animals that received morphine plus ULD atipamezole still had tail flick latencies approximately three times higher than the pre-injection baselines on that day (Figure 3.5A, right panel).

Peak drug anti-allodynic effects (mechanical) of morphine or morphine co-administered with ULD atipamezole were also assessed thirty minutes post-injection on days 4, 7, and 10 post-surgery (Figure 3.5B, left panel) by determining 50% withdrawal thresholds as well as the number of withdrawals to a single 12g filament. The ability of morphine to reverse mechanical allodynia diminished significantly over the ten days following surgery, indicative of an opioid-tolerant state. In contrast to effects on thermal thresholds, ULD atipamezole was more effective in augmenting morphine-induced analgesia on mechanical pain hypersensitivity (Figure 3.5B, left panels). 2-way ANOVA revealed a significant effect of treatment (F(1,10) = 37.48, ***p<0.001), time (F(2,20) = 3.468, *p<0.05) and no interaction (F(2,20) = 0.5259, p=0.599) for
the 50% mechanical withdrawal threshold (fig. 3.5B upper left), and a significant effect of treatment (F(1,34) = 1033, ***p<0.001), time (F(2,68) = 738.9, ***p<0.001), and an interaction (F(2,68) = 522.7, ***p<0.001) for number of positive responses to a 12g filament (fig. 3.5B bottom left). *Post hoc* analysis revealed a significant effect of ULD atipamezole co-treatment compared to morphine alone on days 7 and 10 post-surgery for both mechanical testing paradigms.

To determine whether the time course of morphine anti-allodynic effects was altered by co-administration of ULD atipamezole after neuropathic pain was established, 50% mechanical withdrawal thresholds and number of withdrawals to a 12g filament were assessed over two hours on day ten post-surgery (Figure 3.5B, right panels). Two-way ANOVA revealed a significant effect of treatment (F(2,15) = 30.99, **p<0.01), time (F(3,45) = 4.587, **p<0.01), and an interaction (F(6,45) = 3.760, **p<0.01) for the 50% mechanical withdrawal threshold (fig. 3.5B upper right) and a significant effect of treatment (F(2,42) = 101.4, ***p<0.001), time (F(3.126) = 11.41, ***p<0.001) and an interaction (F(6,126) = 3.652, **p<0.01) for number of positive responses to a 12g filament (fig. 3.5B lower right). Pre-injection baselines of animals that had received daily injections of ULD atipamezole alone showed significantly higher 50% withdrawal thresholds (* P < 0.05) and fewer withdrawals to a 12g filament (*** P < 0.001) compared to morphine alone. This was consistent with the fact that in experiment 2 it was seen that ULD atipamezole had anti-allodynic effects of its own, even in the absence of opioids (see Figure 3.2B), and chronic morphine administration can lead to hyperalgesia. Over the two hour time course, however, mechanical responses in animals that been receiving ULD atipamezole alone over the ten days did not change from the pre-injection baseline on that day, again suggesting the antagonist has no acute effects of its own over that time frame. In contrast, there
was no difference in pre-injection baseline between ULD atipamezole alone or morphine plus ULD atipamezole, but animals that had been co-administered morphine plus ULD atipamezole had significantly fewer 12g withdrawals at 30 and 60 minutes post-injection (φφ p < 0.01) compared to ULD atipamezole alone. Co-administration of ULD atipamezole significantly augmented morphine-induced effects over the entire two hour time course compared to treatment with morphine alone in both mechanical testing paradigms (Figure 3.5B, right panels).
Chronic Co-Administration of ULD Atipamezole Attenuates Development of Morphine Tolerance in Neuropathic Animals

A Thermal

B Mechanical

Fig. 3.5 Chronic co-administration of ULD atipamezole attenuates development of morphine tolerance in neuropathic animals over the ten days following nerve injury (A and B left panels). The two hour time courses following injection on day 10 for thermal (A right panel) and mechanical (B right panels) testing demonstrate that the animals that had been chronically treated with morphine plus ULD atipamezole (5ng) experienced enhanced and prolonged analgesia compared to those that had been treated chronically with morphine alone. The peak analgesic effect was higher in animals chronically co-administered morphine with ULD atipamezole compared to morphine alone and even after two hours, most animals were still experiencing significant analgesia. No acute effects were observed following injection in animals chronically treated with ULD atipamezole alone in either the thermal (A right) or mechanical (B right) testing. Data in left hand panels show tail flick and mechanical responses at thirty minutes (peak effect) post-injection over the ten days post-surgery. Data in right hand panels show two hour time courses after injection on day 10 post-surgery. MS = morphine, Ati = atipamezole. Data displayed as mean +/- S.E.M. N=6 for CCI Ati, N=5 for CCI MS + Ati, N= 10 for CCI MS. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to CCI MS. ϕ = p < 0.05, ϕϕ = p < 0.01, ϕϕϕ = p <0.001 compared to CCI Ati.
3.4 DISCUSSION

In the present study, using several behavioural testing modalities of thermal and mechanical nociception, systemic administration of two chemically distinct $\alpha_2$-AR antagonists attenuated chronic morphine tolerance, attenuated pain hypersensitivities in a model of neuropathic pain, and enhanced morphine analgesia in chronic pain animals. To our knowledge, this is the first study to demonstrate that ULD $\alpha_2$-AR antagonists are effective in alleviating pain hypersensitivities associated with peripheral nerve injury and in enhancing the acute and chronic effects of morphine in a clinically meaningful model. Previous studies have demonstrated that ULD atipamezole and efaroxan attenuate morphine analgesic tolerance but were only tested in pain-naive animals (Lilius et al., 2012; Milne et al., 2013).

Chronic systemic administration of ULD atipamezole or efaroxan had no effect on thermal nociceptive thresholds in either neuropathic or sham control animals. However, atipamezole attenuated the development of mechanical allodynia as evidenced in two different paradigms through which mechanical withdrawal thresholds were assessed. No effect of efaroxan was evident; however, in this study the magnitude of pain hypersensitivity following nerve injury was less compared to the atipamezole experiment (Fig. 3.2) and therefore may have decreased the power to observe a significant effect. Interestingly, ULD $\alpha_2$-AR antagonist treatment enhanced morphine-induced effects to attenuate mechanical allodynia, and atipamezole, but not efaroxan, enhanced morphine-induced antioception in the tail flick thermal nociceptive test. Finally, treatment with atipamezole enhanced the effectiveness of morphine in neuropathic pain animals.

Two $\alpha_2$-AR antagonists were used in the present study. The dose of atipamezole used was based on a pilot study and selected based on optimal responses. Efaroxan has a similar molecular
weight as atipamezole and was therefore administered at the same dose. In competitive binding studies using RX821002 (a selective antagonist at $\alpha_2$-ARs that does not bind to imidazoline receptors [Clarke and Harris, 2002]), atipamezole was shown to have the highest binding affinity for $\alpha_2$-ARs in the rat cortex ($K_i = 0.2\text{nM}$) compared to other selective ligands, and several fold higher than efaroxan (Renouard et al., 1994). Different potencies may explain some variations in behavioural responses to the two drugs, but moving forward in subsequent chapters, ULD atipamezole will be tested rather than efaroxan.

The results in pain-naive animals are consistent with previous studies demonstrating that intrathecal administration of $\alpha_2$-AR antagonists attenuated the development of morphine analgesic tolerance in both acute and chronic opioid tolerance models (Milne et al., 2008, 2013). It is unknown in the present study at which sites the $\alpha_2$-AR antagonists are having their effect, though it is likely partially mediated within the spinal cord. Similarly, Lilius et al. (2012) reported that spinal administration of ULD atipamezole attenuated the development of morphine tolerance in pain-naive animals; however, they reported that systemic administration had no effect. It is unclear why our results differ from that study; however, subcutaneous doses of atipamezole used in the Lilius paper were several-fold higher and their dosing paradigm to induce morphine tolerance differed. Our tolerance model was once daily injection of 5mg/kg morphine for seven days, while Lilius et al. administered morphine twice daily for four days in an escalating dosage paradigm starting at 10mg/kg on day one up to 30mg/kg on day four. We also treated animals chronically with the ULD $\alpha_2$-AR antagonists daily with or without morphine, while Lilius et al. administered atipamezole as a single injection in naive or morphine-tolerant animals at the end of the dosing paradigm.

To date three main subclasses of $\alpha_2$-ARs have been identified, $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$, though
atipamezole and efaroxan have affinity for all three (Pertovaara et al., 2005). Chronic morphine administration increases $\alpha_2$-AR expression in the brains of rats (Hamburg and Tallman, 1981), in the dorsal horn of the spinal cord and peripherally in the dorsal root ganglia (Tamagaki et al., 2010). The change in receptor expression following chronic morphine is thought to underlie the enhanced analgesic effect of spinally administered $\alpha_2$-AR agonists (Tamagaki et al., 2010). This central opioid-adrenergic synergy is dependent on the $\alpha_{2A}$ receptor subtype (Stone et al., 1997), which is predominant in the rat and human CNS (Lawhead et al., 1992; Stone et al., 1998).

Changes in expression or function of the three $\alpha_2$-AR subtypes are not required for the development of pain hypersensitivities (Malmberg et al., 2001). However, expression of $\alpha_{2A}$ increases in dorsal root ganglia neurons (Cho et al., 1997), but a decrease in $\alpha_{2A}$ expression is observed in the spinal cord ipsilateral to the site of peripheral nerve injury (Stone et al., 1999). Spinal nerve ligation was reported to not alter the expression of $\alpha_2$-ARs, but increased G-protein coupling to $\alpha_2$-ARs in the spinal cord (Bantel et al., 2005). These differential changes in expression and coupling to second messenger pathways may explain the enhanced effects of ULD $\alpha_2$-AR antagonists in neuropathic pain compared to pain-naive animals.

### 3.5 CONCLUSION

While the exact mechanisms through which ULD $\alpha_2$-AR antagonists are able to attenuate morphine tolerance and enhance opioid analgesia are not known, this study provides strong evidence that $\alpha_2$-AR antagonists are able to directly or indirectly modulate opioid receptor function in clinically meaningful models.
Chapter 4: Effects of ULD α<sub>2</sub>-adrenergic antagonist atipamezole on morphine and nerve injury-induced gliosis, neuronal activation, and mu-opioid receptor expression

4.1 INTRODUCTION

Considering that ULD α<sub>2</sub>-AR antagonists attenuate the development of neuropathic pain behaviors and inhibited the development of morphine tolerance in pain naïve and nerve injured animals, it may be postulated that ULD α<sub>2</sub>-AR antagonists may prevent the neuroplastic changes associated with the development of neuropathic pain. Moreover, such changes in neuroplasticity are responsible for the development of an opioid tolerant state even in the absence of opioids (Mayer et al., 1999); thus α<sub>2</sub>-AR antagonists may indirectly modulate opioid receptor function by inhibiting such mechanisms. In pain-naive (fig. 3.1) as well as nerve-injured animals (figs. 3.2-3.5), chronic administration of ULD atipamezole on its own had no effect on thermal withdrawal thresholds and was comparable to the effects produced by saline treatment. Hence, it can be argued that atipamezole has no inherent analgesic effects of its own (at least in regards to thermal response thresholds) but must suppress either peripheral or central sensitization associated with the development of neuropathic pain. The fact that there was no change in mechanical or thermal responses following acute administration of either of the α<sub>2</sub>-AR antagonists on their own in any of the experiments suggests that the enhancement of opioid analgesia cannot be entirely attributed to additivity or synergy.

While the mechanisms of action are currently unknown, ULD α<sub>2</sub>-AR antagonists may be attenuating the development of opioid tolerance and neuropathic pain behaviour by modulating neuronal-glial interactions. Both chronic morphine administration (Hutchinson et al., 2007) and
nerve injury (Inoue, 2006) have been shown to cause activation of microglia and astrocytes in the peripheral (Liang et al., 2010) and central nervous system (Hains and Waxman, 2006), contributing to the initiation and maintenance of chronic pain states through the enhanced production of pro-inflammatory cytokines and other inflammatory mediators that can also contribute to the development of opioid analgesic tolerance (Raghavendra et al., 2002). Studies with ULD opioid antagonist naltrexone have been shown to inhibit chronic morphine-induced glial activation (Mattioli et al., 2010). Because the ULD phenomenon appears to be common to several GPCRs involved in pain processing and opioid action, and there is a lot of similarity in terms of the behavioral response and changes in G-protein-coupling mechanisms in response to ULD opioid (Wang et al., 2005), cannabinoid (Paquette et al., 2007), and adrenergic receptor antagonists (Milne et al., 2011), it is likely that the attenuation of tolerance and reduction in pain hypersensitivity seen with ULD atipamezole will correlate with a reduction in spinal gliosis as well.

Pain severity is highly correlated with the amount of glial activation in the spinal cord (O’Callaghan and Miller, 2010) and brain (Loggia et al., 2015), and both nerve injury and chronic morphine result in increased neuronal activity in the spinal dorsal horn (Chen et al., 2005); particularly within the deep (lamina V) and superficial (laminae I/II) regions. Peripheral nerve injury causes an upregulation in microglial activation in the superficial dorsal horn ipsilateral to the site of injury (Taves et al., 2013), and pro-inflammatory cytokines produced by activated glia, including interleukin-1β, interleukin-6, and TNF-α increase neuronal activity and synaptic firing in the superficial dorsal horn (Kawasaki et al., 2008). In the spinal cord, MORs are predominantly expressed in the superficial dorsal horn as well (Arvidsson et al., 1995b) and indirectly modulate the deep dorsal region via inhibitory neuronal projections (Light and
Kavookjian, 1988). Immunoreactive cell counts for c-Fos, a marker of neuronal activation, are significantly higher in the superficial dorsal horn ten days after chronic constriction injury (CCI) compared to sham surgery controls (see fig. 2.2), and chronic intrathecal treatment of nerve injured animals with glial inhibitors like propentofylline and PJ34 reduces spinal gliosis (fig. 2.1) and attenuates the nerve injury-induced upregulation of c-Fos (fig. 2.2. Early pieces of this work can also be found in Grenier, 2010), demonstrating the interconnectedness of pain, glia and neuronal activation and the strength of their feedback loops in maintaining sensitization. Propentofylline is a xanthine-derived astrocyte and microglia inhibitor that is believed to function through activity as a phosphodiesterase and adenosine reuptake inhibitor (Borgland et al., 1998). A recent study, though, has also shown that propentofylline inhibits drug-seeking and cocaine relapse caused by cue- and drug-induced reinstatement, and that the mechanism through which it is able to do so is by attenuating the downregulation of glutamate transporter GLT-1 within the nucleus accumbens (Reissner et al., 2014). The GLT-1 transporter’s function on quiescent astrocytes is to remove excess excitatory glutamate from the synapse, but it becomes downregulated when astrocytes are activated leading to increased glutamatergic activity (Ramos et al., 2010). PJ34 is a microglia inhibitor (Chiarugi and Moskowitz, 2003; Kauppinen and Swanson, 2005) that functions through poly-ADP ribose polymerase-1 (PARP) inhibition and through PARP-independent mechanisms involving cell cycle checkpoint regulation (Madison et al., 2011), thus exerting control over transcriptional regulation of inflammatory cytokine and reactive oxygen species production, and mitotic arrest.

The aim of this chapter was to assess whether co-administration of ULD atipamezole altered chronic morphine and nerve injury-induced spinal gliosis, nerve injury-induced neuronal activity inferred by c-Fos cell counts, and MOR expression in the deep and superficial dorsal
horn in all the animals whose behavior was assessed in the previous chapter. For all animals in chapter 3, spinal cord tissue was collected and perfused at the end of the behavioral testing to perform immunohistochemistry (IHC) in order to determine if there was any molecular evidence to support what has been seen behaviorally with ULD atipamezole. Astrocytes were labelled for glial fibrillary acidic protein (GFAP) and microglia were labelled for CD11b. Fluorescent IHC labeling was quantified in collapsed 2-dimensional images and cell sizes were assessed through 3-dimensional reconstructions. Immunoreactive cell counts for c-Fos were performed by DAB (3,3'-diaminobenzedine) IHC and immunoreactive cell counts were quantified. Previous studies have shown a downregulation in MOR expression following nerve injury in DRG neurons (Zhang et al., 2016; Hervera et al., 2011). Obara et al. (2009; 2010) also saw a nerve injury-induced decrease in MOR expression and functionality in the DRG, but saw no change in the spinal dorsal horn, nor did Herradon et al. (2008), but Zhang et al. (1998) did see a reduction in spinal MOR expression following peripheral axotomy, so we still wanted to determine if ULD atipamezole altered spinal MOR expression in CCI rats. MORs were labeled by DAB IHC as well and cell counts and mean grey scale labeling was quantified.

4.2 METHODS

For all immunohistochemical experiments, tissue was collected from all the animals involved in the behavioral experiments in Chapter Three to assess changes in spinal glial activation (microglia and astrocytes), neuronal activation (via c-Fos), or changes in mu-opioid (MOR) expression. Animals were sacrificed one day after the completion of the behavioral testing; thus, for the morphine tolerance experiments, animals were sacrificed on the eighth day (because the morphine tolerance paradigm was assessed over seven days), and in the neuropathic
pain experiments, animals were sacrificed on the eleventh day post-surgery (because the chronic pain experiments were assessed over ten days, with the longer time frame allowing for more robust development of pain hypersensitivity and spinal gliosis). In the morphine tolerance and neuropathic pain experiments, glial (microglia and astrocyte) activation was assessed and astrocyte volume was quantified. C-Fos immunoreactive cells were not counted in tissue from the morphine tolerance studies because we did not see an upregulation in spinal c-Fos following chronic morphine injections in pilot studies. C-Fos immunoreactive cells were counted in the tissue from the neuropathic pain experiments because we have consistently and repeatedly seen a bilateral upregulation in c-Fos in nerve-injured animals compared to sham controls, and this nerve injury-induced upregulation in c-Fos can be attenuated if animals are chronically treated with a glial inhibitor like PJ34 or propentofylline, confirming the cross-modulation between glial and neuronal activity in chronic pain states (see fig. 2.2). MOR expression was only assessed in tissue from the neuropathic pain experiments simply due to time constraints.

4.2.1 Sacrifice, perfusion, and tissue isolation

On the eighth day (in the morphine tolerance paradigm) or the eleventh day post-surgery (in the neuropathic pain experiments), rats were deeply anesthetized with sodium pentobarbital (75mg/kg, intraperitoneally [i.p.]; MTC Pharmaceuticals, Cambridge, ON, Canada) and perfused transaortically with 500ml of cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Rats were decapitated, spinal cords were ejected, and post-fixed in ice-cold 4% PFA for 30 minutes. Spinal cords were then cryoprotected by immersion in 30% sucrose (dissolved in 0.1M PB) at 4°C for 48-72 hours. Spinal cords were then snap-frozen in -70°C isopentane over dry ice and then placed in a -80°C freezer for storage until the start of immunohistochemical labeling.
4.2.2 Fluorescent immunohistochemistry (IHC) to assess changes in spinal glial activation

4.2.2.1 Tissue sectioning and immunohistochemical labeling

Spinal cords were removed from -80°C storage and the L4,5 lumbar section of the cords was isolated with a razor blade. A small notch was made on the ventral contralateral side (in the case of tissue from the neuropathic pain studies) to allow for lateral identification during imaging. Using a freezing sledge microtome, 30µm thick sections were cut transversely and collected in 24-well plates containing 0.1M tris-buffered saline (TBS). Sections were washed 1 X 5 minutes in 0.1M TBS, then 1 X 5 minutes in 0.1M TBS-Triton (TBS-T) to increase antibody penetration. Tissue was blocked for two hours at room temperature with blocking buffer containing 10% normal goat serum (NGS) and 10% bovine serum albumin (BSA) to reduce the likelihood of non-specific labeling.

Sections were incubated with primary antibody solution containing 1% NGS and 1% BSA in 0.1M TBS-T overnight at 4°C to label microglia (anti-CD11b, raised in mouse, 1:1000 dilution, MLA257R, batch: 0404, AbD Serotec, Raleigh, NC, USA) or astrocytes (anti-glial fibrillary acidic protein [GFAP], raised in rabbit, 1:2500 dilution, Z0334, lot: 00045904, Dako, Glostrup, Denmark). Microglia and astrocyte labeling was performed on separate tissue sections; double labeling was not performed due to problems in the past with bleed-through during confocal imaging.

The next day, sections were washed at room temperature 2 x 5 minutes, then 2 x 10 minutes in 0.1M TBS-T to remove any excess unbound primary antibodies. Sections were then incubated at room temperature for two hours (in the dark) with appropriate secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Invitrogen, ON, CA) at 1:200 dilution in 5% NGS and 5% BSA in 0.1M TBS-T. GFAP-labeled tissue was incubated with a 488nm anti-
rabbit secondary (thus, all GFAP labeled images will appear green), while the CD11b-labeled tissue sections were incubated with 594nm anti-mouse secondary (and all CD11b labeled images will appear red). After incubation, sections were washed 3 x 10 minutes in 0.1M TBS-T, then 1 x 10 minutes in 0.1M TBS. Sections were transferred to a petri dish filled with 0.1M TB and mounted onto glass microscope slides using a paintbrush, then allowed to dry in the dark prior to cover-slipping with Aquamount (Polysciences). Slides were stored in the dark in the fridge at 4°C until confocal imaging was performed.

4.2.2.2 Confocal Imaging

Imaging of CD11b and GFAP fluorescent labeling was performed on a confocal microscope (Leica TCS SP2 Multi Photon Laser Scanning confocal microscope). Images were captured, blind to treatment, within the deep dorsal horn (around lamina V) and the superficial dorsal horn (within laminae I,II), ipsilateral and contralateral to the site of injury (in the case of tissue collected from the neuropathic experiments; laterality would not apply to tissue from the morphine tolerance studies as there is no site of injury). Images were captured in stacks through the z-plane (to allow for later 3-dimensional reconstruction) at 63X magnification under oil immersion. The number of layers varied somewhat for each slice depending on the exact thickness of the tissue but typically each stack contained twenty to thirty layers. Settings such as gain and the number of passes (to clean up background noise) were optimized for each fluorophore at the beginning of the imaging and kept constant across all treatment groups for the entire duration.
4.2.2.3  Quantification of fluorescent labeling intensity

For quantification of average pixel intensity in 2-dimensional images, all the layers of each 3-dimensional stack were collapsed using ImageJ software (open-source, developed by National Institutes of Health) by using its Z Project function (with Maximum Intensity set as the Projection Type). Collapsed images were ‘Measured’ in ImageJ and the mean pixel intensities were compared across groups/treatment.

4.2.2.4  3-dimensional reconstructions and assessment of cell size

Mean pixel intensity in a 2-dimensional image gives a good assessment of the density of the cells in the image, but it doesn’t indicate anything about the size of individual cells; so to compare cell size, 3-dimensional reconstructions of the collected stacks were performed using Image-Pro Plus 6.0 software (a commercial package developed by Media Cybernetics, Rockville, MD). Stacks were imported into Image-Pro Plus and Merge Images was used to create a video representation of the stack. Using the 3D Constructor, voxel and sub-sampling size was set at x=4, y=4, z=1 pixels and kept constant throughout. Global Transparency and Surface Value settings were optimized with the first stack and kept constant for all other stacks. No filters were used for volume measurements. Close Edges was used and Isosurface Simplification was set to Medium as this gave the best and most consistent assessment of individual cells (when set to Low, the software often identified single cells as multiple fragments, and when set to High it often ignored the presence of whole cells). The Volume of all cells was determined throughout the entire stack, not just a small area of interest (AOI) as we have done in the past. Though time consuming, this gives a better assessment of the cells in the whole section, not just a small portion of it and eliminates some of the subjectivity of assigning an AOI. Cell volumes were
sorted from largest to smallest and the volumes of the three largest cells were recorded for each stack. The software also reported mean cell volumes, total number of detected cells, and the total sum of the volumes of all cells in the stack so these were compared and analyzed, but for simplicity only the mean of the three largest cells is reported and compared in the Results as this was the most consistent and reliable indicator of whether cell size was affected by surgery or drug treatment. The stacks were not adjusted or manipulated in any way whatsoever and the volumes were recorded exactly as the software detected them, with all settings kept constant.

4.2.3 Assessment of neuronal activation via c-Fos immunoreactive cell counts and quantification of MOR expression

4.2.3.1 3',3-diaminobenzadine (DAB) IHC to label c-Fos and MOR

Neuronal activation was inferred via immunoreactive cell counts for c-Fos using 3-3’-diaminobenzidine (DAB) IHC and light microscopy. Frozen spinal cords were removed from the -80°C freezer, the L4-L5 lumbar region was isolated with a razor blade and a small notch was cut in the ventral area contralateral to the side of nerve injury to allow for lateral identification during imaging. Sections were cut transversely at 30µm with a freezing sledge microtome and collected in 24-well plate containing 0.1M phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched for ten minutes at room temperature using dilute hydrogen peroxide (1:100 dilution of 30% H₂O₂ in distilled water). 4 x 5 minutes washes with 0.1M PBS were done to ensure no visible sign of hydrogen peroxide (bubbles) remained. Tissue was blocked for two hours at room temperature in blocking solution containing 3% NGS and 3% BSA in 0.1M PBS, then incubated at 4°C with a solution containing the primary antibody for c-Fos (rabbit polyclonal, 1:2000 dilution, ab7963-1, lot: 701686, Abcam, Cambridge, England) in
0.5% NGS and 0.5% BSA in 0.1M PBS for 24 hours. Separate sections were incubated at 4°C for 24 hours with an antibody for mu-opioid receptor (MOR) (raised in guinea pig, 1:500 dilution, GP10106, Neuromics, MN, USA).

The next day, sections were washed 3 x 5 minutes with 0.5% NGS and 0.5% BSA in 0.1M PBS, then incubated for one hour with an appropriate biotinylated secondary antibody (goat anti-rabbit IgG for the c-Fos labeled tissue, anti-guinea pig IgG for the MOR labeled tissue, both 1:200 dilution, Vector Laboratories, Burlingame, CA) dissolved in the washing solution. Sections were then incubated for one hour at room temperature with Vectastain ABC (avidin-biotin complex) (Vector Laboratories, 50µl solution A + 50µl solution B per 5ml of 0.1M PBS). Sections were washed 2 x 10 minutes with 0.1M PBS, then 1 x 10 minutes in 0.1M PB. Sections were incubated under a fume hood for five minutes with 500µl of DAB solution per well (1ml of 5mg/ml DAB, 9ml of 0.1M PB, 100µl of NiCl to increase resolution). Addition of 5µl of H₂O₂ solution (500µl of 30% H₂O₂ in 15ml of distilled water) initiated the reaction, which was timed to ensure equal development time across all groups. The reaction was terminated once a light purple colour was achieved (3 minutes) by removal of the DAB solution and washing with 0.1M PB.

Sections were washed 2 x 10 minutes in 0.1M PB, then transferred to a petri dish containing 0.1M PB. Tissue was mounted on gelatin-coated glass microscope slides using a paintbrush and allowed to dry overnight. The next day, tissue sections were dehydrated by immersing the slides in escalating concentrations of ethanol (5 minutes in 70% EtoH, 5 minutes in 80% EtOH, 5 minutes in 90% EtOH, 2 x 10 minutes in 100% EtoH), then 2 x 15 minutes in CitraSolv™ (a clearing agent and solvent that is less toxic than xylene). Slides were left to dry, then cover-slipped with Permount®.
4.2.3.2 Imaging through light microscopy

Imaging was performed under a light microscope (Leica DM 4000B) and images were captured at 40X resolution (Leica FireCam) within the deep and superficial dorsal horn ipsilateral and contralateral to the site of injury.

4.2.3.2.1 c-Fos immunoreactive cell counts

The number of c-Fos immunoreactive cells was counted, blind to treatment. 2-3 sections were imaged per side per area per animal, for three animals per group/treatment. Sections were randomly selected and were only excluded from counting if they were physically damaged. Statistical analysis was performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA with Tukey post hoc analysis.

4.2.3.2.2 Quantification of MOR expression through cell counts and mean pixel intensity

The number of immunoreactive cells was also counted for MOR labelled tissue in the same manner as was done for c-Fos. Because there was a lot of variability in the amount of MOR labeling on each cell, making counting somewhat difficult as it was hard to decide if the very black cells should be counted in the same way as ones with less labeling, a second method of quantification was performed that was less subjective. Mean pixel intensity was quantified in all the images of MOR labeling with Image J, as was described previously with the 2-d labeling of glia. 3-4 sections were randomly selected per area per side from three animals per group. Sections were only excluded from quantification if they were physically damaged. Statistical analysis was performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA with Tukey post hoc analysis.
4.3 RESULTS

4.3.1 Assessing changes in gliosis in pain naïve animals receiving chronic morphine

In order to quantify changes in microglial and astrocyte activation, CD11b and GFAP were labeled through fluorescent immunohistochemistry and stacks were captured on a confocal microscope within the deep and superficial dorsal horn of the spinal cord in animals treated for seven consecutive days with morphine (5mg/kg), morphine plus ULD atipamezole (5ng), ULD atipamezole alone (5ng), or saline vehicle. Representative micrographs are presented for all groups at 63x magnification under oil immersion (fig. 4.1). For CD11b labeling in the deep dorsal horn (fig. 4.2A), comparison of mean pixel intensity by 2-way ANOVA showed a significant treatment effect ($F_{(1,124)} = 13.30, P = 0.0004$), an effect of vehicle (saline vs atipamezole) ($F_{(1,124)} = 20.14, P < 0.0001$), and no interaction ($F_{(1,124)} = 0.3574, P = 0.5511$). For CD11b labeling in the superficial dorsal horn (fig. 4.2B), comparison by 2-way ANOVA showed a significant treatment effect ($F_{(1,113)} = 13.12, P = 0.0004$), an effect of vehicle ($F_{(1,113)} = 15.60, P = 0.0001$), and no interaction ($F_{(1,113)} = 0.7235, P = 0.3968$). Significantly more CD11b was observed in the morphine-treated animals compared to the saline-treated controls in both the deep (fig. 4.2A, ** $P < 0.01$) and superficial dorsal horn (fig. 4.2B, *** $P < 0.001$). Animals that had been chronically co-administered ULD atipamezole showed a significant attenuation of morphine-induced microgliosis in both the deep (fig. 4.2A, *** $P < 0.001$) and superficial (fig. 4.2B, *** $P < 0.001$) dorsal horn, with the expression of CD11b reduced to levels that did not differ from saline controls. Animals treated with ULD atipamezole alone had significantly lower CD11b expression compared to morphine treated animals in both areas (**** $P < 0.0001$), but did not differ from the saline-treated controls. The reason for the higher n-value in this figure compared to most of the others is simply due to the fact that CD11b expression in this trial was
quantified from single 2d images, not the collapsed stacks as were done for all the other experiments so there was more variability in the mean pixel intensity for each image within groups and the fact that the labeling in single images is low compared to whole stacks necessitated pooling of data from multiple trials. All data collected from multiple trials are represented in this figure and no data points were excluded for any reason.

Quantification and comparison of mean pixel intensity of GFAP labeling in collapsed stacks in the deep dorsal horn (Fig. 4.3A) by two way ANOVA revealed a significant effect of treatment ($F_{(1,40)} = 9.017, P = 0.0046$) and an interaction ($F_{(1,40)} = 22.48, P < 0.0001$). Animals treated with morphine had significantly higher labeling in the deep dorsal horn (Fig. 4.3A) than the saline-treated controls (* $P < 0.05$), while co-administration of ULD atipamezole significantly reduced GFAP labeling compared to animals receiving morphine alone (***, $P < 0.001$). The animals that were co-administered ULD atipamezole with morphine had less GFAP labeling than those receiving ULD atipamezole alone (** $P < 0.01$). No difference in GFAP labeling was observed between the saline-treated controls and the animals receiving ULD atipamezole only. In the superficial dorsal horn (Fig. 4.3B), quantification and comparison of mean pixel intensity of GFAP labeling by two-way ANOVA revealed a significant treatment effect ($F_{(1,143)} = 70.03, P < 0.0001$) and an interaction ($F_{(1,143)} = 4.141, P = 0.0480$). GFAP labeling in animals chronically treated with morphine alone was not significantly different than saline, but animals co-administered ULD atipamezole with morphine had significantly less GFAP labeling compared to animals receiving chronic morphine alone (***, $P < 0.001$), and lower labeling than the saline controls (**** $P < 0.0001$) as well. Animals that received chronic systemic injections of ULD atipamezole alone also had significantly lower GFAP labeling than the saline-treated (**** $P < 0.0001$) rats and the animals that received daily morphine injections...
To assess changes in the size of individual cells, stacks from morphine animals and morphine plus ULD atipamezole animals were reconstructed into 3-dimensional models and cell volumes were quantified within the deep and superficial dorsal horn using Image Pro software. Representative cells are shown as wireframe models drawn to scale to allow for visual comparison (fig. 4.4 A-D). Quantification of reconstructed GFAP-labeled stacks showed co-administration of ULD atipamezole significantly decreased astrocyte volume in both the deep (* P < 0.05) and superficial (**** P < 0.0001) dorsal horn (Fig. 4.4) compared to animals receiving morphine alone (F(3,140) = 13.84).
Microglia (CD11b) and Astrocyte (GFAP) Labeling in the Superficial Dorsal Horn of the Spinal Cord of Pain Naive Rats

Fig. 4.1 Representative micrographs demonstrating microglia (CD11b, left column in red) and astrocyte (GFAP, right column in green) labeling by fluorescent IHC in the superficial dorsal horn in pain naive rats treated chronically for seven days with saline, morphine (5mg/kg), morphine + ULD atipamezole (5ng), or ULD atipamezole alone (Ati). 63X Magnification on confocal microscope under oil immersion.

Ati = atipamezole, GFAP = glial fibrillary acidic protein, IHC = immunohistochemistry, ULD = ultra-low dose.
Co-Administration of ULD Atipamezole Reduces CD11b (Microglia) Labeling in the Deep and Superficial Dorsal Horn Compared to Morphine-Treated Controls

Fig. 4.2 Mean pixel intensity of CD11b labelling in collapsed stacks of the deep (A) and superficial (B) dorsal horn of the L4-L5 spinal cord of pain naïve rats following seven days of injections with saline, morphine alone (5mg/kg), morphine plus ULD atipamezole (5ng), or ULD atipamezole alone. N = 3-6 images were captured per region per animal from at least N= 3 animals per group. Data were pooled from three separate trials and all collected data from those three trials are shown. No data points were excluded for any reason. Total N=12-46 images per group. a.u. = arbitrary units, ULD = ultra-low dose. Bars represent mean +/- S.E.M. ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.
Co-Administration of ULD Atipamezole Reduces GFAP (Astrocyte) Labeling in the Deep and Superficial Dorsal Horn Compared to Morphine-Treated Controls

Fig. 4.3 Mean pixel intensity of GFAP labelling in collapsed stacks of the deep (A) and superficial (B) dorsal horn of the L4-L5 spinal cord of pain naïve rats following seven days of injections with saline, morphine alone (5mg/kg), morphine plus ULD atipamezole (5ng), or ULD atipamezole alone. Three-four stacks per animal were imaged in each area from three animals per group. Total N=10-12/group. a.u. = arbitrary units, ULD = ultra-low dose. Bars represent mean +/- S.E.M. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.
Fig. 4.4 (A-D) Representative 3-dimensional reconstructions of astrocytes in the superficial (A and B) and deep (C and D) dorsal horn of the spinal cord in pain naïve animals treated with morphine (5mg/kg) (A and C) or morphine plus ULD atipamezole (5ng) (B and D) for seven days. Wire frames of the cells are represented to scale. Grey box dimensions are 250 x 250 pixels. (E) Quantification of astrocyte (GFAP) cell volume from 3-dimensional reconstructions of stacks within the deep and superficial dorsal horn of the L4-L5 spinal cord of pain naïve rats following seven days of injections with morphine alone (5mg/kg, red) or morphine plus ULD atipamezole (5ng, blue). Co-administration of ULD atipamezole significantly reduced astrocyte volume in both areas compared to animals treated with morphine alone. The three largest cells were quantified in each area of each stack, with four stacks per animal from three animals per group. Total N=36/group. a.u. = arbitrary units, GFAP = glial fibrillary acidic protein, ULD = ultra-low dose. Bars represent mean +/- S.E.M. * = P < 0.05, **** = P < 0.0001.
4.3.2 Assessing changes in gliosis in nerve-injured animals

4.3.2.1 Quantification of intensity of CD11b and GFAP labeling

To assess changes in microglial activation in the L4,5 lumbar spinal cord, sections were labeled with an antibody against CD11b/OX42, sections were imaged through the z-plane on a confocal microscope, stacks were collapsed into 2-dimensions, and fluorescent intensity was quantified as mean pixel intensity using Image J software. Representative micrographs of collapsed stacks of CD11b labeling within the deep and superficial dorsal horn ipsilateral and contralateral to the site of injury are displayed at 63x magnification under oil immersion (fig. 4.5). CCI animals treated with saline (Fig. 4.6A) showed significantly more CD11b labeling in the superficial compared to the deep dorsal horn ipsilateral to the site of injury (** P < 0.001). Within the superficial dorsal horn (Fig.4.6A) there was much greater labeling on the ipsilateral compared to the contralateral side (**** P < 0.0001) (F(3,28) = 17.21). In CCI animals treated chronically with ULD atipamezole (Fig. 4.6B), there was no significant difference in the expression of CD11b between the deep and superficial dorsal horn, though there was still significantly more labeling on the ipsilateral compared to the contralateral side (** P < 0.01), but only in the deep dorsal horn (F(3,28) = 5.568, P = 0.0040).

Chronic systemic administration of ULD atipamezole in CCI animals significantly reduced the amount of CD11b labeling compared to the saline controls in the superficial dorsal horn (Fig. 4.6D), ipsilateral to the site of injury (* P < 0.05) (F(3,28) = 13.06). Comparing the same groups within the deep dorsal horn (Fig. 4.6C), there was no difference between the CCI animals treated with either saline or ULD atipamezole; in both treatment groups there was more labeling on the ipsilateral side (* P < 0.05 for CCI saline, ** P < 0.01 for ULD atipamezole. F(3,28) = 10.47).
To assess changes in astrogliosis in the L₄,₅ lumbar spinal cord, astrocytes were labeled with an antibody against glial fibrillary acidic protein (GFAP), sections were imaged through the z-plane on a confocal microscope; stacks were collapsed into 2-dimensions, and fluorescent intensity was quantified as mean pixel intensity using Image J software. Representative micrographs of collapsed stacks of GFAP labeling within the deep and superficial dorsal horn ipsilateral and contralateral to the site of injury are displayed at 63x magnification under oil immersion (fig. 4.7). In sham controls (Fig. 4.8A) there was no difference in GFAP labeling in either the deep or superficial dorsal horn, either ipsi- or contralateral to the site of injury ($F_{(3,34)} = 2.041, P = 0.1265$). In the CCI animals (Fig. 4.8B), significantly more labeling was observed in the superficial compared to the deep dorsal horn (* $P < 0.05$), with significantly more labeling on the ipsilateral compared to the contralateral side (** $P < 0.01$) within laminae I/II ($F_{(3,44)} = 6.318, P = 0.0012$). CCI animals exhibited an upregulation in GFAP in the superficial dorsal horn on the ipsilateral side compared to the shams (* $P < 0.05$) ($F_{(3,40)} = 3.677, P = 0.0198$) (Fig. 4.8D), but no significant differences were observed between the CCI and shams in the deep dorsal horn ($F_{(3,38)} = 2.290, P = 0.0939$) (Fig. 4.8C). The nerve-injury induced upregulation of GFAP in the superficial dorsal horn was significantly attenuated in the animals chronically administered ULD atipamezole compared to the saline controls (** $P < 0.01$), ipsilateral to the site of injury ($F_{(3,44)} = 8.379, P = 0.0002$) (Fig. 4.9B), but no differences were seen between saline and ULD atipamezole in the deep dorsal of nerve injured animals ($F_{(3,44)} = 4.070$) (Fig. 4.9A).
CD11b (Microglia) Labeling in the Spinal Dorsal Horn in CCI Animals

Fig. 4.5 Representative micrographs demonstrating microglia (CD11b) labeling by fluorescent IHC in the deep and superficial dorsal horn, contralateral (left column) and ipsilateral (right column) to the side of surgery in CCI animals chronically treated with saline (top two rows) or ULD atipamezole (5ng) (third and fourth row) for ten days post-surgery. 63X Magnification on confocal microscope under oil immersion.

Ati = atipamezole, IHC = immunohistochemistry, ULD = ultra-low dose.
CD11b (Microglia) Labeling is Higher in the Superficial Dorsal Compared to the Deep Horn Ipsilateral to the Site of Injury

Chronic Systemic Administration of ULD Atipamezole Attenuates Nerve Injury-Induced Increase in CD11b (Microglial) Labeling in the Superficial Dorsal Horn Ipsilateral to the Site of Injury

Fig. 4.6 Mean pixel intensity of CD11b labelling in collapsed stacks of the deep and superficial dorsal horn of the L4-L5 spinal cord after ten days following chronic constriction injury (CCI), ipsilateral and contralateral to the site of nerve injury. (A) shows CCI animals treated with saline over the ten days (red). (B) shows CCI animals treated with ULD atipamezole (5ng) over the ten days (blue). (C) and (D) show comparisons between groups within the deep and superficial dorsal horn, respectively. Two-three stacks per animal were imaged in each area from three animals per group giving a total of N=8/group. Ati = atipamezole, a.u. = arbitrary units, CCI= chronic constriction injury, ULD = ultra-low dose. Bars represent mean +/- S.E.M. * = P < 0.05, ** = P < 0.01, **** = P < 0.0001.
Fig. 4.7 Representative micrographs demonstrating astrocyte (GFAP) labeling by fluorescent IHC in the superficial dorsal horn, contralateral (left column) and ipsilateral (right column) to the side of surgery, in sham surgery rats treated with saline (top row), CCI rats treated with saline (middle row) and CCI rats treated with ULD atipamezole (5ng, bottom row) chronically for ten days post-surgery. 63X mag. on confocal microscope under oil immersion. Ati = atipamezole, GFAP = glial fibrillary acidic protein, IHC = immunohistochemistry, ULD = ultra-low dose.
Chronic Constriction Injury (CCI) Increases GFAP (Astrocyte) Labeling in the Superficial Dorsal Horn Ipsilateral to the Site of Injury

Fig. 4.8 Mean pixel intensity of GFAP labelling in collapsed stacks in the deep and superficial dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after surgery in sham (A) and CCI (B) animals. (C and D) show comparisons between sham and CCI animals in the deep and superficial dorsal horn, respectively. CCI caused an upregulation in GFAP labeling in the superficial dorsal horn ipsilateral to the site of injury. Two-four stacks per animal were imaged in each area from three animals per group giving a total of N=8-12/group. a.u. = arbitrary units, CCI = chronic constriction injury, contra = contralateral, ipsi = ipsilateral, GFAP = glial fibrillary acidic protein. Bars represent mean +/- S.E.M. * = P < 0.05, ** = P < 0.01.
Fig. 4.9 Mean pixel intensity of GFAP labelling in collapsed stacks in the deep (A) and superficial (B) dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after surgery in CCI animals chronically treated with saline (red) or ULD atipamezole (blue) over the ten days. Four stacks per animal were imaged in each area from three animals per group giving a total of N=12/group. Ati = atipamezole, a.u. = arbitrary units, CCI = chronic constriction injury, GFAP = glial fibrillary acidic protein, ULD = ultra-low dose. Bars represent mean +/- S.E.M. ** = P < 0.01.
4.3.2.2 Assessing changes in microglial and astrocyte cell size through 3-d reconstruction of CD11b and GFAP labeled stacks

In order to assess changes in microglial cell size, CD11b-labeled stacks were reconstructed using Image Pro software and cell volumes were quantified. Representative models are shown for the deep and superficial dorsal horn on the ipsilateral and contralateral side (fig. 4.10). In animals receiving chronic constriction injury, quantification of microglial cell size revealed that there were significantly larger cells in both the superficial and deep dorsal horn on the ipsilateral compared to the contralateral side (**** P < 0.0001. $F_{(3,92)} = 32.40$) (Fig. 4.11). In the superficial dorsal horn (Fig. 4.12B), CCI animals that had received chronic systemic injections of ULD atipamezole had significantly smaller microglia compared to the CCI saline controls on the ipsilateral side (** P < 0.01. $F_{(3,92)} = 26.89$). Similar results were observed within the deep dorsal horn as well (** P < 0.01. $F_{(3,92)} = 19.20$) (Fig. 4.12A). Unfortunately, no CD11b labeled stacks were collected in the shams so it was not possible to compare microglial cell size between the neuropathic and sham animals.

In order to assess changes in astrocyte cell size, GFAP-labeled stacks were reconstructed using Image Pro software and cell volumes were quantified. Representative models are shown for the deep and superficial dorsal horn on the ipsilateral and contralateral side (fig. 4.13). 3-dimensional reconstruction of the GFAP-labeled stacks and quantification of astrocyte cell size using Image Pro software revealed that chronic constriction injury caused a significant upregulation in astrocyte size compared to the sham controls (** P < 0.01), with much larger cells in the CCI animals on the ipsilateral side compared to the contralateral (*** P < 0.001) in the superficial dorsal horn ($F_{(3,128)} = 7.594$) (Fig. 4.14B). No significant differences were observed between the ipsilateral and contralateral sides in the sham animals (Fig. 4.14 A and B).
In the deep dorsal horn, there was no difference in astrocyte size in either the CCI or sham animals on either the ipsilateral or contralateral sides ($F_{(3,122)} = 0.7012, P = 0.5531$) (Fig. 4.14A).

In the superficial dorsal horn of CCI animals, chronic systemic administration of ULD atipamezole significantly attenuated the nerve injury-induced increase in astrocyte volume seen in the saline-treated controls ipsilateral to the site of injury (** $P < 0.01$) ($F_{(3,140)} = 11.78$) (Fig. 4.15B). No differences in the size of the astrocytes were observed in the deep dorsal horn of CCI animals treated with ULD atipamezole or saline on either side ($P = 0.6593, F_{(3,140)} = 0.5346$) (Fig. 4.15A).
3-Dimensional Reconstructions of Microglia in the Spinal Dorsal Horn of Nerve-Injured Animals

Fig. 4.10 Representative 3-dimensional reconstructions of microglia in the superficial and deep dorsal horn of the spinal cord, ipsilateral and contralateral to the site of injury in CCI animals treated with saline (left) or CCI animals treated with ULD atipamezole (right) for ten days post-surgery. Wire frames of the cells are represented to scale. Grey box dimensions are 250 x 250 pixels. Ati = atipamezole, CCI = chronic constriction injury, contra = contralateral, ipsi = ipsilateral, ULD = ultra-low dose.
Chronic Constriction Injury (CCI) Increases Microglial Size in the Deep and Superficial Dorsal Horn Ipsilateral to the Site of Injury

3-Dimensional Reconstructions of Microglia in CCI Animals

Fig. 4.11 Mean microglial cell volume in CD11b labeled stacks in the deep (hatched red) and superficial (solid red) dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after CCI. The three largest cells were quantified in each stack; two-three stacks per animal in each area from three animals per group giving a total of N=24/group. a.u. = arbitrary units, CCI = chronic constriction injury. Bars represent mean +/- S.E.M. **** = P < 0.0001.
Chronic Systemic Administration of ULD Atipamezole Inhibits the Nerve Injury-Induced Increase in Microglia Cell Size in the Deep and Superficial Dorsal Horn

Fig. 4.12 Mean microglial cell volume in CD11b labeled stacks in the deep (A) and superficial (B) dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after CCI in animals chronically administered saline (red) or ULD atipamezole (blue) throughout the ten days post-surgery. The three largest cells were quantified in each stack; two-three stacks per animal in each area from three animals per group giving a total of N=24/group. Ati = atipamezole, a.u. = arbitrary units, CCI = chronic constriction injury, ULD = ultra-low dose. Bars represent mean +/- S.E.M. ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.
Fig. 4.13 Representative 3-dimensional reconstructions of astrocytes in the superficial and deep dorsal horn of the spinal cord, ipsilateral and contralateral to the site of injury in sham animals treated with saline (left), CCI animals treated with saline (middle) or CCI animals treated with ULD atipamezole (right) for ten days post-surgery. Wire frames of the cells are represented to scale. Grey box dimensions are 250 x 250 pixels.

Ati = atipamezole, CCI = chronic constriction injury, contra = contralateral, ipsi = ipsilateral, ULD = ultra-low dose.
Chronic Constriction Injury (CCI) Increases Astrocyte Cell Size in the Superficial Dorsal Horn of the Spinal Cord Ipsilateral to the Site of Injury

Fig. 4.14 Mean astrocyte cell volume in GFAP labeled stacks in the deep (A) and superficial (B) dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after surgery in sham (yellow) and CCI (red) animals. The three largest cells were quantified in each stack; two-four stacks per animal in each area from three animals per group giving a total of N=24-36/group. a.u. = arbitrary units, CCI = chronic constriction injury, GFAP = glial fibrillary acidic protein. Bars represent mean +/- S.E.M. ** = P < 0.01, *** = P < 0.001.
Chronic Systemic Administration of ULD Atipamezole Inhibits the Nerve Injury-Induced Increase in Astrocyte Cell Size in the Superficial Dorsal Horn

Fig. 4.15 Mean astrocyte volume in GFAP labeled stacks in the deep (A) and superficial (B) dorsal horn on the ipsilateral and contralateral side of the L4-L5 spinal cord ten days after CCI in animals chronically administered saline (red) or ULD atipamezole (blue) throughout the ten days post-surgery. The three largest cells were quantified in each stack; four stacks per animal in each area from three animals per group giving a total of N=36/group. Ati = atipamezole, a.u. = arbitrary units, CCI = chronic constriction injury, GFAP = glial fibrillary acidic protein, ULD = ultra-low dose. Bars represent mean +/- S.E.M. ** = P < 0.01, **** = P < 0.0001.
4.3.3 Assessing changes in neuronal activation via c-fos in nerve-injured animals

The amount of neuronal activation in CCI animals was inferred via immunoreactive cell counts for c-Fos. c-Fos labeling was performed through DAB IHC ten days after surgery and the number of immunoreactive cells were counted within the deep and superficial dorsal horn of the spinal cord, ipsi- and contralateral to the site of injury. Representative micrographs are shown at 40x magnification captured on a light microscope (fig. 4.16). In CCI animals chronically treated with saline over the ten days following surgery, significantly more c-Fos positive cells were counted in the superficial dorsal horn on both the ipsilateral (**** P < 0.0001) and contralateral (* P < 0.05) sides compared to the number of c-Fos positive cells in the deep dorsal horn (Fig. 4.17 A). There were significantly higher c-Fos cell counts on the ipsilateral compared to the contralateral (*** P < 0.001) side in the superficial dorsal horn, but there was no difference between sides in the deep dorsal horn (Fig. 4.17 A) (F_{(3,36)} = 20.69, P < 0.0001).

In CCI animals chronically treated with ULD atipamezole (5ng) over the ten days following surgery (Fig. 4.17 B), the only difference was seen between the superficial and deep dorsal horn on the contralateral side, with significantly more labeling in the superficial dorsal horn (* P < 0.05) (F_{(3,27)} = 4.015, P = 0.0175).

Comparing labeling between the saline and ULD atipamezole groups in the deep and superficial dorsal horn (Figs. 4.17 C and D, respectively), chronic systemic administration of ULD atipamezole significantly reduced c-Fos immunoreactivity in both the deep (Fig. 4.17 C, * P < 0.05) (F_{(3,31)} = 4.116, P = 0.0144) and superficial (Fig. 4.17 D, *** P < 0.001) (F_{(3,32)} = 9.573, P = 0.0001) dorsal horn ipsilateral to the site of injury, suggesting an attenuation of neuronal activation in those areas.
c-Fos Labeling in the Spinal Dorsal Horn in Nerve Injured Animals

Fig. 4.16 Representative micrographs demonstrating c-Fos labeling by DAB IHC in the deep (left column) and superficial (right column) dorsal horn, ipsilateral and contralateral to the site of injury of CCI animals treated chronically with saline or ULD atipamezole (Ati) over the ten days post-surgery. 40X Magnification. CCI = chronic constriction injury, DAB = 3,3’-diaminobenzadine, IHC = immunohistochemistry.
c-Fos Expression in Nerve Injured Animals is Higher in the Superficial Compared to the Deep Dorsal Horn Ipsilateral to the Site of Surgery

Chronic Systemic Administration of ULD Atipamezole Attenuates the Nerve Injury-Induced Upregulation of c-Fos in the Deep and Superficial Dorsal Horn

Fig. 4.17 Immunoreactive cell counts for c-Fos on the ipsilateral and contralateral sides in the deep (hatched bars) and superficial (solid bars) dorsal horn of the L4-L5 spinal cord ten days after CCI in animals chronically treated with saline (A) or ULD atipamezole (B) over the ten days following nerve injury. (C and D) shows comparisons between CCI saline (red) and CCI ULD atipamezole (blue) within the deep and superficial dorsal horn respectively. Chronic administration of ULD atipamezole reduced c-Fos immunoreactive cell counts in the deep and superficial dorsal horn ipsilateral to the site of injury compared to saline treated controls. Two-three sections were counted per animal in each area from three animals per group for a total N=7-10. Ati = atipamezole, CCI = chronic constriction injury, contra = contralateral, ipsi = ipsilateral, ULD = ultra-low dose. Bars represent mean +/- S.E.M. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.
4.3.4 Assessing changes in MOR expression in nerve-injured animals

To assess whether chronic systemic administration of ULD atipamezole altered the levels of mu-opioid receptor (MOR) expression in the deep and superficial dorsal horn, DAB IHC was used to label MORs in CCI animals chronically treated with saline or ULD atipamezole over the ten days. Representative micrographs are shown at 40x magnification captured on a light microscope (fig. 4.18). Immunoreactive cell counts were assessed (Fig. 4.19) and mean grey scale values (Fig. 4.20) of the micrographs were quantified using ImageJ.

CCI animals chronically treated with saline over the ten days post-surgery had higher immunoreactive cell counts for MOR in the superficial compared to the deep dorsal horn on both the ipsilateral and contralateral side (*** P < 0.001), though there was no difference between sides in either area (Fig. 4.19 A) \( F_{(3,51)} = 20.17, P <0.0001 \). The same thing was observed in CCI animals chronically treated with ULD atipamezole (Fig. 4.19 B), with more labeling in the superficial dorsal horn compared to the deep, ipsilateral (*** P < 0.001) and contralateral (** P < 0.01) to the site of injury, but no difference between sides in either area \( F_{(3,44)} = 13.40, P < 0.0001 \).

Comparing between CCI animals chronically treated with saline or ULD atipamezole in the two areas, no differences in MOR immunoreactive cell counts were observed between treatments on either the ipsilateral or contralateral side in either the deep (Fig. 4.19 C, \( F_{(3,48)} = 0.3458, P = 0.7923 \)) or superficial (Fig. 4.19 D, \( F_{(3,47)} = 0.8943, P= 0.4511 \)) dorsal horn.

Looking at mean grey scale intensities of the micrographs labeled for MOR, CCI animals chronically treated with saline over the ten days post-surgery (Fig. 4.20 A) showed no difference in the intensity of labeling in the superficial compared to the deep dorsal horn, and no difference was observed between the ipsilateral and contralateral sides \( F_{(3,51)} = 0.3310, P = 0.8029 \).
Similarly, no difference in labeling intensity was observed in CCI animals chronically treated with ULD atipamezole (Fig. 4.20 B), either between the deep and superficial dorsal horn or between the ipsilateral and contralateral sides in either area ($F_{(3,44)} = 2.890, P = 0.0460$).

Comparing the mean grey scale intensities between CCI animals chronically treated with saline or ULD atipamezole within the deep (Fig. 4.20 C, $F_{(3,48)} = 1.354, P = 0.2682$) and superficial (Fig. 4.20 D, $F_{(3,47)} = 0.06228, P = 0.9794$), no differences were observed between treatment on either the ipsilateral or contralateral side in either area of the spinal cord.
MOR Labeling in the Spinal Dorsal Horn of Nerve Injured Animals

Fig. 4.18 Representative micrographs demonstrating mu-opioid receptor labeling by DAB IHC in the deep (left column) and superficial (right column) dorsal horn, ipsilateral and contralateral to the site of injury of CCI animals treated chronically with saline or ULD atipamezole (Ati) over the ten days post-surgery. 40X Magnification. CCI = chronic constriction injury, DAB = 3,3’-diaminobenzidine, IHC = immunohistochemistry, MOR = Mu opioid receptor.
MOR Immunoreactive Cell Counts Are Higher in the Superficial Compared to the Deep Dorsal Horn in Nerve Injured Animals

C

D

Chronic Systemic Administration of ULD Atipamezole Does Not Alter MOR Immunoreactive Cell Counts in Nerve Injured Animals

Fig. 4.19 Immunoreactive cell counts for mu-opioid receptor (MOR) labeling on the ipsilateral and contralateral sides in the deep (hatched bars) and superficial (solid bars) dorsal horn of the L4-L5 spinal cord ten days after CCI in animals chronically treated with saline (A) or ULD atipamezole (B) over the ten days following nerve injury. Labeling was higher in the superficial than the deep dorsal horn on both sides in both the saline and ULD atipamezole-treated animals. (C and D) show comparisons between CCI saline (red) and CCI ULD atipamezole (blue) animals in the deep and superficial dorsal horn respectively. Chronic administration of ULD atipamezole did not alter MOR immunoreactive cell counts in nerve-injured animals. Four-five sections were counted per animal in each area from three animals per group for a total N=12-14. CCI = chronic constriction injury, MOR = mu-opioid receptor, ULD = ultra-low dose. Bars represent mean +/- S.E.M. ** = P < 0.01, *** = P < 0.001.
Chronic Systemic Administration of ULD Atipamezole Does Not Alter MOR Expression Inferred by Mean Grey Scale Labeling in Nerve Injured Animals

No Differences in MOR Labeling Inferred by Mean Grey Scale Intensity Were Observed Between the Deep and Superficial Dorsal Horn in Nerve Injured Rats

Fig. 4.20 Mean grey scale intensity for mu-opioid receptor (MOR) labeling on the ipsilateral and contralateral sides in the deep (hatched bars) and superficial (solid bars) dorsal horn of the L4-L5 spinal cord ten days after CCI in animals chronically treated with saline (A) or ULD atipamezole (B) over the ten days following nerve injury. No differences in mean pixel intensity of MOR labeling were observed between sides or areas in either the saline or ULD atipamezole-treated animals. (C and D) show comparisons of mean pixel intensity of MOR labeling between CCI saline (red) and CCI ULD atipamezole (blue) treated animals in the deep and superficial dorsal horn respectively. No differences in MOR labeling intensity were observed between groups in either area. Four-five sections were counted per animal in each area from three animals per group for a total N=12-14. CCI = chronic constriction injury, contra = contralateral, ipsi = ipsilateral, MOR = mu-opioid receptor, ULD = ultra-low dose. Bars represent mean +/- S.E.M.
4.4 DISCUSSION

Chronic systemic administration of ULD atipamezole attenuated the chronic morphine-and nerve injury-induced increases in microglial and astrocyte activation which are known to be associated with the development and maintenance of tolerance and chronic pain states. It also blocked the increased expression of c-Fos in CCI animals, a well-established marker of neuronal activity.

Microglial and astrocyte activation were assessed in two ways: by measuring mean pixel intensities of CD11b and GFAP labeling from the 2-dimensional images of the collapsed confocal stacks, as well as through 3-dimensional reconstructions of the stacks to determine cell size. While many studies in the past have relied solely on quantifying average pixel intensity of 2-d images, this method is less than ideal for a number of reasons. If the 2-d images are quantified from a single layer in the z-plane, it is not very representative of everything that has been labelled in the tissue slice, and moving above or below a single layer in the z-plane could give very different measurements depending on the location and orientation of cells within the slice. By capturing dozens of images in a stack, from the upper and lower boundaries of the tissue and using software to collapse the entire stack down to 2-dimensions, all the labeled cells in the slice can be visualized and more reliable measurements can be obtained and compared. Pilot studies comparing the two methods were performed (data not shown) and the collapsed stacks gave a much cleaner, more consistent dataset.

While collapsed stacks are preferable to quantifying single layers, 2-dimensional images alone are still not ideal for inferring a cell’s function based on its morphology. “Activated” glia are those that have shifted from a housekeeping or quiescent to a pro-inflammatory phenotype and often this shift in function is inferred from morphological changes observed in the cells.
Microglia shift their morphology from a highly ramified to an aboeboid shape (Torres-Platas, 2014) and take on phagocytic functions similar to macrophages (see Kettenmann et al., 2011 for an exhaustive review of microglial physiology), while astrocytes increase in size and recruit other cells to the area. By measuring mean pixel intensity only, it can be difficult to tell if the difference one is seeing is due to a change in cell size or an increase in cell number or density as both could hypothetically increase the fluorescent signal being detected and, not all “activated” glia are functionally identical. Different stimuli can cause glia to adopt a spectrum of different phenotypes, and the cellular processes occurring within the cells, the specific receptors being expressed and the factors being released cannot be determined only by measuring the density of cells in an area or their size. Being able to assess changes in intracellular signaling and detect the proteins and other factors being released by the cells will be necessary to characterize a functional phenotype.

In the 3-dimensional reconstruction experiments, the size of the cells can be compared in a number of ways: by comparing the largest, 2nd largest, 3rd largest… cell in each stack, by comparing the mean cell volume in the stack, by comparing the sum of all the cell volumes, and by comparing the total number of cells in each stack exceeding the minimum threshold. Data was obtained for every cell in every stack in every group in all experiments and was compared across all of these parameters, though only the mean cell volume of the 3 largest cells in each stack was reported in the results section. Comparing only the largest cell in each stack was not ideal because the data can so easily be skewed and the variability was higher than it was for the 2nd and 3rd largest cells within the stacks of individual groups. Taking the average of the three largest cells took into account some of the variability within individual stacks and seemed a more robust and representative measure of the volume of the biggest cells within the stack as opposed to just
choosing only the 2\textsuperscript{nd} or 3\textsuperscript{rd} largest cells to compare across groups. The data analysis was performed for the biggest, 2\textsuperscript{nd} biggest, 3\textsuperscript{rd} biggest cell and the results are strikingly similar to the mean of the three largest cells reported in the results section, but only the latter result is reported here. Determining the mean cell volume based on every single cell within a stack was not a reliable or representative measure, as images with many very small cells had severely skewed results toward the lower end. Adding up the sum of all the individual cell volumes within a stack did appear to give a reliable and representative measure of the stack and comparing the stacks across groups showed similar trends as those seen in the results section where only the average of the three biggest cells is shown. Comparing the sum of all the cell volumes in the whole 3-dimensional stack is somewhat analogous to measuring the mean pixel intensity in a 2-dimensional image. Finally, the number of cells exceeding the threshold was determined in every stack and compared across groups, again with similar trends to those reported in the results section. Thus, of all the ways Image Pro can quantify cell volumes in a stack, the most consistent methods were by comparing the average of only the largest cells detected, comparing the total sum of the volume of cells within the stack, and comparing the number of cells detected in excess of the threshold. Of these, the average of the three largest cells was selected to represent the 3-dimensional reconstruction data.

While an argument might be made that reporting total N-values as a function of the number of sections imaged per animal rather than collapsing the data for each animal and only reporting an average for each within a group artificially inflates the power of the statistical analysis, reporting the number of sections imaged per animal and the number of animals per group is not an uncommon way to report this type of immunohistochemical data; indeed there is precedence within our own lab for reporting group numbers in exactly this way (Holdridge et al.,
While we do not disagree that the statistics are more powered with a higher n-value, this does not necessarily lead to an increase in type-I error rates and the effect sizes for many of the figures in this chapter are already quite large.

While CD11b/OX42 is the most commonly used marker of microglia to infer changes in activation state based on the amount of labeling, CD11b is not specific to any phenotype of activated microglia. CD11b is expressed on both quiescent and “activated” microglia in the CNS and is expressed on peripheral monocytes as well (Strauss-Ayali et al., 2007), which could be problematic if any blood remains in the tissue following perfusion. An alternative marker for identifying activated microglia is CD68. CD68 is a lysosomal protein marker that can be used to differentiate ramified quiescent microglia, which express very little CD68, from those in an “activated” or pro-inflammatory state, which express a large amount of CD68 (Graeber et al., 1990; Slepko & Levi, 1996). While it might be argued CD68 would be a better marker than CD11b to measure activated phenotypes, the same issue of non-specificity occurs as is seen with CD11b, where both markers are also expressed on peripheral macrophages (Strobl et al., 1998).

One solution to the problem of trying to determine if the cells in the micrograph are, in fact, CNS microglia or peripheral macrophages or monocytes is to measure expression ratios of another marker, CD45, and sort the cells using flow cytometry, though it could be challenging to get enough from the spinal cords and tissue from many animals might need to be pooled.

Ramified, quiescent microglia of parenchyme origin typically have a low expression of CD45 relative to CD11b based on cell sorting experiments using flow cytometry, while peripheral macrophages express a high amount of CD45 relative to CD11b (Ford et al., 1995). By assessing these ratios and sorting the cells according to the expression levels of these markers, it is possible to differentiate microglia from peripheral monocytes and macrophages.
Alternative markers of microglia such as CD68 or CD45 can be used to complement the CD11b data to give a better assessment of whether there are changes in specific phenotypes of activated microglia and future studies would use these markers to better characterize the cells that are present in the dorsal horn of the spinal cord and how those populations of cells change in morphine tolerant and nerve-injured animals treated with ULD α₂-adrenergic antagonists.

While the evidence we have provided here shows overwhelmingly that ULD atipamezole is attenuating the development of opioid and nerve-injury induced gliosis and neuronal sensitization, the work so far has been correlational rather than causal. Mechanistically it is still not known whether morphine tolerance and neuropathic pain are being attenuated due to ULD α₂-adrenergic antagonists directly blocking neuronal or glial activation, or whether they are inhibiting pain and tolerance in some other distinct manner, and the reduction in c-Fos activation and gliosis are secondary to their true mechanisms. Moving forward, assessing changes in intracellular signaling cascades in glia and neurons (possibly through Western Blotting of kinases or calcium imaging), characterizing cytokine profiles, assessing changes in surface transporters (such as changes in glutamate transporter expression on astrocytes such as GLT-1 and GLAST), and ligand binding studies will give more information about whether ULD α₂-adrenergic antagonists are interacting with glia directly or indirectly and will help determine exactly which phenotypes of “activated” glia are involved.

It is also unknown if these ultralow doses of α₂-AR antagonists are acting directly on α₂-ARs on the glial cells themselves or indirectly by inhibiting the development of tolerance. Studies using RT-PCR and radioligand binding, mRNA and protein expression showed that all α-adrenergic receptors are represented in astrocytes (Aoki, 1992; Chen and Hertz, 1999; Hertz et al., 2004; Porter and McCarthy, 1997; Hutchinson et al., 2011), therefore the potential of these
effects being direct is plausible.

In chapter three it was shown that chronic systemic morphine administration in pain naïve animals caused rapid development of tolerance, which was attenuated in animals co-administered ULD atipamezole. Here we show that co-administration of ULD atipamezole reduces the morphine-induced upregulation in microglia (CD11b, fig. 4.2) and astrocyte (GFAP, fig. 4.3) expression, and reduced astrocyte cell volume (Fig. 4.4) in both the deep and superficial lumbar dorsal horn, suggesting that morphine tolerance may be at least partially attenuated by ULD atipamezole though the inhibition of spinal glial activation.

In chapter three, it was also shown that in nerve-injured animals, chronic systemic administration of ULD atipamezole was able to attenuate the development of mechanical allodynia on its own, and animals chronically treated with it showed an increased analgesic response to an acute injection of morphine on the tenth day post-surgery in both thermal and mechanical testing paradigms. Here we provided molecular support by showing that ULD atipamezole attenuated the development of microglial and astrocyte activation associated with nerve injury, both in terms of reducing expression of CD11b and GFAP (figs. 4.5-4.9) and decreasing cell volumes (figs. 4.10-4.15), and attenuated nerve injury-induced neuronal activation inferred by a decrease in the number of c-Fos immunoreactive cell counts in nerve injured animals compared to saline-treated controls (Fig. 4.17).

The fact that chronic systemic administration of ULD atipamezole did not alter either the number of immunoreactive cell counts (Fig. 4.19) or the mean intensity of MOR labeling (Fig. 4.20) in either the deep or superficial dorsal horn compared to saline suggests that any differences in behaviour caused by chronic ULD atipamezole following nerve injury are not a result of alterations in MOR expression levels. Attempts were made to assess changes in the
expression of $\alpha_2$-adrenergic receptor subtypes (specifically $\alpha_{2A}$ and $\alpha_{2C}$, as they have anti-
nociceptive functions), both through DAB IHC and fluorescent IHC, but unfortunately the
antibodies used did not label consistently and $\alpha_2$-adrenergic receptor expression could not be
quantified in these studies.

In most of the immunohistochemical experiments there was consistently more labeling
within the superficial compared to the deep dorsal horn, particularly on the ipsilateral side, which
may explain why more consistent results were observed within laminae I/II as there were larger
windows in which to observe a change in expression with the ULD atipamezole treatments.
More GFAP and CD11b labeling was observed in nerve-injured animals, the size of the glial
cells were bigger in the superficial dorsal horn, and there was higher labeling of c-Fos and MOR
as well compared to the deep dorsal horn. The higher labeling within laminae I/II is consistent
with much of the literature, as this has been reported for microglial activation (Taves et al.,
2013), for neuronal activity and synaptic firing following nerve injury (Kawasaki et al., 2008),
and MOR expression levels (Arvidsson et al., 1995b).

Another possibility for the observed behavioural effects could be due to a change in the
expression of receptor heteromers between $\alpha_2$-adrenergic and mu opioid receptors following
peripheral nerve injury. Heterodimerization or direct functional interactions between opioid and
$\alpha_2$-ARs is possible (Jordan et al., 2003), and cross-conformational changes impact intracellular
signalling (Vilardaga et al., 2008). ULD antagonists have been shown to modulate opioid
receptor heteromer function, therefore it is feasible that ULD $\alpha_2$-AR antagonists may modulate
the function of an $\alpha_2$/mu complex in neuropathic pain states. Another potential mechanism of
ULD $\alpha_2$-AR antagonists is through modification of G-protein coupling. Chronic morphine
exposure leading to a G-protein coupling shift to excitatory states and changes in adenylyl
cyclase activation is a possible mechanism to explain development of tolerance and opioid-induced hyperalgesia (Chakrabarti et al., 2005). Studies with ULD opioid antagonists have suggested that enhanced opioid analgesia and attenuation of tolerance might be attributed to prevention of this change in G-protein coupling (Wang et al., 2005, 2006). Adrenergic receptors are GPCRs that are structurally similar to opioid receptors, though it is not known if similar changes in receptor coupling occur following chronic ULD α2-AR antagonist administration.

Thinking ahead to possible therapeutic applications of ULD α2-adrenergic receptor antagonists in humans, it is not possible to predict with certainty whether or not they would prove efficacious in a clinical setting in the treatment of chronic pain, either on their own or in combination with opioids, until pre-clinical data is collected in human patients. Application of ULD opioid antagonists like naloxone and naltrexone, however, have translated well from basic science to clinical settings (Cepeda et al., 2004; Chindalore et al., 2005; Gan et al., 1997; Hay et al., 2011; La Vincente et al., 2008) and it appears the ULD phenomenon is common to other GPCRs, not just opioid receptors. It has been demonstrated that ULD CB1 antagonists attenuate the development of cannabinoid tolerance (Paquette et al., 2007), and that there is cross-modulation between opioids and cannabinoid systems, whereby ULD opioid antagonists enhance the analgesic effects of CB1 agonists (Paquette and Olmstead, 2005), but it is not known if the converse is true. We are currently assessing whether or not chronic administration of ULD CB1 (cannabinoid) receptor antagonists are also able to attenuate the development of morphine tolerance and inhibit the development of gliosis as well. While those studies are ongoing and are outside the purview of this dissertation, early results show chronic systemic ULD rimonabant (5ng/kg), a CB1-receptor antagonist inhibits morphine-induced gliosis in the deep dorsal horn of the spinal cord, but this did not result in a change in the development of morphine tolerance at any
of the doses tested (1ng/kg, 5ng/kg, 50ng/kg). This discrepancy is consistent with what was seen in Chapter 2; while chronic morphine and chronic pain increase gliosis, reduction in gliosis does not necessarily equate to a decrease in pain. CB₁ receptors are expressed predominantly on presynaptic nerve terminals, exerting control over synaptic transmission (Schlicker and Kathmann, 2001), though they are also expressed on neuronal soma and dendrites (Tsou et al., 1998). The wide distribution of CB₁ receptors throughout the peripheral and central nervous system on both excitatory and inhibitory neurons (Domenici et al., 2006) may have resulted in no net effect in terms of behavioral responses (Kano et al., 2004).

Currently efaroxan is available for research purposes only and while atipamezole is commonly used in veterinary practice to reverse anesthesia or sedation (Pertovaara et al., 2005), it has not been approved for use in humans despite minimal side effects in phase 1 clinical trials, especially at lower doses (Karhuvaara et al., 1990). In addition to its affinity for α₂-adrenergic receptors, efaroxan also has antagonistic effects at non-adrenergic imidazoline (I₁) receptors (Schäfer et al, 2002), which are also involved in blood pressure regulation (Bousquet, 2001), and agonist effects at I₃ receptors (Mayer and Taberner, 2002), which play a role in the regulation of insulin secretion and blood glucose metabolism (Eglen et al., 1998). While the imidazoline receptors have yet to be cloned and their name has not been recognized as official nomenclature, there is a large body of evidence to suggest that they have adrenergic-like effects, but are distinctly different than the recognized α- and β-adrenergic receptors and their sub-types (Bektas et al., 2015). Yohimbine, a non-selective α₂-adrenergic receptor antagonist is currently approved for use in humans, so early pre-clinical trials could possibly be carried out with that compound. For research purposes, however, use of yohimbine is not ideal due to lack of specificity, making it difficult if not impossible to deduce a mechanism of action. Yohimbine has been used as a
stressor to induce drug relapse (Banna et al., 2010) and has a broad side effect profile. While yohimbine has a high affinity for $\alpha_2$-adrenergic receptors, it is not selective to any sub-type; it acts as an antagonist at $\alpha_1$-adrenergic receptors, has weak to moderate affinity as an antagonist for various 5-HT (5-hydroxytryptamine/serotonin) and dopamine receptors (Millan et al., 2000), and has activity as a partial agonist at 5-HT$_{1A}$ (Arthur et al., 1993). While atipamezole is very selective to $\alpha_2$-adrenergic receptors in general, it is not specific to any sub-type of the receptor. BRL44408, a compound whose properties we have studied in the past (Milne et al., 2014) is highly selective for $\alpha_{2A}$ over the other $\alpha_2$-adrenergic receptor sub-types and would thus be a very useful tool moving forward in terms of elucidating a mechanism of action.
4.5 CONCLUSION

Despite these molecular results correlating positively with the effects seen of ULD atipamezole in terms of pain behaviors and responsiveness to opioids, they are not causative. It remains unknown if glial and neuronal activation in the spinal dorsal horn are attenuated as a result of ULD atipamezole acting directly on glial cells or if the change in the activity of these cells is secondary to a decrease in pain mediated through other mechanisms. Still, the fact that these studies have provided molecular support to our previous findings is still a good stepping stone from which functional assays can be performed moving forward. The next step would be to perform cytokine assays, assess CD68 and CD45 labeling in microglia, and it would be interesting to determine if ULD atipamezole altered glutamate GLT-1 and GLAST transporters on astrocytes, as these are responsible for removing excitatory glutamate from the synapse and are down-regulated when astrocytes are “activated”. Reissner et al., (2014) have observed that the glial inhibitor propentofylline inhibits cocaine seeking behaviours and relapse by preventing the downregulation of GLT-1 in the nucleus accumbens, a brain region within the dopaminergic reward pathway. Emotional processing in such higher brain regions is important in terms of pain and relief from it. All the studies presented thus far have looked at acute nociceptive behaviors in chronic pain and morphine tolerant states, but pain has an important negative affective component as well. So to better understand how pain is being altered by ULD α₂-adrenergic antagonists, reward and analgesia can be assessed in rats through a conditioned place preference (CPP) paradigm.
5.1 INTRODUCTION

Pain is not only a sensory phenomenon; there is an important affective or emotional component to the processing and interpretation of nociceptive stimuli that takes place within the brain after signals are relayed to certain regions from the periphery and spinal cord (Price, 2000). Chronic pain drives changes in neural circuitry associated with cognitive and emotional processing including alterations in endogenous opioid and dopaminergic systems (Martikainen et al., 2015), and thus managing pain in clinical populations is more difficult than simply reducing the intensity of sensory inputs (Bushnell et al., 2013). Negative affective states increase the perception of pain, while positive emotional states decrease it (Zeidan et al., 2011). Environmental and sensory cues impact expectations that alter pain perception (Lobanov et al., 2014). For example, positive expectation of pain relief can lead patients to report lower pain scores in the absence of active pharmacological agents (Kam-Hansen et al., 2014), while negative expectations (Rodriguez-Raecke et al., 2010) and uncertainty (Yoshida et al., 2013) can mask the antinociceptive effects of analgesic drugs.

The previous two chapters have shown the efficacy of ULD $\alpha_2$-AR antagonists like atipamezole in attenuating the development of morphine tolerance in both pain naive and neuropathic pain animals, in preventing the development of mechanical allodynia following nerve injury, and in enhancing acute opioid analgesia after neuropathic pain was left to develop. The fact that ULD atipamezole, when administered alone, had no acute effects over the two hour
testing window post-injection suggests that its effects on pain and opioid analgesia are not merely a result of additivity or synergy with opioids, but suggests that the neuroplastic changes associated with the development of analgesic tolerance and neuropathic pain are somehow being altered. Molecular support of this theory was provided in the previous chapter, which showed ULD atipamezole attenuated the development of chronic morphine- and nerve injury-induced gliosis, and prevented the increase in neuronal activation in the spinal dorsal horn.

While the models used in previous chapters were models of chronic morphine tolerance and chronic pain, the testing paradigms used were acute nociceptive assays compared over time. While used for decades in pain research in animal models, these types of sensory assays essentially show reflex responses at the level of the spinal cord and brain stem, and are incapable of measuring the more complex affective component of pain processing in the brain (see Li, 2013 for review). Thus, it is important to know whether chronic systemic administration of ULD atipamezole is capable of altering the emotional component of pain as well, which is ultimately more clinically relevant, as pain is associated with a negative affective state (Hummel et al., 2008a), and in humans is commonly co-morbid with depressive disorders (Holmes et al., 2012) with an elevated risk of suicide in chronic pain sufferers (Juurlink et al., 2004), while removal or reduction in pain induces an opposite and powerful positive affective state (Franklin et al., 2013). Environmental cues play an extremely important role in the subjective experience of pain or freedom from it (Bayer et al., 1991; Malenbaum et al., 2008), and the psychoactive effects of many drugs, whether rewarding or aversive are often at least partially associated with the environment in which they are administered (Siegel, 2005). For example, the reward associated with cocaine use is higher in animals exposed to stress early in life (Koob, 2008; Kreibich et al., 2009) and elevated following removal of environmental enrichment (Nader et al., 2012). Similar
results are observed with opioids in terms of stress (Shaham et al., 1992; Shaham and Stewart, 1994) and environmental cues (Gracy et al., 2000; McFarland and Ettenberg, 1997). These context-sensitive triggers are often the biggest cause of relapse in people with prior drug addictions, even after complete detoxification (Otto et al., 2007; Self and Nestler, 1998). In animals, environmental stressors are more powerful inducers of heroin reinstatement than withdrawal itself (Shaham et al., 1996). Thus, the emotional component of pain processing and opioid reward and analgesia is at least as important as the sensory inputs that carry nociceptive signals from the periphery and spinal cord (Cahill et al., 2014a), and context-sensitive cues impact how the pain is interpreted. The affective component of pain can be effectively assessed in animals through use of the conditioned place preference (CPP) paradigm (Prus et al., 2009).

In the CPP paradigm, animals are conditioned with a particular drug and, over time, learn to associate the visual and tactile cues of the chamber with reward or pain relief and will return to the environment in which they received the drug in the past when provided the choice to do so (Cunningham et al., 2006). One of the difficulties in assessing morphine CPP in nerve injured animals is that the two processes may complicate the expected outcomes and it can be difficult to determine if a CPP exists as a result of reward associated with the drug or a result of its analgesic properties. Thus, the dose of morphine for these experiments was selected based on data that we have published previously whereby we showed an inverted CPP dose-response curve to morphine in nerve-injured animals (Cahill et al., 2013; see Appendix C). At higher doses of 4mg/kg and 8mg/kg, morphine induced a CPP in both shams and neuropathic animals, but at 2mg/kg CPP did not develop in the shams, but remained in the neuropathic rats. Because the naïve and sham animals were no longer in pain that long after surgery, any CPP in those groups with the higher morphine doses was likely a result of opioid reward. Conversely, the neuropathic
animals were likely associating the drug-paired chamber with pain relief rather than reward because the 2mg/kg dose was analgesic in those animals in both thermal and mechanical testing paradigms.

The purpose of this study was to determine i) whether ULD atipamezole (5ng) would induce a CPP on its own in pain naïve, sham and neuropathic rats, and ii) to determine what effect co-administration of ULD atipamezole would have on morphine CPP in those same surgical groups. Based on the positive results in the previous chapters, both in terms of the sensory behavioral testing and the molecular immunohistochemical experiments, it was hypothesized that co-administration of ULD atipamezole would increase morphine CPP in all treatment groups (since co-administration of ULD atipamezole prevented morphine tolerance in pain naïve and nerve-injured animals, see fig. 3.1 and fig. 3.5 respectively, and attenuated mechanical allodynia following nerve injury, see fig. 3.2). It was hypothesized that conditioning with ULD atipamezole alone would not induce a CPP in naïve or sham animals since pain naïve animals treated with ULD atipamezole alone had no change in thermal tail flick latencies either acutely or compared to pre-trial baselines following chronic dosing (see fig. 3.1). A crucial part of the conditioning is that the animal must have the ability to associate the drug injection and its effects with the contextual cues, but since the timeframe for conditioning is only thirty minutes post-injection and ULD atipamezole has no acute effects, it should not be able to induce CPP in sham and naïve animals. In the CCI animals, ULD atipamezole alone attenuates mechanical allodynia, but this occurs over the long-term and thus the animals are unlikely to associate its analgesic effects with exposure to the boxes’ visual and tactile cues immediately post-injection.
5.2 METHODS

5.2.1 Animals

Male Long Evans rats (Charles River, QC) were used for all CPP experiments because they are believed to be better able to differentiate visual cues than strains such as the Sprague-Dawley (Prusky et al., 2002). Upon arrival, animals were allowed to acclimatize to the new environment for six days and handled several times over this period. All experiments were performed in accordance with guidelines and regulations set by the Queen’s University Animal Care Committee and The Canadian Council on Animal Care. Animals were housed two per cage and allowed free access to food and water.

5.2.2 Surgery to induce neuropathic pain

Animals were randomly assigned to a neuropathic pain group (receiving chronic constriction injury of the sciatic nerve of the hindpaw as described by Bennett & Xie), a sham surgery control group receiving the same skin and blunt muscle dissection but without manipulation or ligation of the sciatic nerve, or a pain-naïve (no surgery) group. The Bennett & Xie model was used as it caused more robust and consistent development of mechanical allodynia compared to the Mosconi-Kruger model (see chapter 2).

Rats were administered 0.6ml of liquid acetaminophen (32mg/ml) orally immediately prior to surgery and were then placed in a Plexiglas® induction box and anesthetized with isoflurane (5L/min induction). Once a surgical plane of anaesthesia was attained, the flow rate of isoflurane was reduced to 2.5L/min. for maintenance. Eye drops were placed in the rats’ eyes for lubrication (Hypotears). Lactated Ringer’s Solution was administered to maintain hydration (2 X 2.5mL bolus, subcutaneous).
The surgical site was shaved and sterilized with isopropyl alcohol followed by iodine. A small incision (approximately 2cm) was made in the skin with a scalpel and small scissors were used to bluntly dissect the underlying muscle layers. For the CCI groups, the sciatic nerve was located and carefully exposed by placing curved forceps under it and lifting it out of the cavity. Connective tissue was removed with pointed forceps. The nerve was kept hydrated to keep it from dying. Using 4-0 chromic gut suture thread, four ligatures were tied loosely around the nerve. The nerve was carefully replaced and gently worked back into position, and the muscle and skin layers were sutured with 3-0 Monocryl suture thread. Rats were given acetaminophen dissolved in gelatin (50mg/cube, one cube each) immediately after surgery and once more the following morning. Animals were allowed to recover for seven days before the start of the CPP protocol. They were weighed daily and observed carefully for any signs of unusual distress.

5.2.3 Confirmation of mechanical allodynia using von Frey filaments

Nociceptive responses were assessed in the ipsilateral hindpaw prior to surgery, and four and seven days post-surgery to confirm the development of mechanical allodynia by assessing 50% withdrawal thresholds to a series of von Frey filaments applied in an up-down fashion to the plantar portion of the hindpaw. Any animal that did not develop mechanical allodynia was excluded from the experiment. Only the 50% withdrawal threshold method was used in this experiment, not the # of responses to a 12g filament, as this was only done to confirm the surgeries were successful. Animals were allowed to recover for seven days before the start of the CPP paradigm. Seven days was chosen because it was a long enough time frame to allow mechanical allodynia to develop and peak effects in terms of behavior and spinal gliosis occur seven to ten days post-surgery.
5.2.4 Conditioned place preference (CPP) paradigm

On the eighth day post-surgery, animals began the CPP protocol. The CPP testing apparatus consisted of three-chambered boxes with a small neutral, centre chamber acting as a tunnel between two larger chambers, each with different visual and tactile cues (striped vs solid walls, large vs small floor gratings, see fig. 5.1). Guillotine doors separated the larger chambers from the tunnel and could be opened or closed depending on the testing phase.

5.2.4.1 Habituation

Animals were habituated to the CPP apparatus for thirty minutes with all doors open, allowing access to the two main compartments and the tunnel. Figure 5.1 shows a visual representation of a three-chambered CPP box. During this animals were allowed to explore freely and were undisturbed. Habituation time is important because the animal should be familiar with the environment before the start of conditioning. New environments are stressful for animals so they should not be experiencing the boxes for the first time on conditioning day. Conversely, the animal should not be too familiar with boxes before the start of conditioning because novel association between the visual and tactile cues and drug administration is crucial for conditioning to occur. For these experiments only one thirty minute habituation session occurred.
5.2.4.2 Pre-conditioning

The following day, pre-conditioning was performed to determine if any of the animals had an initial bias to one side of the test chambers. The guillotine doors were opened, the rats were placed in the neutral, central compartment and their movement was tracked over thirty minutes to determine the time spent in each of the compartments. Ideally rats would not show a preference to either side at the start of the paradigm and would spend an equal amount of time exploring both sides. If an animal spends more time in one compartment than the other during pre-conditioning, a decision needs to made about whether the animal should be excluded or whether it will stay in the study but have drug administration paired to the opposite side (a biased approach, whereby the animal not only needs to like getting the drug, but must learn to like its least-preferred side more over time). A third possibility exists whereby the initial preference is noted but assignment to boxes and pairing of the drug to a compartment remains randomized. There are pros and cons to each decision which will talked about in more detail in the discussion, but for these studies, animals that had a preference to one side initially were paired drug with the opposite side. While excluding animals completely would be ideal, this is not practical or ethical since the animals had already undergone surgery at this point.
5.2.4.3 Conditioning

The following day the conditioning phase began and continued for eight days. Proper counter-balancing of the boxes and the sides on which the animal will receive drug or saline is crucial so that the data do not become skewed in one direction or the other. Animals in each surgical group were assigned to a morphine only group (2mg/kg), atipamezole only group (5ng), or morphine plus atipamezole. The dose of morphine used was based on earlier experiments where we performed dose-response curves in neuropathic, sham and pain naïve animals (Cahill et al., 2013). Injections were administered systemically (subcutaneously, 0.9% saline vehicle) and animals were immediately placed in their respective compartments for thirty minutes with all doors closed. Injections alternated daily between drug and saline so animals would always receive drug on one side of the test boxes and saline on the opposite side. The boxes were counterbalanced appropriately (see table 5.1).

5.2.4.4 Post-conditioning

Following the eight day conditioning phase, two days of post-conditioning were performed to determine whether or not the animals had developed a preference for the drug-paired compartment. This was assessed with the guillotine doors open, allowing the animal free access to all compartments for thirty minutes. The first post-conditioning day was assessed drug-free, while the second conditioning day was assessed following re-administration of the drug the animal had been receiving during the conditioning phase (state-dependent testing).

Time spent in the saline-paired and drug-paired compartment were compared across each surgical group by one-way ANOVA followed by Bonferroni post hoc analysis. P<0.05 was considered statistically significant.
Fig. 5.1 Diagram showing a three chambered conditioned place preference (CPP) box with two conditioning sides and a neutral tunnel separating them through which the animal must pass to enter the other side. Each side has distinct patterns on the wall and different mesh flooring so the animal can learn to associate drug administration with visual and tactile cues. During habituation and pre-conditioning (to assess whether or not there is an initial bias to one side before dosing begins), the guillotine doors separating the chambers are opened to allow the animal to explore. During the conditioning phase, the doors are closed and the animal is confined to one side of the box. On alternating days the animal is given drug or saline, each of which is paired with one side of the box. During post-conditioning testing the doors are again opened and the amount of time each animal spends in each compartment is measured to see if a CPP to the drug has occurred.
### Counter-Balancing of CPP Boxes

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Surgery</th>
<th>Box</th>
<th>Odd Days (1,3,5,7)</th>
<th>Even Days (2,4,6,8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCI</td>
<td>1</td>
<td>MS (Left)</td>
<td>Sal (Right)</td>
</tr>
<tr>
<td>2</td>
<td>CCI</td>
<td>2</td>
<td>Sal (Left)</td>
<td>MS (Right)</td>
</tr>
<tr>
<td>3</td>
<td>CCI</td>
<td>3</td>
<td>MS + Ati. (Right)</td>
<td>Sal. (Left)</td>
</tr>
<tr>
<td>4</td>
<td>CCI</td>
<td>4</td>
<td>Sal. (Right)</td>
<td>MS + Ati. (Left)</td>
</tr>
<tr>
<td>5</td>
<td>CCI</td>
<td>1</td>
<td>Sal. (Left)</td>
<td>MS + Ati (Right)</td>
</tr>
<tr>
<td>6</td>
<td>CCI</td>
<td>2</td>
<td>MS + Ati (Left)</td>
<td>Sal (Right)</td>
</tr>
<tr>
<td>7</td>
<td>CCI</td>
<td>3</td>
<td>Sal (Right)</td>
<td>MS (Left)</td>
</tr>
<tr>
<td>8</td>
<td>CCI</td>
<td>4</td>
<td>MS (Right)</td>
<td>Sal (Left)</td>
</tr>
<tr>
<td>9</td>
<td>Sham</td>
<td>1</td>
<td>MS (Left)</td>
<td>Sal (Right)</td>
</tr>
<tr>
<td>10</td>
<td>Sham</td>
<td>2</td>
<td>Sal (Left)</td>
<td>MS (Right)</td>
</tr>
<tr>
<td>11</td>
<td>Sham</td>
<td>3</td>
<td>MS + Ati. (Right)</td>
<td>Sal. (Left)</td>
</tr>
<tr>
<td>12</td>
<td>Sham</td>
<td>4</td>
<td>Sal. (Right)</td>
<td>MS + Ati. (Left)</td>
</tr>
<tr>
<td>13</td>
<td>Sham</td>
<td>1</td>
<td>Sal. (Left)</td>
<td>MS + Ati (Right)</td>
</tr>
<tr>
<td>14</td>
<td>Sham</td>
<td>2</td>
<td>MS + Ati (Left)</td>
<td>Sal (Right)</td>
</tr>
<tr>
<td>15</td>
<td>Sham</td>
<td>3</td>
<td>Sal (Right)</td>
<td>MS (Left)</td>
</tr>
<tr>
<td>16</td>
<td>Sham</td>
<td>4</td>
<td>MS (Right)</td>
<td>Sal (Left)</td>
</tr>
</tbody>
</table>

Table 5.1 Table represents an example showing even counter-balancing of 16 animals from 2 surgical groups (CCI and sham) among four different conditioning boxes (with different wall patterns and floor textures), half of which are being conditioned with morphine and half of which are being conditioned with morphine plus ULD atipamezole. Animals always receive drug on the same side of their assigned box and saline on the opposite side. In this example, animals are being conditioned over eight days, so four of those days they would receive drug and on the alternate days they would receive saline. The dosing needs to be counter-balanced so half the animals in each group get drug on odd days and half get drug on even days, and vice-versa, and the sides of the boxes in which animals are receiving drug or saline must be equally counter-balanced to eliminate potential bias. Ati = atipamezole, CCI = chronic constriction injury, MS = morphine sulfate, Sal. = saline, ULD = ultra-low dose.
5.3 RESULTS

5.3.1 Confirmation of mechanical allodynia prior to commencement of CPP

Development of mechanical allodynia was confirmed in CCI animals over the seven days following nerve injury. Two-way ANOVA revealed a significant effect of time ($F_{(2,28)} = 24.74$) and surgery ($F_{(1,14)} = 40.82$), and an interaction ($F_{(2,28)} = 24.74$). 50% withdrawal thresholds in the ipsilateral hindpaw decreased rapidly and steadily over this time period with the lowest thresholds seen on day seven, just prior to the start of the CPP protocol (approximately 4.8g). Mechanical allodynia did not develop in the sham animals, as expected. The sham animals recovered from surgery quickly and their 50% withdrawal threshold remained at the 15g cut-off both four and seven days post-surgery (**p < 0.001 compared to CCI animals at both time points).
Fig. 5.2 Assessment of 50% withdrawal thresholds to a calibrated series of von Frey filaments applied in an up-down manner to the plantar portion of the ipsilateral hindpaw over the seven days following surgery in CCI (red) and sham (blue) animals to assess the development of allodynia prior to beginning the CPP protocol. Bars represent means +/- S.E.M. N=8/group. CCI = chronic constriction injury, CPP = Conditioned Place Preference. *** P < 0.001.

Mechanical Allodynia Was Allowed to Develop in CCI Animals Prior to Commencement of the CPP Protocol
5.3.2 Assessment of initial bias in pre-conditioning

To determine if any animals had an initial bias to one compartment or the other before conditioning began, pre-conditioning times were determined in all animals and the cumulative time spent in what would later be assigned as drug- and saline-paired compartments were compared. Half the animals with an initial bias to one side were assigned to receive drug in the opposite compartment while half were assigned to receive saline in that side in an effort to balance the boxes and chambers as much as possible without excluding animals. Following the shuffling of several animals, there was no difference in the time spent in the respective drug- and saline-paired side in any of the surgical groups before the start of the conditioning phase.

5.3.3 Post-conditioning times

Following eight days of conditioning, post-conditioning times were determined whereby the guillotine doors were opened and the animals were allowed free access to all compartments to measure the time spent in both sides. The first post-conditioning day was drug-free while the second was state-dependent whereby the animals were re-administered the drug they were originally conditioned with.

5.3.3.1 Effect of conditioning with ULD atipamezole alone

In the animals conditioned with ULD atipamezole alone (fig. 5.3), there was no difference in the amount of time the animals spent in the drug- or saline-paired compartment within the CCI or sham groups, and the CCI and sham animals did not differ significantly from each other on either the first (drug-free, fig. 5.3A, \( F_{(3,28)} = 1.312, P = 0.2902 \)) or second (state-dependent fig. 5.3B, \( F_{(3,28)} = 1.177, P = 0.3364 \)) post-conditioning day. \( N = 8 \text{/group}. \)
Chronic Systemic Administration of ULD Atipamezole Does Not Induce Conditioned Place Preference in CCI or Sham Surgery Animals

Figure 5.3 Cumulative time spent in the drug- and saline-paired compartments over thirty minutes in CPP boxes in CCI (red) and sham animals (blue) conditioned with systemic injections of ULD atipamezole (5ng) on the first (drug-free) conditioning day (A) and the second (state-dependent) conditioning day following drug administration (B). N=8/group. Bars represent means +/- S.E.M. CCI = chronic constriction injury, CPP = conditioned place preference, ULD = ultra-low dose.
5.3.3.2 Effect of co-administration of ULD atipamezole on development of morphine CPP

All animals that were conditioned with morphine alone (2mg/kg) spent significantly more time in the drug-paired compared to the saline-paired compartment (*** p < 0.01 for pain naïve animals, *** p < 0.001 for sham animals, ** p < 0.001 for CCI animals) (fig. 5.4 A, B, and C, respectively). Pain naïve (fig. 5.4A) and sham animals (fig. 5.4B) that were co-administered ULD atipamezole during conditioning also spent more time in the drug-paired compared to the saline-paired compartment (*** p < 0.001 for pain naïve animals, * p < 0.05 for sham animals), though the time spent in the drug-paired compartment in naïve and sham animals conditioned with morphine and ULD atipamezole was not different compared to naïve and sham animals conditioned with morphine alone. In CCI animals, however, co-administration of ULD atipamezole during conditioning disrupted morphine CPP and there was no difference in the amount of time spent in the drug-paired vs the saline-paired compartment (fig. 5.4C).

For pain naïve rats (fig. 5.4A), $F_{(3,28)} = 25.54$, $P < 0.0001$. For sham surgery rats (fig. 5.4B), $F_{(3,28)} = 11.24$, $P < 0.0001$. For CCI rats (fig. 5.4C), $F_{(3,26)} = 6.300$, $P = 0.0023$. N = 8/group.
Figure 5.4 Cumulative time spent in the drug- and saline-paired compartments of the CPP boxes on the post-conditioning day (drug-free) in pain naïve (A), sham (B) and CCI (C) animals conditioned with systemic injections of morphine (2mg/kg) (solid bars) or morphine plus ULD atipamezole (5ng) (hatched bars). Co-administration of ULD atipamezole prevented the development of morphine CPP, but only in the CCI animals (C), not the shams (B) or pain naives (A). Ati = atipamezole, CCI = chronic constriction injury, CPP = conditioned place preference, ULD = ultra-low dose. Bars represent means +/- S.E.M. N=8/group. * P < 0.05, ** P < 0.01, *** P < 0.001.
5.4 DISCUSSION

5.4.1 Summary

Chronic co-administration of ULD atipamezole inhibited morphine CPP in CCI animals, but not in sham or pain naives, and ULD atipamezole on its own did not induce a CPP, nor was it aversive. The results were unexpected. Based on the fact that ULD atipamezole attenuated the development of mechanical allodynia on its own, attenuated the development of morphine tolerance (chapter three), and attenuated astrogliosis and the upregulated expression of c-Fos following nerve injury (chapter four) in earlier experiments, it was hypothesized that the same dose of atipamezole might induce a CPP on its own in nerve-injured animals, and would increase the amount of time CCI animals spent in the morphine-paired compartment. Paradoxically, co-administration of ULD atipamezole actually disrupted morphine CPP in CCI animals, while having no effect on morphine CPP in the shams or pain naïve rats. The rats conditioned with ULD atipamezole alone had no preference for one compartment over the other, and there was no difference between the CCI and sham groups. The reason for these paradoxical effects is unknown.
5.4.2 Methodological validity and study limitations

One limitation of the current study is that Long-Evans rats were used in all CPP experiments, whereas all previous chapters used Sprague-Dawley rats. One reason for the change was the fact that albino rat strains like the Sprague-Dawley have inferior visual acuity compared to pigmented strains like the Long-Evans (Prusky et al., 2002). Because the CPP paradigm relies partly on learning to associate visual cues with drug effects, Long-Evans rats were a better model. Also, the dose of morphine used in these experiments was based on an earlier study that used Long-Evans rats (Cahill et al., 2013).

In addition to differences in the visual acuity among different rat strains, spatial learning and memory is also strain-dependent. Inbred and albino rats perform worse in spatial learning tasks than domesticated breeds. For example, in the Morris Water Maze task in which the animal is placed in a tub of water and must learn and remember the location of a platform they can climb onto to get out of the water, Long-Evans rats performed at the same level as wild-type animals, while Wistar and Fischer 344 performed the worst (Harker and Whishaw, 2002). Of the four inbred and albino strains tested Sprague-Dawley rats performed the best but were not up to the level of performance seen in the Long-Evans and wild-type animals. These studies highlight the importance of appropriate strain selection for behavioural testing and underlie the selection of the Long-Evans strain for the conditioned place preference (CPP) experiments, despite the fact that earlier chapters used Sprague-Dawley rats for nociceptive testing in the morphine tolerance and neuropathic pain models.

The dose of morphine used in all CPP experiments was 2mg/kg. This dose was selected based on a paper we previously published that demonstrated an inverted dose-response curve in CCI animals. At higher doses, morphine induced a CPP in both sham and CCI animals. As the
dose was decreased, morphine no longer induced a CPP in sham animals, but CCI animals still spent significantly more time in the drug-paired compartment. Based on this finding we concluded at lower doses (2mg/kg) a place preference could be obtained that was likely due to relief of pain which was distinct from morphine’s rewarding effects, since the same dose had no effect on the sham animals. Unfortunately, this effect was not replicated in the present study and all three surgical groups demonstrated a CPP to the 2mg/kg dose of morphine, not just the CCI animals. One reason for the discrepancy could be due to the fact that the morphine used here was from a different source than the published study. The previous dose-response curve was obtained using a commercial solution of liquid morphine, whereas in this study morphine was made into solution from its salt.

One of the benefits of using a three-chamber compared to a two-chambered CPP box is that the neutral tunnel allows for the rat to make a choice about which side it prefers. In a two-chambered box without a neutral tunnel, the animals must pass into the opposite side directly to leave the side it is in (this is a forced choice) and thus it can be difficult to tell if it has a preference for one side or it finds the other side aversive, which is an important distinction, especially with a new drug or combination of drugs where the outcome is hard to predict. Drugs like morphine have been studied for a long time and are known to induce CPP due to rewarding or pain-relieving properties and they have predictable effects, but it was not known whether ULD atipamezole would cause a CPP on its own, whether it would be aversive due to peripheral effects, or if it would just not have any impact one way or another, so a two-chambered box would not have been ideal for this particular experiment. In a two-chambered CPP box, the time spent on both sides is dependent (they can only be on one side or the other), while in a three-chambered box it is not. One problem, however, with three-chambered CPP boxes that contain a
tunnel is that the animals will sometimes spend very long periods of time in this neutral chamber without moving, which can heavily skew the data. The small tunnel may feel more secure to the rat than the wider open spaces of the two larger compartments. Because of the presence of the tunnel, the time spent in the drug- and saline-paired sides are not dependent and the choice is not forced so three-chambered boxes were seen as the best choice for this experiment.

Another important consideration in designing a CPP trial is whether the design will be biased or unbiased based on an animal’s initial preference for one side of the box or the other during the pre-conditioning phase (before any drug is administered to the animals). Prior to conditioning, the animal is placed in the boxes and allowed to explore, and the amount of time spent in each compartment is recorded. If an animal has an initial bias for one side, the experimenter must decide if the animal will be excluded from the experiment completely, whether it will be assigned to receive the drug in the opposite compartment than the one it initially preferred, or whether the animal will still be included in the experiment but the assignment to one side or the other will remain randomized (but still properly counter-balanced). While there does not appear to be a clear consensus in the literature about the best course of action should an initial preference exist, some studies have shown that animals that do exhibit a CPP when they are assigned to receive drug in the opposite side to the one initially preferred (and thus need to overcome that initial preference), do not show a CPP if the assignment of the drug-paired side is completely randomized (Calcagnetti and Schechter, 1994). While it is not understood why this may be the case, for the CPP experiments in this chapter animals that showed an initial bias to one side were assigned the opposite compartment as the drug-paired side. So while the assignment for the animals that did not show an initial preference to one side was randomized, the few animals that did show an initial preference were assigned the opposite
side as the drug-paired compartment in a biased manner while still maintaining proper counter-balancing in terms of boxes, sides and dosing days, as this seemed to be the most appropriate course of action in this study. Also, while it is possible to look at the difference between the time spent in the drug-paired compartment on the post-conditioning day and the time spent in the drug-paired compartment during pre-conditioning (before drug administration begins), differences were not shown for this experiment and instead cumulative time spent in each compartment on test days is reported as raw numbers. Though CPP data is also sometimes represented as a ratio of the time spent in the drug-paired:saline-paired compartments, this is more appropriate when two-chambered CPP boxes are employed, as the time spent in one side is completely dependent on the time spent in the other. With three-chambered CPP boxes, on the other hand, the rat can spend time in the tunnel and the fact that time spent in either side is not completely dependent on time spent in the opposite side, so it seemed more appropriate to report cumulative times rather than ratios in these experiments.
5.4.3 Alterations in receptor expression and functionality in tolerance and chronic pain states underlie changes in CPP

Mechanistically, the reasons for the unexpected results in this experiment are difficult to parse. Changes in expression and functional competence of opioid and adrenergic receptors occur following repeated stimulation from chronic dosing, and nerve injury induces changes in opioid and adrenergic receptor expression, altering the analgesic response to opioid and adrenergic receptor ligands so it is difficult to interpret the results of this CPP in terms of mechanism. Desensitization and internalization of MORs following repeated stimulation is agonist-dependent (Whistler et al., 1999) and some of the literature is not in agreement in regards to changes in MOR expression and function (Fábián et al., 2002). For example, one study showed chronic morphine administration results in an increase in G-protein-coupling, increased expression of the α subunit of the G-proteins, an increase in ligand binding at the plasma membrane after ten days of repeated dosing, and an increase in surface and intracellular MOR stores that may be a result of a combination of post-translational modification and newly synthesized receptors (Fábián et al., 2002). This work is in contrast to earlier studies that postulated chronic opioid tolerance resulted from desensitization and internalization of MORs from the plasma membrane through β-arrestin-mediated mechanisms, thus decreasing their functional competence. In reality, the mechanisms of tolerance appear to be much more complex and involve many changes in intracellular signaling and G-protein-coupling, and tolerance might actually be reduced by receptor internalization mechanisms (Kieffer and Evans, 2002). Unlike certain other MOR agonists (such as DAMGO or etorphine), morphine does not cause internalization of the receptor to a large degree (Keith et al., 1996), nor does it cause significant phosphorylation of the receptor after binding or desensitization, at least in HEK 293 cells.
(Whistler et al., 1999); effects in vivo are less clear. Internalization does not necessarily lead to
degradation of the receptor in the lysosome as previously thought, but it is important for
recycling of the receptor to be inserted back into the membrane to regain functionality
(Lefkowitz et al., 1998; Trapaidze et al., 2000). This type of receptor internalization and
recycling is actually protective, while the lack of internalization induced by morphine can be viewed as detrimental as it causes prolonged activation of the receptor and abnormally long
periods of cell signaling that might be responsible for the adverse effects of chronic exposure
related to tolerance and opioid withdrawal upon abrupt discontinuation (He et al., 2002). When
morphine is co-administered with another MOR agonist that does induce receptor internalization
(eg: DAMGO), the functional competence of MORs is enhanced as more are able to return to the
plasma membrane, the analgesic effects are potentiated and less opioids are needed to maintain
adequate pain relief (Hashimoto et al., 2006).

The rewarding effects of opioids are lower in neuropathic pain states in rodents (Ozaki et
al., 2002, 2003; Oe et al., 2004). Opioid self-administration is lower in neuropathic rats and
spinal administration of clonidine, an α2-adrenergic receptor agonist further reduces opioid
consumption in those animals (Martin et al., 2007). In mice, nerve injury induces opioid
tolerance by causing an increase in the release of endogenous β-endorphin and phosphorylation
and desensitization of MORs, causing them to be removed from the plasma membrane (Niikura
et al., 2008; Petraschka et al., 2007). Behaviorally, this results in a rightward shift in the dose-
response curve in thermal tail flick testing and an attenuation of morphine CPP in nerve injured
animals (Petraschka et al., 2007).

Similar to the results we have published showing the inverted morphine CPP dose-
response curve following chronic constriction injury of the sciatic nerve (Cahill et al., 2013,
Appendix C), other groups have seen that certain doses of spinally-administered clonidine, an α₂-AR agonist induce CPP in nerve-injured animals, but not in sham control animals at the same dose (King et al., 2009). Interesting paradoxical effects are seen in the CPP paradigm involving cross-modulation between opioid and noradrenergic receptor systems. For example, we have seen that clonidine appears to be aversive in the CPP paradigm following chronic morphine conditioning in nerve-injured animals, but not in sham controls (unpublished observations) despite the fact that clonidine is analgesic in pain naïve and nerve-injured rats (Duflo et al., 2002), can induce a CPP when administered on its own (Asin and Wirtshafter, 1985), synergizes when co-administered with opioids in chronic pain states (Ossipov et al., 1997), and is used to help treat symptoms of opioid withdrawal (Gowing et al., 2014). Based on the results in the previous chapters in terms of behavior, ULD atipamezole exhibits behavioral effects similar to what would be observed with a partial agonist, and the results in terms of CPP to morphine in nerve injured animals are similar to what we have observed in the past with clonidine. This is not to say an ULD antagonist functions mechanistically in the same way as an agonist, but in terms of behavioral effects the two do appear to share a lot of similarities.

Isobolographic analyses show morphine and clonidine synergize to produce a greater amount of analgesia than would be expected based on either drug alone, though synergy of adverse events such as sedation or negative effects on the cardiovascular system is not observed (Stone et al., 2014). Despite this fact, clonidine does cause side effects ranging from minor problems like dry mouth, constipation, and diuresis (Maze and Tranquilli, 1991), to sedation, memory and cognitive impairment (Hall et al., 2001) and slowed heart rate at higher doses. Abrupt discontinuation following prolonged use can result in death due to rebound hypertension and increased heart rate due to neuroplastic changes in the basal tone of the noradrenergic system.
(Jamadarkhana and Gopal, 2010). Due to the very low concentration of ULD α₂-AR antagonists, co-administration with morphine should be expected to induce fewer side effects than if a high dose agonist like clonidine is administered with an opioid, though this remains to be tested in humans.

5.4.4 Disruption of morphine CPP in nerve injured animals appears to be a result of decreased reward

It is not known why ULD atipamezole disrupted morphine CPP in the nerve-injured animals. One argument that could be made is that perhaps the ULD atipamezole disrupts learning and memory, which is crucial for the establishment of a CPP. This is unlikely, however, since animals that are unable to learn to associate the visual and tactile cues associated with pain relief and possible reward would spend an equal amount of time in both compartments. This was not the case in the sham and pain naïve animals which spent significantly more time in the morphine-paired compartment regardless of whether or not they were co-administered atipamezole during the conditioning phase.

Another possibility that we considered was that maybe ULD atipamezole was causing unpleasant adverse effects in the CCI animals that were leading them to avoid the drug-paired compartment. This is likely not the case, however, as the animals administered ULD atipamezole alone did not show aversion to the drug-paired side. It was suggested that perhaps the co-administration of ULD atipamezole might be inducing a type of opioid withdrawal in the nerve injured animals following chronic morphine administration that was causing them to spend less time in the drug-paired side. While assessment of naloxone-precipitated withdrawal has not been performed in the ULD atipamezole study, quantification of withdrawal symptoms was assessed
in clonidine-treated animals conditioned with morphine to try to explain similar paradoxical effects with the α₂-AR agonist. Rats that were administered clonidine were highly sedated and very few withdrawal-like symptoms were observed so it is not possible to draw further parallels (data not shown). The possibility remains that because there are no acute effects with ULD atipamezole and since the conditioning is performed over the thirty minutes post-injection, it may be that any positive effects of the drug are not being associated with the contextual cues, though this is unlikely to explain the difference between the groups.

5.5 CONCLUSION

No obvious reason exists to explain why morphine CPP is disrupted by ULD atipamezole in neuropathic animals. Further studies will need to be carried out to determine a new dose-response curve with the morphine from this new source. The 2mg/kg dose was selected because it was supposed to induce CPP in neuropathic but not sham or pain naïve rats, but unfortunately a CPP was induced in all groups in this experiment making it difficult to tease apart the rewarding effects of the morphine from its analgesic effects. At present, though, it does appear that the only explanation is a reduction in morphine reward in nerve injured animals since it was shown in earlier chapters that ULD atipamezole unequivocally alleviates pain and enhances opioid analgesia; they are not spending less time in the drug-paired compartment because they have less pain relief. Thus, the current data suggest that not only does ULD atipamezole attenuate opioid tolerance in pain naïve and nerve-injured animals, enhance the antinociceptive effects of opioids and attenuate mechanical allodynia on its own, thus decreasing opioid requirements and reducing risk of opioid side effects by allowing use of lower doses, it may reduce opioid reward in chronic pain states as well, though further study in this area is needed to better understand the role of ULD atipamezole in the affective processing of pain.
CHAPTER 6: General Discussion

6.1 SUMMARY

It was hypothesized, based on the small amount of ULD α₂-AR antagonist literature and the larger body of ULD opioid antagonist work that systemic administration of ULD atipamezole would attenuate the development of opioid tolerance and enhance opioid analgesia in pain naïve and nerve-injured animals, that it would attenuate spinal gliosis associated with chronic morphine administration and nerve injury, and that it would increase morphine CPP. While the hypotheses were correct in regards to attenuation of opioid tolerance and gliosis, some surprising outcomes were observed. It was not expected that ULD atipamezole would be able to attenuate mechanical allodynia on its own following nerve injury, even in the absence of opioids, and the fact that morphine CPP was disrupted, not enhanced, but only in nerve injured animals was surprising.

In Chapter 3 it was demonstrated that chronic systemic administration of ULD α₂-adrenergic antagonists attenuates the development of morphine tolerance in pain naïve rats (fig. 3.1), consistent with what previous studies have shown through intrathecal administration in acute tolerance models. The ability to systemically administer drugs is important in a clinical setting, and the chronic morphine tolerance paradigm used is more analogous to repeated dosing in humans, where the problem with tolerance to opioids develops over a longer period of time, not just after a few doses. Previous studies have mainly used acute tolerance paradigms where the α₂ antagonists are administered repeatedly over a few hours. The fact that we were able to reproduce previous findings by using the drug in more clinically relevant ways provided incentive to try using the drugs in a chronic pain model, which has not been done previously.

Following chronic constriction injury to induce neuropathic pain, chronic systemic
administration of ULD atipamezole alone was able to attenuate the development of mechanical allodynia in the ipsilateral hindpaw as evidenced in two different testing modalities (fig. 3.2B); this result was unexpected. Previous studies with ULD BRL44408, an antagonist selective for the $\alpha_{2A}$-adrenergic receptor subtype showed that it too had analgesic effects when administered alone (Appendix 2, fig. 2), though its effects were observed acutely, and they were delayed and long-lasting. No acute effects were observed in our studies with ULD atipamezole or efaroxan, either on the first day of administration or following repeated dosing, though behavioral time courses were only assessed over two hour time intervals. It is possible that the acute analgesic effects, if any, were delayed, but even at two hours post-injection no change in baseline had occurred (fig. 3.5, right panels for ULD atipamezole alone). The fact that no acute analgesic effects were seen with ULD atipamezole or efaroxan suggests that prolongation of opioid analgesia following an acute injection of morphine in CCI animals previously treated for ten days with the ULD $\alpha_2$-adrenergic antagonists is very likely not a result of additivity or synergy with opioids, but is probably a result of modulation of the neuroplastic changes that occur following nerve injury. While no explicit isobolographic analyses have been performed, it is hard to imagine additivity or synergy as a possibility as no change from pre-injection baseline was observed in either the thermal tail flick or the two mechanical paw withdrawal tests for either atipamezole or efaroxan.

The fact that greater effects were observed in the # of 12 g withdrawal method of assessing mechanical allodynia compared to 50% withdrawal method (eg: see fig. 3.4) further highlights the importance of testing modality that was initially observed in chapter 2 (see fig. 2.3) with the glial inhibitor drugs. Additionally, while ULD atipamezole alone attenuated the development of mechanical allodynia in the ipsilateral hindpaw, no change in thermal tail flick
was observed over the ten days of repeated injections (fig. 3.2). This suggests that either the ULD $\alpha_2$-adrenergic antagonists have greater effects on mechano-sensitive neurons or that the mechanical tests themselves are more sensitive than the thermal tail flick assay. This interpretation, however, is complicated by the fact that animals did not develop thermal hyperalgesia in the tail (fig. 3.2A), so this could only really be confirmed by selecting a thermal test to use on the hindpaw and repeating the study. Regrettably we do not have thermal data for the paw. It would be interesting and worthwhile to test the effects of the ULD $\alpha_2$-adrenergic antagonists in a wider battery of nociceptive testing paradigms (many of which are described in table 1.6), though we chose to focus on only three. The two mechanical tests were used because differential effects were seen in Chapter 2 (fig. 2.3) and we concluded it was possible that different populations of mechano-sensitive afferent neurons might be preferentially engaged with the method comparing the # of 12g withdrawals which involves repeated stimulation of the affected paw. We chose to assess thermal responses in the tail because we wanted to assess nociception in an area not directly associated with the injury itself, as well as the fact that the tail is the main thermoregulatory organ in the rat. While repeated stimulation (which happens in the mechanical test with the 12g filament) could hypothetically trigger the release of endogenous opioids that could change nociceptive responses, this is unlikely as CCI saline controls do not show a decrease in the # of withdrawals across several bins. The question of whether repeated stimulation changes nociceptive responses was investigated previously (see Appendix 2, fig. 7) and no differences in thermal or mechanical testing were observed over a four hour time course between animals tested frequently at regular intervals (every 30 minutes) compared to animals tested only at four time points.

To take it one step further toward clinical relevance, nerve injured animals were
chronically treated with morphine alone or morphine & ULD atipamezole. Again, co-
administration of ULD atipamezole attenuated the loss of morphine analgesia as evidenced in
both the thermal tail flick and both mechanical paw pressure tests (fig. 3.5). Thus, systemic ULD
atipamezole attenuates morphine tolerance both in pain naïve and nerve injured animals, though
interestingly the attenuation of tolerance is much greater in the neuropathic animals compared to
the pain naives (fig. 3.1) providing further evidence that at least some effects of the antagonists
are likely due to modulation of neuroplastic changes associated with the development of
neuropathic pain.

In addition to assessing changes in sensory responses following nerve injury, the negative
affective or emotional component of pain was assessed as well through the CPP paradigm. ULD
atipamezole had no effective on place preference on its own, but disrupted morphine CPP in the
neuropathic animals, but not the shams or pain naives (fig. 5.4), suggesting that perhaps opioid
reward was reduced in the nerve-injured animals, as the dose of morphine used (2mg/kg) was an
analgesic dose in the CCI rats.

It is highly likely that the effects of ULD $\alpha_2$-adrenergic antagonists are due, at least
partially, to direct in interactions with the $\alpha_2$-adrenergic receptors. At high doses, efaroxan blocks
clonidine analgesia and the effects are stereoselective. When chirality is reversed and the inactive
terteroisomer is administered, clonidine analgesia is unaltered (see Appendix 1, fig.1). Only the
active stereoisomer or a racemic mixture of efaroxan blocks the development of acute morphine
tolerance and prevents hyperalgesia caused by low dose morphine administration (Appendix 1,
fig. 3 and fig.4, respectively). Additionally, co-administration of high dose BRL44408, selective
for $\alpha_{2A}$, blocks clonidine analgesia (Appendix 2, fig. 1), and analgesia induced by ULD
BRL44408 is antagonized when high dose atipamezole is administered at the same time, and
reversed when high dose atipamezole is administered near peak effect (see Appendix 2, fig. 6). The latter studies suggest that the $\alpha_{2A}$-subtype is directly involved in the action of ULD $\alpha_2$-antagonists.

While $\alpha_2$-adrenergic receptors appear to be directly responsible for at least some of the effects of ULD atipamezole and efaxoroxan, this does not discount the possibility of direct or indirect effects on other receptor systems and cell types. In Chapter 4 we showed molecular evidence to support the behavioral studies by demonstrating that spinal glial activation induced by chronic morphine administration (figs 4.1-4.4) or nerve injury (figs. 4.5-4.15) are attenuated by chronic systemic ULD atipamezole. Additionally, nerve injury-induced upregulation of c-Fos, a marker of neuronal activation, was attenuated in CCI animals chronically treated with ULD atipamezole (fig. 4.16-4.17). Chapter 2 highlighted the complicated interplay between behavior and glial and neuronal activation associated with the development of neuropathic pain, but ceded that while gliosis is correlated with chronic pain states, we did not see evidence of it as the driving force behind it, since administration of glial inhibitors propentofylline and PJ34 attenuated spinal glial and neuronal activation (figs 2.1 and 2.2) with only moderate to negligible changes in pain responses (fig. 2.3). Thus, while Chapter 4 provided evidence of gliosis and neuronal activation being altered with ULD atipamezole, it is difficult to say whether there is a causal or only correlational relationship with the changes in pain behavior in Chapter 3. Is pain and opioid tolerance attenuated because of glial inhibition in the spinal cord, or is there less gliosis in the spinal cord because there is less pain?

While most $\alpha_2$-adrenergic receptors are expressed pre-synaptically on neurons, they are also expressed post-synaptically (Zhang et al., 2009) and extra-synaptically, and are also expressed on astrocytes (Enkvist et al., 1996) and microglia (Mori et al., 2002) as well, so it is
difficult to determine at this stage exactly whether the effects of ULD atipamezole are primarily mediated through neuronal or glial mechanisms; indeed it is likely that both play crucial roles in the mechanism of action of ULD α2-adrenergic antagonists and that these actions feed into and modulate one another.

GPCR dimerization further complicates mechanistic interpretation at this point as opioid and adrenergic receptors homodimerize among themselves and heterodimerize with each other, as well as other GPCRs involved in pain processing and opioid reward such as the substance P NK1 receptor, dopamine and cannabinoid receptors, altering their signaling and internalization properties (see table 1.4).
6.2 CURRENT AND FUTURE DIRECTIONS

While ULD atipamezole attenuated neuropathic pain behaviors, opioid tolerance and spinal gliosis, further studies will need to be carried out if a more precise mechanism of action is to be deduced.

One of the most obvious limitations of this thesis work is that, regrettably, knock-out studies were not performed. Had time and means permitted, genetic deletion of the $\alpha_2$-adrenergic receptor or specific sub-types would have provided meaningful insights into their direct roles in the mechanism of action. This, however, would have required replication of the ULD $\alpha_2$-adrenergic antagonist effects in mice before moving forward as these and all previous studies were performed in rat models. It is likely the effects would be preserved across rodent species, but not guaranteed. Also, genetic deletion of other GPCRs involved in pain processing and reward pathways that interact directly (eg: through dimerization) or indirectly with adrenergic receptor systems could provide important insight in terms of mechanism.

Another obvious path to investigate is whether or not G-protein signaling pathways are altered. At this stage it is unknown whether similar changes in G-protein-coupling are induced by ULD $\alpha_2$-adrenergic antagonists as is the case with ULD opioid antagonists, which makes receptor binding studies an interesting avenue of exploration.

While an attenuation of spinal and nerve injury-induced gliosis was observed, only changes in marker expression and cell size were characterized, so altered function was inferred from morphological changes in the cells, as is common in much of the pain literature. It will be useful, however, to be able to better characterize changes in glial phenotype, not only visually, but through direct measurement of intracellular signalling proteins and quantification of proteins released from the cells. A choice was made at the start of the molecular studies whether to look at
changes in receptor binding, as has been done with ULD opioid antagonists, or to investigate the involvement of spinal glia. Since previous work in our lab has shown a role of glial cells in the mechanism of ULD opioid antagonists (Mattioli et al., 2010), and the fact that we had performed work with the glial inhibitors in Chapter 2, this was a more obvious route of investigation. Thus, the next step would be to perform Western Blotting to measure changes in kinases involved in inflammatory signalling pathways in glia (eg: p38 or ERK), perform cytokine arrays to quantify changes in pro- and anti-inflammatory cytokines and other mediators, and to perform more immunohistochemistry using markers like CD68, which will allow characterization and comparison of microglial populations.

Another important research question is how common are the paradoxical effects of ULD GPCR antagonists? Do they extend to all GPCRs or is the effect common only to certain types that share similar function or sequence homology? Most of the literature is focused on the effects of ULD opioid antagonists in animal studies and clinical investigations, while this thesis work builds on the small amount of literature concerning ULD adrenergic antagonists and is the first to show positive effects in clinically meaningful models of chronic pain and reward. To date, the paradoxical effects of ULD antagonists have all been observed in GPCRs coupled to inhibitory G proteins and pathways, and thus it appears that the ULD antagonist effects might be a property that is generalizable to several GPCRs involved in pain and reward processing. Currently assessment of whether ULD CB₁ receptor antagonist rimonabant is able to attenuate morphine tolerance and gliosis is underway and could lend support to this hypothesis.
6.3 CONCLUSION

While the mechanism of action is not currently known and more molecular studies need to be undertaken, the data provided in this thesis show both behaviorally and molecularly that ULD α₂-adrenergic receptor antagonists like atipamezole: i) inhibit the development of morphine tolerance in pain naïve animals, ii) attenuate tolerance much more dramatically in chronic pain states, iii) are effective in attenuating mechanical allodynia on their own, and iv) may reduce the rewarding effects of opioids following nerve injury. The models used in these studies are the most clinically relevant assessed to date, and investigate not only the sensory component of pain, but the affective or emotional component as well, which, considering the high co-morbidity of chronic pain with depressive mood disorders and heightened risk of suicide in sufferers, may ultimately be more clinically important in human populations.

It is difficult to predict whether positive results at the basic science level will ultimately translate, but there is a very clear need for new drugs or combination therapies for chronic pain sufferers. Current options for the treatment of chronic pain are often ineffective and inconsistent. Some drug classes work better than others for treating neuropathic pain arising from a specific etiology, but what may prove effective for one sufferer may have no effect in another. In humans, prevention of tolerance would mean less dose escalation and lower risk of side effects that occur at high doses; use of other drug classes with broad adverse effect profiles might be minimized if ULD α₂ antagonists can attenuate pain on their own in clinical populations, and lowered opioid reward in chronic pain states would reduce abuse potential, improving the quality of life of many while at the same time removing a huge burden on the social and economic systems that affect us all.
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Appendix A: (Publication) Stereo-selective inhibition of spinal morphine tolerance and hyperalgesia by an ultra-low dose of the alpha-2-adrenoceptor antagonist efaroxan

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Stereo-selective inhibition of spinal morphine tolerance and hyperalgesia by an ultra-low dose of the alpha-2-adrenoceptor antagonist efaxoxan

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Abstract
Ultra-low doses of α2-adrenoceptor antagonists augment spinal morphine antinociception and inhibit tolerance, but the role of receptor specificity in these actions is unknown. We used the stereo-isomers of the α2-adrenoceptor antagonist, efaxoxan to evaluate the effect of receptor specificity on the induction of spinal morphine tolerance and hyperalgesia. Tail flick and paw pressure tests were first used to evaluate high dose efaxoxan (12.6 μg) and its stereo-isomers on spinal analgesia in intrathecally catheterized rats. Ultra-low doses of individual isomers (1.3 ng) were then co-administered with morphine (15 μg) to determine their effects on acute antinociceptive tolerance and hyperalgesia induced by low dose spinal morphine (0.06 ng). Results demonstrate that high dose (+)-efaxoxan antagonized clonidine-induced antinociception, while (-)-efaxoxan had minimal effect. In addition, an ultra-low dose of (+)-efaxoxan (1.3 ng), substantially lower than required for receptor blockade, inhibited the development of acute morphine tolerance, while (-)-efaxoxan was less effective. Racemic (−) efaxoxan effects were similar to those of (+)-efaxoxan. Furthermore, low dose morphine (0.06 ng) produced sustained hyperalgesia in the tail flick test and this was blocked by co-injection of (+) but not (−) efaxoxan (1.3 ng). Given the isomer-specific efaxoxan effects and their different receptor potencies, we suggest that inhibition of opioid tolerance by ultra-low dose efaxoxan involves a specific interaction with spinal α2-adrenoceptors in this model. Likewise, inhibitory effects of adrenoceptor antagonists on morphine tolerance may be due to blockade of opioid-induced hyperalgesia.

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1. Introduction
Repeated systemic or spinal opioid administration produces analgesic tolerance (Christie, 2008), a phenomenon linked to the induction of progressive and latent hyperalgesia with a consequent loss of drug potency (Chu et al., 2006). Indeed, the outcome of analgesic tolerance is a complex phenomenon resulting in the activation of various opiate receptor antagonists (Harrison et al., 1998; Zeng et al., 2006) including the onset of mechanisms leading to opioid-induced hyperalgesia (Chu et al., 2008) and opioid receptor desensitization (Connor et al., 2004).

Ultra-low dose of the opioid antagonist, naltrexone paradoxically inhibits development of opioid tolerance (Shen and Cran, 1997) (ultra-low dose is defined as a dose several log units lower than that required to produce functional antagonism at the respective receptors), a finding many other studies have replicated (Powell et al., 2002; Terner et al., 2006; McNaul et al., 2007). For example, ultra-low doses of competitive opioid receptor antagonists such as naltrexone have been shown to paradoxically augment spinal morphine analgesia and inhibit or reverse the development of opioid tolerance (Powell et al., 2002; Chindalore et al., 2005; Mattioli et al., 2010). Interestingly, the ultra-low dose phenomenon is not restricted to opioid antagonists but is also produced by ultra-low dose adrenergic antagonists. Hence, we have shown that ultra-low dose α2-adrenoceptor antagonists both prevent and reverse established analgesic tolerance to morphine (Milne et al., 2008), a finding subsequently confirmed by Liu et al. (2012). We have shown that ultra-low doses of structurally diverse α2-adrenoceptor antagonists (atipamezole, yohimbine, mitrazapine, and idazoxan) also increase the acute antinociceptive effects of morphine. Morphine blocks the induction of tolerance as well as chronic tolerance, and effectively reverse established tolerance to spinal morphine in tests of
thermal and mechanical noceception (Milne et al., 2008). The basis of these unusual crossover effects of the adrenergic antagonists remains unknown, but may involve action on G-protein coupled receptor heteromeric complexes and/or conformational cross-talk (Jordan et al., 2003; Vilardega et al., 2008).

In the present study, we aimed to determine whether the ultra-low dose effects of an α2 receptor antagonist to block acute morphine tolerance and opioid-induced hyperalgesia are receptor-mediated by using stereoselective isoforms. Efaroxan is a potent and selective α2--adrenoceptor antagonist whose dextro isomer (+) shows greater potency and activity than the levo isomer (−) at this receptor. The compound is a 2-ethylsubstituted lidazoxan analog, possessing a dihydrobenzofuran ring instead of a benzodiazoxan ring. In α2-adrenoceptor binding experiments on the human frontal cortex, IC50 values for efaroxan at the α2-adrenoceptor were reported to be 27 ± 1.9 nM for the (+) and 11,000 ± 580 nM for the (−) enantiomer (Flamme et al., 1997). In rats, bearing 6-hydroxydopamine-induced lesions of the dopaminergic nigrostriatal pathway, stereoselective facilitating effects of the (+) enantiomer of efaroxan were demonstrated on circling behavior (Chopin et al., 1999). Thus, using the racemic (±) efaroxan and its stereoisomers we investigated whether (+) and (−) efaroxan produce stereoselective antagonistic effects on clonidine-induced antinociception in the acute thermal and mechanical noceception tests, (ii) doses of efaroxan stereoisomers substantially below those producing α2-adrenoceptor antagonism block the development of acute spinal morphine tolerance, and (iii) ultra-low doses of efaroxan exhibit a stereoselective action on hyperalgesia induced by a low dose of morphine (Crain and Shen, 2000; McNaul et al., 2007).

2. Materials and methods

2.1. Subjects

All experiments were performed on male Sprague–Dawley rats (250–300 g) obtained from Charles River Laboratories, Montreal QC, Canada following approval by the Queen’s University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. Animals were given ad libitum access to food and water, and were maintained under a 12 h light/dark cycle at room temperature (21–23°C). Animals were acclimatized for 3–4 days before surgery.

2.2. Intrathecal catheterization

Intrathecal catheters were implanted under halothane anesthesia using the method described by Yalcin and Rudy (1976). Briefly, the animal was placed prone in a stereotactic frame, a small incision was made in the atlanto-occipital membrane of the cisterna magna and a polyethylene catheter (PE10; 7.5 cm) inserted through the opening such that the tip reached the lumbar enlargement of the spinal cord. To avoid potential interaction with the test compounds, rats did not receive an analgesic pre-surgery, but did receive lactated Ringer’s solution to prevent dehydration (5 ml, s.c.) and 0.04 ml/100 g Tribrissen 24% s.c. peri-operatively. Following surgery and recovery from the anesthetic, rats were returned to their cage with food and water available ad libitum (soft food was provided to any rat that did not appear to be eating well). Animals were monitored daily to inspect general appearance and any animal that showed signs of distress such as matted hair, spontaneous vocalization upon handling, or showing visible neurological deficits (forelimb or hind limb paralysis) was sacrificed immediately. Nylon fiber chews and a section of polyvinyl chloride (PVC) tubing was provided for environmental enrichment. Minor skin lesions were treated with gentamicin violet solution. All animals were allowed to recover for 4–5 days prior to experimentation. Investigational drugs were injected in a single blind fashion, through the rostral exteriorized portion of the catheter in a 10 µl volume and flushed with 10 µl of normal saline.

2.3. Nociception assessment

Following conditioning to the testing environment, antinociception was assessed using thermal tail flick and mechanical paw pressure tests. The tail flick test (D’Amour and Smith, 1941) measured the response to a brief thermal stimulus applied 5 cm from the base of the tail with the use of an antinociception meter (Owen et al., 1981). Time for tail removal from the thermal stimulus was recorded with baseline latency set at 2–3 s and a cut-off time of 10 s to prevent tissue damage. The paw pressure test measures response to a brief mechanical nociceptive stimulus applied to the dorsal hind paw using an inverted air-filled syringe connected to a pressure gauge (Loomis et al., 1987). Pressure was gradually increased until withdrawal occurred (baseline 70–90 mmHg, cut-off 300 mmHg) (Milne et al., 2008). All animals were habituated to the testing apparatus for at least 3 days prior to experimentation (Milne et al. 2008). Tail flick testing preceded paw pressure testing in each animal and prior experience has shown no significant interaction between responses in these tests (Loomis et al., 1987). In hyperalgesia experiments, only the tail flick test was utilized, using a lower stimulus intensity yielding a baseline latency response of 9–10 s (cut off 24 s), as holding and restraining these animals for paw pressure testing leads to increased variability in withdrawal thresholds such that data are inconsistent in demonstrating a mechanical hyperalgesic effect.

All behavioral testing was performed without knowledge of the treatments, and testing occurred between 0800 and 1400 h during the light cycle. Drug, drug isomer, and vehicle treatments were administered in the same experiment.

2.4. Experiment 1: effects of racemic efaroxan and its stereoisomers on clonidine antinociception

To establish the antagonist effects of efaroxan at α2 receptors, efaroxan (12.6 µg or 1.3 µg) was administered concomitantly with clonidine (13.3 µg) via intrathecal (i.t.) injection through chronically implanted catheters. The effect of stereoselective enantiomers (+) efaroxan (12.6 µg) and (−) efaroxan (12.6 µg) on clonidine-induced antinociception was also determined in both the tail flick and paw pressure tests.

2.5. Experiment 2: effect of ultra-low dose efaroxan and its stereoisomers on acute spinal morphine tolerance

Acute tolerance to spinal morphine was induced by administration of three successive injections of intrathecal morphine (15 µg) delivered at 90 min intervals (McNaul et al., 2007). Thermal and mechanical response thresholds were recorded prior to and following drug injection using the tail flick and paw pressure tests respectively. Latencies to respond were evaluated at 30-min intervals since peak morphine effect in the tail flick and paw pressure tests occurs at this interval following drug injection (Powell et al., 2002; Milne et al., 2008).

To determine the effects of ultra-low dose efaroxan on morphine tolerance racemic (+) efaroxan (1.3 ng or 0.13 ng) or (+) efaroxan (1.3 ng) or (−) efaroxan (1.3 ng) were injected concomitantly with the first, second and third dose of intrathecal morphine in the acute morphine tolerance paradigm described.
above. The ability of efaroxan to attenuate acute morphine tolerance was determined by the ability of the drug to influence both the magnitude of the morphine-elicited response on day 1 of the testing period and on the morphine ED₉₀ values obtained 24 h after repeated injections. Cumulative dose-response curves for the acute action of morphine were obtained 24 h after the repeated injections of morphine or morphine and efaroxan iso- 
somers to derive quantitative estimates of the opioid agonist potency (ED₂₀ values). Dose-response curves were obtained by administering ascending cumulative doses of morphine (2.5, 5, 10 and 20 μg morphine in efaroxan (1.3 or 0.13 ng) or efaroxan plus morphine treatment groups and 12.5, 25, 50 and 100 μg morphine in the repeated morphine-treatment group) at 30 min intervals until a maximal antinociceptive response was obtained. Morphine ED₂₀ values were derived from the dose-response curves obtained in these tests and calculated by linear regression using the Prism Graphpad software (version 4.0). The occurrence of antinociceptive tolerance was indicated by a progressive decrease in the magnitude of the antinociceptive effect produced by successive morphine injections (day 1), and a significant increase in the morphine ED₂₀ value (day 2) reflecting a loss of the agonist potency (Millne et al., 2008).

2.6. Experiment 2: effects of ultra-low doses of efaroxan stereo-isomers and other z₂-adrenoceptor antagonists on morphine hyperalgesia

Morphine hyperalgesia was induced by a single intrathecal injection of low dose morphine (0.05 ng) and analgesia assessed using the tail flick test (McNaul et al., 2007). In subsequent tests, morphine was co-injected with a dose (1.3 ng) of the efaroxan isomers evaluated in preceding experiments on tolerance, or with ultra-low doses of other z₂ receptor antagonists, atipamezole (0.08 ng) or yohimbine (0.02 ng), previously found to modulate acute morphine tolerance (Millne et al., 2008).

2.7. Data analysis

All tail flick and paw pressure values were converted to percentage of maximum possible effect (MPE) (MPE = 100 × ([post-drug response−baseline response]/cutoff response−baseline response))). Data are expressed as mean ± S.E.M. for N = 4–8 per group. ED₂₀ values were determined using nonlinear regression analysis. A 2-way repeated-measures analysis of variance (ANOVA) with time as a with-in-subject factor and treatment as a between-subject factor was used to account for repeated measures design. Time X treatment interaction was included to test for differences in longitudinal response. Where applicable, Tukey's post-hoc tests were conducted.

2.8. Drugs

All drugs were dissolved in 0.9% sterile saline. Morphine sulfate (BDH Pharmaceuticals, Toronto, Canada), atipamezole (Farns, Turku, Finland), yohimbine and clonidine (Sigma Chemical Co., St. Louis, MO, USA). The racemic efaroxan was obtained from Toxris Bioscience and efaroxan isomers were kindly provided by Dr. Marc Marien, Institut de Recherché Pierre Fabre, Castres, France.

3. Results

3.1. Actions of efaroxan and its stereo-isomers on z₂-adrenoceptor mediated analgesia

Intrathecal injection of clonidine (13.3 μg) produced an increase in withdrawal thresholds in both the tail flick and paw
pressure tests, with peak antinociceptive effects observed 30 min post-injection and a slow return towards baseline over the 180 min time course (Fig. 1A,B). The antinociceptive effects of clonidine were attenuated by co-administration of racemic (±) efaroxan (12.6 µg and 1.3 µg) compared to clonidine alone and this effect was greater with the higher dose (Fig. 1A, B). The effects produced by co-injection of stereo-isomers of efaroxan with clonidine demonstrate that (+)efaroxan (12.6 µg), the isomer with high binding affinity for κ-opioid receptors, inhibited clonidine-induced antinociception (Fig. 1C, D) in both the tail flick and paw pressure tests. In contrast, when the (−) isomer of efaroxan was co-injected with clonidine, the resulting antinociceptive effects in the tail flick test were not significantly different compared to clonidine alone (Fig. 1C). However, in the paw pressure test (Fig. 1D), inhibition occurred with the (−) isomer, but to a lesser extent than with the (+) isomer. There was shorter onset of analgesia with clonidine and the (−) efaroxan combination.

3.2. Action of racemic (±) efaroxan on acute morphine tolerance

Intrathecal administration of morphine (15 µg) produced an increase in withdrawal thresholds in both the tail flick and paw pressure tests. Three successive morphine injections administered at 90-min intervals represents a valid protocol for acute opioid antinociceptive tolerance (McNaul et al., 2007) (Fig. 2A). Morphine co-administered with ultra-low dose racemic (±) efaroxan (1.3 ng) produced augmented antinociceptive effects at 60 min and 90 min following morphine injections at 90 and 180 min but there was no difference in peak antinociceptive effects at 30 min following each morphine injection compared to morphine alone (Fig. 2A). In the paw pressure test, morphine combined with ultra-low dose racemic (±) efaroxan produced sustained antinociceptive effects that were not different than peak antinociceptive effects of morphine at 30 min (Fig. 2B). Racemic (±) efaroxan alone (1.3 ng) did not produce significant effects on thermal tail flick latencies or paw withdrawal thresholds (Fig. 2A,B). Twenty-four hours following the acute morphine tolerance paradigm, all animals were exposed to cumulative injections of morphine to establish dose–response curves. Saline controls were not conducted for the current investigation but have been completed numerous times previously with no observed effect (Abul-Husn et al., 2007; Milne et al., 2008, 2011). Previous ED50 values obtained from cumulative dose–response curves in saline-treated rats were established at 5.5 and 5.9 µg for tail flick and paw pressure tests respectively (Milne et al., 2008). Co-treatment of animals with efaroxan (1.3 ng or 0.13 ng) significantly shifted the dose–response curves to the left indicating a reduction of antinociceptive tolerance in both the tail flick and paw pressure tests (Fig. 2C,D). The curve was apparently further left-shifted with 0.13 ng efaroxan.

Fig. 2. Effect of racemic efaroxan on acute morphine tolerance: Tail flick (A) (n=4–8) and paw pressure (B) tests (n=4–8) were performed over a 240 min time course. Efaroxan injections were administered at 0, 90, and 180 min. Cumulative dose–response curves for tail flick (C) (n=4–8) and paw pressure (D) (n=4–8) tests were obtained by administering ascending cumulative doses of morphine at 30 min intervals until a maximal antinociceptive response was obtained. Morphine ED50 values were derived from the dose–response curves using linear regression analysis. **P < 0.001, ***P < 0.001 and ****P < 0.005.
Table 1
Effect of low dose (+) efaroxan and its stereoisomers on the induction of acute tolerance to morphine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tail flick ED50 (µg L.T.) (mean ± S.E.M.)</th>
<th>Paw pressure ED50 (µg L.T.) (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine (15 µg)</td>
<td>28.92 ± 1.39</td>
<td>31.18 ± 1.62</td>
</tr>
<tr>
<td>Morphine (15 µg) + Efaroxan (1.3 ng)</td>
<td>4.42 ± 0.24</td>
<td>3.78 ± 0.27</td>
</tr>
<tr>
<td>Efaroxan (1.3 ng)</td>
<td>4.82 ± 0.42</td>
<td>5.45 ± 0.31</td>
</tr>
<tr>
<td>Morphine (15 µg) + Efaroxan (0.13 ng)</td>
<td>2.55 ± 0.30</td>
<td>4.72 ± 1.30</td>
</tr>
<tr>
<td>Efaroxan (0.13 ng)</td>
<td>3.60 ± 0.19</td>
<td>3.60 ± 0.39</td>
</tr>
</tbody>
</table>

Fig. 2
Morphine (15 µg) | 28.92 ± 1.39 | 31.18 ± 1.62 |
Morphine (15 µg) + Efaroxan (1.3 ng) | 4.42 ± 0.24 | 3.78 ± 0.27 |
Efaroxan (1.3 ng) | 4.82 ± 0.42 | 5.45 ± 0.31 |
Morphine (15 µg) + Efaroxan (0.13 ng) | 2.55 ± 0.30 | 4.72 ± 1.30 |
Efaroxan (0.13 ng) | 3.60 ± 0.19 | 3.60 ± 0.39 |

Morphine ED50 values for the tail flick and paw pressure tests obtained from the cumulative dose-response curves in Figs. 2 (C, D) and 3 (C, D). 24 h following termination of drug treatment. Data are presented as mean ± S.E.M.

* Significant difference from morphine (15 µg) group (P < 0.001).
* Significant difference from morphine (15 µg) + Efaroxan (1.3 ng) group (P < 0.001).
N = 4-8 animals per treatment group.

3.3. Action of ultra-low dose efaroxan stereo-isomers on acute morphine tolerance

To determine if the effects of efaroxan were stereospecific, both active and inactive isomers were co-administered with morphine in the acute morphine tolerance paradigm. In the thermal nociceptive test, co-administration of morphine (15 µg) with the (+) efaroxan isomer (1.3 ng) attenuated the loss of opioid antinociception throughout the 4-h time course, where after 4 h animals were still exhibiting significant antinociception (approximately 75-80% M.P.E., Fig. 3A). In contrast, co-administration with the (-) efaroxan isomer (1.3 ng) had no effect on the loss of opioid-induced antinociception in the acute tolerance paradigm in the thermal test (Fig. 3A). In the paw pressure test, co-administration of morphine with either the (+) efaroxan or the (-) efaroxan isomer significantly attenuated the loss of morphine antinociception throughout the time course. However, the effects of the (+) isomer were significantly different from those produced by the (-) isomer whereby the antinociceptive effect produced by co-injection of morphine with (+) efaroxan were significantly augmented compared to co-treatment with (-) efaroxan (Fig. 3B). Twenty-four h following the acute morphine tolerance paradigm all animals were exposed to cumulative injections of morphine to establish dose-response curves. Co-treatment of animals with (+) efaroxan significantly shifted the dose-response curves to the left indicating the prevention of antinociceptive tolerance (Fig. 3C). Co-treatment of animals with (-) efaroxan also significantly shifted morphine dose-response curves to the left although not as far left as non-opioid treated animals (Fig. 3C). Calculation of the ED50 from the dose-response curves showed that the ED50 of morphine alone was significantly higher than morphine co-administered with (+) efaroxan isomer (1.3 ng) by approximately 50%, but was nearly ten-fold higher than morphine co-administered with (-) efaroxan isomer (1.3 ng) in both the tail flick and paw pressure tests. Table 1 lists the ED50 values from the dose-response curves in both the tail flick and paw pressure tests from Fig. 3.

3.4. Attenuation of low dose morphine hyperalgesia with ultra-low-dose α2 receptor antagonists

To investigate changes in the development of opioid hyperalgesia, an ultra-low dose of morphine (0.05 ng) was administered intrathecally, alone or in combination with a low dose of an α2 receptor antagonist. Opioid hyperalgesia was observed over the first 90 min after morphine injection (0.05 ng) as evidenced by a negative change in P M.P.E. After 120 min antinociceptive effects were produced with maximal effect observed 210 min post injection (Fig. 4A). Morphine co-administered with low dose (+) efaroxan stereo-isomer (1.3 ng) significantly attenuated morphine-induced hyperalgesia, while co-administration of the (-) efaroxan isomer (1.3 ng) had no effect (Fig. 4A). Interestingly, co-administration with an ultra-low dose of other α2-adrenoceptor antagonists (atipamezole (0.08 ng) or yohimbine (0.02 ng)) also inhibited morphine-induced hyperalgesia causing analgesia at approximately 60 min after their administration (Fig. 4B). The maximal effect of morphine-induced antinociception was delayed but the maximal response achieved after 120 min was not influenced.

4. Discussion

The current investigation demonstrates that the α2-adrenoceptor antagonist efaroxan effectively inhibits the antinociceptive effects of clonidine in thermal and mechanical nockiceptive tests. In addition, repeated acute administration of spinal morphine induced antinociceptive tolerance was effectively blocked by an ultra-low dose of the active (+) isomer of efaroxan. The dose that was effective in attenuating the development of antinociceptive tolerance also suppressed thermal hyperalgesia elicited by a low dose of intrathecal morphine.

The present study used a model of acute morphine tolerance and efaroxan stereo-isomers (with different receptor affinities) to investigate whether the actions of an α2-adrenoceptor antagonist in modulating opioid analgesia and tolerance are due to interaction at the α2-adrenoceptor. In the present study, ultra-low doses of the α2 antagonist efaroxan inhibited the development of acute morphine tolerance, an effect reflected in both the maintenance of the opioid-induced response to repeated drug administration and prevention of the loss of agonist potency. Previous evidence implicating α2 receptors in opioid analgesic tolerance was based on the actions of atipamezole, a highly selective α2 antagonist (Milne et al., 2008, Lilius et al., 2012) and reinforced by replication of these major findings with other ligands including yohimbine, idazoxan and mirizazine that are less selective, but have the common ability to block α2 receptors (see Milne et al., 2008). The present study demonstrates that the effects of ultra-low dose efaroxan were stereo-specific in the thermal acute tolerance test, suggesting that the inhibition of tolerance by ultra-low doses of the antagonist indeed involves a specific interaction with spinal α2-adrenoceptors. In this study, efaroxan was chosen because of strong evidence for its stereo-selectivity in both binding studies (Flamez et al., 1997) and in vivo studies not involving nociception (Chopin et al., 1999).
Overall, the data demonstrate that development of both tolerance and hyperalgesia is largely stereospecific, suggesting the effects of efaroxan were produced by inhibition of $\alpha_2$-adrenoceptors. While the stereo-selectivity displayed by pharmacological systems constitutes the best evidence that receptors exist and that they incorporate concrete molecular entities as integral components of their active sites (Lehmann, 1982), the stereo-selective action of isomers is not absolute. This may explain some of the partial effects of (-) efaroxan in its effect on acute morphine tolerance in the paw pressure test. (-) efaroxan is the "less active" isomer as reflected in binding studies (Flamer et al., 1997). The use of ultra-low doses, however, potentially calls into question whether the effects are mediated via activity at adrenoceptors, since much larger doses are required to antagonize an adrenergic agonist. While stereo-selectivity is confirmed, the precise receptor remains elusive in the absence of binding studies to confirm significant binding affinity with $\alpha_2$-adrenoceptors at such low concentrations, as those used in our experiments.

Ultra-low dose racemic efaroxan alone produced delayed analgesia in the paw pressure test at 240 min. We do not have a good explanation for this effect although the delayed antinociception may possibly be due to an interaction of efaroxan with endogenous opiates released during repeated testing. Interestingly, BR44408, a highly selective $\alpha_2$-adrenoceptor antagonist has been recently shown to exhibit analgesia in a model of visceral pain. The authors (Dwyer et al., 2010) suggest that selective $\alpha_2$-adrenoceptor antagonists, either by direct inhibition of $\alpha_2$-adrenoceptors or through a hetero-receptor function of $\alpha_2$-adrenoceptors may be useful in pain therapy. It is conceivable that efaroxan may have similar actions.

The mechanism by which ultra-low dose $\alpha_2$ antagonists inhibit the development of acute morphine tolerance is unknown, although it is well accepted that there are interactions between these G-protein coupled receptors. Thus, agonists of mu opioid and $\alpha_2$ receptors produce a synergistic effect in that the activation with $\alpha_2$ agonists augments opioid-induced antinociception in rodents (Fairbanks et al., 2002; Tajar et al., in press), and such combination has been proven beneficial in clinical practice whereby effective pain treatment was reported with reduced side effects when clonidine was combined with an opioid agonist (Eisbach et al., 1994; Paech et al., 2004). Additionally, morphine-induced antinociception recruits $\alpha_2$ receptors as demonstrated by reduced analgesic potency in $\alpha_2$ null mutant mice (D75N point mutation) compared to wild type animals (Stone et al., 1997). In addition to functional synergistic interactions, mu opioid receptors have been shown to form heteromers with several G protein coupled receptors involved in pain regulation including the $\alpha_2$ receptors (Gupta et al., 2006; Jordan et al., 2003). Such interactions have been reported to occur not only in the spinal cord but...
also at the level of the primary afferent neurons and other CNS sites (Iles and Norenberg, 1990). These receptors, either singly or as a heterodimer, activate common signal transduction pathways mediated through the inhibitory G proteins G(i) and G(o). However, there is evidence that continued opioid exposure in culture (Cain and Shen, 1996) or prolonged administration of opioids in vivo (Cain and Shen, 2000) could paradoxically produce facilitatory effects via activation of stimulatory G proteins G(s)). Similarly, the hyperalgesic effects produced by low dose intrathecal morphine may also involve opioid receptor activation of Gs (McNaul et al., 2007; Esmaili-Mahani et al., 2008). One of the mechanisms that may underlie the effects of the ultra-low dose &zeta; antagonists is to prevent the mu opioid receptor from coupling to stimulatory effector systems that are initiated via activation of Gs. Such an effect could account for the ability of the stereoselective effects of efaroxan to block the induction of thermal hyperalgesia resulting from a low dose of spinal morphine, as well as acute opioid tolerance. Alternatively, conformational cross-talk controlling cell signaling between &zeta; and mu-opioid receptors (Vilardaga et al., 2008) may allow for the ultra-low dose of &zeta; antagonists to augment the interaction of the mu receptor with its ligand. It is also relevant to consider the possibility of efaroxan producing its effects via the imidazoline receptor. Hence, clonidine is a agonist at &zeta; as well as imidazoline receptors (Reis and Pilietz, 1997). However both stereo-isomers of the alkoxy-substituted imidazoline derivative efaroxan displays low affinity for imidazoline receptors (Vaa Quinn et al., 1999), thus making it unlikely that this would be a potential mechanism for the effects produced in the present study.

It is also worth considering the aspect of opioid-induced hyperalgesia and whether such phenomenon occurs in an acute opioid tolerance model. Previous studies have suggested the induction of hyperalgesia as a contributing factor in the development of acute opioid tolerance (see McNaul et al., 2007). Many mechanisms have been proposed to mediate opioid-induced hyperalgesia (Lee et al., 2011; Angst and Clark, 2006) and involve the activation of opponent processes (Bryant et al., 2005). A single dose of morphine (Goldfarb et al., 1978) or heroin (Celerier et al., 2001) can generate naloxone-precipitated hyperalgesia that has been replicated in non-addicted humans following a single injection of morphine (Compton et al., 2003). Similar effects are reported following mechanical tests for anesthesia (Guignard et al., 2000). Under these conditions, hyperalgesia has been associated with increased amplitude of spinal cord reflexes (Goldfarb et al., 1978) and increased activity of nociceptive facilitatory neurons in the medulla (Neubert et al., 2004), each of which effectively results in increased pain behaviors. Hence, the doses of morphine used in the acute tolerance study may recruit opponent processes that initiate a hyperalgesic state, and whether such mechanisms are similar to low-dose morphine-induced hyperalgesia remains unknown. However, ultra-low dose &zeta; antagonists appear to mitigate the genesis of such processes.

It is noteworthy that the less active stereoisomer of efaroxan partially inhibited the development of opioid tolerance in the mechanical nociceptive test and partially shifted the dose response curve for morphine following the acute tolerance paradigm. This is consistent with stereo-selectivity not being absolute as stated previously and may potentially explain the absence of complete stereo-specificity across the tests. There is no evidence to suggest that mechanisms of opioid tolerance differ between nociceptive modalities, however, it is not uncommon that opioid-induced mechanical hyperalgesia is reported more often than warm thermal hyperalgesia in clinical studies of healthy human subjects (Schmidt et al., 2012). Thus, mechanical tests may be more sensitive to detect the presence of opioid-induced hyperalgesia. In the present study, all animals were catheterized for spinal delivery of drugs and such catheterization causes neuroinflammation on its own (Dello et al., 1997) and can facilitate the development of opioid tolerance (Mattioda et al., 2012). Therefore, the catheter-induced neuro-inflammation may have sensitized nociceptive neurons that facilitated opioid-induced hyperalgesia in the acute opioid tolerance model.

5. Conclusion

The present study shows concomitant administration of an unexpectedly low dose of an &zeta; antagonist can inhibit the development of acute opioid analgesic tolerance and low dose morphine-induced thermal hyperalgesia in a stereo-selective manner. This result suggests that the effects are indeed via an interaction between the opioid and adrenergic system rather than an alternative receptor pathway. It is not known if such processes occur in a chronic tolerance model although our previous experiments have demonstrated the ability of similar low doses of diverse, chemically distinct &zeta; antagonists to prevent and reverse established antinociceptive tolerance following chronic morphine administration (Milne et al., 2008). It is also unknown if this interaction is specific to spinal sites and merits further investigation as to whether systemic administration of these ligands will produce similar effects.

Disclosures/conflicts of interest

The research reported in this manuscript is similar in nature to that for which patent applications have been filed by PARTEQ, Queen's University, Kingston, Ontario, Canada presented in part at the Society for Neuroscience Annual meeting, Abstract 678, San Diego, California, USA, November 13–17, 2010.
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References


Appendix B: (Publication)

Analgesia, enhancement of spinal morphine antinociception, and inhibition of tolerance by ultra-low dose of the $\alpha_{2A}$-adrenoceptor selective antagonist BRL44408.

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Neuropharmacology and analgesia

Analgesia, enhancement of spinal morphine antinociception, and inhibition of tolerance by ultra-low dose of the α2A-adrenoceptor selective antagonist BRL44408

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1. Introduction

Spinal administration of morphine and related opioid agonists evokes powerful analgesia; however, repeated exposure to these agents induces tolerance, a phenomenon indicated by reduced potency of the agonist (McNaul et al., 2007). It has long been known that α2-adrenoceptor agonists are antinociceptive and can enhance the analgesic effects of morphine (Eisenach et al., 1996; Maze and Trangulli, 1991; Ospilov et al., 1989). More recently, the structurally diverse non-selective α2-adrenoceptor antagonists atipamezole, yohimbine, mitrazapine, and idazoxan have been shown to augment acute spinal morphine antinociception, block induction of morphine tolerance, and reverse established tolerance when given at ultra-low doses several log units lower than those producing receptor antagonism (Milne et al., 2008). These effects are reminiscent of those produced by ultra-low doses of the competitive opioid receptor antagonists naloxone (Powell et al., 2002) and naloxone (Mattioi et al., 2010). Some of these observations were recently verified when low doses of intrathecal (i.t.) atipamezole were shown to attenuate and shift the bi-phasic effect of morphine in opioid naïve and tolerant rats (Lilus et al., 2012).

When administered alone, ultra-low doses of α2-adrenoceptor antagonists have delayed but sustained weak antinociceptive actions (Milne et al., 2008). Ultra-low doses of atipamezole, influencing spinal morphine actions, similarly augment clonidine- or norpinephrine-induced antinociception, and inhibit acute tolerance to norpinephrine in the spinal model (Milne et al., 2011). These 'pro-opioid' effects of ultra-low dose α2-adrenoceptor antagonists are stereo-selective, since tolerance to repeated acute injections of spinal morphine is inhibited by an ultra-low dose of
the active (+) but not the inactive (−) isomer of the α2-adrenoceptor antagonist efaroxan (Milne et al., 2013). This stereo-selectivity suggests that the observed crossover effects of adrenergic antagonists are specifically mediated via spinal α2-adrenoceptors.

Considerable evidence supports the existence of three distinct α2-adrenoceptor subtypes (2A, 2B, and 2C) present in the dorsal spinal cord (Fairbanks et al., 2008), suggesting their potential role in pain modulation. Studies using transgenic mice and pharmacological analyses support a primary role of the α2A-adrenoceptor in pain mediation, although the α2C subtype may also medulate nociceptive transmission (Gentili et al., 2007). In addition, studies in a mouse line expressing a point mutation in the α2A-adrenoceptor indicate that the α2A subtype is the primary mediator of α2-adrenergic agonist-induced spinal analgesia, and is necessary for analgesic synergy with opioids (Stone et al., 1997). Given the existence of several α2-adrenoceptors, the role they play in pain modulation, and their interaction with morphine in the analgesia model, it is of interest to determine whether any of these receptor subtypes mediate pain modulation in the presence of antagonists in ultra-low doses.

Recent work has shown that BRL 44408, an α2-adrenoceptor antagonist with high affinity for the α2A-adrenoceptor, has anti-nociceptive actions as well as anxiolytic-like qualities in a model of visceral pain, suggesting that antagonism of this receptor subtype may present an effective treatment strategy for mood disorders and pain (Dwyer et al., 2010). Thus, we sought to investigate whether ultra-low doses of this antagonist influence morphine- and norepinephrine-induced acute analgesic efficacy, and whether it modulates analgesic tolerance to norepinephrine and morphine.

In addition, we investigated whether ultra-low dose BRL 44408 alone can produce characteristic delayed analgesic actions, and whether its effects are influenced by the repeated nociceptive testing involved in the acute tolerance model.

2. Material and methods

This study was approved by the Queen's University Animal Care Committee and conducted under Guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250–300 g) housed in normal light-dark cycles were implanted with indwelling i.t. catheters (7.5 cm, PF-10) under isoflurane anesthesia (Yaksh and Rudy, 1976). In each animal, the catheter was inserted through a small slit in the cisterna magna, with the tip terminating at the lumbar enlargement of the spinal cord as in prior experiments (Milne et al., 2011, 2008). Animals had a recovery period of 4–5 days following surgery prior to experimentation. All drugs were injected i.t. via the exteriorized catheter into 10 μl volumes with a 10 μl 0.9% saline flush. BRL 44408 (Tocris Bioscience, Bristol, United Kingdom), clonidine, norepinephrine (Sigma-Aldrich, St. Louis, MO, USA), and atipamezole (Farmos, Turku, Finland) were dissolved in 0.9% saline. Drug combinations were given as one solution after being prepared on the day of experimentation. Analgesia testing was performed between 0800 and 1400 hours with the experimenter being blind to drug treatment. Each animal was used for only one experiment.

In the tail flick test (D’Amour and Smith, 1941), a thermal stimulus from a light source was applied 5 cm from the tail base using an analgesia meter (Owen et al., 1981). The light intensity was adjusted to give baseline latencies of 2–3 s and a cutoff time of 10 s in order to prevent tissue damage. In the paw pressure test, mechanical pressure was applied to the dorsal surface of the hindpaw with an inverted air-filled syringe attached to a pressure gauge (Loomis et al., 1987). A cutoff of 300 mmHg was used in this test, with a baseline of 70–90 mmHg.

2.1. Acute analgesia experiments

To determine the antagonistic effects of BRL 44408 on analgesia induced by clonidine (an α2-adrenoceptor agonist), animals were administered a single injection of clonidine (15.3 μg) or clonidine with a high (antagonist) dose of BRL 44408 (16.5 μg), and tail flick and paw pressure responses were assessed for 180 min post-drug injection.

To investigate the effects of ultra-low dose BRL 44408 on acute morphine- and norepinephrine-induced analgesia, animals were administered a single injection of morphine (15 μg), morphine plus BRL 44408 (1.65 μg), norepinephrine (30 μg), norepinephrine plus BRL 44408 (1.65 μg), or BRL 44408 alone. Tail flick and paw pressure responses were assessed for 180 min post-drug treatment.

2.2. Acute tolerance experiments

To determine the effects of ultra-low dose BRL 44408 on the development of acute analgesic tolerance to morphine or norepinephrine, animals were administered three repeated injections of either morphine (15 μg), morphine plus BRL 44408 (1.65 μg), BRL 44408 alone (1.65 μg), norepinephrine alone (30 μg), norepinephrine plus BRL 44408 (1.65 μg), or saline vehicle every 90 min. Tail flick and paw pressure responses were assessed over 4 h. Twenty-four hours following the three repeated drug injections, cumulative dose response curves were performed to test morphine or norepinephrine potency (ED50 values). Dose response curves were derived using cumulative doses of 2.5–20 μg in the saline group, 12.5–100 μg in the morphine treatment groups, 15–120 μg in the norepinephrine group, and 3.75–45 μg in the norepinephrine plus BRL 44408 group.

2.3. Antagonism and limited testing experiments

To determine the potential of an α2-adrenoceptor antagonist to influence the actions of BRL 44408 alone on antinociception, animals were administered a single injection of BRL 44408 (1.65 μg), and tail flick and paw pressure responses were assessed for 180 min. At 180 min, animals were administered a single injection of the α2-adrenoceptor antagonist atipamezole (10 μg), or saline vehicle, and responses were assessed again 30 min later (at 210 min).

To determine if the repeated nociceptive testing in the tail flick and paw pressure tests confounded behavioral nociceptive outcomes, two groups of animals were compared following an injection of BRL 44408 (1.65 μg) at 0, 90 and 180 min. One group received regular testing every 30 min for 4 h post-injection, while the other group received only limited testing, with responses assessed only at 30 min post-injection and again at 210 and 240 min.

2.4. Statistical analyses

Percentage of maximum possible effect (M.P.E.) [(M.P.E. = 100 × [postdrug response – baseline response])/(cutoff response – baseline response)] was calculated for the results of both nociceptive tests. Nonlinear regression was used to calculate ED50 values, and two-way repeated-measure analysis of variance (ANOVA) using time as a within-subject factor and treatment as a between-subjects factor was utilized to factor into account the repeated measures design. Time × treatment interaction tested for longitudinal response pattern differences, and Tukey’s post-hoc tests
were utilized where appropriate (Milne et al., 2011). All data are reported as mean ± S.E.M., and the α level was set to 0.05.

3. Results

3.1. Effects of BRL44408 on clonidine-induced antinociception

Intrathecally clonidine (13.3 μg) increased noxious thresholds in both the tail flick and paw pressure assays, with peak effects observed 30 min post-injection in both tests (Fig. 1). Clonidine had much greater analgesic effects in the tail flick assay compared to those of the paw pressure assay, reaching approximately 85% MPE and 35% MPE, respectively. In both tests, animals treated with clonidine alone returned to baseline response levels by 180 min. Co-administration of BRL44408 (16.5 μg) blocked the analgesic effects of clonidine and responses were significantly lower over the first 60 min in the tail flick test (P < 0.001) and over the first 90 min in the paw pressure test (P < 0.001 from 20–50 min, P < 0.01 at 60 and 90 min). Two-way ANOVA revealed a significant effect of time (F(6,66) = 51.24, P < 0.001), treatment (F(2,35) = 24.79, P < 0.01), and interaction (F(12,66) = 58.90, P < 0.001) in the tail flick assay. In the paw pressure test, a significant effect of time (F(6,66) = 9.683, P < 0.001), treatment (F(2,35) = 6.450, P < 0.01), and interaction (F(12,66) = 29.03, P < 0.001) was observed.

3.2. Effects of ultra-low dose BRL44408 on acute morphine and norepinephrine antinociception

Intrathecal morphine (15 μg) increased both the thermal latency and mechanical nociceptive threshold, with all animals experiencing nearly 100% MPE in both paradigms with a peak effect at 30 min post-injection (Fig. 2a and b). After 30 min, the antinociception decreased steadily over time, and by 180 min responses returned to pre-injection baselines. Co-administration of ultra-low dose BRL44408 (1.65 μg) with morphine resulted in a slightly delayed antinociceptive effect, with animals reaching peak effect around 50 min post-injection in the tail flick assay and 60 min in the paw pressure test. Despite the delayed peak effects, antinociception was prolonged in the animals co-administered ultra-low dose BRL44408 compared to morphine alone, and responses were significantly higher from 60 min onward in both testing paradigms (P < 0.001). By 180 min, responses had begun to decrease, but were still significantly higher than pre-injection baselines (50–60% MPE), and significantly higher than animals treated with morphine alone. Administration of ultra-low dose BRL44408 alone also produced an increase in nociceptive thresholds, and although the effect was delayed in onset, a significant effect was apparent at 60–80 min post-injection. At 180 min, animals treated with BRL44408 alone had reached nearly 100% MPE in the tail flick test, but only 40% MPE in the paw pressure test. Two-way ANOVA revealed a significant effect of time (F(6,66) = 7.365, P < 0.001), treatment (F(2,35) = 4.981, P < 0.001), and interaction (F(12,66) = 38.42, P < 0.001) for thermal nociceptive testing. In the paw pressure test, a significant effect of time (F(6,66) = 9.683, P < 0.001), treatment (F(2,35) = 6.450, P < 0.001), and interaction (F(12,66) = 29.03, P < 0.001) was observed. Acute ln. norepinephrine (30 μg) increased both thermal latency and mechanical nociceptive threshold (Fig. 2c and d). Peak effects were observed in both cases 30 min post-injection, although norepinephrine was more effective in the tail flick compared to the paw pressure test (approximately 80% MPE vs. 65% MPE, respectively). Animals receiving norepinephrine alone did not produce maximal antinociception in either test. Animals that were co-administered BRL44408 (1.65 μg) with norepinephrine experienced delayed peak analgesia in the tail flick assay, which occurred 60 min post-injection compared to 30 min for norepinephrine alone. The peak effect in the paw pressure test, however, occurred at the same time as it did in animals given norepinephrine alone (30–40 min post-injection). Peak MPE in the tail flick test reached almost 100% in rats co-administered norepinephrine with ultra-low dose BRL44408, and prolonged the antinociceptive effects compared to norepinephrine alone. In the tail flick test, significantly higher %MPE was observed from 50–180 min (all P < 0.05) post-injection in the animals co-administered norepinephrine with ultra-low dose BRL44408 compared to norepinephrine alone. No difference was observed in the paw pressure test in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone, which was significantly lower than the peak effect observed. For norepinephrine (Fig. 2c and d), two-way ANOVA revealed significant effects of time (F(6,66) = 24.49, P < 0.001), treatment (F(2,35) = 22.81, P < 0.001), and interaction (F(12,66) = 10.68, P < 0.001) in the tail flick test, and significant effects of time (F(6,66) = 26.21, P < 0.001) and interaction (F(12,66) = 6.552, P < 0.001) in the paw pressure test.

3.3. Effects of ultra-low dose BRL44408 on the development of acute morphine and norepinephrine tolerance

Repeated injection of morphine (15 μg) produced a significant decrease in thermal latencies indicative of acute tolerance (Fig. 3a). Compared to morphine alone, animals administered morphine with ultra-low dose BRL44408 had a significantly lower antinociceptive effect at 30 min post-injection (P < 0.001), but demonstrated attenuation of the acute opioid antinociceptive tolerance. Co-administration of morphine with ultra-low dose BRL44408
Fig. 2. Effects of ultra low-dose BRL44408 on acute morphine and norpaphinephrine (NE) antinociception. Morphine administration was analgesic in both the tail flick and paw pressure tests (A, B) (n=4). Co-administration of ultra low-dose BRL44408 with morphine (n=5) resulted in delayed analgesia with a peak effect slightly lower than the morphine only group. Analgesia was prolonged in the animals co-administered ultra-low dose BRL44408 compared to morphine alone and responses were significantly higher from 60 min onward (P < 0.001). Administration of ultra-low-dose BRL44408 alone demonstrated delayed and increasing analgesia over the time. At 180 min, animals treated with BRL44408 alone (n=3) had reached nearly 100% MPE in the tail flick but only around 40% MPE in the paw pressure test, both of which were significantly higher than morphine treated animals (P < 0.001 and P < 0.05 respectively). *** indicates significant difference compared to morphine alone. # indicates significant difference compared to ULB98. Two-way ANOVA revealed a significant effect of time (F(9,135)=7.962, **P < 0.001), treatment (F(2,135)=4.981, ***P < 0.001), and interaction (F(18,270)=38.42, ****P < 0.001). In the paw pressure test, a significant effect of time (F(9,135)=6.602, ***P < 0.001), treatment (F(2,135)=4.650, ***P < 0.001), and interaction (F(18,270)=29.63, ****P < 0.001) was observed.

Acute intrathecal norpaphinephrine (30 μg) was analgesic in both the tail and paw pressure tests (C, D) (n=7). Animals co-administered ultra low-dose BRL44408 (1.65 ng, n=5) with norpaphinephrine experienced delayed peak analgesia in the tail flick assay compared to norpaphinephrine alone. Peak effect in the paw pressure test, however, occurred at the same time as norpaphinephrine alone. Peak 50 MPE in the tail flick test reached almost 100% in the rats co-administered morphine with ultra-low dose BRL44408 and animals were still experiencing significant analgesia even 180 min post-injection, whereas animals receiving norpaphinephrine alone were not. In the tail flick test, significantly higher MPE was observed at 60 (P < 0.001), 120 (P < 0.001), 180 (P < 0.001), 240 (P < 0.001), and 300 (P < 0.001) min post-injection in the animals co-administered morphine with ultra-low dose BRL44408 compared to norpaphinephrine alone. No difference was observed in the paw pressure test in animals co-administered ultra-low dose BRL44408 compared to norpaphinephrine alone. For norpaphinephrine alone, a two-way ANOVA revealed significant effects of time (F(9,135)=24.40, ***P < 0.001), treatment (F(2,10)=22.81, ****P < 0.001), and interaction (F(18,270)=10.68, ****P < 0.001) in the tail flick test, and significant effects of time (F(9,135)=26.21, ***P < 0.001) and interaction (F(18,270)=6.552, ****P < 0.001) in the paw pressure test.

Assessment of morphine potency 24 h following the acute tolerance paradigm revealed a rightward shift in the dose response curves in the animals that had been repeatedly treated with morphine compared to those treated with saline. Ultra-low dose BRL44408 prevented the rightward shift in morphine dose response curve in both the tail flick (Fig. 3c) and paw pressure (Fig. 3d) assays. Calculation of morphine ED50 from the dose response curves revealed that animals that had received morphine alone had significantly higher ED50 values in both the tail flick and paw pressure tests compared to animals that had received saline, morphine with ultra-low dose BRL44408, or BRL44408 alone (P < 0.001 for all three), suggesting that ultra-low dose BRL44408 prevented the decline in morphine potency (Fig. 5a).

There was no difference in morphine ED50 values between morphine plus ultra-low dose BRL44408, BRL44408 alone, or saline in either test, and values for all groups were similar in both tests (Fig. 5a).

Acute antinociceptive tolerance to repeated norpaphinephrine injections (30 μg) was observed in both the tail flick and paw pressure tests (Fig. 4a and b). After the first injection of norpaphinephrine, thermal latencies increased to 80% MPE and mechanical thresholds increased to 50-60% MPE, with peak effect observed 30 min post-injection. Co-administration of norpaphinephrine with ultra-low dose BRL44408 (1.65 ng) attenuated the development of
acute tolerance to norepinephrine in both the tail flick and paw pressure tests. Thermal latencies were significantly higher in animals co-administered ultra-low dose BRL44408 compared to norepinephrine alone (60-240 min, all P < 0.05). Similarly, in the paw pressure test, co-administration of ultra-low dose BRL44408 with norepinephrine compared to norepinephrine alone resulted in significantly higher nociceptive thresholds from 120-240 min (all P < 0.001). In the tail flick test, statistical analysis by two-way ANOVA revealed a significant effect of time (F(7,77) = 16.16, P < 0.001), treatment (F(3,231) = 16.98, P < 0.001), and interaction (F(21,77) = 13.73, P < 0.001). In the paw pressure test, a significant effect of time (F(7,77) = 12.30, P < 0.001), treatment (F(3,111) = 39.61, P < 0.001), and interaction (F(21,77) = 20.05, P < 0.001) was observed.

Norepinephrine dose–response curves, assessed 24 h following the acute tolerance paradigm, revealed a leftward shift in the curves of the animals that had received norepinephrine with ultra-low dose BRL44408 (1.65 ng) on day one compared to norepinephrine alone in both the tail flick and paw pressure tests (Fig. 4c and d). Calculating the ED50 from the dose response curves (Fig. 5b) showed that animals treated with norepinephrine and ultra-low dose BRL44408 on day one had ED50 values nearly seven-fold lower than those treated with norepinephrine alone on day one (approximately 5 µg i.t. compared to 35 µg i.t., respectively, P < 0.001) in the tail flick test, and around 50% lower in the paw pressure test (20 µg i.t. compared to 45 µg i.t., respectively, P < 0.001).

3.4. Effects of the α-2-adrenoceptor antagonist atipamezole on ultra-low dose BRL44408 antinociception

BRL44408 antinociception peaked at 180 min post-injection in both the tail flick and paw pressure tests (Fig. 6). At this time point, animals were injected with either saline or atipamezole (10 µg). Following injection of the antagonist, there was a significant decrease in BRL44408 antinociception in both the tail flick and paw pressure tests compared to saline controls (P < 0.001). However, the reversal was only partial in both tests, atipamezole reducing SMPF by approximately 20% in the tail flick test and by approximately 30% in the paw pressure test. The BRL44408 antinociception continued to increase in the control animals that were injected with saline at 180 min. Two-way ANOVA revealed significant effects of time (F(3,231) = 70.10, P < 0.001), treatment (F(3,231) = 3.246, P < 0.05), and interaction (F(21,77) = 7.422, P < 0.001).

In the paw pressure test, a significant effect of time (F(3,231) = 43.51, P < 0.001) and interaction (F(21,77) = 12.49, P < 0.001) was observed.
3.5. Effects of repeated vs. limited testing on ultra-low dose BRL44408 antinociception

There was no significant difference at any time point between the repeated or limited testing groups in either the tail flick (Fig. 7a) or paw pressure (Fig. 7b) test. For both testing paradigms, BRL44408 significantly increased thermal and mechanical nociceptive thresholds throughout the entire testing period and did not show evidence of decline at 240 min. Assessing morphine potency 24 h following injection of BRL44408 for repeated and limited testing (Fig. 7c) revealed that there was no difference in the morphine ED₅₀ values for the regular or limited testing groups that had received ultra-low dose BRL44408 the previous day.

4. Discussion

Previous studies from our laboratory have provided evidence that extremely low doses of several non-selective α₂-adrenoceptor antagonists, well below those producing the adrenoceptor blockade, can augment spinal morphine antinociception and inhibit the development of tolerance (Milne et al., 2008). Such effects of low dose antagonists also extend to the acute antinociception and tolerance produced by the spinal injections of the α₂-adrenoceptor agonists clonidine or norepinephrine (Milne et al., 2011). These actions of the adrenoceptor antagonists on morphine-induced responses thus parallel the previously documented actions of ultra-low dose competitive opioid receptor antagonists, such as naloxone (McNaul et al., 2007; Powell et al., 2002) and naloxone (Mattiodi et al., 2014, 2010). While the mechanisms underlying the crossover effects of the adrenergic antagonists on opioid agonist-induced responses remain unclear, they are not without precedence. Similar effects have been observed in other models, most notably in the peripheral nociception model (Aley and Levine, 1997). The effects observed in the spinal model used in the present study apparently involve a specific interaction of antagonists with the spinal α₂-adrenoceptors, since extremely low doses of the enantiomers of efaroxan, a non-selective α₂-adrenoceptor antagonist, have recently been reported to exhibit a stereo-selective enhancement of morphine antinociception and inhibition of acute tolerance to repeated doses of i.t. morphine (Milne et al., 2013). Since all the antagonists tested in previous experiments are known to be non-selective α₂-adrenoceptor ligands, the nature of the α₂-adrenoceptor subtype mediating their unusual pro-opioid actions at the spinal level remains unclear. The availability of BRL44408, a receptor antagonist that is highly selective for the α₂A-adrenoceptor type (Young et al., 1989), prompted us to determine in the present
study whether this receptor has a role in expression of the low dose effects of antagonists on the antinociception and acute tolerance produced by the spinal administration of morphine or norepinephrine.

The present study established that it. BRL44408 behaves as an $\alpha_2$-adrenergic antagonist since at a higher dose it effectively blocked the antinociceptive actions of clonidine. However, when administered at an ultra-low dose, BRL44408 effectively prolonged morphine antinociception in tests of thermal and mechanical nociception, and inhibited the acute morphine tolerance produced by three successive maximal doses of spinal morphine. The action of BRL44408 on morphine tolerance was reflected in: a) a significant attenuation of the progressive decline of the peak pharmacological response, and b) a marked inhibition of the agonist potency lost, as evidenced by a significant increase in the morphine $\text{ED}_{50}$ values derived from the dose–response curves obtained 24 h post-drug treatment. The results of this study also showed low dose BRL44408 to exert similar effects on the responses produced by L-t. norepinephrine, although in this case its action on antinociception was significant only in the tail flick test. As observed in our previous work (Milne et al., 2013), the paw pressure test demonstrated reduced sensitivity to the spinal actions of adrenergic agonists when compared with the effectiveness of morphine, and this factor might partly contribute to the poor actions of BRL44408 observed in this test. However, this discrepancy notwithstanding the ability of BRL44408 to largely replicate the effects of non-selective adrenoceptor antagonists observed in earlier studies (Milne et al., 2013, 2008) suggests that the $\alpha_2$-adrenoceptor subtype in the spinal cord likely plays a major role in expression of the modulatory actions of such antagonists on opioid antinociception and tolerance.

Consistent with the actions of non-selective antagonists, the ultra-low dose of BRL44408 in the present study showed intrinsic activity, eliciting a slowly developing antinociceptive response that attained peak levels towards the end of the 240 min testing period. However, in contrast with the low level response produced by non-selective antagonists (Milne et al., 2008), BRL44408 produced a stronger response that reached a maximal value in the tail flick test. The basis of this incremental response over the test period remains unclear. We sought to determine if the application of a repeated test stimulus, entailed in derivation of the time-response relationship of the drug effect over a 240 min period, could be a factor in the production of this antinociceptive effect. Such application of a repeated test stimulus could lead to a local release of endogenous factors (for example, opioid or adrenergic transmitters), and the ultra-low dose of BRL44408 might augment their action to yield an antinociceptive response. Thus, the action of BRL44408 was re-examined using only limited

Fig. 5. Morphine (Mor) and norepinephrine (NE) $\text{ED}_{50}$ values following acute tolerance testing. Calculation of morphine $\text{ED}_{50}$ from the dose response curves revealed that animals that had received morphine alone on day one had significantly higher $\text{ED}_{50}$ values in both the tail flick and paw pressure tests compared to animals that had received morphine with ultra-low dose (ULD) BRL44408 or BRL44408 alone on day one ($* * * P < 0.001$). There was no difference in morphine $\text{ED}_{50}$ values between morphine plus ultra-low dose BRL44408 or BRL44408 alone in either test (a). Calculating the $\text{ED}_{50}$ from the dose response curves (b) showed that animals treated with norepinephrine and ultra-low dose BRL44408 on day one had $\text{ED}_{50}$ values nearly seven-fold lower than those treated with norepinephrine alone on day one (approximately 5 μg, i.t. compared to 35 μg, i.t. respectively; $* * * P < 0.001$) in the tail flick test, and around 50% lower in the paw pressure test (20 μg, i.t. compared to 45 μg, i.t. respectively; $* * * P < 0.001$). $\text{ED}_{50}$ values for both groups were significantly lower in the tail flick compared to the paw pressure test.

Partial reversal

Fig. 6. Effects of the $\alpha_2$-adrenergic antagonist atipamezole (Atipam) on ultra-low dose BRL44408 antinociception. BRL44408 (1.65 mg, given at time zero) antinociception increased steadily, and at 180 min, just prior to antagonist (n=7) or saline (arrow, n=4) administration, there was no difference in BRL44408 antinociception in either of the groups. Following injection of atipamezole there was a significant decrease in BRL44408 antinociception in both the tail flick and paw pressure tests ($P < 0.001$ compared to saline). Two-way ANOVA revealed significant effects of time ($F_{3,527}=70.6; P < 0.001$), treatment ($F_{2,32}=3.24; P < 0.05$), and interaction ($F_{2,32}=7.422; P < 0.001$). In the paw pressure test, a significant effect of time ($F_{3,48}=45.1; P < 0.001$) and interaction ($F_{2,32}=12.49; P < 0.001$) was observed.
Fig. 7. Effects of regular vs. limited testing on an ultra-low dose BRL44408 antinociception. Animals received a single injection of BRL 44408 (1.65 mg) at time zero and had antinociception testing every 30 min (regular) for 4 h or limited testing at 30, 210 and 240 min post-injection. There was no significant difference at any time point for either the regular (n = 4) or limited (n = 5) testing group in either the tail flick (A) or paw pressure (B) assay. Assessing morphine potency 24 h later (C), there was no difference in the morphine ED50 values for either the regular or limited testing groups that had received ultra-low dose BRL44408 the previous day, either in the tail flick or paw pressure test.

nociceptive testing. Interestingly, the timing of the stimulus completely failed to influence the action of BRL44408, suggesting that repeated stimulation was not a contributory factor in the intrinsic activity of BRL44408. Also, neither mode of stimulation influenced the potency of morphine determined 24 h after the low dose BRL44408 treatment. This suggests that the specific mode of stimulation is an unlikely factor in the intrinsic actions of BRL44408. We also considered that such actions of BRL44408 may originate from its interaction with alpha2-adrenoreceptors, either via a direct action on the receptor or via an indirect release of norepinephrine from terminals of the noradrenergic neurons (Umetsu et al., 1997). This possibility was suggested by the observation that the antinociceptive action of BRL44408 alone, like those of the adrenergic agonists (clonidine and norepinephrine), was weaker in the paw pressure than in the tail flick test. Indeed, administration of the non-selective antagonist atenolol, at a dose shown to produce adrenergic receptor blockade in analgesia tests (Milne et al., 2008), partially reversed the delayed antinociceptive action of BRL44408 in both tests. However, the reversal of BRL44408 action was incomplete, suggesting that some other factors also contribute to its origin. Thus, analysis of its action merits exploration in future tests. Although BRL44408 can elicit analgesic-like activity in the visceral nociception model (Dwyer et al., 2010), its action has been attributed to blockade of the alpha2-adrenoreceptor. In the current study the ultra-low dose BRL44408 did not produce such blockade, as evident by its inability to antagonize the norepinephrine-induced responses.

An interesting question raised by the intrinsic actions of BRL44408 is its additivity or synergy with morphine is the basis of its pro-opioid actions. While this possibility cannot be excluded in the acute antinociception experiments, it is unlikely to explain BRL44408 effects on the development of tolerance, especially its ability to influence the loss of agonist potency, a hallmark indicator of this phenomenon. As indicated, low dose BRL44408 produced a strong inhibition of the loss of morphine potency, the estimates of which were made 24 h after the drug treatment, a time point at which BRL44408 is likely to be absent from the spinal space. Thus, it would appear that BRL44408 likely induces a change in the processes that contribute to the genesis of tolerance in response to repeated opioid agonist exposure.

While it is known that the alpha2-adrenoreceptor subtype is the primary mediator of delta spinal analgesia, and is necessary for analgesia synergy with opioids (Stone et al., 1997), the mechanism of action of the alpha2-adrenoreceptor antagonist BRL44408 on morphine antinociception and tolerance is unknown. Considering the co-localization of alpha2-adrenoreceptors and opioid receptors in the spinal cord (Jordan et al., 2003), it is likely that conformational cross-talk between alpha2-adrenoregic and mu opioid receptors controlling cell signaling with the G protein-coupled receptor heterodimers may facilitate these changes (Vilardaga et al., 2008). Other possibilities include the ability of these alpha2-adrenoreceptor antagonists to counteract the stimulatory hyperalgesic-like responses of opioid agonists (Crain and Shen, 2000, 1995; Milne et al., 2013), or to influence glial activation since ultra-low doses of the opioid antagonist naltrexone affect activation of glia by morphine (Mattioli et al., 2010). Similarly, prolonged spinal delivery of the alpha2-adrenoreceptor agonists such as clonidine, resulting in loss of the antinociceptive response, produces a hyperalgesic thermal hyper-sensitivity (Quartilha et al., 2004) and thus BRL44408 may be acting to influence this response.

5. Conclusion

In summary, ultra-low doses of BRL44408 were found to significantly augment acute morphine and norepinephrine analgesia, and to inhibit the loss of drug potency from repeated exposure, implicating involvement of the alpha2-adrenoreceptor in the action of low-dose alpha2-adrenoreceptor antagonists on morphine and norepinephrine antinociception and tolerance. BRL44408 produces significant analgesia that is unrelated to testing frequency and is only partially dependent on its interaction with alpha2-adrenoreceptors,
suggesting that this agent class may be potentially useful in the treatment of pain.

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References


Appendix C: (Publication)

Changes in morphine reward in a model of neuropathic pain

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Changes in morphine reward in a model of neuropathic pain
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In addition to sensory disturbances, neuropathic pain is associated with an ongoing and persistent negative affective state. This condition may be reflected as altered sensitivity to rewarding stimuli. We examined this hypothesis by testing whether the rewarding properties of morphine are altered in a rat model of neuropathic pain. Neurogenic pain was induced by chronic constriction of the common sciatic nerve. Drug reward was assessed using an unbiased, three-compartment conditioned place preference (CPP) paradigm. The rats underwent two habituation sessions beginning 6 days after surgery. Over the next 8 days, they were injected with drug or vehicle and were confined to one CPP compartment for 30 min. On the following test day, the rats had access to all three compartments for 30 min. Consistent with the literature, systemic administration of morphine dose-dependently increased the CPP in pain-naive animals. In rats with neuropathic pain, however, the dose-dependent effects of morphine were in a bell-shaped curve, with a low dose of morphine (2 mg/kg) producing a greater CPP than a higher dose of morphine (8 mg/kg). In a separate group of animals, acute administration of morphine reversed mechanical allodynia in animals with neuropathic pain at the same doses that produced a CPP. The increased potency of systemic morphine to produce a CPP in animals with neuropathic pain suggests that the motivation for opioid-induced reward is different in the two states. Behavioral Pharmacology 24:207–213 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: chronic pain, conditioned place preference, neuropathic pain, opioid, reward

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Introduction
Neuropathic pain, caused by various central and peripheral nerve disorders, results in chronic and intractable pain. Treatment of this condition is particularly challenging as it is typically refractory to both conventional (opioids) and nonconventional (antidepressants and antiepileptics) analgesics; the use of these medications is limited, further, by intolerable side-effects (Gifford et al., 2006; Dworkin et al., 2010). In addition to the marked sensory disturbances that result in pain hypersensitivity (allodynia and hyperalgesia), neuropathic pain is associated with tonic ongoing pain and affective and emotional states. The negative affect, or how much the pain is ‘bothersome’, significantly impacts the quality of life of the sufferer and leads to the common comorbidities of psychiatric disorders such as depression. Comorbidity between chronic pain and axis I disorders of the DSM-IV has been well documented, where depression is the most common comorbidity, with some studies finding a prevalence rate approaching 100% among clinical chronic pain samples (reviewed by Nicholson and Verma, 2004). In fact, chronic pain is second only to bipolar disease as the major cause of suicide among all medical illnesses, further highlighting the importance of negative affect in this condition (Juurink et al., 2004).

Although it can be argued that treating the affective aspect of pain has such clinical relevance as alleviating the sensory aspects of pain, much less is known about pain affect compared with the vast knowledge of mechanisms underlying sensory disturbances in pain transmission. Drug reward is frequently assessed in animals using the conditioned place preference (CPP) paradigm, which measures time spent in an environment previously associated with motivationally salient stimuli (e.g. drugs or food). In recent years, this test has also been used to measure the tonic aversive component of ongoing pain (King et al., 2009). Using this paradigm, spinal administration of a local anesthetic or the \(\alpha\)2 adrenergic agonist clonidine, a drug used clinically to alleviate pain, elicited a CPP in neuropathic pain, but not sham animals (King et al., 2009). This suggests that neural circuitry engaged by analgesic drugs is distinct in neuropathic and pain-naive states. In contrast, opioids, including morphine, a strong analgesic and drug of abuse that activates hedonic reward circuits, are reported to be less rewarding in animals with neuropathic pain (Ozaki et al., 2002, 2003; Oc et al., 2004; Martin et al., 2007; Petsakchos et al., 2007; Niikura et al., 2008). In addition, neuropathic pain suppresses the opioid-induced potentiation of electrical self-stimulation in the ventral tegmental area (Ewan and Martin, 2011). One interpretation of these findings is that the blunted analgesic effect of opioids in chronic pain is because of alterations in neural systems responsible for reward as their analgesic...
effects are strongly linked to their rewarding capacity (Franklin, 1989).

Although our understanding of the neural circuitry in reward states has advanced significantly (Wise, 2008; Volkow et al., 2012), how motivation is modulated by pain remains relatively unexplored. Such studies would be informative not only in understanding the neural mechanisms of affective states in chronic pain but also in helping to understand why analgesics have limited efficacy in treating neuropathic pain. In this study, we used a CPP paradigm in neuropathic pain and surgery control animals to determine the effectiveness of systemic morphine in eliciting a reinforcing effect. This involved determining the dose-dependent effects of morphine in neuropathic pain states.

Methods

Subjects

Adult male Long–Evans rats (220–250 g at the beginning of experimentation; Charles River, St Constant, Quebec, Canada) were maintained on a reverse 12-h light/dark cycle and allowed free access to food and water. Experiments were conducted during the dark phase and in accordance with protocols approved by the Queen’s University Animal Care Committee and according to the guidelines set forth by the Canadian Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues. After arrival within the housing facility, animals were allowed to acclimatize for 3–4 days before any handling. The experimenter performed all handling procedures including cage changing, food and water replacement, surgery, and drug injections.

Surgery

Rats were assigned randomly to either a surgery to induce neuropathic pain, sham, or naive (no surgery) groups. Rats undergoing sham or peripheral nerve injury received an analgesic preoperatively (acetaminophen 32 mg/ml, 0.25 ml/100 g orally) and were anesthetized with gaseous isoflurane (induction at 3% and maintenance at 2.0–2.5% in oxygen). Rats received 5 ml lactated ringers saline and 0.04 ml/100 g tribriissen 2% subcutaneously perioperatively. The lateral left thigh was shaved, and surgery was performed under aseptic conditions. A skin incision was made, followed by blunt dissection to expose the sciatic nerve. Peripheral nerve injury was performed as described previously (Bennett and Xie, 1988). Briefly, four ligatures (4.0 chronic gut) were tied loosely around the nerve so that the total length of the nerve affected was 3–5 mm. Care was taken to ensure that the nerve was not pinched by the ligatures and that these were not too tight to prevent the occlusion of perineural blood flow. The separated muscle was stitched and the incision was closed with 3-0 mononly. The sham animals received similar surgery, but without manipulation of the nerve. After surgery and recovery from anesthesia, rats were returned to their cage with food and water freely available (soft food or Jello was provided to any rat that did not appear to be eating well). Rats also received a Jello cube containing 50 mg of acetaminophen the evening of surgery and on day 1 postoperatively. Because of the nature of the study, no opioid analgesic was administered postoperatively as these may affect the outcome of the results.

Conditioned place preference apparatus

CPP experiments were conducted in a three-compartment apparatus consisting of two large compartments of equal size (45 × 45 × 30 cm) joined by a gray tunnel (18 × 18 × 30 cm). The two large compartments were distinguished by visual cues (white and white stripes on unpainted wood). Different floor textures (e.g., grid versus sawdust) could evoke more pain in animals with neuropathic pain; therefore, tactile cues in the large compartments were distinguished by different patterns of wire-grid flooring. Beam breaks were recorded when animals entered and left each compartment. This information was sent to a PC computer with software written in-house.

Conditioned place preference paradigm

The CPP protocol included habituation, conditioning, and testing phases. The first was initiated 6 days after surgery (or not, in naive animals): on 2 consecutive days, rats were placed in the tunnel and allowed to explore the entire apparatus for 30 min. None of the rats showed a significant preference for one of the large compartments, indicating that the boxes were unbiased (Table 1). During the subsequent eight daily conditioning sessions, rats in each group (neuropathic pain, sham, and naive) were injected with either drug or vehicle and confined to one of the large compartments for 30 min. The order of injection (drug vs. vehicle) as well as the drug-paired compartment were counterbalanced within groups. Drug-free testing occurred on the day immediately after conditioning when animals were placed in the tunnel and allowed free access to the entire compartment. The amount of time spent in each compartment was assessed over a 30-min session.

Pain-naive (no surgery), sham, or animals with neuropathic pain were assigned randomly to one of the four doses of morphine (1, 2, 4, and 8 mg/kg, subcutaneously), administered during conditioning sessions. A dose–response curve was generated by conducting multiple experiments, where at least one animal from each group [surgery or not (3 × morphine dose × 4)] was included. This helped to minimize the impact of extraneous variables such as time of the year, shipping incident, etc. Animals were used only once and were not retested with a different dose of morphine.

Mechanical withdrawal thresholds

Mechanical withdrawal responses to von Frey filament stimulation were assessed in rats with peripheral nerve
injury as described previously by Chaplan et al. (1994). Rats were placed under opaque Plexiglas boxes positioned on a wire-grid platform (5 × 5 mm mesh), through which von Frey filaments were applied to the plantar surface of the hindpaw in an up-down manner. In brief, filaments were applied in either ascending or descending force as necessary to most accurately determine the threshold of response. The intensity of stimuli ranged from 0.25 to 15 g. From the resulting response patterns, the 50% response thresholds (g) were calculated. Paw withdrawal thresholds were assessed before and on days 4, 7, and 10 after nerve injury in addition to testing 30, 60, and 90 min following systemic morphine injection on day 10 after nerve injury.

**Statistical analysis**

Data are expressed as the mean (±SEM) amount of time (s) spent in the drug-paired and vehicle-paired compartments on the test day for place-preference experiments. A paired student t-test was used to compare the amount of time spent in the drug-paired versus the vehicle-paired compartment for each group. In addition, a two-way analysis of variance (ANOVA), with drug dose and group as between-subjects factors, was used to examine differences in the magnitude of preference for the morphine-paired compartment across neuropathic pain, sham, and naive groups. A two-way ANOVA was also used to determine the effects of morphine (1, 2, and 4 mg/kg subcutaneously) on mechanical withdrawal thresholds in animals with neuropathic pain.

**Results**

The conditioning apparatus was deemed unbiased as there was no significant difference between the times spent in each of the large compartments during the habituation and preconditioning phase (Table 1). Preference for the drug-paired compartment after conditioning to systemic morphine administration is presented in Fig. 1. In sham animals (Fig. 1a), the only dose that produced a significant place preference was the highest dose of morphine tested (i.e., 8 mg/kg) (t = 5.66, P < 0.001; all other P values > 0.05). In contrast, morphine produced a CPP in animals with neuropathic pain (Fig. 1b) at all four doses tested: 1 mg/kg (t = 4.56, P < 0.0035), 2 mg/kg (t = 4.54, P < 0.0035), 4 mg/kg (t = 4.55, P < 0.002), and 8 mg/kg (t = 3.12, P < 0.02). Data from Fig. 1 were transformed into dose-response curves by plotting the time spent in the drug-paired compartment for each dose of morphine in sham and neuropathic pain animals. A naive (nonsurgical) group was also included in this data set to determine any effect of sham surgery on the place preference. Figure 2 shows the dose-dependent effect of morphine in all three groups. A two-way ANOVA showed a significant effect of group [F(2,114) = 9.49, P < 0.001] and dose [F(3,114) = 2.73, P < 0.05]. A significant interaction was also present [F(6,114) = 3.29, P < 0.01].

In a separate group of animals, the ability of systemic administration of morphine to attenuate pain hypersensitivity associated with peripheral nerve injury was evaluated. Mechanical withdrawal thresholds were significantly lower 7 and 10 days after nerve injury as determined by a two-way ANOVA [time: F(2,20) = 51.36, P < 0.001, Fig. 3a], but there was no significant difference between groups [treatment assignment: F(3,20) = 0.74]. Acute administration of all three doses of morphine tested attenuated mechanical allodynia in neuropathic animals, as assessed by stimulation with von Frey filaments [treatment assignment: F(3,20) = 17.91, P < 0.001, Fig. 3b]. Post-hoc analysis showed that the antiallodynic effects produced by the two lowest doses of morphine were only significant at the 30-min timepoint, whereas 4 mg/kg morphine attenuated mechanical pain hypersensitivity at 60 and 90 min after the morphine injection.

**Discussion**

The principal findings of this study are that morphine can elicit a rewarding effect in neuropathic pain, but the dose dependency of this effect was bell shaped, where low doses that did not produce a CPP in naive or sham animals produced a CPP in chronic pain animals. In addition, preference for the drug-paired compartment was greater with lower, rather than higher, doses of morphine in animals with neuropathic pain. These effects correlate with the ability of morphine to alleviate
Fig. 1

Dose-dependent effects of morphine (1, 2, 4, and 8 mg/kg, subcutaneously)-induced conditioned place preference in (a) sham and (b) neuropathic (NP) animals. Data are expressed as a scatter plot with mean and SEM of the time spent in the morphine-paired and vehicle-paired compartment. Statistical analysis showed a significant effect of only the highest dose of morphine (8 mg/kg) in sham animals (**P<0.001). However, statistical analysis showed that all doses of morphine tested were significantly different compared with the time spent in the vehicle-paired compartment in the neuropathic animals (1 mg/kg, **P<0.005; 2 mg/kg, ***P<0.0005; 4 mg/kg, **P<0.002; 8 mg/kg, *P<0.02). n=8-15/group.

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mechanical allodynia, as evidenced by an increase in mechanical withdrawal thresholds.

The primary finding that low doses of opioids produced a CPP in neuropathic pain, but not in pain-naive, animals was unexpected. This finding is not consistent with the reports showing a reduced place preference to opioid analgesics compared with sham animals. Specifically, a CPP to morphine (4 and 8 mg/kg, subcutaneously) was attenuated in neuropathic pain, compared with sham, rats (Ozaki et al., 2002). The methodological differences between the present study and that of Ozaki and colleagues are that their study was on a normal light-dark cycle, used Sprague-Dawley rats, nerve injury was a spared nerve injury rather than chronic constriction, conditioning was for 1 h rather than 30 min, conditioning occurred for 6 days rather than 8 days, and commenced 4 days after surgery. They did not report the time spent in the non-drug-paired compartment, only the difference between morphine and saline compartments in sham and neuropathic pain animals. In addition, the same research group reported that the time spent in the drug-paired compartment was significantly reduced after sciatic nerve ligation when mice were conditioned to tramadol or its active metabolite (Nakamura et al., 2008). A reduction in morphine CPP was also reported in mice with peripheral nerve injury (Petraschka et al., 2007). However, this latter study exposed animals to morphine daily (conditioning twice a day) and implicated desensitization of opioid receptors because of the release of endogenous opioids in

The acute antiallodynic effects of morphine in peripheral nerve-injured animals were evaluated using the von Frey test. Mechanical withdrawal thresholds were assessed 30, 60, and 90 min after morphine administration (1, 2, and 4 mg/kg, subcutaneously) in separate groups of animals (b). Data represent mean ± SEM for n = 4–6/group. Statistical analyses using a two-way analysis of variance showed a significant effect of time and treatment (**P < 0.01, ***P < 0.001).
This latter study used doses similar to the present study, where low doses between 1 and 2 mg/kg produced a CPP in chronic pain, but not in sham or naïve groups. It remains unknown why our results differ from those of other previously published studies. However, a few methodological differences are worth reflecting on. First, a potential explanation for the reduced CPP seen in our experiments is that the occurrence of opioid receptor desensitization (Ozaki et al., 2002, 2003; Oe et al., 2004) or that the protocols used for conditioning to morphine exposed animals to opioids daily rather than every other day (Petrash et al., 2007). In addition, endogenous opioid release in the brain of animals with neuropathic pain may facilitate exogenous opioid-induced desensitization of μ-opioid receptors. In support of this hypothesis, there is a reduction in opioid-induced GTP γS activation in the ventral segmental area (Ozaki et al., 2002) and an increase in μ-opioid receptor phosphorylation in the striatum of animals with neuropathic pain (Petrash et al., 2007). Moreover, it has been proposed that the release of endogenous opioids partially mediates this decreased effect, as animals with neuropathic pain lacking the opioid peptide β endorphin show similar reinforcing effects to morphine as pain-naive animals (Petrash et al., 2007; Niikura et al., 2008). Second, another possible explanation for the discrepancies between the present study and the previous literature may involve circadian rhythms, as our studies were carried out in the active (dark) phase, whereas previous studies were carried out on rodents in the light phase. Pain because of several pathological conditions shows temporal variations in intensity throughout the circadian cycle. This diurnal variation is multifactorial and may be affected by endogenous fluctuations in neuroendocrine or other factors, as well as external influences, which affect touch-induced pain levels and physical activity. Clinical impressions suggest that neuropathic pain is often worse at night (Belgrade, 1999; Oderich et al., 2006). Indeed, a clinical study identified that neuropathic pain intensity increases progressively throughout the day and this temporal profile appears to be unaffected by treatment with gabapentin and/or morphine (Oderich et al., 2006). To our knowledge, no study has directly compared pain hypersensitivities associated with neuropathic pain in the light and dark phases; however, it is well established that opioid-induced analgesia is shifted to the left in the active (dark) phase (Bornschein et al., 1977). Nevertheless, these results suggest that the motivation for opioid-induced reward is different in animals with neuropathic pain compared with pain-naive groups, and this warrants further research on how chronic pain alters reward circuitry.

Conclusion
It remains unknown why low doses of opioids produce a place preference in animals with neuropathic pain; however, two possibilities prevail. The presence of morphine-induced CPP could be because of the association of a positive affective state of the drug with contextual cues in the drug-paired compartment. Positive affect is equated with hedonic pleasure but it seems unlikely that lower doses of morphine would induce mechanisms that enhance this state. Rather, we propose that μ-opioid activation induces a CPP because it alleviates pain associated with neuropathy. In this conceptualization, the rewarding effect of the conditioned stimuli is pain relief that most likely encompasses the negative affective (subjective), cognitive, and sensory discriminative components of the pain experience.

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Conflicts of interest
There are no conflicts of interest.

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