

Ultra-Low Dose Antagonist Effects on Cannabinoids and Opioids in Models of Pain:

Is Less More?

By

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A thesis submitted to the Department of Psychology in conformity

with the requirements for the degree of

Doctor of Philosophy

Queen's University

Kingston Ontario, Canada

November 2007

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Abstract

An ultra-low dose of a drug is approximately 1000-fold lower than the dose range traditionally used to induce a therapeutic effect. The purpose of the present thesis was to broaden the knowledge of the ultra-low dose effect, that was previously identified in the opioid receptor system, by looking at whether opioids and cannabinoids interact at the ultra-low dose level, whether cannabinoid receptors themselves demonstrate the ultra-low dose antagonist effect, and whether the opioid ultra-low dose effect is maintained in a model of persistent, unavoidable pain. For experiment 1, separate groups of Long Evans rats were tested for antinociception following an injection of vehicle, the cannabinoid agonist WIN 55 212-2 (WIN), the opioid antagonist naltrexone (an ultra-low or a high dose), or a combination of WIN and naltrexone doses. Ultra-low dose naltrexone elevated WIN-induced tail flick thresholds without extending its duration of action. In experiment 2, antinociception was tested in rats following either acute or sub-chronic (7 days) injections of vehicle, WIN, ultra-low doses of the CB1 receptor antagonist rimonabant (SR 141716), or a combination of WIN and ultra-low dose rimonabant. Following the chronic experiment, striatal tissue was rapidly extracted and subjected to co-immunoprecipitation to analyse CB1 receptor coupling to G-protein subtypes. Ultra-low dose rimonabant extended the duration of WIN-induced antinociception, and attenuated the development of WIN-induced tolerance. Animals chronically treated with WIN alone had CB1 receptors predominately coupling to Gs proteins, whereas all other groups had CB1 receptors predominately coupling to Gi proteins. For experiment 3, all animals were subjected to the formalin test following either acute or sub-chronic injections of vehicle, the opiate morphine, ultra-low doses naltrexone, or a combination

of morphine and ultra-low dose naltrexone. Ultra-low dose naltrexone had no significant effect on morphine-induced pain ratings in either the acute, or sub-chronic drug treatments. This thesis provides evidence that the ultra-low dose effect, including the agonist-induced G-protein coupling switch, extends to another receptor type. This effect may, therefore, be part of a generalized principle that applies to many G-protein coupled receptors.

Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices. Mary C. Olmstead, my supervisor, contributed to all aspect of this thesis from brainstorming research ideas to assisting with the manuscripts, and she is therefore a co-author on these chapters. In experiment 2 (chapter 3), Dr. Hoau-Yan Wang and his graduate student Kalindi Bakshi from the City University of New York were responsible for running the co-immunoprecipitation experiments and writing the methodology associated with these tests, and thus they are co-authors on this chapter. In experiment 3 (chapter 4), Mallory Griffith assisted with running the experiments. Ms. Griffith was not, however, involved with formulating hypotheses, planning the experimental design, or production of the manuscript, and for these reasons is not a co-author of this chapter.

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November 2007

Acknowledgements

There are many people that have been invaluable to me over the course of completing this thesis. Their assistance, patients, and support are the foundation of my persistence.

My supervisor, Dr. Mary C. Olmstead (Cella), is without question the single most important person involved with this thesis. Cella encouraged me to follow my interests, and motivated me during times when I thought my studies were going to be fruitless. She has an admirable ability of returning my manuscript drafts covered in corrections in a matter of hours – not days (including this thesis!). I have watched Cella successfully manage a busy laboratory, a busy teaching schedule, and a busy family, and because of this she has given me hope that there is life outside the laboratory. I couldn't have asked for a better supervisor. Thank you for sharing your wisdom.

My thesis committee members Dr. Richard J. Beninger and Dr. Hans C. Dringenberg provided excellent feedback on my writing and experimental design throughout the course of my thesis. I highly value their input and I feel privileged to have worked so closely with them.

Dr. Hoau-Yan Wang and Kalindi Bakshi were collaborators for the second experiment of this thesis. I am most proud of this experiment. Thank you for your expertise.

The Department of Psychology at Queen's University has the best support staff, but I would like to specifically thank the departmental technical supervisor, Steve Ferguson, the general technician, Rick Eves, and the departmental animal health

technician, Lisa Miller. Without the three of them, I wouldn't have any experimental apparatuses or subjects. Thank you for ensuring that our laboratory operates efficiently.

Behind every successful graduate thesis is the help from many undergraduates who enthusiastically choose to work and volunteer in the laboratory. Thank you Aydin Tavakoli, Jessica Nee, Katharine Tuerke, and Mallory Griffith for your assistance on many aspects of this thesis.

Over the past 7 years, I have been fortunate to have become friends with, and learned from, the many graduate students that comprise the Olmstead Lab. Thank you Kim Hellemans and Tracie Paine for showing me what it takes to successfully complete a Ph. D. degree. I would also like to thank Stephanie Hancock, Joanna Pohl, Scott Hayton, and Jessie Lubeski for their friendship and support in the laboratory.

When things are not going well, I rely on my family for support. Thank you Mom and Dad for just being there and having confidence in me. Additionally, thank you Joseph L. Paquette. Without his forethought I probably would not have enrolled in university or graduate school. I would especially like to thank Jolene. I don't know why she tolerates me, but she does and I love her for that. Also, thank you Conrad for being the best dog ever, and for the facials☺!

Finally, I would like to acknowledge the person who instilled in me a fascination for science. When I was much younger he would spend hours talking about the wonders of science, from what it is like in outer space to what is happening inside my body. It was because of these talks that I became interested in science. Thus, I dedicate this thesis to my first mentor, and big brother, Dr. Scott J. Paquette.

Table of Contents

ABSTRACT.....	ii
STATEMENT OF ORIGINALITY.....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vii
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
LIST OF ABBREVIATIONS.....	xiv
CHAPTER 1. GENERAL INTRODUCTION.....	1
1.1. Meeting Mary Jane (Marijuana).....	1
1.2. Cannabinoid Physiology.....	2
1.3. Cannabinoids and Pain.....	4
1.4. Opioids and the Ultra Low Dose Story.....	7
1.5. Cannabinoid-Opioid Interactions.....	9
1.6. Biphasic Effects of Cannabinoids.....	10
1.7. Tonic Pain and Opioid Ultra-Low Dose Combinations.....	12
CHAPTER 2. EXPERIMENT 1 – ULTRA-LOW DOSE NALTREXONE ENHANCES	
CANNABINOID-INDUCED ANTINOCICEPTION	13
2.0. Abstract.....	14
2.1. Introduction.....	15
2.2. Method.....	17
2.2.1. <i>Subjects</i>	17

2.2.2. <i>Apparatus</i>	18
2.2.3. <i>Procedure</i>	18
2.2.4. <i>Drugs and Administration</i>	19
2.2.4.1. <i>Intraperitoneal injections</i>	19
2.2.4.2. <i>Intravenous injections</i>	19
2.2.5. <i>Statistical Analysis</i>	21
2.3. <i>Results</i>	21
2.3.1. <i>Intraperitoneal Injections of WIN With Ultra-Low Dose Naltrexone</i>	21
2.3.2. <i>Intravenous Injections of WIN With Ultra-Low Dose Naltrexone</i>	23
2.4. <i>Discussion</i>	27
 CHAPTER 3. EXPERIMENT 2 – CANNABINOID-INDUCED TOLERANCE IS	
ASSOCIATED WITH A CB1 RECEPTOR G-PROTEIN COUPLING	
SWITCH THAT IS PREVENTED BY ULTRA-LOW DOSE RIMONABANT.....	
3.0. <i>Abstract</i>	32
3.1. <i>Introduction</i>	33
3.2. <i>Experimental Procedures</i>	35
3.2.1. <i>Subjects</i>	35
3.2.2. <i>Drugs and Administration</i>	36
3.2.2.1. <i>Single injection testing</i>	36
3.2.2.2. <i>Repeated injection testing</i>	37
3.2.3. <i>Apparatus</i>	37
3.2.4. <i>Nociceptive Testing</i>	37
3.2.5. <i>Tissue Sampling</i>	38

3.2.5.1. <i>Western blot analysis</i>	41
3.2.6. <i>Statistics</i>	43
3.3. Results	44
3.3.1. <i>Tail-flick Latencies Following an Acute Injection</i>	44
3.3.2. <i>Tail-flick Latencies Following Repeated Injections</i>	47
3.3.3. <i>Co-immunoprecipitation of CB1R-G Protein Complexes</i>	49
3.4. Discussion	50
 CHAPTER 4. EXPERIMENT 3 – ULTRA-LOW DOSE NALTREXONE DOES NOT	
 ENHANCE MORPHINE-INDUCED ANALGESIA IN THE FORMALIN	
 TEST OF PAIN	58
4.0. Abstract	59
4.1. Introduction	60
4.2. Experimental Procedures	62
4.2.1. <i>Subjects</i>	62
4.2.2. <i>Drugs and Administration</i>	63
4.2.2.1. <i>Single injection testing</i>	63
4.2.2.2. <i>Repeated injection testing</i>	63
4.2.3. <i>Apparatus</i>	64
4.2.4. <i>Behavioural Procedures</i>	64
4.2.4.1. <i>Acute injections</i>	64
4.2.4.2. <i>Chronic injections</i>	65
4.2.5. <i>Statistics</i>	66
4.3. Results	67

4.3.1. <i>Acute Formalin Test</i>	67
4.3.1.1. <i>Acute morphine dose response</i>	67
4.3.1.2. <i>Acute morphine + naltrexone doses response</i>	67
4.3.2. <i>Chronic Morphine + Naltrexone Dose Response</i>	73
4.3.2.1. <i>Morphine alone on testing day 7</i>	73
4.3.2.2. <i>Combination treatment on testing day 7</i>	78
4.4. Discussion.....	78
CHAPTER 5. GENERAL DISCUSSION	87
REFERENCES	94
APPENDICES	119

List of Tables

TABLE 4.1. ACUTE FORMALIN TEST STATISTICS.....	70
TABLE 4.2. TAIL-FLICK TESTING ACROSS CHRONIC DRUG ADMINISTRATION DAYS	75
TABLE 4.3. FORMALIN TESTING FOLLOWING CHRONIC DRUG ADMINISTRATION	79

List of Figures

FIGURE 2.1. THE ANTINOCICEPTIVE PROPERTIES OF WIN 55 212-2 ARE ENHANCED BY ULTRA-LOW DOSE NALTREXONE, BUT NOT BY HIGH DOSE NALTREXONE.....	22
FIGURE 2.2. THE ANTINOCICEPTIVE PROPERTIES OF WIN 55 212-2 ARE DOSE DEPENDENT, AND ARE ENHANCED BY ULTRA-LOW DOSE NALTREXONE	26
FIGURE 3.1. CB1R ANTIBODY IS RELATIVELY SPECIFIC TO THE TARGETED CANNABINOID RECEPTOR	42
FIGURE 3.2. ULTRA-LOW DOSE RIMONABANT ENHANCES WIN-INDUCED ANTINOCICEPTION	46
FIGURE 3.3. ULTRA-LOW DOSE RIMONABANT ATTENUATES THE DEVELOPMENT OF TOLERANCE TO WIN-INDUCED ANTINOCICEPTION.....	48
FIGURE 3.4. CHRONIC WIN-INDUCED Gs– CB1R COUPLING IS ATTENUATED BY CO- TREATMENT WITH ULTRA-LOW-DOSE RIMONABANT.....	52
FIGURE 4.1. MORPHINE PRODUCES DECREASED PAIN RESPONSES ONLY IN PHASE 2 OF THE FORMALIN TEST.....	69
FIGURE 4.2. NALTREXONE, WHEN COMBINED WITH MORPHINE, DOSE DEPENDENTLY INCREASES PAIN RESPONSES COMPARED TO MORPHINE ALONE IN PHASE 2 OF THE FORMALIN TEST.....	72
FIGURE 4.3. ULTRA-LOW DOSE NALTREXONE, WHEN COMBINED WITH MORPHINE, PRODUCED GREATER ANTINOCICEPTION COMPARED TO MORPHINE ALONE	74
FIGURE 4.4. MORPHINE-INDUCED TOLERANCE CAN BE SEEN IN PHASE 2 OF THE FORMALIN TEST, BUT THIS EFFECT IS NOT ALTERED BY NALTREXONE	77

FIGURE 4.5. ULTRA-LOW DOSE NALTREXONE, WHEN COMBINED WITH MORPHINE, DOES NOT PRODUCE GREATER ANTINOCICEPTION COMPARED TO MORPHINE ALONE	80
FIGURE 4.6. NALTREXONE, WHEN COMBINED WITH MORPHINE ON DOSING AND TESTING DAYS, HAS NO EFFECT ON MORPHINE-INDUCED TOLERANCE IN THE FORMALIN TEST	82
FIGURE A1. FORMALIN TEST TIME COURSE DATA FROM THE MORPHINE DOSE RESPONSE	119
FIGURE A2. FORMALIN TEST TIME COURSE DATA FROM THE MORPHINE (2 MG/KG) PLUS NALTREXONE DOSE RESPONSE	120
FIGURE A3. FORMALIN TEST TIME COURSE DATA FROM THE SUB-CHRONIC MORPHINE (10 MG/KG) PLUS NALTREXONE DOSE RESPONSE GROUPS	121
FIGURE A4. FORMALIN TEST TIME COURSE DATA FROM THE SUB-CHRONIC MORPHINE (10 MG/KG) PLUS NALTREXONE DOSE RESPONSE GROUPS	122

List of Abbreviations

2-AG	2-arachidonoyl-glycerol
2-AGE	2-arachidonoyl-glyceryl ether
5HT-1A	serotonin 1A receptor
AEA	N-arachidonoyl-ethanolamine
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid CB1 receptor
DMSO	dimethyl sulfoxide
GABA	γ -aminobutyric acid
G-protein	guanine nucleotide regulatory protein
MANOVA	multivariate analysis of variance
MPE	maximal possible effect
NADA	N-arachidonoyl-dopamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 1% Tween20
SDS	sodium dodecyl sulfate
TRPV1	transient receptor potential vanilloid subtype 1 receptor
WIN	WIN 55,212-2
Δ^9 -THC	Δ^9 -tetrahydrocannabinol

Chapter 1. General Introduction

1.1. Meeting Mary Jane (Marijuana)

Marijuana and opium have been used recreationally and medicinally for thousands of years. In 1908, the Opium and Narcotic Act mandated that opium and its derivatives were prohibited from being sold, cultivated or imported into Canada for non-medical purposes. Fifteen years later, an amendment to this act included marijuana and its derivatives, initiating decades of marijuana prohibition. In the last five years there has been a shift in Canadian marijuana laws, favouring a less strict policy for marijuana possession and legalizing medicinal marijuana. Now that marijuana and marijuana-like drugs are becoming marketable pharmaceuticals, a better understanding of their mechanisms of action and improved clinical utility is necessary (see: Canada. Parliament. Senate. Special Committee on Illegal Drugs, 2002).

Marijuana is the common name for the plant *Cannabis sativa*. When ingested, *C. sativa* produces physiological changes (e.g., tachycardia, hypotension, hypothermia), perceptual changes (e.g., analgesia, altered time perception), and cognitive-emotional changes (e.g., euphoria, anxiety, impaired learning and memory) (see Dewey, 1986, Hampson & Deadwyler, 1998, and Pertwee, 1988 for reviews). Although *C. sativa* contains a plethora of compounds, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) is the psychoactive ingredient that is largely responsible for the behavioural effects (Gaoni & Mechoulam, 1964; Mechoulam, Shani, Edery, & Grunfeld, 1970). Since the discovery of Δ^9 -THC, many variants of this compound and other synthetic compounds have been created that mimic the action of Δ^9 -THC with varying potencies. Because these drugs exert similar effects as *C. sativa* they are referred to as cannabinoids.

1.2. Cannabinoid Physiology

Cannabinoids exert their effects by activating guanine nucleotide regulatory protein (G-protein) coupled receptors in neurons (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988). Since this discovery, two genes that code for receptors that are activated by cannabinoids have been cloned. The CB1 receptor (CB1R) is almost exclusively expressed in neural membranes with the exception of trace amounts in the testes (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). The CB2 receptor is expressed in macrophages in the marginal zone of spleen (Munro, Thomas, & Abu-Shaar, 1993), peripheral nerve tissue (Stander, Schmelz, Metze, Luger, & Rukwied, 2005), and potentially on microglia of the lumbar spinal cord in a model of chronic neuropathic pain (Zhang et al. 2003; but see also Wotherspoon, et al. 2005).

The activation of cannabinoid receptors by cannabinoids exerts a range of G-protein mediated intracellular events. Because this thesis focuses on the CB1R, the remainder of the discussion of receptor action will focus on the CB1R (see Howlett et al. 2002, and Pertwee, 1997, for reviews of CB2 receptor activation). When nM- μ M concentrations of a cannabinoid agonist are applied, activation of the CB1R results in an intracellular guanosine diphosphate- to guanosine triphosphate-protein exchange. The CB1R activates pertussis toxin sensitive $G_{\alpha i}$ and $G_{\alpha o}$ associated G-proteins (Howlett, Qualy, & Khachatrian, 1986; Mukhopadhyay & Howlett, 2005; Prather, Martin, Breivogel, & Childers, 2000). This G-protein activation by cannabinoid agonists typically leads to inhibition of adenylyl cyclase and reduced production of the second messenger cyclic adenosine monophosphate (cAMP; Howlett, 1985; Howlett & Fleming 1984; Wade, Tzavara, & Nomikos, 2004). With cells cotransfected with CB1Rs and the

nine adenylyl cyclase isoforms, cannabinoid agonists inhibit the I, V, VI and VII isoforms, but surprisingly stimulate the II, IV, and VII isoforms (Rhee, Bayewitch, Avidor-Reiss, Levy, & Vogel, 1998). Whether CB1Rs couple to both inhibitory and stimulatory adenylyl cyclase isoforms in natural neurons has yet to be determined.

The discovery of receptors activated by cannabinoid compounds initiated the search for endogenous ligands for these receptors. The first discovered endogenous cannabinoid (a.k.a. endocannabinoid) was N-arachidonoyl-ethanolamine (AEA) which was given the name anandamide (Devane et al. 1992). Shortly thereafter 2-arachidonoyl-glycerol (2-AG), 2-arachidonoyl-glyceryl ether (2-AGE), and N-arachidonoyl-dopamine (NADA) were discovered (Bisogno et al. 2000; Hanus et al. 2001; Huang et al. 2002; Mechoulam et al. 1995). AEA and 2-AG bind to both CB1 and CB2 receptors with 3-4 times greater affinity for the CB1R, NADA binds with 40 times greater affinity to the CB1R, and 2-AGE binds almost exclusively to the CB1R (Bisogno et al. 2000; Hanus et al. 2001; Hillard et al. 1999; Mechoulam et al. 1995). *O*-arachidonoyl-ethanolamine (virodhamine) is the most recently discovered endocannabinoid, and has the most unconventional action. Virodhamine is a full agonist at CB2 receptors, but it acts as a partial agonist on CB1Rs serving as an endogenous CB1R antagonist (Porter et al. 2002).

The use of endocannabinoids to study the effects of cannabinoid receptor activation is limited by the fact that these compounds activate non-cannabinoid receptors, such as the transient receptor potential vanilloid subtype 1 receptor (TRPV1; Golech et al. 2004; Lam, McDonald, & Lambert, 2005; Sagar et al. 2004). For this reason, natural and synthetic exogenous cannabinoids may be better tools to study cannabinoid receptor mechanisms and therapeutics. For example, when measuring the inhibition of evoked vas

deference contractions by the endocannabinoid AEA and the synthetic cannabinoid WIN, a TRPV1 antagonist blocks the effects of AEA but not WIN (Ross, Gibson, et al. 2001). Thus, WIN does not appear to stimulate TRPV1 receptors, and is therefore a more suitable ligand for studying the effects of cannabinoid receptor activation.

1.3. Cannabinoids and Pain

Knowledge that ingesting cannabis exerts effects on the body dates back 5 000 years to the oral traditions of the Emperor Shên-nung (as cited in Russo, 1998). The earliest reference to cannabis use as an analgesic dates back 2 000 years, for the treatment of headaches in India (Dwarakanath, 1965). Despite continued use of cannabis ever since, controlled experiments testing the efficacy of cannabis to treat pain in humans did not begin until the 1970s (Noyes & Baram, 1974). Shortly thereafter, researchers demonstrated Δ^9 -THC -induced dose-dependent pain relief in cancer patients (Noyes, Brunk, Baram, & Canter, 1975), thus showing the therapeutic potential of cannabinoid compounds. This line of research eventually led to the Canadian approval of Sativex®, a marijuana extract composing of Δ^9 -THC and cannabidiol in an approximate 1:1 ratio, for alleviating neuropathic pain in patients suffering from multiple sclerosis (Sibbald, 2005).

Preclinical research on the pain relieving properties of cannabinoids has intensified since the 1970s (see Pertwee, 2001 for review). In acute pain models, Δ^9 -THC and other synthetic cannabinoid agonists reduce avoidance behaviours of painful stimuli in rats including intense thermal (Gallager, Sanders-Bush, & Sulser, 1972), mechanical pressure (Smith, Fujimori, Lowe, & Welch, 1998), and electrical stimulation (Weissman, Milne, & Melvin, 1982). Cannabinoid agonists are also effective analgesics in inflammatory pain models. In these models, noxious chemicals (e.g., formalin or

capsaicin) are injected into sensitive areas (e.g., under the skin of the plantar surface of the paw) to produce unavoidable pain lasting for hours or, in some cases, days. The formalin test of pain produces bi-modal pain behaviours wherein the first phase occurs within 10 min post-injection, and the second phase occurs from 15-60 min post-injection. Cannabinoid agonists reduce first and second phase formalin-induced pain behaviours (Tsou et al 1996). Likewise, cannabinoids reduce capsaicin-induced mechanical and thermal hyperalgesia (Li et al. 1999). Recent research demonstrates that cannabinoid agonists are very effective in reducing neuropathic pain. In this pain model, surgery is performed to expose a nerve (e.g., sciatic nerve) and it is loosely tied with a suture. This process produces a long-term sensitization of that nerve characterized behaviourally by allodynia and hyperalgesia¹ (Bennett & Xie, 1988). Cannabinoid agonists reduce allodynia and hyperalgesia in this model of neuropathic pain (Herzberg, Eliav, Bennett, & Kopin, 1997). Thus, cannabinoids have therapeutic potential for most types of pain.

Cannabinoid agonists are probably effective in all animal models of pain because cannabinoid receptors can inhibit nociceptive signaling at almost every neural junction where pain signals are transmitted or moderated. For instance, within the periphery, CB1Rs are located on primary afferent sensory neurons (Hohmann & Herkenham, 1999): This is the point where noxious stimuli are first detected and transmitted to the spinal cord. In the dorsal root ganglion, CB1Rs are primarily located in small and medium diameter cells, and are preferentially co-expressed in TRPV1 positive neurons, thus indicating that these are nociceptive primary afferent neurons (Ahluwalia, Urban, Capogna, Bevan, & Nagy, 2000). Inside the spinal cord, CB1Rs are densely populated in

¹ *Hyperalgesia – the reporting of greater than normal pain intensity to a painful stimulus.
Allodynia – the detection of pain from a normally non-painful stimulus.*

lamina I and lamina II regions of dorsal horn (Farquhar-Smith et al. 2000; Salio, Fischer, Franzoni, & Conrath, 2002): These areas are highly innervated by nociceptive primary afferents (Sugiura, Lee, & Perl, 1986). Ultrastructural localization of these dorsal horn labeled CB1Rs place them pre-synaptically in unmyelinated axons, and post-synaptically in dendrites and somas (Salio et al. 2002). Pre-synaptic CB1Rs likely attenuate nociception by inhibiting neurotransmitter release (Richardson, Aanonsen, & Hargreaves, 1998; Ross, Coutts, et al. 2001). CB1Rs may also inhibit pain supraspinally. For instance, microinjecting a cannabinoid agonist into the central amygdala, nucleus submedius, or superior colliculus – all are areas associated with processing nociception (Bernard, Huang, & Besson, 1990; Craig & Burton, 1981; Stein & Dixon, 1978) – produces antinociception (Martin et al. 1999). Unfortunately, CB1R antagonist controls were not performed in the latter study, making it difficult to verify whether these effects were CB1R mediated. Finally, in the periaqueductal gray and rostral ventromedial medulla – midbrain and brainstem areas known to produce analgesia by projecting descending inhibiting efferents to the spinal cord (Fields, Basbaum, Clanton, & Anderson, 1977; Mayer, Wolfle, Akil, Carder, & Liebeskind, 1971; Oliveras, Besson, Guilbaud, & Liebeskind, 1974) – CB1 agonists inhibit pain responses to acute thermal and inflammatory stimuli, and their effects are blocked by CB1R antagonists (de Novellis et al. 2005; Martin, Tsou, & Walker, 1998; Walker, Huang, Strangman, Tsou, & Sanudo-Pena, 1999). Together, these data strongly implicate the CB1R as an important modulator of pain. Investigation of the similarities in cannabinoid receptor functioning, and the interaction with other pain-associated receptor systems, is needed to better understand the cannabinoid-pain relationship.

1.4. Opioids and the Ultra Low Dose Story

Opioids have been used for centuries to reduce pain, an effect that is mediated through activation of endogenous opioid receptors. With μM doses of morphine, μ -opioid receptors are activated and couple to G_i/o -proteins (Crain, Crain, & Makman, 1986; Lujan et al. 1984; Tucker, 1984), thereby inhibiting sensory neurotransmission of the pain signal in the spinal cord (Einspahr & Piercey, 1980; Homma, Collins, Kitahata, Matsumoto, & Kawahara, 1983; Le Bars, Menetrey, Conseiller, & Besson, 1975). Opioids at μM doses produce a shortening of the action potential duration in dorsal root ganglion neurons (Werz & MacDonald, 1982). These action potential duration shortening effects are prevented by naloxone and pertussis toxin (Crain, Crain, & Makman, 1987). When opioids are administered at much lower doses, however, they have opposing effects. For instance, fM to nM doses of opioids produced action potential duration prolongation in neurons (Higashi, Shinnick-Gallagher, & Gallagher, 1982; Shen & Crain, 1989; Crain & Shen, 1995), an effect that is blocked by naloxone and cholera toxin (Shen & Crain, 1989; 1990a b). Opioids are thus capable of producing inhibitory and stimulatory effects on sensory neurons that are mediated by opioid receptors coupling to G_i/o - and G_s -proteins, respectively. Remarkably, both opioid mediated effects may occur in the same neurons (Shen & Crain, 1989). Shen and Crain (1989) postulate that opioid receptors which couple to stimulatory G-proteins may be high in affinity but very low in quantity, whereas receptors which couple to inhibitory G-proteins may be lower in affinity but very high in quantity. If this is true, low doses of opioids would preferentially stimulate receptors that couple to stimulatory G-proteins, whereas higher doses would saturate the stimulatory receptors and activate a large number of receptors

that couple to inhibitory G-proteins (Shen & Crain, 1990a). This would explain why opioid agonists at nM doses can produce hyperalgesia, but in μ M doses produce analgesia (Kayser, Besson, & Guilbaud, 1987; Crain & Shen, 2001).

Because opioids can stimulate and inhibit neurons depending on the dose administered, opioid agonists may be tonically inhibiting their own analgesic activity. Interestingly, if an ultra-low dose (nM to pM) of an opioid receptor antagonist is administered with a hyperalgesic dose (ultra-low dose) of an opioid agonist, the antagonist blocks the expression of hyperalgesia and reveals potent analgesia (Crain & Shen, 2001; Shen & Crain, 2001). Similarly, analgesic doses of opioids are more potent when administered in conjunction with an ultra-low dose of an opioid antagonist (Crain & Shen, 1995; Shen & Crain, 1997; Powell et al. 2002). Together, these data suggest that ultra-low dose opioid antagonists occlude the opioid receptors that would couple to stimulatory effectors, therefore preventing activation of this tonically opponent system.

Repeated administration of opioids leads to a progressive loss of analgesic potency, a process called opioid tolerance. This phenomenon can be explained by a switch in the population of opioid receptor coupling from the predominantly inhibitory Gi/o-proteins to the predominantly stimulatory Gs-proteins (Wang et al. 2005). The development of analgesic tolerance is prevented when an ultra-low dose of an opioid antagonist is co-administered with the agonist (Wang et al. 2005; Powell et al. 2002; Shen & Crain 1997). This suggests that opioid receptors that couple to stimulatory G-proteins are responsible for tolerance that develops to the analgesic effect of opioid agonists.

The few studies investigating the impact of ultra-low dose opioid antagonists on morphine-induced analgesia in humans have produced conflicting results. For example, combination treatment reportedly decreases opioid-induced side effects (Cepeda et al. 2004; Gan et al. 1997), opioid requirements (Gan et al. 1997), and pain severity (Joshi et al. 1999), whereas other studies reported no effect of combination treatment (Sartain et al. 2003), or increased pain and opioid requirements (Cepeda et al. 2002). These discrepancies appear, primarily, to be a factor of antagonist dose, wherein higher daily doses were the least effective. Moreover, a recent randomized and controlled clinical trial demonstrated that an ultra-low dose of the opioid antagonists naltrexone in combination with the opioid agonist oxycodone (Oxytrex®) produce greater analgesia in osteoarthritic patients compared to opioid treatment alone (Chindalore et al. 2005). Thus, opioid agonist and ultra-low dose antagonist combinations have the potential to become an effective clinical treatment for pain.

1.5. Cannabinoid-Opioid Interactions

Opioid and cannabinoid agonists produce comparable drug effects including catalepsy, hypothermia, tolerance, dependence, reward and analgesia (Dewey, 1986; Olson, Olson, Vaccarino, & Kastin, 1998). In addition, opioid and cannabinoid systems interact (Maldonado & Valverde, 2003; Manzanares et al. 1999) and their agonists have synergistic properties in analgesia (Cichewicz, 2004; Smith, Cichewicz, Martin, & Welch, 1998). Furthermore, cannabinoid and opioid agonists exhibit ‘cross-’ precipitated withdrawal by opioid and cannabinoid antagonists, respectively (Navarro et al. 2001), and cannabinoid and opioid agonists also show interactions in measures of tolerance (Thorat & Bhargava, 1994; Rubino, Tizzone, Vigano, Massi, & Parolaro, 1997;

Cichewicz & Welch, 2003). Together, these studies strongly demonstrate an opioid-cannabinoid interaction.

Cannabinoid and opioid interactions may be related to the ability of cannabinoid agonists to induce opioid gene expression in the brain and spinal cord (Corchero, Avila, Fuentes, & Manzanares, 1997; Corchero, Fuentes, & Manzanarez, 1997), and to increase the release of spinal dynorphins (Mason, Lowe, & Welch, 1999; Pugh, Mason, Combs, & Welch, 1997) and enkephalins in the brain (Valverde et al. 2001). The interrelationship between these systems may be further explained by the fact that μ -opioid and CB1Rs are co-localized on spinal (Salio et al. 2001) and supraspinal (Rodriguez, Mackie, & Pickel, 2001) neurons, and may even form heterodimers (Rios, Gomes, & Devi, 2006).

Moreover, cannabinoid and opioid receptors have comparable signal-transduction mechanisms whereby both μ -opioid and CB1Rs predominately couple to pertussis toxin sensitive Gi/o-proteins (Howlett et al. 1986; Standifer & Pasternak, 1997), leading to either decreases in adenylyl cyclase production of cAMP, or decreases in Ca^{+2} and increases in K^{+} channel conductance. Given the close relationship between opioid and cannabinoid systems, the focus of experiment 1 was on whether the cannabinoid and opioid systems interact at the ultra-low dose level. Because cannabinoid agonists increase the release of endogenous opioids (Mason et al. 1999; Pugh et al. 1997; Valverde et al. 2001), we hypothesize that ultra-low doses of the opioid antagonist naltrexone will enhance the antinociceptive potency of the cannabinoid agonist WIN.

1.6. Biphasic Effects of Cannabinoids

Similar to opioid-induced effects, cannabinoid agonists produce opposing behavioural effects at different dose ranges. For instance, low doses of the

endocannabinoid anandamide (10 $\mu\text{g}/\text{kg}$) produce hyperlocomotion and near hyperalgesia, but higher doses (10 mg/kg) produce hypolocomotion and analgesia (Sulcova et al. 1998). These authors argued that the opposing behavioural effects induced by different dose ranges of the same drug might be a result of differential activation of Gs and Gi proteins by cannabinoid receptors. Supporting this contention, CB1Rs activate stimulatory Gs-proteins (Glass & Felder, 1997; Calandra et al. 1999) in addition to the Gi activation (Howlett, 1985; Howlett & Fleming, 1984; Howlett, Qualy, & Khachatrian, 1986).

Because of the similarity between opioid and cannabinoid receptor activation, and the dual activation of inhibitory and stimulatory G-proteins, the second experiment investigated whether cannabinoid agonists could be made more potent with an ultra-low dose of a CB1R antagonist using the tail flick test of antinociception. In experiment 2, we hypothesize that the duration of antinociception induced by the cannabinoid agonist WIN would be prolonged by co-injecting an ultra-low dose of the CB1R antagonist rimonabant (previously named SR 141716), and that ultra-low dose rimonabant would attenuate WIN-induced analgesic tolerance. Then we investigated the coupling of CB1Rs to G-protein subunits following repeated drug treatments. It was hypothesized that, like the μ -opioid receptor (Wang et al., 2005), the CB1R would switch G-protein coupling preference, from inhibitory-type to stimulatory-type, following sub-chronic WIN administration, and that this effect would be prevented by ultra-low dose rimonabant co-treatment. Thus, this second proposed study attempted to provide a model of cannabinoid-induced analgesic tolerance, and a way of preventing this tolerance.

1.7. Tonic Pain and Opioid Ultra-Low Dose Combinations

Virtually all of the pre-clinical research on ultra-low dose opioid antagonist effects has used animal models of acute and escapable pain, such as the tail flick or hot plate tests. In contrast, clinical pain states are persistent or chronic and, by definition, unavoidable. To further our understanding of the ultra-low dose phenomenon, we investigated the effects of morphine in combination with ultra-low doses of the opioid antagonist naltrexone in the formalin test of persistent inflammatory pain following either acute, or sub-chronic drug administrations (experiment 3). We hypothesized that morphine-induced analgesia would be enhanced, and the development of tolerance would be attenuated, by ultra-low doses of the antagonist.

Chapter 2. Experiment 1

Ultra-Low Dose Naltrexone Enhances Cannabinoid-Induced Antinociception

By

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Published in

Behavioural Pharmacology, 2005, 16, 597-603.

2.0. Abstract

Both opioids and cannabinoids have inhibitory effects at micromolar doses, which are mediated by the receptor coupling to Gi/o-proteins. Surprisingly, the analgesic effects of opioids are enhanced by ultra-low doses (nanomolar to picomolar) of the opioid antagonist, naltrexone. Because opioid and cannabinoid systems interact, this study investigated whether ultra-low dose naltrexone also influences cannabinoid-induced antinociception. Separate groups of Long Evans rats were tested for antinociception following an injection of vehicle, a sub-maximal dose of the cannabinoid agonist WIN 55 212-2 (WIN), naltrexone (an ultra-low or a high dose), or a combination of WIN and naltrexone doses. Tail-flick latencies were recorded for three hours, at 10- min intervals for the first hour, and at 15-min intervals thereafter. Ultra-low dose naltrexone elevated WIN-induced tail flick thresholds without extending its duration of action. This enhancement was replicated in animals receiving intraperitoneal or intravenous injections. High dose naltrexone had no effect on WIN-induced tail flick latencies, but a high dose of the CB1 receptor antagonist rimonabant blocked the elevated tail flick thresholds produced by WIN + ultra-low dose naltrexone. These data suggest a mechanism of cannabinoid-opioid interaction whereby Gs-protein coupled opioid receptor activation may attenuate cannabinoid-induced antinociception and/or motor functioning.

2.1. Introduction

Opioid and cannabinoid drugs induce comparable effects when administered alone, including catalepsy, hypothermia, tolerance, dependence, reward and analgesia (Dewey, 1986; Olson et al. 1998). In addition, opioid and cannabinoid systems interact (Maldonado & Valverde, 2003; Manzanares et al. 1999) in that cannabinoid and opioid agonists have synergistic properties in analgesia (Cichewicz, 2004; Smith, Cichewicz, et al. 1998), cannabinoid antagonists decrease opiate self-administration, and opioid antagonists decrease self administration of cannabinoid agonists (Navarro et al. 2001). Cannabinoid and opioid agonists also exhibit ‘cross-’ precipitated withdrawal by opioid and cannabinoid antagonists, respectively (Navarro et al. 2001), and oral co-administration of low doses of Δ^9 -tetrahydrocannabinol and morphine reduces naloxone-precipitated withdrawal (Cichewicz & Welch, 2003). Cannabinoid and opioid agonists also show interactions in measures of tolerance (Thorat & Bhargava, 1994; Rubino et al. 1997; Cichewicz & Welch, 2003). Together, these studies strongly demonstrate an opioid-cannabinoid interaction.

The interaction between cannabinoid and opioid systems may be related to the ability of cannabinoid agonists to induce opioid gene expression in the brain and spinal cord (Corchero, Avila, et al. 1997, Corchero, Fuentes, et al. 1997), and to increase the release of spinal dynorphins (Mason et al. 1999; Pugh et al. 1997) and enkephalins in the brain (Valverde et al. 2001). The interrelationship between cannabinoid and opioid systems may be further explained by the fact that μ -opioid and CB1Rs are co-localized on spinal (Salio et al. 2001) and supraspinal (Rodriguez et al. 2001) neurons. Moreover, cannabinoid and opioid receptors have comparable signal-transduction mechanisms

whereby both μ -opioid and CB1Rs predominately couple to pertussis toxin sensitive Gi/o-proteins (Howlett et al. 1986; Standifer & Pasternak, 1997), leading to either decreases in adenylyl cyclase production of cAMP, or decreases in Ca^{+2} and increases in K^{+} channel conductance.

Opioid and cannabinoid agonists are both effective analgesics that produce pain relief by inhibiting cellular activity (Kelly & Chapman, 2001; Werz & Macdonald, 1983). In the last 10-15 years, it has become clear that ultra-low doses (i.e., picomolar to nanomolar range) of opioid receptor agonists stimulate, rather than inhibit, cellular activity (Shen & Crain, 1989). This effect appears to be mediated through a sub-population of high affinity opioid receptors that couple to pertussis toxin insensitive Gs-proteins (Shen & Crain, 1990a). With micromolar morphine doses, the opioid receptor coupling to inhibitory Gi/o-proteins prevails over the sub-population of opioid receptors coupling to stimulatory Gs-proteins (Crain & Shen, 1995, 2001). In addition, opioid antagonists in micromolar doses prevent many of the behavioural effects of opioid agonists but, in ultra-low concentrations, these drugs enhance the effects of opioid agonists (Crain & Shen, 1995). For instance, morphine-induced analgesia is enhanced by co-injecting ultra-low doses of the non-specific opioid receptor antagonist naltrexone; the duration of analgesia is extended, the development of tolerance is blocked, and established tolerance is reversed (Powell et al. 2002). One proposed mechanism of this ultra-low dose opioid receptor antagonist effect involves the prevention of the switch in μ -opioid receptor coupling, from Gi/o proteins in naïve animals to Gs proteins in morphine tolerant animals, by ultra-low dose opioid receptor antagonists (Wang et al. 2005). With single injection enhancement of morphine by ultra-low dose naltrexone, the

antagonist may be selectively antagonizing pre-existing opioid receptors that couple to Gs-proteins, however this is only an assumption.

Given the interaction between opioid and cannabinoid systems at micromolar doses, this study investigated whether the interaction is also expressed at the ultra-low dose level. More specifically, the influence of ultra-low doses of an opioid antagonist on the antinociceptive effects of a micromolar dose of a cannabinoid agonist was tested. It was hypothesized that ultra-low doses of naltrexone would enhance the antinociceptive effects of the cannabinoid agonist WIN 55 212-2 (WIN). In the first experiment, an ultra-low dose of naltrexone was co-administered with WIN via an intraperitoneal route of administration. The second set of experiments elaborated on the first, whereby two doses of WIN were co-administered with various ultra-low doses of naltrexone using an intravenous route of administration.

2.2. Method

2.2.1. Subjects

Male Long-Evans rats (N=175) from Charles River (Montreal, QC, Canada) ranging from 230-380 g, were housed in polycarbonate cages in pairs and given free access to food (Lab Diet, PMI Nutrition International, Inc., Brentwood, MO, USA) and water. Animal quarters were kept on a reverse light-dark cycle (lights on from 7 pm to 7 am) and maintained at 22 ± 2 °C and 45 ± 20 % relative humidity. Animals were given a minimum of 3 days prior to the experiment to acclimatize to the animal quarters. All procedures were approved by Queen's University Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care and the Animals for Research Act.

2.2.2. Apparatus

The tail-flick apparatus consists of a projection lamp that creates radiant heat located just below the animal-testing surface (D'Amour & Smith, 1941). The light from the lamp projected through a small hole in the testing surface and was aimed at a photocell located 25 cm above the testing surface. A digital timer, connected to the apparatus, started when the heat source was activated. When the animal flicked its tail away from the heat source, the light from the projection lamp activated the photocell, simultaneously stopping the timer and turning off the lamp. The heat intensity was calibrated to result in baseline tail flick latencies of 2-3 s and a 10 s cutoff was used to minimize tissue damage.

2.2.3. Procedure

Nociceptive reflexes to a thermal stimulus were tested using the tail-flick analgesia meter. This apparatus focuses a hot beam on the animal's tail. The time it takes for the rat to flick its tail away from the heat source is a measure of nociception; the longer the animal leaves its tail on the hotspot, the greater the degree of pain relief. On the day prior to tail flick testing, animals were handled on the tail flick apparatus for 5-10 min to reduce stress-induced analgesia (Kelly & Franklin, 1985; Terman, Shavit, Lewis, Cannon, & Liebeskind, 1984). On testing day, animals were restrained in a small towel and a baseline tail flick latency was measured. Following the baseline measure, animals were given a drug injection and tail-flick latencies were assessed every 10 min for the first hour post-injection, and every 15 min for the following two hours. Tail-flick latencies were converted into a percent of maximal possible effect (MPE) using the equation:

$$\text{MPE} = [(\text{post-injection latency} - \text{baseline latency}) / (10 \text{ s cutoff} - \text{baseline latency})] \cdot 100$$

2.2.4. *Drugs and Administration*

All injections were administered in a volume of 1 ml/kg.

2.2.4.1. *Intraperitoneal injections*

All chemicals were dissolved in 99.5% dimethyl sulfoxide (DMSO; Sigma, Oakville, ON, Canada). DMSO alone was used as a control injection (n=8). The non-specific cannabinoid receptor agonist WIN 55 212-2 [(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; Tocris Cookson, Ellisville, MO, USA), at a dose of 2.0 mg/kg (adapted from Fox et al. 2001, and Herzberg et al. 1997), was administered alone (n=8), or in combination with either an ultra-low dose (20 ng/ml; n=8) or a high dose (5 mg/kg; n=7) of the non-specific opioid receptor antagonist naltrexone hydrochloride dihydrate (Sigma, Oakville, ON, Canada). The ultra-low dose of naltrexone selected was based on the systemic dose (10 ng/kg) used by Powell et al. (2002) that enhanced the analgesic effects of morphine, and on our pilot research replicating this effect. In addition, an ultra-low dose of naltrexone (20 ng/kg; n=8) was administered alone.

Only one dose of WIN and ultra-low dose naltrexone were used because this was the first test of this drug combination, and consequently the first demonstration of differences between WIN and WIN plus ultra-low dose naltrexone. To further analyse these ultra-low dose effects including extending the dose-response curve, intravenous injections were used for the remainder of the experiments.

2.2.4.2. *Intravenous injections*

All intravenously injected agents were suspended in 0.3% polyoxyethylenesorbitan monooleate (Tween® 80; Sigma, Oakville, ON, Canada) vehicle and administered in the posterior 1/3 of the lateral tail vein. WIN was administered alone at doses of 0.25, 0.125, 0.09375 and 0.0625 mg/kg. The 0.0625, 0.125, and 0.25 mg/kg intravenous doses were chosen because they were able to reduce the firing rate of ventroposterolateral thalamic nucleus neurons to a noxious pressure stimulus (Martin et al. 1996), and to increase tail-flick latencies (Meng, Manning, Martin, & Fields, 1998). Ultra-low doses of naltrexone (1.5, 0.75, 0.15 or 0.075 ng/kg) were combined with WIN (0.09375 mg/kg) and administered as a single injection. These combinations produce WIN to naltrexone molar ratios of 50 000:1, 100 000:1, 500 000:1, and 1 000 000:1, respectively. Also, WIN (0.0625 mg/kg) was combined with ultra-low doses of naltrexone (0.5, 0.1, 0.05, or 0.01 ng/kg) producing WIN to naltrexone molar ratios of 100 000:1, 500 000:1, 1 000 000:1, and 5 000 000:1, respectively. WIN (0.0625 mg/kg) was also combined with a high dose of naltrexone (0.15 mg/kg) forming a 1:3 WIN to naltrexone molar ratio. This dose was based on the molar ratio the WIN and high dose naltrexone used in the intraperitoneal injection experiment. The control group received 0.05 ng/kg ultra-low dose of naltrexone alone.

Different ultra-low dose naltrexone dose ratios were tested for 0.09375 mg/kg WIN, and 0.0625 mg/kg WIN associated groups because of how our original tests were executed. For the 0.09375 mg/kg and the 0.0625 mg/kg WIN associated combination groups, WIN to naltrexone dose ratios of 100 000:1 and 500 000:1 were the first to be tested. Because the 0.09375 mg/kg WIN combination groups showed no significant differences from WIN alone, one lower and one higher dose ratio was tested to see if the

effective dose range was previously missed. With the 0.0625 mg/kg WIN combination groups, however, there was a significant difference only between the 500 000:1 dose ratio and WIN alone groups. Two lower dose ratios were tested to produce a dose response curve.

The CB1R antagonist rimonabant [N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] was generously donated by the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Bethesda, MD, USA). Rimonabant (0.15 mg/kg) was administered alone, or with the WIN (0.0625 mg/kg) and ultra-low dose naltrexone (0.05 ng/ml) combination.

2.2.5. Statistical Analysis

Separate two-way repeated measure multivariate analysis of variance (MANOVA) tests were performed on the intraperitoneal injection groups, and intravenous injection groups. Post-injection time (10-180 min) was the within-subjects factor and drug group as the between-subjects factor. Because many drug group comparisons were irrelevant, *a priori* multiple comparisons were used to analyze the main drug effect using the Dunn's critical *t*-ratio. A corrected per-comparison α of 0.01 was used for the intraperitoneal injection groups because there were 4 comparisons, and a per-comparison corrected α of 0.001 was used for the intravenous injection groups because there were 19 comparisons in order to control the familywise error.

2.3. Results

2.3.1. Intraperitoneal Injections of WIN With Ultra-Low Dose Naltrexone

The post-injection tail-flick measures (MPE) taken across 180 min for each drug group are shown in Figure 2.1. When all drug groups were combined, a main

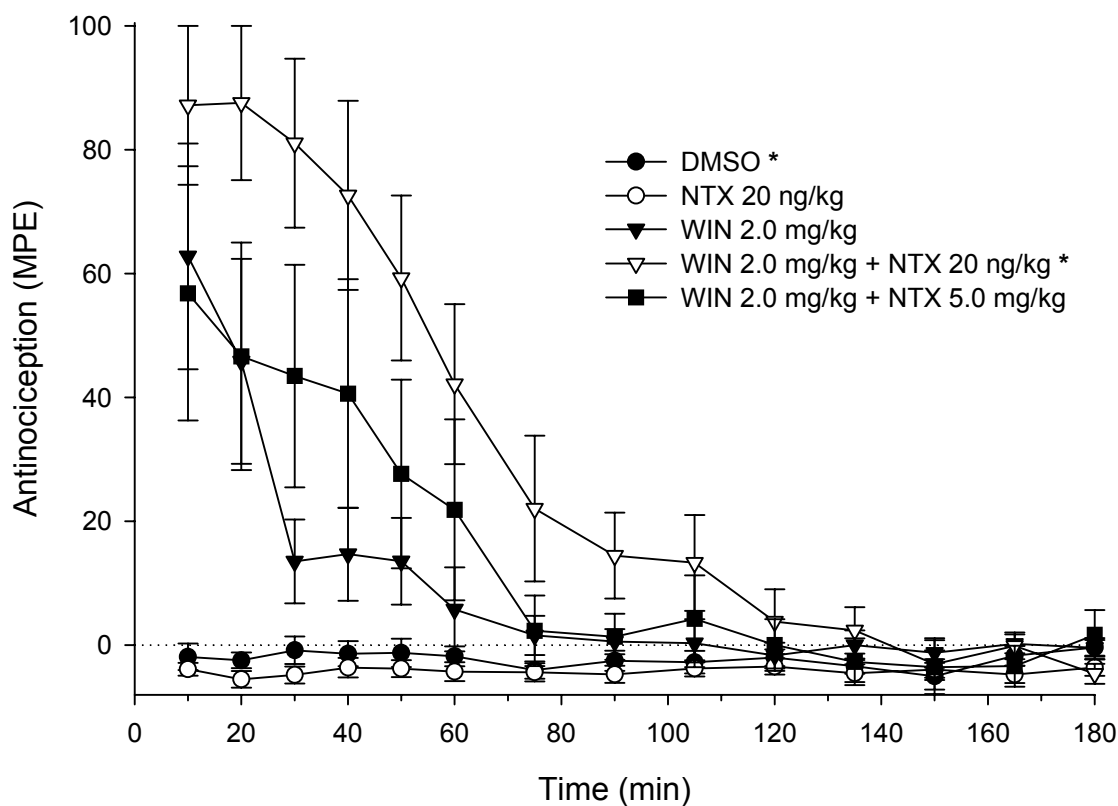


Figure 2.1. The antinociceptive properties of WIN 55 212-2 (WIN) are enhanced by ultra-low dose naltrexone (NTX), but not by high dose naltrexone. Group mean (\pm SEM) antinociception represented as percent maximal possible effect (MPE) using the tail-flick test. $n=8$ per group except WIN + naltrexone 5.0 mg/kg for which $n=7$. All injections administered intraperitoneally.

* significant difference from WIN 2.0 mg/kg.

effect of time [$F(13,422)=32.98$, $P<0.001$] was revealed, whereby tail-flick latency MPEs decreased across time. Furthermore, there was a main drug effect [$F(4,34)=9.53$, $P<0.001$], and a drug X post-injection time interaction, [$F(52,422)=7.76$, $P<0.001$]. Multiple comparisons analyzing the main effect of drug show that there was a significant difference between the DMSO and WIN alone groups [$t(14)=6.12$, $P\leq 0.01$]. Most importantly, there was a significant difference in tail-flick latencies between the WIN alone group and the WIN plus ultra-low dose naltrexone group [$t(14)=10.57$, $P\leq 0.01$], due to the fact that the combination treatment induced greater tail-flick latencies than WIN alone. This effect occurred despite the lack of difference between ultra-low dose naltrexone alone and vehicle groups [$t(14)=0.90$]. Furthermore, there was no significant difference between the WIN alone and co-injection of WIN with high dose naltrexone groups [$t(13)=2.58$].

2.3.2. Intravenous Injections of WIN With Ultra-Low Dose Naltrexone

Figure 2.2 shows the tail-flick data from animals given intravenous injections. A main effect of time indicated that there was a change in tail-flick latencies across time [$F(13,1547)=206.88$, $P<0.001$]. Furthermore, there was a main effect of drug [$F(16,119)=10.65$, $P<0.001$], and a time x drug group interaction [$F(208,1547)=8.72$, $P<0.001$].

When analyzing the main effect of drug, multiple comparisons show that there was no difference between vehicle and 0.0625 mg/kg WIN-treated animals [$t(14)=2.34$], but vehicle-treated animals were different from animals injected with 0.09375, 0.125, or 0.25 mg/kg WIN [$t(14)=5.39$, $P\leq 0.001$; $t(14)=8.74$, $P\leq 0.001$; and $t(14)=9.99$, $P\leq 0.001$, respectively; Figure 2.2.A].

The tail-flick latency MPEs were also compared between WIN alone groups and the combined WIN and ultra-low dose naltrexone groups. There were no significant differences between the 0.09375 mg/kg WIN alone group and the same dose of WIN mixed with the 1.5, 0.75, 0.15, or 0.075 ng/kg ultra-low dose of naltrexone [$t(14)=1.67$; $t(14)=1.16$; $t(14)=0.16$; and $t(14)=2.11$, respectively; Figure 2.2.B]. In contrast, ultra-low doses of naltrexone showed a dose-dependent effect when co-administered with 0.0625 mg/kg WIN (Figure 2.2.C). Thus, animals showed longer tail-flick latencies at the middle two doses (0.1 and 0.05 ng/kg) of ultra-low dose naltrexone in combination with 0.0625 mg/kg WIN, compared to the 0.0625 mg/kg WIN alone [$t(14)=5.89$, $P\leq 0.001$; and $t(14)=7.52$, $P\leq 0.001$, respectively]. In contrast, at the highest and lowest doses (0.5 and 0.01 ng/kg) of ultra-low dose naltrexone administered with 0.0625 mg/kg WIN there were no significant differences in tail-flick latencies compared to 0.0625 mg/kg WIN alone [$t(14)=1.95$; and $t(14)=0.12$, respectively]. The enhancement of the analgesic effect of 0.0625 mg/kg WIN by ultra-low dose naltrexone occurred even though ultra-low dose naltrexone (0.05 ng/kg) was not different from vehicle [$t(14)=0.13$] or 0.0625 WIN alone [$t(14)=2.47$]. Furthermore, combining a high dose of naltrexone (0.15 mg/kg) with 0.0625 mg/kg WIN had no effect [$t(14)=0.42$; Figure 2.2.D].

To determine whether the ultra-low dose naltrexone and WIN combination was dependent on CB1R stimulation, the CB1R antagonist rimonabant at a dose three times the molarity of WIN was co-injected with WIN and ultra-low dose naltrexone. The 0.05 ng/kg naltrexone – 0.0625 mg/kg WIN – 0.15 mg/kg rimonabant drug injection group was significantly different from the 0.05 ng/kg naltrexone – 0.0625 mg/kg WIN injection group [$t(14)=10.06$, $P\leq 0.001$], but not the vehicle group [$t(14)=0.20$]. Furthermore,

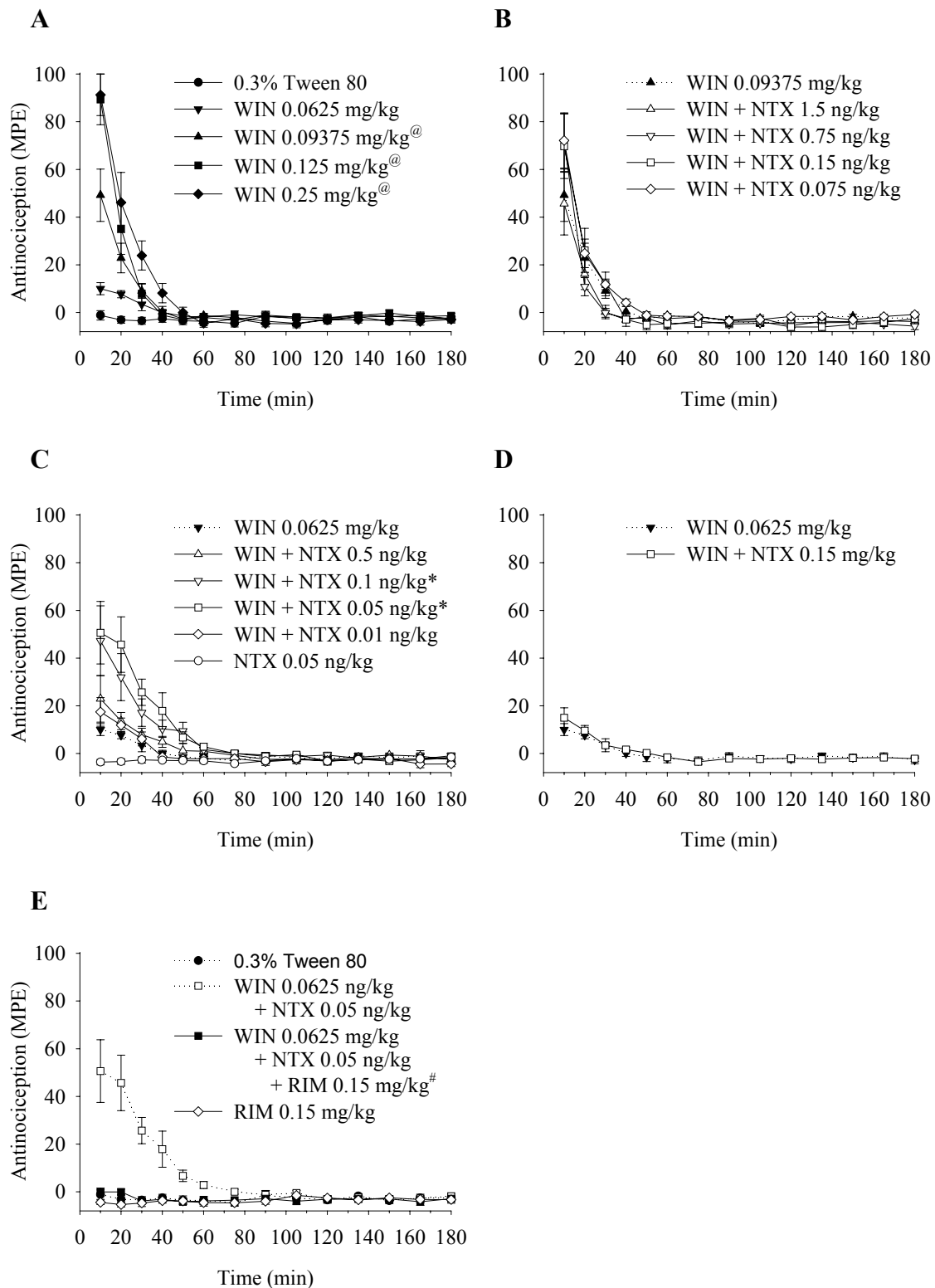


Figure 2.2. The antinociceptive properties of WIN 55 212-2 (WIN) are dose dependent, and are enhanced by ultra-low dose naltrexone (NTX). Group mean (+/- SEM) antinociception is represented as percent maximal possible effect (MPE) using the tail-flick test. (A) The cannabinoid WIN produces dose-dependent analgesia. (B) WIN 0.09375 mg/kg is not affected by ultra-low dose naltrexone. (C) WIN 0.0625 mg/kg is dose-dependently enhanced by ultra-low dose naltrexone. (D) High dose naltrexone does not affect WIN-induced antinociception. (E) The CB1 receptor antagonist rimonabant (RIM) blocks WIN + ultra-low dose naltrexone-induced antinociception. Dotted lines represent data re-plotted from Figure 2.2.A or C. n=8 per group. All injections are administered intravenously.

@ significant difference from 0.3% Tween 80

* significant difference from WIN 0.0625 mg/kg

significant difference from WIN 0.0625 mg/kg + naltrexone 0.05 ng/kg

rimonabant alone (0.15 mg/kg) was not different from vehicle or from the ultra-low dose naltrexone – WIN – rimonabant drug combination [$t(14)=0.78$; and $t(14)=0.57$, respectively; Figure 2.2.E].

2.4. Discussion

Ultra-low doses of the opioid receptor antagonist naltrexone enhance the analgesic properties of the cannabinoid agonist WIN. The effective WIN to naltrexone molar ratios were 80 000:1 for intraperitoneal injections, and 100 000 – 500 000:1 for intravenous injections. The ultra-low dose effect is specific to this dose range because a higher dose of naltrexone (WIN to naltrexone molar ratio of 1:3) did not affect WIN-induced tail-flick latencies. The high dose of naltrexone in the intraperitoneal injection group does appear to enhance WIN-induced tail-flick latencies, but this effect was not significant and was not replicated with intravenous injections. Although it has been previously reported that typically blocking doses of opioid antagonists do not affect cannabinoid-induced analgesia (Massi, Vaccani, Romorini, & Parolaro, 2001; Vivian et al. 1998), others report that opioid antagonists reduce cannabinoid-induced analgesia (Smith, Fujimori, et al. 1998; Tulunay, Ayhan, Portoghese, & Takemori, 1981). Regardless of this controversy, our study is the first evidence for enhanced cannabinoid-induced analgesia by an opioid antagonist. Our data also demonstrate that the WIN and ultra-low dose naltrexone effect is dependent on the CB1R. This suggests that WIN must be activating CB1Rs, and that the ultra-low dose effect is mediated through this system.

Our intravenous injected doses have comparable molar ratios to those used by Powell et al. (2002) who showed that systemic morphine and ultra-low dose naltrexone treatments have enhanced analgesic potency over morphine alone at a 410 000:1 molar

ratio. Similarly, our intraperitoneal injected molar ratio is comparable to the 55 000:1 morphine to naltrexone molar ratio used by Crain and Shen (1995): that study demonstrated enhanced analgesic duration compared to morphine alone in the mouse hot water immersion test. Thus, our studies looking at cannabinoid enhancement by ultra-low dose naltrexone relate to other studies looking at the paradoxical enhancement of morphine by ultra-low dose naltrexone.

Given that WIN and ultra-low dose naltrexone produce comparable behavioural effects to that of morphine and ultra-low dose naltrexone, the two effects may be mediated by similar mechanisms. Morphine produces hyperalgesia at low doses (nanomolar), but analgesia at higher doses (micromolar) (Crain & Shen, 2001), because opioid receptors can couple to both stimulatory and inhibitory G-proteins (Cruciani, Dvorkin, Morris, Crain, & Makman, 1993). When the G-protein $G_{\alpha s}$ subunit is inhibited by cholera toxin-B subunit, low dose morphine that typically produces hyperalgesia reveals an analgesic effect (Shen & Crain, 2001). This suggests that there is a subpopulation of high affinity opioid receptors that couple to G-proteins having the stimulatory $G_{\alpha s}$ subunit, and producing effects in opposition to the majority of opioid receptors that couple to G-proteins having the inhibitory $G_{\alpha i/o}$ subunit. It is hypothesized that ultra-low dose naltrexone enhances the effects of morphine because it preferentially antagonizes opioid receptors that are coupled to the stimulatory G-proteins. As a result, ultra-low dose naltrexone would relieve tonic inhibition of the effects of opioids, and therefore unmask greater analgesia.

In contrast to the studies of opioid mechanisms, our study involved activation of the CB1R (i.e., by WIN). Because cannabinoid agonists enhance the release of

endogenous opioids (Mason, Lowe, & Welch, 1999; Pugh et al. 1997; Valverde et al. 2001), WIN most likely induces elevated levels of endogenous opioids which then act on opioid receptors. The addition of ultra-low dose naltrexone enhances the analgesic effect of WIN presumably because naltrexone preferentially antagonizes the opioid receptors that are coupled to stimulatory G-proteins, allowing for the elevated endogenous opioids to enhance CB-induced antinociception. If this is true, our study may be the first indirect evidence that ultra-low dose opioid antagonists enhance the effects of endogenous opioids.

An alternative explanation for the WIN-induced tail-flick latency enhancement by ultra-low dose naltrexone may be that naltrexone interferes with WIN metabolism. Because this experiment is a behavioural analysis of the effects of ultra-low dose naltrexone on WIN-induced antinociception, any mechanistic explanation for the reported effects are speculative. The important point is that high dose naltrexone did not significantly affect WIN-induced tail-flick latencies. Thus, if naltrexone interferes with WIN metabolism, it only does so in ultra-low dose concentrations.

Our data may not reflect a pure analgesic enhancement of WIN by ultra-low dose naltrexone because measures of antinociception in the tail-flick test are affected by motor processes. The tail-flick analgesia meter relies on the rat's ability to move its tail away from the heat stimulus, and cannabinoids have strong inhibitory control over measures of motor functioning like locomotor activity and catalepsy (Compton, Aceto, Lowe, & Martin, 1996; Cosenza et al. 2000; Varvel et al. 2005). Our demonstration of greater tail-flick latencies in animals receiving the combination treatment may therefore reflect greater motor impairments and not greater analgesia. We are currently testing this

hypothesis. Regardless of the interpretation (analgesia or motor impairment), the uniqueness of this ultra-low dose opioid-cannabinoid interaction is still noteworthy.

Whether ultra-low dose opioid antagonists will be useful clinically is still up for debate. The few studies investigating ultra-low dose opioid antagonists on morphine-induced analgesia in humans have produced conflicting results (Cepeda et al. 2002, 2004; Gan et al. 1997; Joshi et al. 1999; Sartain et al. 2003). These conflicting findings may be due to a number of factors. For example, administration of the ultra-low dose antagonist differed (i.e., as part of the morphine patient controlled administration injection, or as a separate injection or infusion), and there was no consistency in selecting the effective ultra-low dose of the antagonist. Another limitation of these clinical studies is the lack of dose response relationship with ultra-low dose antagonists, and all of these studies deal with postoperative pain. In contrast, recent research examining the effects of opioid agonist-antagonist combinations on osteoarthritic pain has produced promising results (L.H. Burns, personal communication, June 2005). Taken together, we feel that the clinical research on this topic is still in its infancy, and it is too soon to draw any firm conclusions as to whether the effects of ultra-low dose antagonists are unique to rodents.

This is the first study to show an opioid-cannabinoid interaction at the ultra-low dose level. Ultra-low dose naltrexone likely enhances the effects of endogenous opioids that are released as a result of the WIN administration. As medicinal cannabinoid drugs are being discovered and marketed, this combination treatment may be useful to enhance therapeutic effects of these agents.

Chapter 3. Experiment 2

**Cannabinoid-induced tolerance is associated with a CB1 receptor G-protein
coupling switch that is prevented by ultra-low dose rimonabant**

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Published in

Behavioural Pharmacology, in press.

3.0. Abstract

The analgesic effect of opioids is enhanced, and tolerance is attenuated, by ultra-low doses (nanomolar to picomolar) of an opioid antagonist, an effect that is mediated by preventing the receptor from coupling to Gs-proteins. Recently, we demonstrated a cannabinoid-opioid interaction at the ultra-low dose level, suggesting that the effect may not be specific to opioid receptors. The purpose of the present study was to examine, both behaviourally and mechanistically, whether the cannabinoid CB1 receptor is also sensitive to ultra-low dose effects. Antinociception was tested in rats following an injection of vehicle, the CB1 receptor agonist WIN 55 212-2 (WIN), ultra-low doses of the CB1 receptor antagonist rimonabant (SR 141716), or a combination of WIN and ultra-low dose rimonabant. In the acute experiment, tail-flick latencies were recorded at 10-min intervals for 90 min; in the chronic experiment, tail-flick latencies were recorded 10 min after a daily injection across 7 days. Ultra-low dose rimonabant extended the duration of WIN-induced antinociception. WIN produced maximal tolerance by day 7 whereas WIN + ultra-low dose rimonabant continued to produce strong antinociception, demonstrating that ultra-low dose rimonabant prevented the development of WIN-induced tolerance. Animals chronically treated with WIN alone had CB1 receptors predominately coupling to Gs receptors in the striatum, whereas vehicle, ultra-low dose rimonabant and WIN + ultra-low dose rimonabant groups had CB1 receptors predominately coupling to Gi receptors. Thus, cannabinoid-induced tolerance is associated with a G-protein coupling switch from the inhibitory Gi-protein, to the excitatory Gs-protein, an effect which is prevented by ultra-low dose rimonabant.

3.1. Introduction

Agonists of the cannabinoid CB1 receptor (CB1R) are effective analgesics in a variety of pain tests. These include acute pain induced by thermal, electrical, or mechanical stimulation, persistent/chronic pain induced by formalin or capsaicin administration, as well as spinal nerve injury models of neuropathic pain (Pertwee, 2001). CB1R agonists produce their effects through activation of guanine nucleotide regulatory protein (G-protein)- coupled CB1Rs (Devane et al. 1988). *In vitro* application of nM- μ M concentrations of CB1R agonists stimulates CB1Rs, resulting in activation of pertussis toxin-sensitive Gi proteins (Howlett et al. 1986; Prather et al. 2000; Mukhopadhyay & Howlett, 2005). Activation of this G-protein by cannabinoid agonists typically leads to inhibition of adenylyl cyclase and reduced production of the second messenger cyclic adenosine monophosphate (cAMP) (Howlett & Flemming, 1984; Howlett, 1985; Wade et al. 2004).

Under certain circumstances, the CB1R may activate Gs proteins, resulting in increased adenylyl cyclase production of cAMP (Glass & Felder, 1997; Calandra et al. 1999). In these situations, CB1Rs may be coupling predominately to stimulatory G-proteins thereby exerting effects in the opposite direction to those predicted by traditional (G α i-mediated) CB1R activation. This may explain the *in vivo* biphasic effects of the endocannabinoid anandamide: systemically administered doses of anandamide in the μ M/kg range produce hypolocomotion and analgesia, but nM/kg doses produce hyperlocomotion and near hyperalgesia (Sulcova et al. 1998). Receptor activation of stimulatory G-proteins may also explain the antagonistic effects of low dose anandamide on behavioural and intracellular effects of cannabinoid agonists (Fride et al. 1995).

Opiates, such as morphine, also produce biphasic effects on neurons and measures of pain. For instance, μM doses of morphine produce analgesia and dorsal root ganglion action potential duration shortening, whereas ultra-low doses (pM-nM range) produce hyperalgesia and dorsal root ganglion action potential duration lengthening (Crain & Shen, 1990a, b; Shen & Crain, 1992). The finding that morphine analgesia could be paradoxically enhanced by ultra-low dose opioid antagonists led to the hypothesis that there is a subpopulation of high affinity μ -opioid receptors that couple to stimulatory G-proteins, and that they could be preferentially blocked by ultra-low dose antagonists (Crain & Shen, 1992). This hypothesis is further supported by reports demonstrating that morphine-induced somatic withdrawal, and the development of analgesic tolerance are attenuated by ultra-low dose naltrexone or naloxone (Crain & Shen, 1995; Powell et al. 2002; Wang et al. 2005). The molecular underpinnings of this ultra-low dose opioid effect involve a prevention of excitatory signaling of opioid receptors, mediated by a switch in G_i/o to G_s coupling that occurs during chronic administration of the opiate alone (Wang et al. 2005).

Opioid and cannabinoid agonists produce similar behavioural effects (Dewey, 1986; Olson et al. 1998), synergistic properties in antinociception (Smith et al. 1998; Cichewicz, 2004), affect tolerance (Thorat & Bhargava, 1994; Rubino et al. 1997; Cichewicz & Welch, 2003), and exhibit cross-precipitated withdrawal by cannabinoid and opioid antagonists, respectively (Navarro et al. 2001). These studies provide convincing evidence for an opioid-cannabinoid interaction (Manzanares et al. 1999; Maldonado & Valverde, 2003) and provide a rationale for studying opioid-cannabinoid interactions at the ultra-low dose level. Our initial study confirmed that ultra-low doses

of the opioid antagonist naltrexone enhance the analgesic potency of the cannabinoid agonist, WIN 55,212-2 mesylate (WIN), in the tail-flick test (Paquette & Olmstead, 2005). Here, we examine whether the ultra-low dose phenomenon extends to the cannabinoid system alone by testing whether the analgesic properties of WIN are altered by ultra-low dose co-administration of the CB1R antagonist, rimonabant (previously named SR 141716). Given that ultra-low dose naltrexone blocks the development of tolerance and reverses established tolerance to the analgesic effect of morphine (Crain & Shen, 1995; Powell et al. 2002), we also tested whether tolerance to repeated WIN administration is blocked by ultra-low dose rimonabant co-treatment. We investigated the molecular underpinnings of this latter effect by examining cannabinoid receptor coupling to Gi and Gs proteins in the striatum of rats chronically treated with vehicle, WIN, WIN plus ultra-low-dose rimonabant or rimonabant alone.

3.2. Experimental Procedures

3.2.1. Subjects

Male Long-Evans rats (N = 112) from Charles River (Montreal, QC, Canada) ranging from 235-400 g, were housed in polycarbonate cages in pairs and given free access to food (Lab Diet, PMI Nutrition International, Inc., Brentwood, MO, USA) and water. Animal quarters were kept on a reverse light-dark cycle (lights on from 7 pm to 7 am) and maintained at 22 ± 2 °C and 45 ± 20 % relative humidity. Animals were given a minimum of 3 days to acclimatize to the animal quarters prior to the experiment. All procedures were approved by Queen's University Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care and the Animals for Research Act.

3.2.2. *Drugs and Administration*

All injections were administered in a volume of 1 ml/kg. All chemicals were dissolved in 5% dimethyl sulfoxide (DMSO; Sigma, Oakville, ON, Canada), 0.3% polyoxyethylenesorbitan monooleate (Tween® 80; Sigma, Oakville, ON, Canada) and saline vehicle, and administered intravenously in the posterior 1/3 of the lateral tail vein.

3.2.2.1. *Single injection testing*

Vehicle alone was used as a control injection (n=8). The non-specific CB1R agonist WIN 55 212-2 [(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; Tocris Cookson, Ellisville, MO, USA), was administered alone at doses of 62.5 and 93.75 µg/kg (these doses are referred to as 60 and 90 µg/kg, respectively, throughout this paper for simplicity of reporting). These doses were chosen because they were previously shown to produce sub-maximal antinociception following intravenous administration in the tail-flick test (Meng et al. 1998; Paquette & Olmstead, 2005).

The CB1R antagonist rimonabant [N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] was generously donated by the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Bethesda, MD, USA). Ultra-low doses of rimonabant (550 or 55 pg/kg) were combined with WIN (60 µg/kg) and administered as a single injection. These combinations produce WIN to rimonabant molar ratios of 100 000:1 and 1 000 000:1, respectively. Also, WIN (90 µg/kg) was combined with ultra-low doses of rimonabant (830 or 83 pg/kg) producing WIN to rimonabant molar ratios of 100 000:1 and 1 000 000:1, respectively. The control group received 550 and 830 pg/kg of rimonabant alone.

3.2.2.2. *Repeated injection testing*

Vehicle alone was used as a control injection (n=8). WIN was administered alone at doses of 125 µg/kg. This dose was chosen because preliminary data demonstrated that this dose produces maximal antinociception following intravenous administration in the tail-flick test (Meng et al. 1998; Paquette & Olmstead, 2005). Ultra-low doses of rimonabant (1.1 ng/kg or 110 pg/kg) were combined with WIN (125 µg/kg) and administered as a single injection. These combinations produce WIN to rimonabant molar ratios of 100 000:1 and 1 000 000:1, respectively. The control group received 1.1 ng/kg of rimonabant alone.

3.2.3. *Apparatus*

The tail-flick apparatus consists of a projection lamp that creates radiant heat located just below the animal-testing surface (D'Amour & Smith, 1941). The light from the lamp projected through a small hole in the testing surface and was aimed at a photocell located 25 cm above the testing surface. A digital timer, connected to the apparatus, started when the heat source was activated. When the animal flicked its tail away from the heat source, the light from the projection lamp activated the photocell, simultaneously stopping the timer and turning off the lamp. The heat intensity was calibrated to result in baseline tail-flick latencies of 2-3 s and a 10 s cutoff was used to minimize tissue damage.

3.2.4. *Nociceptive Testing*

Nociceptive reflexes to a thermal stimulus were tested using the tail-flick antinociceptive meter. This apparatus focuses a hot beam on the animal's tail. The time it takes for the rat to flick its tail away from the heat source is a measure of pain; the

longer the animal leaves its tail on the hotspot, the greater the degree of pain relief. On the day prior to tail-flick testing, animals were handled on the tail-flick apparatus for 5-10 min to reduce stress-induced analgesia (Terman et al. 1984; Kelly & Franklin, 1985). For single injection tested groups, animals were restrained in a small towel and a baseline tail-flick latency was measured. Following the baseline measure, animals were given a drug injection and tail-flick latencies were assessed every 10 min for 90 min, similar to previous protocols (Meng et al. 1998; Damaj, Glassco, Aceto, & Martin, 1999; Powell et al. 2002; Paquette & Olmstead, 2005). For the repeated injection tested groups, animals were given one drug injection every day for seven days. Prior to the first injection, a baseline tail-flick latency was measured. The baseline latency was only given on the first day. Following the baseline measure, animals were given a drug injection and tail-flick latencies were assessed 10 min post-injection. This time point was selected from preliminary data showing maximal WIN-induced antinociception in this protocol. Post injection tail-flick latencies were assessed on days 1, 3, 5, and 7. For all animals, tail-flick latencies were converted into a percent of maximal possible effect (MPE) using the equation:

$$\text{MPE} = \left[\frac{(\text{post- injection latency} - \text{baseline latency})}{(10 \text{ s cutoff} - \text{baseline latency})} \right] \cdot 100$$

3.2.5. *Tissue Sampling*

On the day following the last injection, animals were sedated with CO₂, and then decapitated. The brain was quickly extracted on ice, and a sample of the striatum was extracted, immersed in liquid nitrogen, and stored at -80 °C until the receptor coupling assay and Western blotting could be performed. Striatal tissue punches were extracted by

first taking a coronal section using a coronal slice rat brain matrix (VWR International Ltd; Mississauga, Ontario, Canada) that was kept cold with packed dry ice. Sectioning blades were then placed approximately 1.0 mm anterior and 0.8 mm posterior to bregma to obtain a full coronal section of the rat brain (Paxinos & Watson, 1998). This section was placed anterior side up on glass that was kept cold on dry ice. A blunted 16 gauge needle (1.2 mm inner diameter; VWR International Ltd; Mississauga, Ontario, Canada) was used to obtain a cylindrical section of the right striatum with approximate borders from bregma of 4.1 to 2.9 lateral and medial, respectively, and 4.6 to 5.8 mm dorsal and ventral, respectively (Paxinos & Watson, 1998).

3.2.6. *CB1R-G protein coupling*

To investigate the linkage between CB1Rs and G proteins directly, synaptic membranes were prepared from striatal tissue. Enriched synaptic membranes (200 µg) were incubated with 1 µM methanandamide in Tocrisolve™100 (Tocris Bioscience, Ellisville, MO), a CB1R agonist for 5 min at 37°C in Krebs's Ringer solution or vehicle (Tocrisolve™100). Membranes were solubilized in immunoprecipitation buffer (25 mM HEPES, pH7.5; 200 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.2% 2-mercaptoethanol, 50 µg/ml leupeptin, 25 µg/ml pepstatin A, 0.01 U/ml soybean trypsin inhibitor, 0.04 mM phenylmethylsulfonyl fluoride [PMSF]) containing 0.5% digitonin, 0.2% sodium cholate and 0.5% NP-40 at 4°C with end-over-end shaking for 60 min. After centrifugation at 50,000 X g for 5 min to remove insoluble debris, the obtained supernatant was used to assess CB1R-G protein coupling by co-immunoprecipitation of CB1Rs and G proteins. The protein concentrations in supernatant were determined using the Bradford method according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Similar to the methodology described in our previous publications (Wang et al. 2005; Wang & Burns, 2006), G protein-coupled CB1R was immunopurified together with its associated G protein using immobilized anti-G α antibodies to prevent interference from immunoglobulins. Anti-G α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were covalently cross-linked to protein-A conjugated resin in Seize-X protein A immunoprecipitation kit (Pierce-ENDOGEN, Rockford, IL) according to the manufacturer's instructions. CB1R-G protein complexes in solubilized brain lysates were isolated by immunoprecipitation in which 165 μ g solubilized brain membrane extracts were incubated with immobilized anti-G α -protein A-resin at 4°C overnight. After centrifugation and three washes with 25 mM Na-HEPES-buffered saline (pH 7.4) at 4°C, the CB1R-G protein complexes were eluted with 200 μ l of the neutral pH buffer antigen elution buffer (Pierce-ENDOGEN, Rockford, IL). To achieve complete solubilization of the obtained proteins, the eluate was combined with 35 μ l of 6X polyacrylamide gel electrophoresis (PAGE) sample preparation buffer [62.5 mM Tris-HCl, pH6.8; 60% glycerol, 12% sodium dodecyl sulfate (SDS); 30% 2-mercaptoethanol, 0.3% bromophenol blue] and boiled for 5 min. The level of specific G protein-associated CB1Rs in 50% of anti-G α column eluate was determined by Western blotting using a specific antibody directed against the amino-terminal region of the CB1R (Santa Cruz Biotechnology). The blots were then stripped and re-probed with a mixture of anti-G α antibodies to assess the efficiency of immunoprecipitation and loading.

Specificities of the four anti-G α antibodies have been extensively characterized and shown in our published work (Wang et al. 2005). Other than minor cross-reactivity between G α i and G α o, these anti-G α antibodies detect only their respective target

proteins. Likewise, the specificity of the anti-CB1R was demonstrated here by pre-adsorption of anti- CB1R with 25 μ g antigen peptides for CB1R and CB₂ receptor (Santa Cruz Biotechnology, Santa Cruz, CA) individually. The result showing that CB1R but not CB2 receptor antigen peptides nearly abolished the detection of CB1Rs indicates the relative specificity of anti- CB1R for the intended target (figure 3.1.). An additional control experiment showing that anti- CB1R immunoprecipitate contains [³H]WIN55212-2 but not [³H]DAMGO (a μ -opioid receptor agonist) or [³H]SCH23390 (a D₁-dopamine receptor antagonist) binding capacity further support the notion that a 75-kDa protein that is recognized exclusively by the anti- CB1R antibody is the functional, glycosylated form of the receptor.

3.2.5.1. *Western blot analysis*

Striatal membranes were prepared as described above and protein concentration was determined by the Bradford method. Membranes were solubilized by boiling for 5 min in SDS-PAGE sample preparation buffer. A 20- μ g aliquot of solubilized membranes was size-fractionated on 10% or 12% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes. The membranes were washed with phosphate buffered saline (PBS) and blocked overnight at 4°C with 10% milk followed by washing with PBS with 0.1% Tween-20 (PBST) and incubation at room temperature for 2 hrs with antibodies against specific G α proteins (separately, at 1:1,000 dilutions) and anti- CB1R receptor antibodies (1:500 dilution). After washing, membranes were incubated for 1 hr with anti-rabbit IgG-HRP (1:5,000 dilution) and washed with 0.3% PBST followed by washing with 0.1% PBST. Immunoreactivity was detected by reacting with enhanced chemiluminescent reagent (Pierce-ENDOGEN) for exactly 5 min and immediately

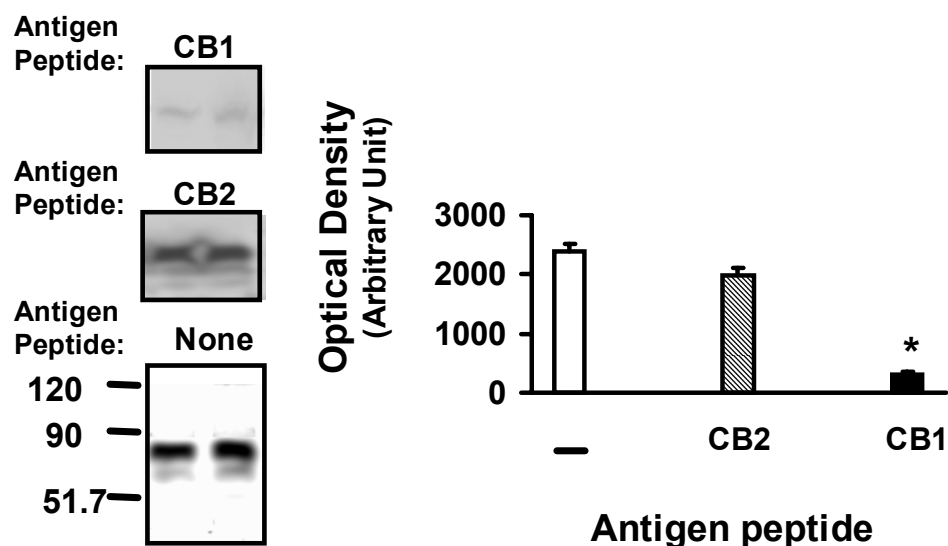


Figure 3.1. CB1R antibody is relatively specific to the targeted cannabinoid receptor subclass. The specificity of anti-CB1R antibodies were evaluated by Western blotting with CB1R antibodies that have been pre-absorbed with antigen peptides specific for CB1 or CB2 receptors. The bottom blot shows the detection of CB1R by anti- CB1R antibodies. The blots were stripped and re-probed with the same antibodies that were pre-absorbed for 30 min at 25°C with 25 µg of the CB2 antigen peptide [middle blots]. The blots were stripped once again and re-probed with the same anti-CB1R antibodies after they were pre-absorbed for 30 min at 25°C with 25 µg of CB1R antigen peptides [top blots]. The obtained blots were quantified using densitometric scanning. The blots shown are the representative of 4 individual experiments each using 50 µg of solubilized striatal membranes/lane in duplicate. The specificity of the anti- CB1R antibody is supported by the demonstration that pre-adsorption with the CB1R but not CB2 receptor antigen peptides reduced the detection of the intended CB1R proteins by 87.6 %.

*p < 0.01, Student's t-test.

exposing to X-ray film. Specific bands were quantified by densitometric scanning (GS-800 calibrated densitometer, Bio-Rad Laboratories) and the optical intensity in arbitrary units for each of the protein bands was recorded and summarized.

To ascertain even loading, blots were stripped and re-probed with anti- β -tubulin (Chemicon). Immunoreactivity was similarly detected using the chemiluminescent method and quantified by Densitometric scanning. The quantitative data of G α proteins are normalized according to the β -tubulin signal and expressed as the ratios of optical intensity of G α to β -tubulin.

3.2.6. Statistics

Separate two-way repeated measure analysis of variance (ANOVA) tests were used to analyze tail-flick latencies in the single and repeated injection studies. Drug group was a between-subjects factor in both studies; post-injection time (10-90 min) and day of testing (days 1-7) were the within-subjects factors for the single and repeated studies, respectively. Whenever there were violations of sphericity, P-values from the Greenhouse-Geisser correction to the within-group degrees of freedom were reported for all ANOVA tests. Because many drug group comparisons were irrelevant, *a priori* multiple comparisons were used to analyze the main drug effect and the interaction using the Bonferroni *t* test.

All quantitative data derived from CB1R-G protein coupling experiment and Western blotting are presented as mean \pm standard error from the mean. Treatment effects were evaluated by two-way ANOVA followed by Newman-Keul's test for multiple comparisons. Two-tailed Student's *t* test was used for *post hoc* pairwise comparisons. The threshold for significance was $p < 0.05$.

3.3. Results

3.3.1. Tail-flick Latencies Following an Acute Injection

The post-injection tail-flick measures (MPE) taken across 90 min for each drug group are shown in figure 3.2. When all drug groups were combined, a main effect of time [F(8,504)=66.64, P<0.001] was revealed, whereby MPE values decreased across time. Furthermore, there was a main effect of drug [F(8,63)=16.00, P<0.001], and a drug X post-injection time interaction, [F(64,504)=7.62, P<0.001].

There were a total of 8 *a priori* multiple comparisons tests performed, producing a critical *t*-value of 3.30. WIN alone produced a dose-dependent effect on tail-flick latencies as demonstrated by the fact that the MPE for the WIN (90 µg/kg) group was significantly different from the vehicle [*t*(14)=4.63, P<0.01] group, whereas the WIN (60 µg/kg) group was not [*t*(14)=2.63]. Most importantly, the MPE of the WIN alone (60 µg/kg) group was significantly different from the WIN (60 µg/kg) plus rimonabant (550 pg/kg) group [*t*(14)=6.72, P<0.05], and the WIN (90 µg/kg) group was significantly different from the WIN (90 µg/kg) plus both rimonabant (830, and 83 pg/kg) groups [*t*(14)=9.63, P<0.01 and *t*(14)=8.16, P<0.01, respectively]. As can be seen in figure 3.2.A and B, these combination treatment groups showed prolonged antinociception compared to their respective WIN alone control groups, although the analgesic potency at later time points is relatively minor. The ultra-low dose rimonabant effect is dose dependent since there was no significant difference between the WIN (60 µg/kg) group and the WIN (60 µg/kg) plus rimonabant (55 pg/kg) group [*t*(14)=3.28]. The combination treatment effects occurred despite the fact that there were no significant

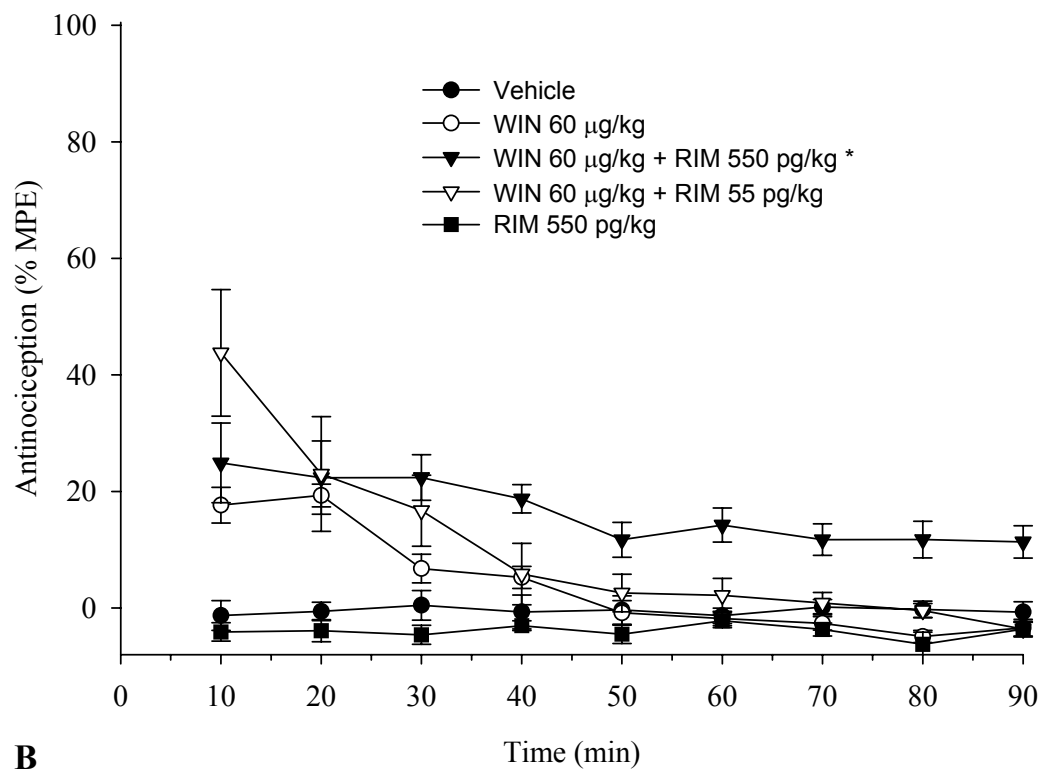
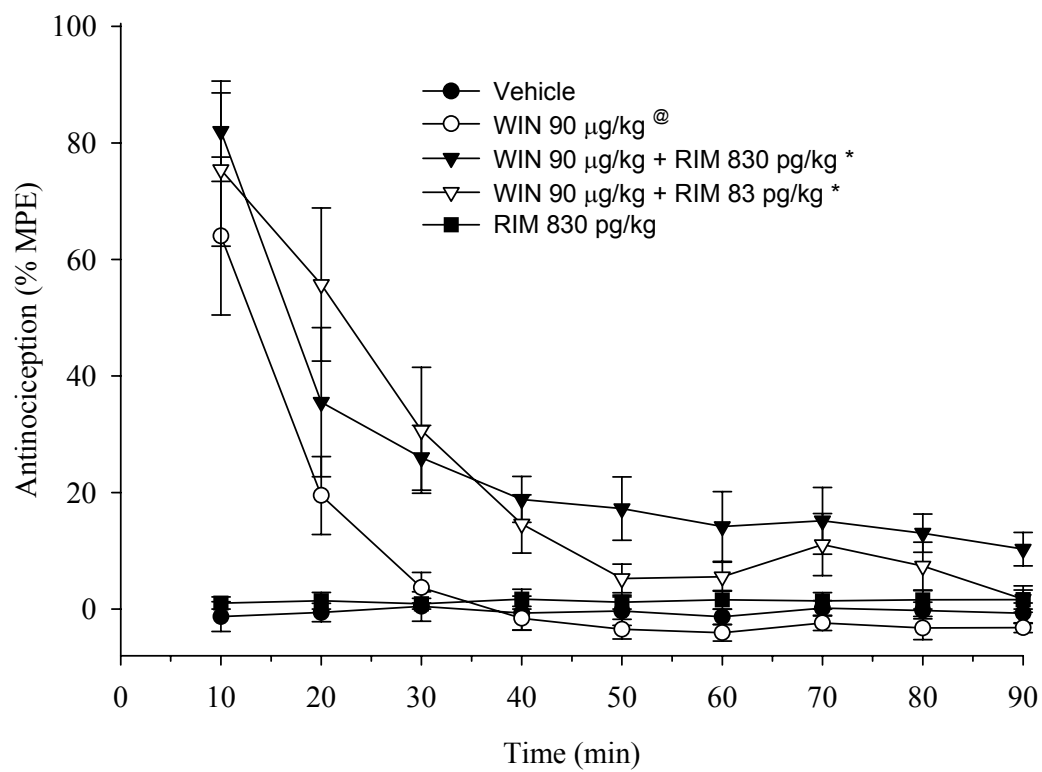
A**B**

Figure 3.2. Ultra-low dose rimonabant enhances WIN-induced antinociception. The acute antinociceptive properties of 60 $\mu\text{g}/\text{kg}$ WIN (Fig. 3.2.A) and 90 $\mu\text{g}/\text{kg}$ WIN (Fig. 3.2.B) are enhanced by ultra-low dose rimonabant. Following a single i.v. injection, tail-flick latencies were tested every 10 min for 90 min. Group mean (\pm SEM) antinociception is represented as percentage MPE. Symbols indicate between-group differences resulting from the main effect of drug analyses. $n=8$ per group.

@ Significantly different from vehicle

* Significantly different from WIN alone.

WIN = WIN 55 212-2 mesylate; RIM= rimonabant; MPE = maximal possible effect.

differences between the rimonabant alone (550 and 830 pg/kg) and the vehicle groups [$t(14)=1.59$, and $t(14)=1.00$, respectively].

3.3.2. Tail-flick Latencies Following Repeated Injections

The post-injection tail-flick measures (MPE) taken on days 1, 3, 5, and 7 for each drug group are shown in figure 3.3. When all drug groups were combined, a main effect of injection day [$F(3,105)=59.36$, $P<0.001$] was revealed, whereby MPE values decreased across days. Furthermore, there was a main effect of drug [$F(4,35)=73.05$, $P<0.001$] and a drug X injection day interaction, [$F(12,105)=13.92$, $P<0.001$].

Four *a priori* multiple comparisons tests were performed on the main drug effect data. WIN (125 $\mu\text{g}/\text{kg}$) alone produced greater antinociception than vehicle [$t(14)=10.44$, $P<.01$]. More importantly, both the WIN (125 $\mu\text{g}/\text{kg}$) plus rimonabant (1.1 ng/kg and 110 pg/kg) groups displayed greater antinociception compared to the WIN alone (125 $\mu\text{g}/\text{kg}$) group [$t(14)=9.77$, $P<0.01$ and $t(14)=14.35$, $P<0.01$, respectively]. The combination treatment effects occurred despite the fact that there was no significant difference between the rimonabant alone (1.1 ng/kg) group and the vehicle group [$t(14)=0.02$].

Four additional *a priori* contrasts were performed that describe the injection X day interaction. On injection day one, WIN (125 $\mu\text{g}/\text{kg}$) alone produced near maximal antinociception; antinociception in this group was significantly different from the vehicle group [$t(14)=16.54$, $P<0.01$], which displayed baseline-like tail-flick latencies. By injection day 7, however, WIN produced tail-flick latencies at baseline values that were not significantly different from vehicle treatment [$t(14)=0.05$]. The combination treatment (both groups combined) produced near maximal antinociception on injection

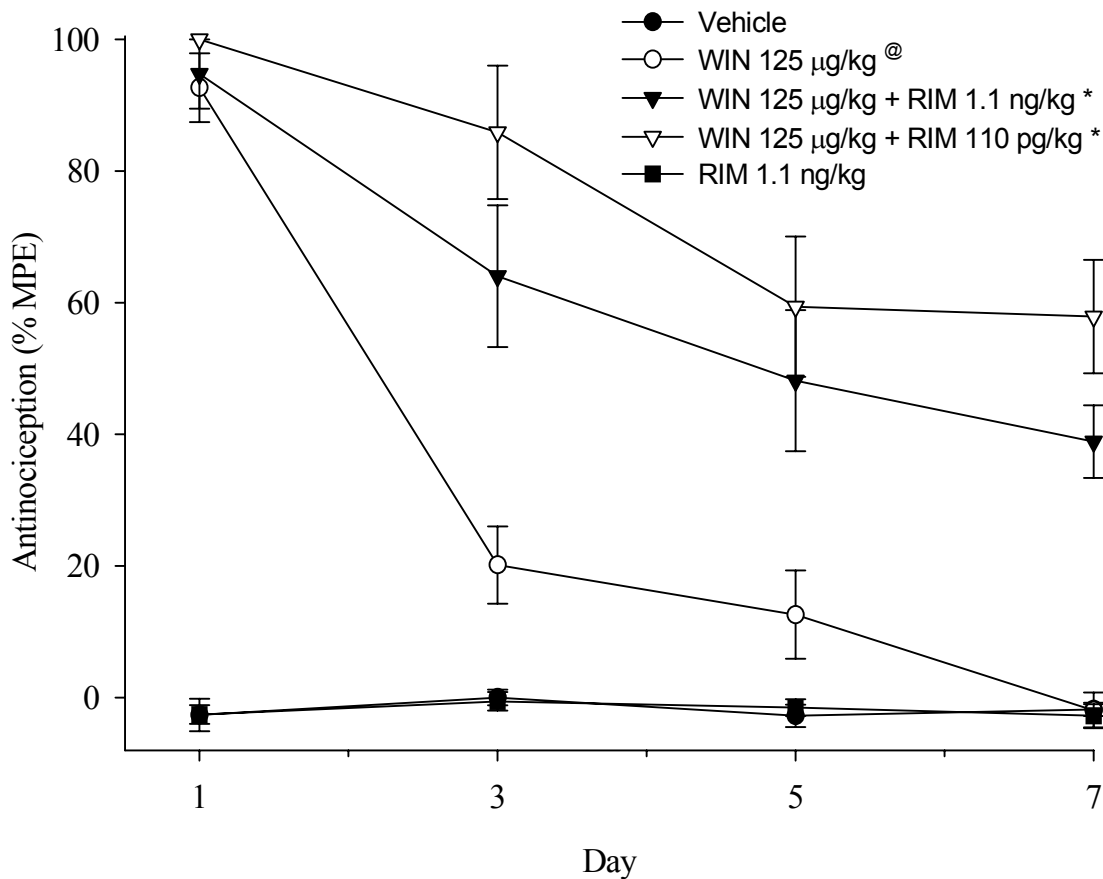


Figure 3.3. Ultra-low dose rimonabant attenuates the development of tolerance to WIN-induced antinociception. Antinociceptive tolerance is attenuated dose-dependently by ultra-low dose rimonabant. Rats received one i.v. injection per day for seven days; tail-flick latencies were assessed 10 min post-injection. Group mean (\pm SEM) antinociception is represented as percentage MPE using the tail-flick test. Symbols indicate between-group differences resulting from the main effect of drug analyses. $n=8$ per group.

@ Significantly different from vehicle

* Significantly different from WIN alone.

WIN = WIN 55 212-2 mesylate; RIM = rimonabant; MPE = maximal possible effect.

day 1 that was not significantly different from WIN alone [$t(22)=0.90$]. By injection day 7, however, the combination treatment groups displayed longer tail-flick latencies compared to the WIN alone group [$t(20)=8.19$, $P<0.01$]¹. It should be noted, however, that the combination treatment groups displayed longer tail flick latencies on injection day 1 compared to injection day 7 [$t(30)=8.01$, $P<0.01$]. Thus, the combination treatment group demonstrated some tolerance to the repeated injection regime, albeit much reduced compared to WIN alone group.

3.3.3. Co-immunoprecipitation of CB1R-G Protein Complexes

To determine whether cannabinoid agonist-induced analgesic tolerance is associated with alterations in CB1R-G protein coupling, and whether ultra-low-dose CB1R antagonists affect such changes, we isolated CB1R-expressing CNS tissue from rats receiving chronic injections of vehicle, 125 $\mu\text{g}/\text{kg}$ WIN, 1.1 ng/kg rimonabant or 125 $\mu\text{g}/\text{kg}$ WIN + 1.1 ng/kg rimonabant. Under non-denaturing conditions that keep CB1R-G protein complexes intact, specific G proteins (Gi and Gs/olf) together with their coupled receptors were immunoprecipitated with selective anti-G α antibodies from solubilized synaptic membranes obtained from striatum of the four different treatment groups ($n=6$), under both basal and methanandamide-stimulated conditions. Similar to other G-protein coupled receptors that recruit G-proteins when stimulated (Jin, Wang, & Friedman, 2001; Wang et al. 2005), *in vitro* methanandamide stimulation consistently increased CB1R-Gi protein coupling well above basal levels, although an identical pattern of CB1R-G protein coupling was observed under both conditions.

¹ This comparison was subjected to a Welch-Satterthwaite correction to the degrees of freedom due to a violation of homogeneity of variance and unequal sample sizes.

The relative amounts of CB1Rs coupling to each of the G protein subtypes in the four treatment groups are shown in Western blots of the G α immunoprecipitates probed with the CB1R-specific antibody (figure 3.4.). In the striatum, CB1Rs coupled exclusively to Gi in vehicle- and rimonabant -treated rats, and to both Gi and Gs in WIN-treated rats. The Gi-coupled CB1R was, however, decreased in the chronic WIN-treated group compared to the vehicle group ($p < 0.01$; figure 3.4.A and B). In striatum of rats treated with WIN + rimonabant, coupling to Gs was markedly decreased from that in the WIN-treated animals, whereas coupling to Gi was increased toward control levels (figure 3.4.A and B). CB1R coupling to Go or Gq/11 G-proteins was not detected in any of the treatment groups (data not shown). There were no discernible changes in the immunoprecipitation efficiency and loading as demonstrated by similar levels of G α proteins were immunoprecipitated with respective immobilized anti-G α antibodies (data not shown). The alterations in G-protein coupling were not due to changes in expression of either CB1R or G α proteins as comparable G α and CB1R levels were detected in all treatment groups (data not shown).

3.4. Discussion

Here, we demonstrate that CB-induced antinociceptive effects are prolonged by ultra-low doses of a CB1R antagonist, rimonabant, with no effect on antinociceptive potency. Furthermore, co-application of ultra-low doses of rimonabant dramatically attenuated the development of CB-induced tolerance. Using CB1R-enriched striatal tissue, we found that chronic cannabinoid agonist treatment leads to a switch in CB1R G-protein association, from inhibitory Gi- to excitatory Gs-proteins, in CB-tolerant rats. This CB1R coupling switch was likewise attenuated by co-administration of ultra-low

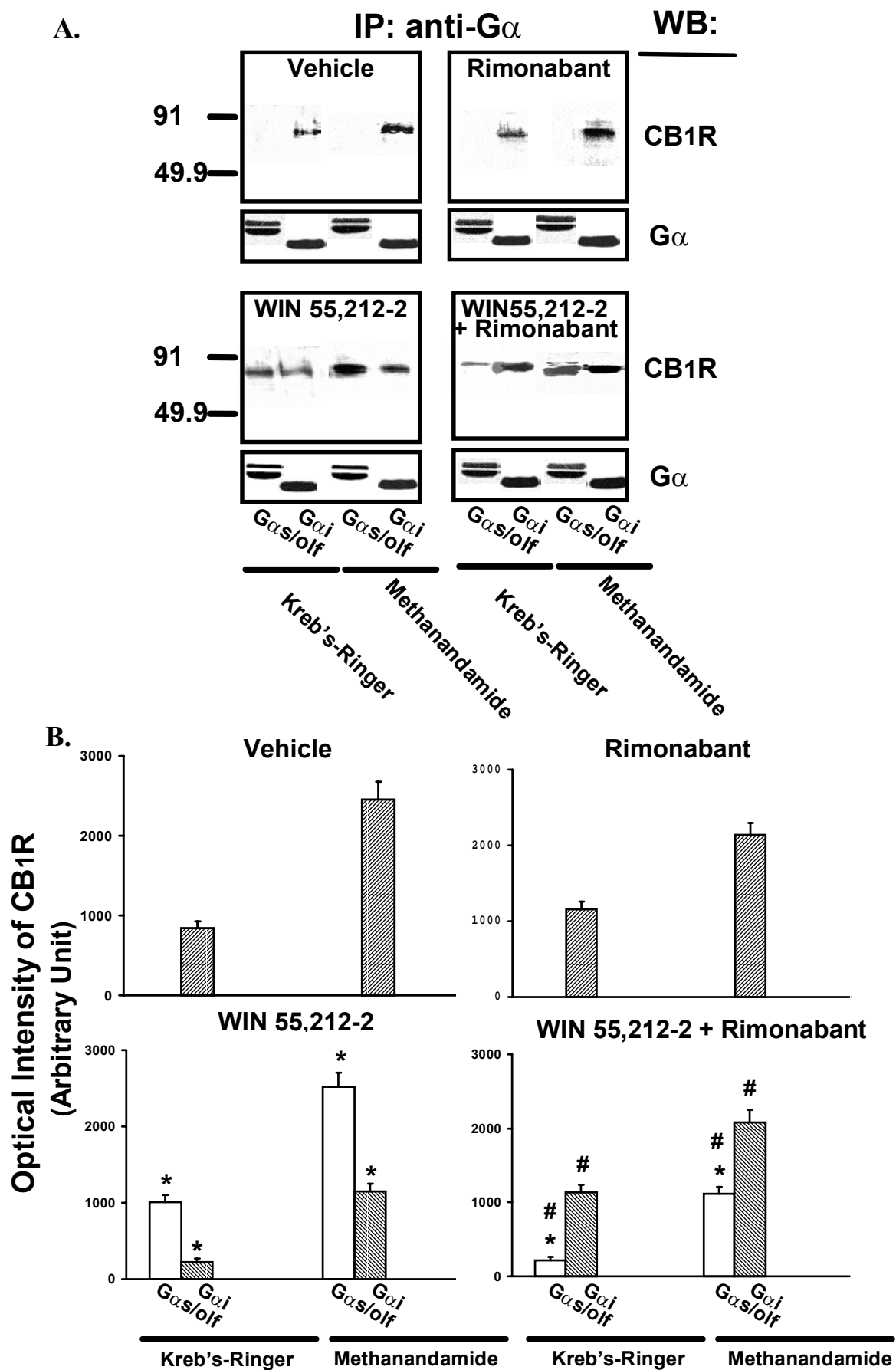


Figure 3.4. Chronic WIN-induced Gs– CB1R coupling is attenuated by co-treatment with ultra-low-dose rimonabant as demonstrated by co-immunoprecipitation of G α proteins with CB1R. A. The representative blots show that CB1R protein detected in immunoprecipitates of G α i and G α s/olf of striatum from rats treated chronically with saline, WIN (0.125 mg/kg), WIN + rimonabant (1.1 ng/kg, i.v.) or rimonabant alone (1.1 ng/kg, i.v.). Striatal membranes were incubated with vehicle or 1 μ M methanandamide, solubilized and the G protein-coupled CB1Rs were immunoprecipitated using indicated immobilized anti-G α antibodies as described in Method section in detail. The level of CB1Rs in 50 % of G α immunoprecipitants obtained from each *in vivo* and *ex vivo* treatment conditions was determined by Western blotting with specific anti-CB1R. The blots were stripped and re-probed with a mixture of antibodies against various G α proteins, demonstrating similar amounts of G α protein precipitated regardless of *in vitro* methanandamide exposure or *in vivo* treatments. B. Densitometric quantification of CB₁R protein bands of blots shown in (a) and five additional individual experiments that each used striata from one rat in each of four treatment groups. Data are expressed as means (\pm SEM) of optical intensity. $n=6$ striata from rats in each of the four treatment groups. * $P<0.01$ versus same G α protein in vehicle and rimonabant-treated group. # $P<0.01$ versus same G α protein in WIN-treated group. Methanandamide-stimulated coupling was significantly greater ($P<0.01$) than basal coupling for each G α protein within each treatment group. WIN, WIN 55 212-2 mesylate.

* $p < 0.01$ versus same G α protein in vehicle and rimonabant-treated group.

$p < 0.01$ versus same G α protein in WIN-treated group. Methanandamide-stimulated coupling was significantly greater ($p < 0.01$) than basal coupling of each G α protein within each treatment group.

doses of a CB1R antagonist. Thus, the biochemical data may provide a mechanistic explanation for the behavioural data.

The most notable finding in this study is that chronic exposure to a cannabinoid agonist results in a switch in coupling of CB1R from the Gi to the Gs subtype. Although previous reports have suggested that the CB1R can exert excitatory influences on the cell (Glass & Felder, 1997; Sulcova et al. 1998; Hampson, Mu, & Deadwyler, 2000), our findings presented here directly demonstrate CB1R can couple to stimulatory Gs-proteins. This is important because it shows that CB1R to G-protein coupling profiles can change as a result of repeated agonist administration. Considering that these signaling changes occur concurrent with functional changes, it is conceivable that the G-protein coupling switch in CB1R can result in behavioural adaptations such as drug tolerance.

Our investigation of ultra-low dose cannabinoid antagonist effects is an extension of Crain and Shen's original finding on opioid-induced tolerance and hyperalgesia (Crain & Shen, 1995). This work demonstrated that the analgesic effects of morphine are more potent when the drug is co-administered with an ultra-low dose opioid antagonist, and that this combination prevents the development of morphine-induced tolerance. Subsequent studies indicated that the mechanism underlying this ultra-low dose effect involves the blockade of an increase in excitatory signaling by the μ -opioid receptor with chronic opioid treatment (Wang et al. 2005). Our original study (Paquette & Olmstead, 2005) extended the ultra-low dose antagonist effect by demonstrating that a cannabinoid agonist could be made more potent by co-administering ultra-low doses of an opioid antagonist. It was unclear previously, however, whether this enhancement was acting via

an opioid receptor because cannabinoid agonists enhance the release of endogenous opioid peptides (Pugh et al. 1997; Mason et al. 1999; Valverde et al. 2001). Our current findings provide mechanistic evidence that ultra-low dose effects apply to another pain-modulated G-protein coupled receptor that favors excitatory signaling upon chronic agonist exposure. The parallels between ultra-low dose effects in opioid and cannabinoid systems include: 1) similar effective agonist to antagonist molar ratios, 2) similar inhibitory to excitatory G-protein coupling switch following chronic agonist administration, 3) similar prevention of tolerance and G-protein coupling switch by ultra-low dose antagonist.

The analgesic enhancement we observed with co-administration of ultra-low dose CB1R antagonist is arguably confounded by motor deficits because the tail-flick test relies on the animal's ability to move its tail away from the heat source. Because cannabinoid agonists have strong inhibitory control over behavioural measures of motor functioning (Compton et al. 1996; Cosenza et al. 2000; Varvel et al. 2005), increases in tail-flick latencies may reflect changes in motor function, rather than antinociception. Some researchers believe that the tail-flick test is a spinal reflex which is not inhibited by supraspinal input sites (Wright, 1981; Ghorpade & Adnokat, 1994; Gleeson & Atrens, 1982) whereas others suggest that supraspinal pathways may affect this measure (Jones, 1991; Guimaraes, Guimaraes, & Prado, 2000; Ma, Dohi, Wang, Ishizawa, & Yanagidate, 2001). In light of this debate, we are currently investigating whether cannabinoid agonist-induced changes in motor responses may be altered by ultra-low dose CB1R antagonists. Our preliminary data indicate that tolerance to the cataleptic effect of

morphine is not altered by ultra-low dose naltrexone, even though antinociceptive tolerance is blocked in the same animals (K. Tuerke, unpublished findings).

Our biochemical data could be taken as evidence that ultra-low dose CB1R antagonists exert their effects through motor systems because we observed changes in a G-protein coupling switch in the striatum. We elected to conduct biochemical analyses on striatal tissue because it has high levels of CB1R expression (Herkenham et al. 1990), and we have extensive experience demonstrating an opioid-induced ultra-low dose G-protein coupling switch in this area (Wang & Burns, 2006; Wang et al. 2005). Moreover, although the striatum is most known for its control over motor function (Grillner, Hellgren, Menard, Saitoh, & Wikstrom, 2005; Pisa, 1988), it is also involved in pain perception (Hagelberg et al. 2004; Lin, Wu, Chandra, & Tsay, 1981). We are planning a series of future experiments in which we conduct biochemical analyses on brain regions more typically associated with pain, such as the periaqueductal grey and dorsal horn of the spinal cord. Regardless of whether the combination of agonist and ultra-low-dose antagonist is influencing motor systems, pain perception, or both, it is still notable that ultra-low dose CB1R antagonist co-administration dramatically reduces excitatory signal-mediated behaviours.

There is still question, however, as to the mechanism by which the G-protein coupling switch occurs, and how ultra-low dose rimonabant treatment prevents this switch. Because CB1R G-protein coupling regulates intracellular signaling and the concentration of rimonabant we used could only occupy a small portion of the CB1Rs, it is unlikely that prevention of G-protein switch by ultra-low-dose rimonabant is the result of CB1R blockade. Although the precise mechanism underlying ultra-low-dose

rimonabant mediated G-protein coupling switch remains unknown, we speculate that ultra-low-dose rimonabant binds to a site upstream of the CB1R and G proteins, such as the synaptic scaffolding protein, resulting in conformational change in favor of CB1R-Gi coupling. Given that the CB1R switches from the preferred G-protein coupling when forming a heterodimer (Kearn, Blake-Palmer, Daniel, Mackie, & Glass, 2005), ultra-low dose rimonabant may prevent oligomerization. Alternatively, the inverse agonist properties of rimonabant could be vital in preventing the G-protein coupling switch of CB1Rs. Further research needs to be directed at understanding the mechanism by which ultra-low dose agonist or antagonists affect G-protein coupling.

Preventing a switch in G-protein coupling with chronic cannabinoid administration has obvious clinical utility, especially in countries (including Canada) where marijuana and other synthetic cannabinoid drugs are legally prescribed for medicinal use. Ultra-low dose cannabinoid antagonists may be an effective way to enhance the cannabinoid agonist efficacy, thereby reducing the quantity and dosing frequency of agonist required for pain relief. Further supporting the potential clinical utility of ultra-low dose combination therapy is Oxytrex, an opioid-based ultra-low dose combination therapy that is currently undergoing clinical trials (Chindalore et al. 2005; Webster et al. 2006). The usefulness of opioid ultra-low dose combinations to treat pain in humans is debatable (Cepeda et al. 2002, 2004; Gan et al. 1997; Joshi et al. 1999; Sartain et al. 2003), as the research is still in its infancy. Regardless of the clinical efficacy of these compounds, elucidation of the underlying mechanisms and generality of the ultra-low dose phenomena may provide novel insights into neuropharmacology and synaptic plasticity.

In summary, this study demonstrates that CB1R antagonists in ultra-low dose concentrations extend the analgesic duration of a cannabinoid agonist and, when administered chronically, prevent the development of cannabinoid agonist-induced analgesic tolerance. This study also demonstrates that cannabinoid agonist-induced tolerance is associated with an increase in Gs-coupled CB1Rs which potentially increases excitatory signaling-mediated pain outputs, and that this G-protein coupling switch is prevented by ultra-low dose cannabinoid antagonist co-treatment. Future research will need to focus on understanding the mechanism by which these G-protein coupled receptor populations switch their preferred G-protein subtypes, and how ultra-low dose antagonists prevent this switch. As medicinal cannabinoid drugs are being discovered and marketed, this combination treatment may be useful to enhance the therapeutic effects of these agents in a wide variety of pathologies involving cannabinoid receptors.

Chapter 4. Experiment 3

**Ultra-Low Dose Naltrexone Does Not Enhance Morphine-Induced Analgesia in the
Formalin Test of Pain**

By

Jay J. Paquette & Mary C. Olmstead

4.0. Abstract

The antinociceptive effects of opioid agonists are enhanced, and the development of tolerance is attenuated, by ultra-low doses of opioid antagonists. To date, these studies have typically employed animal models of acute and escapable pain, such as the tail-flick test. The purpose of the present study was to examine whether opioid ultra-low dose effects are present in the formalin test of persistent inflammatory pain. Rats received an intraplantar injection of dilute (2%) formalin, and were immediately placed into the observation chamber. Thereafter, pain ratings were recorded at 1-min intervals for 60 min. In the acute drug conditions, animals received an injection (s.c.) of vehicle, the opiate morphine, ultra-low doses of the opioid receptor antagonist naltrexone, or a combination of morphine and ultra-low dose naltrexone, 30 min prior to formalin administration. In the sub-chronic conditions, animals received daily injections of vehicle, morphine, ultra-low doses of naltrexone, or morphine and ultra-low dose naltrexone combinations across 6 days, and antinociceptive reflexes were assessed using the tail-flick test 30 min post-injection. On day 7, animals received either a morphine injection, or the same treatment as their previous injections, 30 min prior to formalin testing. Ultra-low dose naltrexone had no significant effect on morphine-induced pain ratings in either the acute, or sub-chronic drug treatments. Notably, morphine-induced antinociceptive tolerance was replicated in one chronic testing group but not another, despite identical methodologies. Although previous studies demonstrate that ultra-low dose opioid antagonists enhance antinociception and prevent the development of tolerance in tests of acute/phasic pain, these effects do not appear to generalize to the formalin test of tonic pain.

4.1. Introduction

Opiates have been used for centuries to reduce pain, an effect that is mediated through activation of opioid receptors. The prototypical opiate, morphine, is a potent analgesic, but its use is fraught with serious side effects such as addiction, dependence, constipation, and tolerance. There is a pressing need, therefore, to provide safer analgesics that produce minimal side effects.

The cellular mechanisms mediating the analgesic effects of morphine are well established. Micromolar doses activate μ opioid receptors that couple to Gi/o-proteins (Crain et al. 1986; Lujan et al. 1984; Tucker, 1984), thereby inhibiting sensory neurotransmission of the pain signal (Einspahr & Piercey, 1980; Homma et al. 1983; Le Bars et al. 1975). There are, however, a small population of μ -opioid receptors that couple to Gs-proteins (Chakrabarti, Regec, & Gintzler, 2005; Shen & Craine, 1990a b; Wang et al. 2005) which may explain why ultra-low doses (fM - nM) of morphine produce hyperalgesia and a lengthening of dorsal root ganglion action potential duration (Crain & Shen, 1995; Crain & Shen, 2001; Higashi et al. 1982; Shen & Crain, 1989). Interestingly, combining ultra-low doses of opioid receptor antagonists with μ M doses of morphine enhances the analgesic potency of morphine alone (Crain & Shen, 1995; Powell et al. 2002; Shen & Crain, 1997). This paradoxical effect is explained by ultra-low dose antagonists preferentially blocking opioid receptors that couple to stimulatory G-proteins.

Repeated administration of morphine produces opioid tolerance, manifested as a progressive loss in analgesic potency. Although alternative mechanisms have been proposed (Finn & Wistler, 2002; Johnson & Fleming, 1989), morphine-induced analgesic

tolerance can be explained by a switch in the population of opioid receptor coupling from the predominantly inhibitory Gi/o-proteins to the predominantly stimulatory Gs-proteins (Wang et al. 2005). Furthermore, the development of morphine-induced analgesic tolerance is prevented by co-administration of ultra-low dose opioid antagonist (Powell et al. 2002; Shen & Crain, 1997; Wang et al. 2005). Thus, opioid receptors that couple to stimulatory G-proteins may be responsible for opioid-induced tolerance. One interpretation is that ultra-low dose opioid antagonists are preferentially occluding the opioid receptors that couple to stimulatory G-proteins, thus preventing the development of opioid-induced analgesic tolerance.

A small number of studies have investigated the impact of ultra-low dose opioid antagonists on morphine-induced analgesia in humans. Most notably, a recent randomized and controlled clinical trial demonstrated that ultra-low dose opioid antagonists in combination with opioid agonist treatment (Oxytrex) produce greater analgesia in osteoarthritic patients compared to opioid treatment alone (Chindalore et al. 2005). Other research on opioid ultra-low dose combination therapy in humans has produced conflicting results. For example, combination treatment reportedly decreases opioid-induced side effects (Cepeda et al. 2004; Gan et al. 1997), opioid requirements (Gan et al. 1997), and pain severity (Joshi et al. 1999), whereas other studies reported no effect of combination treatment (Sartain et al. 2003), or increased pain and opioid requirements (Cepeda et al. 2002). These discrepancies appear, primarily, to be a factor of antagonist dose, wherein higher daily doses were the least effective.

Despite the obvious clinical application, virtually all of the pre-clinical research on ultra-low dose opioid antagonist effects has used animal models of acute and

escapable pain, such as the tail flick or hot plate tests. In contrast, clinical pain states are persistent or chronic and, by definition, unavoidable. To further our understanding of the ultra-low dose phenomenon, we investigated the effects of morphine in combination with ultra-low doses of the opioid antagonist naltrexone in the formalin test of persistent inflammatory pain. In the first study, an acute sub-analgesic dose of morphine was co-administered with a range of naltrexone doses, prior to testing in the formalin test. We hypothesized that morphine-induced analgesia would be blocked by high doses of naltrexone and enhanced by ultra-low doses of the antagonist. In the second study, we examined whether ultra-low dose naltrexone alters the development of morphine-induced tolerance in the formalin test. In this study, animals were repeatedly injected with a high dose of morphine combined with a range of naltrexone doses over a 7-day period. We hypothesized that morphine alone would produce little or no analgesia at the end of the dosing regime, whereas animals injected with morphine plus ultra-low dose naltrexone combinations would continue to display analgesia in the formalin test.

4.2. Experimental Procedures

4.2.1. Subjects

Male Long-Evans rats (N = 293) from Charles River (Montreal, QC, Canada) ranging from 270-502 g were housed in polycarbonate cages in pairs and given free access to food (Lab Diet, PMI Nutrition International, Inc., Brentwood, MO, USA) and water. Animal quarters were kept on a reverse light-dark cycle (lights on from 7 pm to 7 am) and maintained at 22 ± 2 °C and 45 ± 20 % relative humidity. Animals were given a minimum of 3 days to acclimatize to the animal quarters prior to the experiment. All procedures were approved by the Queen's University Animal Care Committee and were

in accordance with the guidelines of the Canadian Council on Animal Care and the Animals for Research Act.

4.2.2. Drugs and Administration

Opioid agonists and antagonists were dissolved in saline and administered subcutaneously in a volume of 1 ml/kg. For formalin pain testing, 10% formalin (Sigma-Aldrich Inc., Oakville, ON, Canada) was diluted v/v with saline to make a 2% formalin concentration. A volume of 50 μ l of dilute formalin (2%) was administered subcutaneously in the plantar surface of the left hind paw using a 300- μ l syringe with a 29-gauge needle (286 μ m in diameter). This concentration of formalin induces significant nociceptive behavioural responses and demonstrates distinct first (1-10 min) and second (15-60 min) phase nociceptive behaviours (Abbott, Ocvirk, Najafee, & Franklin, 1999).

4.2.2.1. Single injection testing

Saline alone was used as a control injection. The opiate morphine sulphate (Medisca Pharmaceutique Inc. St.-Laurent, PQ, Canada) was administered alone at doses of 1, 2, and 4 mg/kg. These doses of morphine were selected because 2 mg/kg morphine has an half maximal analgesic effect in the formalin test using 1% formalin (Abbott et al. 1999). The opioid receptor antagonist naltrexone (Sigma Aldrich Inc., Oakville, ON, Canada) was prepared in a range of doses (1 pg/kg to 10 mg/kg) and co-administered with morphine (2 mg/kg). These combinations produce morphine to naltrexone molar ratios of 1×10^9 :1 to 1:10. The control group received 100 ng/kg naltrexone alone.

4.2.2.2. Repeated injection testing

Saline alone was used as a control injection. Morphine was administered alone at a dose of 10 mg/kg. This dose was chosen because preliminary data demonstrated that

similar doses produce maximal antinociception in the tail-flick test when administered acutely, and produce full analgesic tolerance when administered daily for 7 days (Powell et al. 2002; Wang et al. 2005). Naltrexone (50 pg/kg to 5 µg/kg) was combined with morphine (10 mg/kg) and administered as a single injection. These combinations produce morphine to naltrexone molar ratios of $1 \times 10^8:1$ to $1 \times 10^3:1$.

4.2.3. Apparatus

The tail-flick apparatus consists of a projection lamp that creates radiant heat located just below the animal-testing surface (D'Amour & Smith, 1941). The light from the lamp projected through a small hole in the testing surface and was aimed at a photocell located 25 cm above the testing surface. A digital timer, connected to the apparatus, started when the heat source was activated. When the animal flicked its tail away from the heat source, the light from the projection lamp activated the photocell, simultaneously stopping the timer and turning off the lamp. The heat intensity was calibrated to result in baseline tail flick latencies of 2-3 s and a 10 s cutoff was used to minimize tissue damage.

The formalin test observation chamber consists of a polycarbonate box (30 cm³) with a mirror placed under the observation chamber on a 45-degree angle. The mirror allows for easy observation of the testing compartment floor surface.

4.2.4. Behavioural Procedures

4.2.4.1. Acute injections

Behavioural responses to a persistent inflammatory nociceptive stimulus were tested using the formalin test. This test involves an intraplantar injection of the inflammatory agent formalin. A dilute formalin injection produces a characteristic set of

nociceptive behaviours that persist for approximately one hour. The bimodal nociceptive behaviour includes a first phase lasting up to 10 min post injection, and a delayed second phase that starts around 10 min, peaks around 20 min, and ends around 60 min post-injection

Two days prior to testing, animals were placed in the formalin testing chambers in order to minimize stress-induced analgesia (Kelly & Franklin, 1985; Terman et al. 1984). On testing days, animals were first given their drug injection (saline, morphine, naltrexone, or morphine + naltrexone). Thirty minutes later, animals were restrained in a towel, injected with formalin, and immediately placed in the observation chamber for 60 min. We used a time sampling method in which behavioural ratings were taken once every minute (Abbott et al. 1999). Animals were rated on a 0-3 scale wherein 0 = normal weight distribution on all paws, 1 = favouring of the formalin-injected paw with foot pads still on the floor, 2 = lifting the formalin-injected paw with foot pads off the floor with, at most, the toes touching the floor, and 3 = licking/biting or shaking the formalin-injected paw. After the 60-min observation period, animals were returned to their homecage. Behavioural pain ratings were averaged across 1-10 min and 11-60 min post-formalin injection to create a pain score for phase 1 and phase 2, respectively. A research assistant that was blinded to drug group conducted all behavioural pain ratings.

4.2.4.2. Chronic injections

This procedure had two parts: 1) six dosing days, and 2) one testing day. On each dosing day, animals were injected with drug, assessed for antinociceptive responses in the tail-flick test, and habituated to the formalin test observation chamber. Prior to the first dosing, baseline nociceptive reflexes to a thermal stimulus were assessed using the tail-

flick test of antinociception. On each of the 6 dosing days animals were injected, and tail-flick latencies were assessed 30 min later to ensure that the dosing regiment was replicating the morphine tolerance effects and the ultra-low dose naltrexone co-treatment effects previously reported (Crain & Shen, 1995; Powell et al. 2002; Shen & Crain, 1997; Wang et al. 2005). For all animals, tail-flick latencies were converted into a percent of maximal possible effect (MPE) using the equation:

$$\text{MPE} = \left[\frac{(\text{post- injection latency} - \text{baseline latency})}{(10 \text{ s cutoff} - \text{baseline latency})} \right] \cdot 100$$

The day following the last dosing day, animals were tested in the formalin test as described above. In one experiment, animals were injected with morphine (10 mg/kg) alone, whereas in the other experiment, animals were injected with the same drug that they received during the dosing days. These animals were then subjected to formalin testing 30 min post-injection.

4.2.5. Statistics

Data from the formalin tests were analysed using separate one-way ANOVA tests on global pain scores for phase 1 (1-10 min post-formalin injection) and phase 2 (11-60 min post-formalin injection). For the tail-flick data, injection day (dosing days 1-6) was the within-subjects factor and drug group was the between-subjects factor. Because many drug group comparisons were irrelevant in the single and repeated injection experiments, *a priori* multiple comparisons were used to analyze significant drug effects using the Dunn's critical *t*-ratio with a corrected α . Whenever there were violations of sphericity, the Greenhouse-Geisser correction to the within-group degrees of freedom was reported.

4.3. Results

4.3.1. Acute Formalin Test

4.3.1.1. Acute morphine dose response

There was no significant difference in pain scores for groups injected with different doses of morphine in phase 1 of the formalin test [$F(3, 28)=1.02$]. In phase 2, however, there was a significant group effect [$F(3,28)=7.77, P<0.001$; figure 4.1.], in that the 4 mg/kg morphine group exhibited a lower pain rating than the vehicle group (table 4.1.). Both the 1 mg/kg and 2 mg/kg morphine dose groups did not differ significantly from the vehicle group (table 4.1.). Because we hypothesized that ultra-low dose naltrexone would enhance the analgesic effects of morphine, the 2-mg/kg dose of morphine was selected for the agonist-antagonist combination studies.

4.3.1.2. Acute morphine + naltrexone doses response

There was a between drug group effect in phase 1 of the formalin test [$F(12,138)=1.95, P<0.05$], however, none of the *a priori* comparisons performed on these data demonstrated any statistical difference¹(table 1). Thus, within phase 1, naltrexone had no significant effect on morphine. In phase 2, there was also a between drug groups effect [$F(12,42)=2.96, P<0.01^2$; figure 4.2.]. The morphine (2mg/kg) + naltrexone (10 mg/kg, 1 mg/kg, 100 µg/kg, and 10 µg/kg) dose groups produced greater pain behaviours compared to the morphine (2mg/kg) alone group (table 4.1.), whereas the morphine (2mg/kg) + naltrexone (1 µg/kg, 100 ng/kg, 10 ng/kg, 1 ng/kg, 100 pg/kg, 10 pg/kg, and 1 pg/kg) dose groups were not significantly different from the morphine (2mg/kg) alone

¹ Further analysis of this phenomenon suggested that the significant ANOVA resulted from the difference between the morphine + naltrexone (1 pg/kg) group, and the morphine + naltrexone (10 mg/kg) group.

² Here, a Welch test is reported because there was a violation of homogeneity of variance and there were unequal sample sizes.

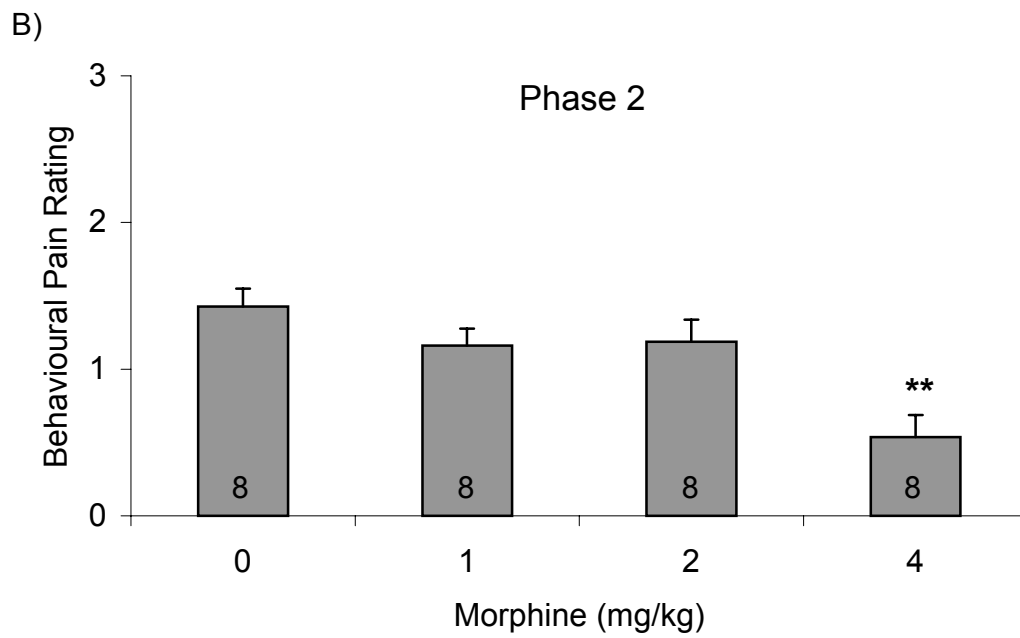
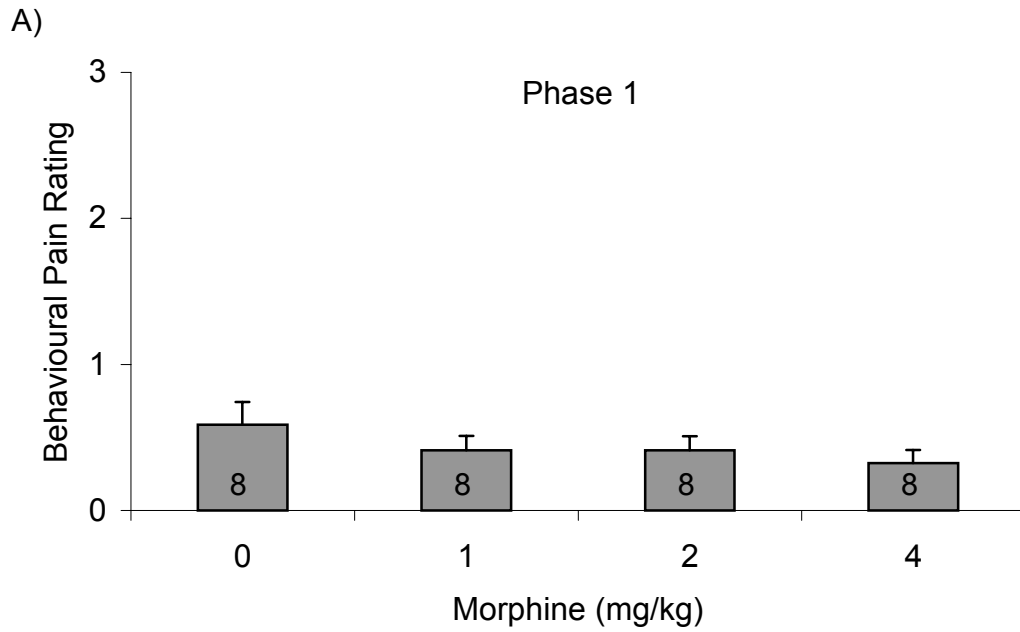


Figure 4.1. Morphine produces decreased pain responses only in phase 2 of the formalin test. Bars represent mean (\pm SEM) behavioural pain ratings following morphine administration (s.c.). A) Phase 1 represents the average of ratings from 1-10 min post-formalin injection. B) Phase 2 represents the average of ratings from 11-60 min post-formalin injection. Animals received a drug injection 30 min prior to a 2% formalin injection in the hind left paw. Values within bars represent group sizes. See appendix I for time course figure.

** $P < 0.01$ compared to vehicle.

Table 4.1. Acute formalin test statistics

Drug Group	df	Phase 1		Phase 2	
		t-statistic	P-value	t-statistic	P-value
Vehicle vs.					
Morphine					
1 mg/kg	28	1.09	n.s.	1.39	n.s.
2 mg/kg	28	0.47	n.s.	1.25	n.s.
4 mg/kg	28	1.64	n.s.	4.62	<0.01
Morphine (2 mg/kg) alone vs.					
Morphine (2 mg/kg) + Naltrexone					
1 pg/kg	38	1.25	n.s.	0.24	n.s.
10 pg/kg	38	0.13	n.s.	0.80	n.s.
100 pg/kg	38	0.13	n.s.	0.42	n.s.
1 ng/kg	46	1.11	n.s.	1.05	n.s.
10 ng/kg	45	0.11	n.s.	1.12	n.s.
100 ng/kg	46	0.72	n.s.	2.58	n.s.
1 µg/kg	38	0.73	n.s.	2.14	n.s.
10 µg/kg	38	1.07	n.s.	3.43	<0.01
100 µg/kg	38	2.02	n.s.	4.30	<0.01
1 mg/kg	38	1.59	n.s.	3.47	<0.01
10 mg/kg	38	2.79	n.s.	3.62	<0.01
Morphine (2 mg/kg) + Naltrexone (100 ng/kg)					
vs. Naltrexone (100 ng)	22	0.16	n.s.	0.88	n.s.

Note: n.s. = not significant ($P > 0.05$); df = degrees of freedom

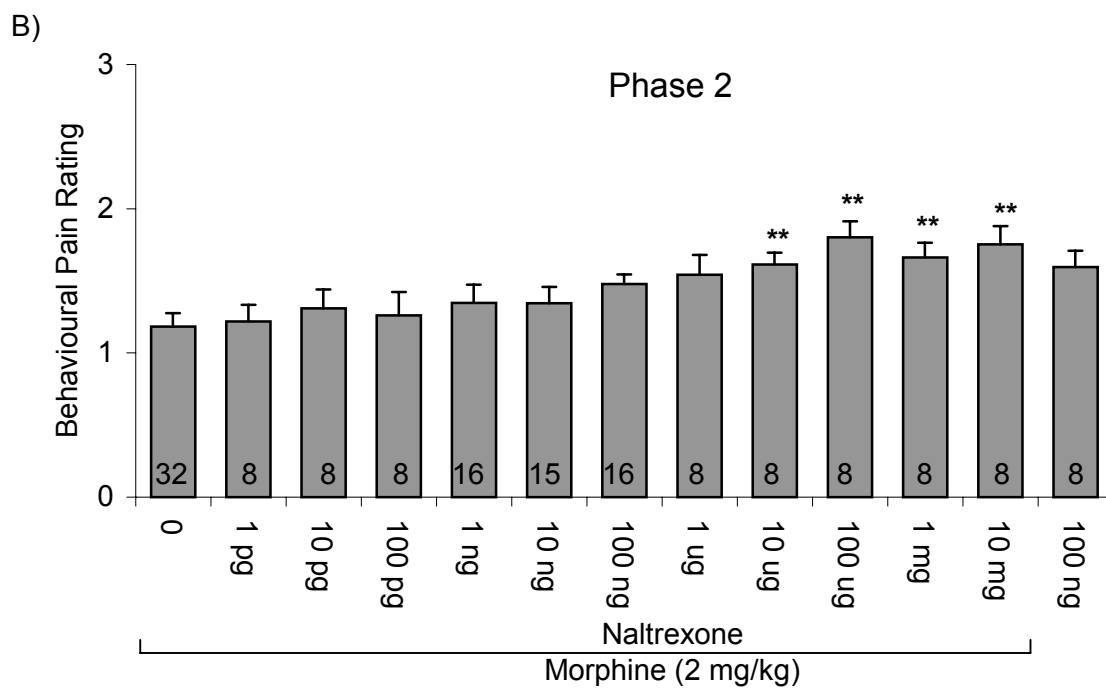
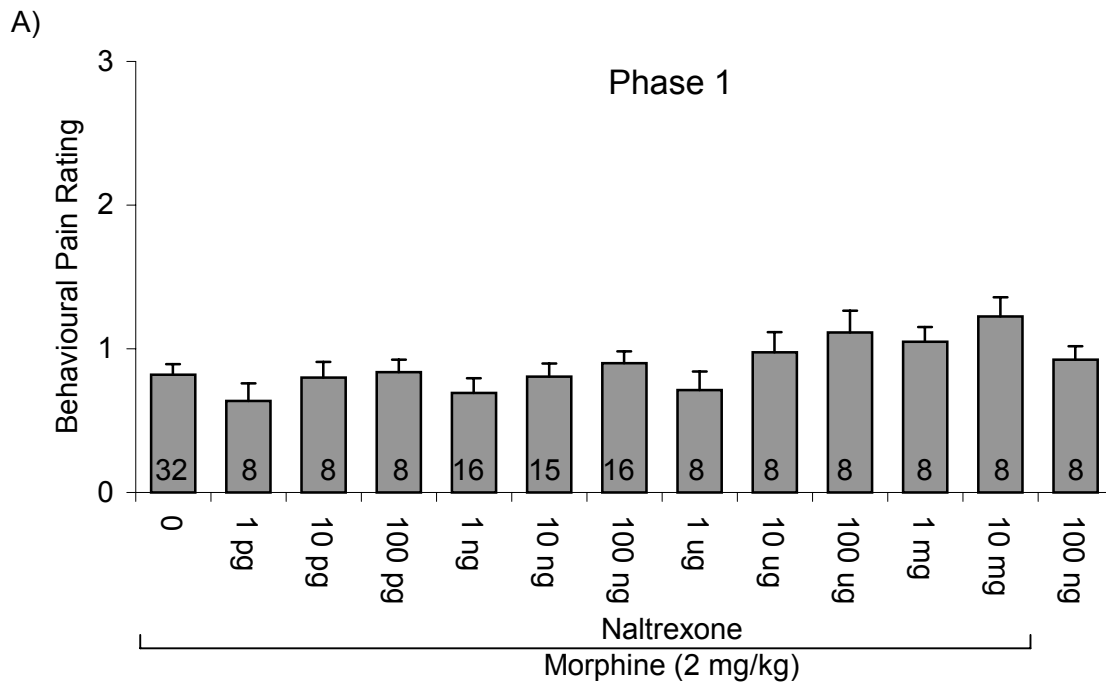


Figure 4.2. Naltrexone, when combined with morphine, dose dependently increases pain responses compared to morphine alone in phase 2 of the formalin test. Bars represent mean (\pm SEM) behavioural pain ratings following administration of morphine alone or morphine combined with various doses of naltrexone (s.c.). A) Phase 1 represents the average of ratings from 1-10 min post-formalin injection. B) Phase 2 represents the average of ratings from 11-60 min post-formalin injection. Animals received a drug injection 30 min prior to a 2% formalin injection in the hind left paw. Values within bars represent group sizes. See appendix II for time course figure.

** $P < 0.01$ compared to morphine alone.

group (table 4.1.). Thus, acute morphine in combination with higher doses of naltrexone (μg to mg/kg) produced greater formalin-induced pain behaviours in phase 2 compared to morphine alone. On the other hand, acute administration of ultra-low doses of naltrexone had no effect on morphine as measured in the formalin test.

4.3.2. Chronic Morphine + Naltrexone Dose Response

4.3.2.1. Morphine alone on testing day 7

The post-injection tail-flick measures (MPE) taken across the 6 dosing days for each drug group are shown in figure 4.3. When all drug groups were combined, a main effect of injection day [$F(5,360)=13.30$, $P<0.001$] was revealed, whereby MPE values decreased across days. Furthermore, there was a main effect of drug [$F(7,72)=52.41$, $P<0.001$], but there was no drug X injection day interaction, [$F(35,360)=1.15$]. To further analyse the drug group main effect, *a priori* comparisons were performed comparing each group to the morphine (10 mg/kg) group. The morphine (10 mg/kg) alone group produced longer tail-flick latencies than the vehicle group (table 4.2.), whereas the morphine + naltrexone (5 ng/kg) group produced longer tail-flick latencies than the morphine (10 mg/kg) alone group (table 4.2.). None of the other drug groups were significantly different from morphine (10 mg/kg) alone.

The day following the last dosing day was the formalin test day. Regardless of the dosing history of the animals, all animals received morphine (10 mg/kg) prior to formalin testing. There was no effect of drug history on pain behaviours in phase 1 [$F(7,72)=1.62$], but there was a significant drug-history group effect in phase 2 [$F(7,72)=3.36$, $P<0.01$; figure 4.4.]. The morphine (10 mg/kg) alone group produced

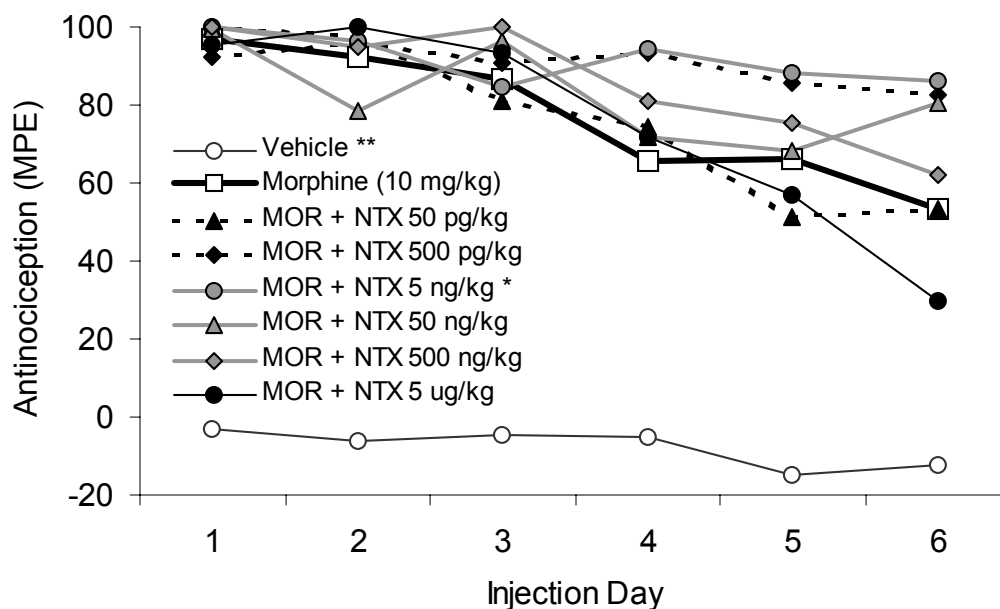


Figure 4.3. Ultra-low dose naltrexone, when combined with morphine, produced greater antinociception compared to morphine alone. Lines represent mean (\pm SEM) tail-flick latencies, reported as percent mean possible effect (MPE), across six injection days. Animals were tested following subcutaneous administration of morphine (MOR) alone or combined morphine + various doses of naltrexone (NTX). Drug injections were given once a day, 30 min prior to tail-flick testing. Group sizes can be found in figure 4.4.

* $P < 0.05$ compared to morphine alone.

** $P < 0.01$ compared to morphine alone.

Table 4.2. Tail-flick testing across chronic drug administration days

Drug Group	df	t- statistic	P- value
<i>Morphine alone on test day 7</i>			
Morphine (10 mg/kg) alone vs.			
Vehicle	30	14.59	<0.01
Morphine (10 mg/kg) + Naltrexone			
50 pg/kg	30	0.11	n.s.
500 pg/kg	30	2.80	n.s.
5 ng/kg	30	3.15	<0.01
50 ng/kg	30	1.18	n.s.
500 ng/kg	30	1.85	n.s.
5 µg/kg	30	0.47	n.s.
<i>Combination treatment on test day 7</i>			
Morphine (10 mg/kg) alone vs.			
Morphine (10 mg/kg) + Naltrexone			
500 pg/kg	14	1.12	n.s.
5 ng/kg	14	0.85	n.s.
50 ng/kg	14	0.81	n.s.

Note: n.s. = not significant ($P > 0.05$); df = degrees of freedom

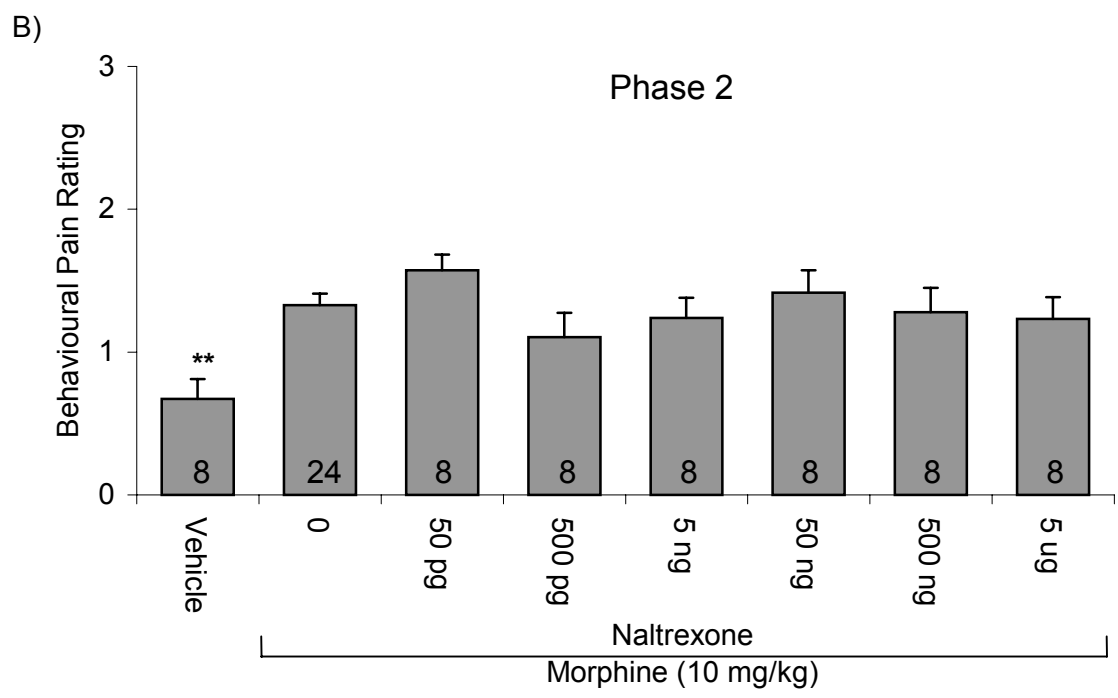
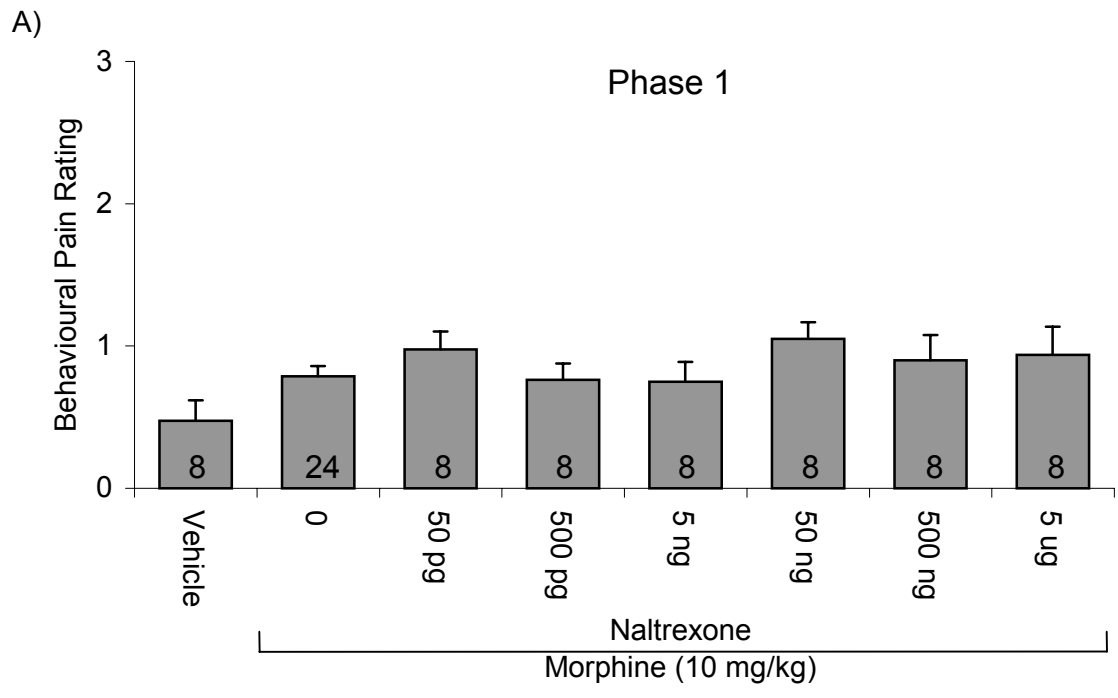


Figure 4.4. Morphine-induced tolerance can be seen in phase 2 of the formalin test, but this effect is not altered by naltrexone. Bars represent mean (\pm SEM) behavioural pain ratings in the formalin test following 6 days of chronic administration of morphine alone or combined morphine + various doses of naltrexone (s.c.). All animals received morphine alone (10 mg/kg) on day 7, 30 min prior to a 2% formalin injection in the hind left paw. A) Phase 1 represents the average of ratings from 1-10 min post-formalin injection. B) Phase 2 represents the average of ratings from 11-60 min post-formalin injection. Values within bars represent group sizes. See appendix III for time course figure.

** $P < 0.01$ compared to morphine alone.

greater pain behaviours than the vehicle group in phase 2 only. All other comparisons were not statistically significant (table 4.3.).

4.3.2.2. *Combination treatment on testing day 7*

The post-injection tail-flick measures (MPE) taken across the 6 dosing days for each drug group are shown in figure 4.5. When all drug groups were combined, a main effect of injection day [$F(5,130)=5.73$, $P<0.01$] was revealed, whereby MPE values decreased across time. Furthermore, there was no main effect of drug [$F(3,26)=0.35$], nor a drug X injection day interaction, [$F(15,130)=0.87$]. Statistics from the *a priori* comparisons are presented in table 4.2., none of which are significant.

The day following the last dosing day was the formalin test day. In this experiment, animals continued to receive the drug combination they received during the previous 6 dosing days. There was no significant effect of drug group on phase 1 [$F(3,26)=1.42$], nor phase 2 pain behaviours [$F(3,26)=0.03$; figure 4.6.]. Morphine (10 mg/kg) in combination with ultra-low dose naltrexone had no significant effect compared to morphine alone. Statistics from the *a priori* comparisons, none of which are significant, are presented in table 4.3.

4.4. Discussion

Here, we demonstrate that the antinociceptive effects of morphine are not influenced by ultra-low dose naltrexone in the formalin test of persistent inflammatory pain, contrary to results obtained in tests of acute pain (Crain & Shen, 1995; Powell et al. 2002; Wang et al., 2005). More specifically, a single injection of morphine produces dose dependent analgesia, but the analgesic effect of morphine was not enhanced by ultra-low dose naltrexone. Following chronic drug treatment, morphine produced

Table 4.3. Formalin testing following chronic drug administration

Drug Group	df	Phase 1		Phase 2	
		t-statistic	P-value	t-statistic	P-value
<i>Morphine alone on test day 7</i>					
Morphine (10 mg/kg) alone vs.					
Vehicle	30	1.91	n.s.	3.88	<0.01
Morphine (10 mg/kg) + Naltrexone					
50 pg/kg	30	1.15	n.s.	1.44	n.s.
500 pg/kg	30	0.15	n.s.	1.32	n.s.
5 ng/kg	30	0.23	n.s.	0.53	n.s.
50 ng/kg	30	1.61	n.s.	0.51	n.s.
500 ng/kg	30	0.69	n.s.	0.29	n.s.
5 µg/kg	30	0.92	n.s.	0.57	n.s.
<i>Combination treatment on test day 7</i>					
Morphine (10 mg/kg) alone vs.					
Morphine (10 mg/kg) + Naltrexone					
500 pg/kg	14	1.52	n.s.	0.16	n.s.
5 ng/kg	14	0.25	n.s.	0.60	n.s.
50 ng/kg	14	0.29	n.s.	0.19	n.s.

Note: n.s. = not significant ($P > 0.05$); df = degrees of freedom

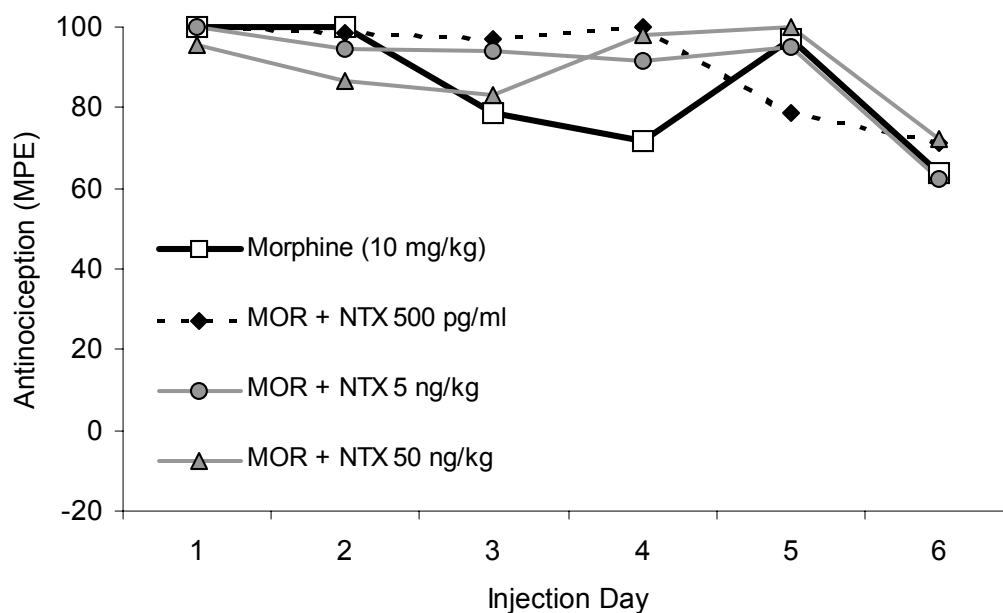


Figure 4.5. Ultra-low dose naltrexone, when combined with morphine, does not produce greater antinociception compared to morphine alone. Lines represent mean (\pm SEM) tail-flick latencies, reported as percent mean possible effect (MPE), across six injection days. Animals were tested following subcutaneous administration of morphine (MOR) alone or morphine combined with various doses of naltrexone (NTX). Drug injections were given once a day, 30 min prior to tail-flick testing. Group sizes can be found in figure 4.6.

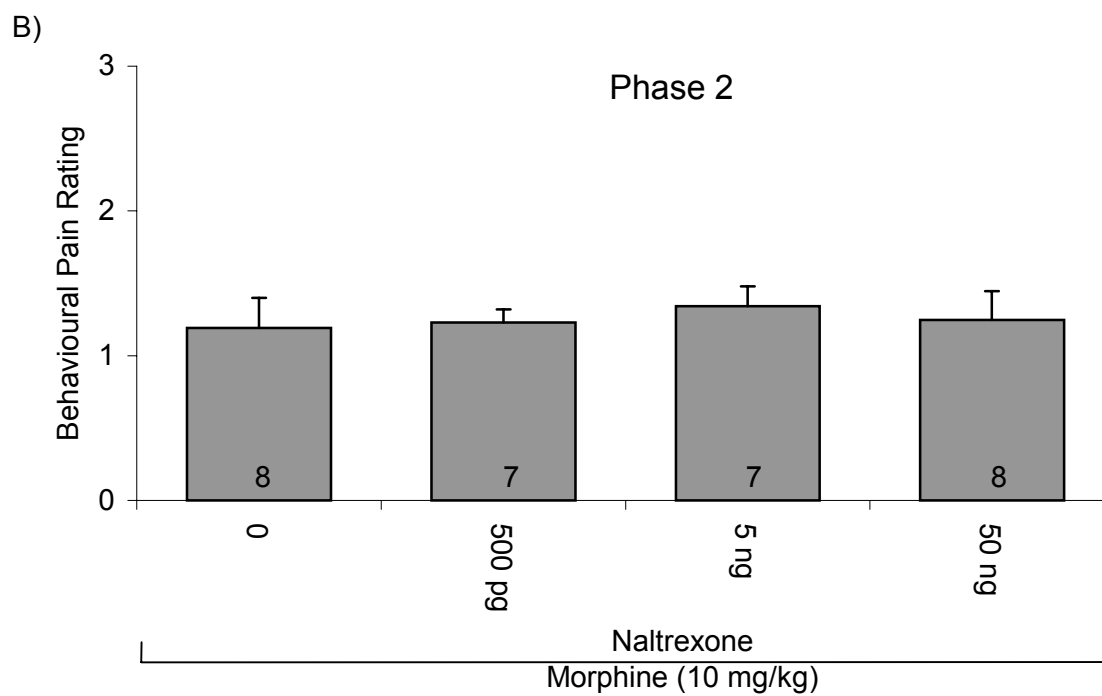
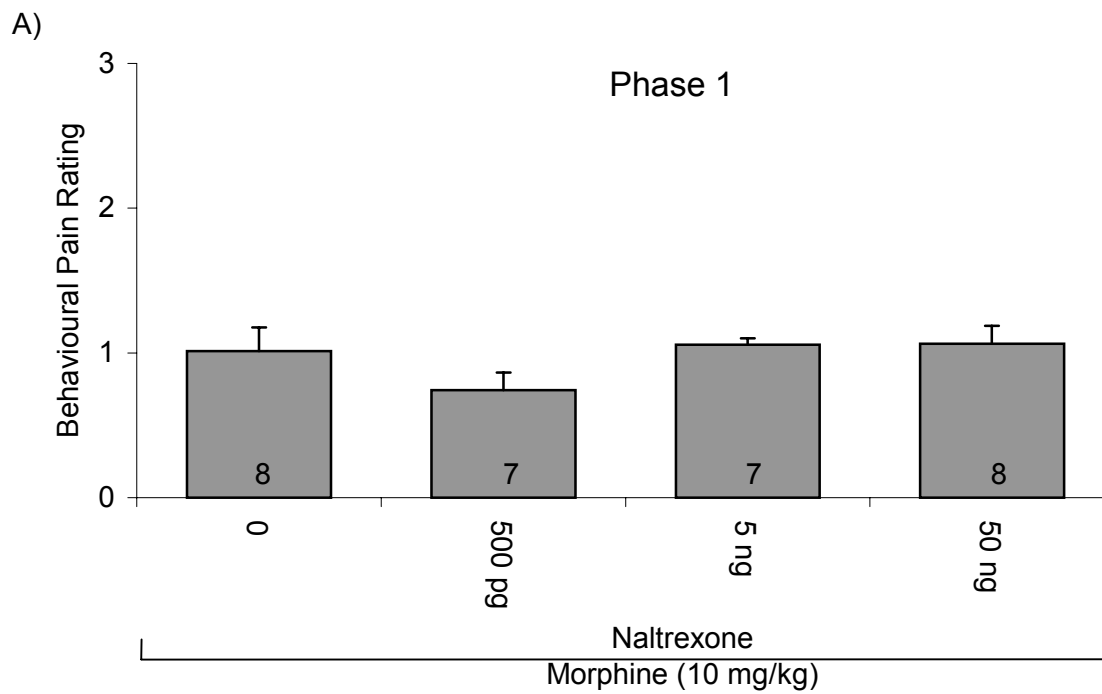


Figure 4.6. Naltrexone, when combined with morphine on dosing and testing days, has no effect on morphine-induced tolerance in the formalin test. Bars represent mean (\pm SEM) behavioural pain ratings in the formalin test following 6 days of chronic administration of morphine alone or morphine combined with various doses of naltrexone (s.c.). Animals continued to receive their respective drugs on day 7, 30 min prior to a 2% formalin injection in the hind left paw. A) Phase 1 represents the average of ratings from 1-10 min post formalin injection. B) Phase 2 represents the average of ratings from 11-60 min post formalin injection. Values within bars represent group sizes. See appendix IV for time course figure.

analgesic tolerance, but this tolerance was not prevented by co-application of ultra-low dose naltrexone. Taken together, we report that there is no opioid associated ultra-low dose effect in the formalin test.

This study is the first to examine morphine and ultra-low dose naltrexone combination treatment in an animal model of persistent/tonic, non-avoidable, inflammatory pain. Arguably, this is a more appropriate model to develop pharmacological tools for pain relief in humans because it more closely reflects the type of pain that requires intervention. For example, people use oven mitts instead of taking pain medication when removing a hot casserole dish from the oven, but will take pain medications instead of using oven mitts to treat the pain from a twisted ankle. Other persistent/chronic pain models need to be considered before rejecting the possibility that morphine + ultra-low dose opioid antagonist combination treatment could be an effective therapy for clinical pain. This is particularly true given that a preliminary study using a model of neuropathic pain showed that ultra-low dose naltrexone enhanced morphine-induced anti-hyperalgesia (Armstrong, Jhamandas, & Cahill, 2006).

We are still left questioning why ultra-low dose opioid antagonists enhance the effects of morphine in some pain models (i.e., tail-flick test, neuropathic pain model), but not in others (i.e., formalin test). Morphine-induced antinociception is enhanced by ultra-low doses of an opioid antagonist in studies of acute avoidable mechanical and thermal pain (Powell et al. 2002; Crain & Shen, 1995), and ultra-low dose opioid antagonist produce analgesia when co-administered with hyperalgesic doses of morphine (Crain & Shen, 2001). The non-significant effect of ultra-low dose naltrexone in the formalin test may be due to one of three explanations: 1) the destruction of primary afferent neurons

by formalin occludes the ultra-low dose effect, 2) endogenous chemical communicators involved in the sensitization of the nociceptive pathway occlude the ultra-low dose effect, and/or 3) there are fundamentally different testing methodologies employed in these pain models (i.e., phasic avoidable pain stimuli vs. passive reaction to tonic pain) that are measuring separate pain mechanisms, which may be differentially affected by ultra-low dose treatments.

First, it is unlikely that the destruction of neurons by formalin would be responsible for occluding the ultra-low dose effect because, in a model of neuropathic pain which also involves peripheral afferent destruction, ultra-low dose opioid antagonists enhance the anti-hyperalgesic effects of morphine (Armstrong et al. 2006). Second, the formalin test induces sensitization of the nociception pathway, a process involving the release of a host of inflammatory mediators (i.e., bradykinin, prostaglandin, adenosine, etc.) which result in this pathway becoming hypersensitive (Doak & Sawynok, 1995; Malmberg, Rafferty, & Yaksh, 1994; Sufka & Roach, 1996; Woolf & Salter, 2000). It may be the case that this sensitization process occludes the ultra-low dose effect. On the other hand, in osteoarthritic patients, a clinical pain syndrome involving inflammation (Bonnet & Walsh, 2005), the opioid ultra-low dose combination therapy, Oxytrex®, has a greater therapeutic index compared to the opioid agonist alone (Chindalore et al. 2005). At the very least, if there is a particular inflammatory chemical that is responsible for occluding the opioid ultra-low dose combination effect in the formalin test, this chemical is probably not a predominate factor in clinical osteoarthritis.

The most likely reason that we observed no enhancement of morphine by ultra-low dose naltrexone in the formalin test is that this pain test is fundamentally different

from other previously used tests of pain (i.e., tail-flick, von Frey filaments). The acute/phasic tests of nociception, whether thermal or mechanical, involve the assessment of an avoidance response following the brief application of a stimulus. In contrast, in the formalin test, the stimulus is non-avoidable persistent pain; rather than stimulus detection, behavioural reaction to the inflamed paw is assessed. Avoidance pain studies, especially the tail-flick test, are predominately dependant on the spinal cord reflex (Ghorpade & Advokat, 1994; Gleeson & Atrens, 1982; Wright, 1981), whereas the formalin test is mediated by supraspinal circuits (Ryan, Watkins, Mayer & Maier, 1985; Tasker, Choiniere, Libman & Melzack, 1987; Vaccarino & Melzack, 1989). Although the ultra-low dose antagonist enhancements of opioid-induced analgesia is mediated in the spinal cord (Powell et al. 2002), no studies have looked at the effects of local injections in supraspinal nuclei. This may be an important distinction explaining why the ultra-low dose combination treatment effects do not extend to the formalin test. Assessing reflexive avoidance behaviours following the induction of inflammatory pain, either in the formalin test or other inflammatory pain models, could potentially resolve this debate.

One major limitation of this study arose when comparing the tail-flick data from the two tolerance experiments (figures 4.3. and 4.5.). The procedures for these two experiments were identical for the first six days, yet in one instance morphine produced tolerance and in the other instance it did not. Coincidentally, the morphine used for these two experiments was obtained from different sources, and this difference may be responsible for the discrepancy in the data. Because others have demonstrated that similar doses of morphine administered once a day for 7 days will produce tolerance

(Powell et al. 2002; Wang et al. 2005), we can only assume that the dataset that does not show morphine tolerance is erroneous, and that the formalin test data for these groups is not interpretable. This test should be attempted again once the tolerance regime is under experimenter control. Thus, the question of whether ultra-low dose naltrexone modifies tolerance in the formalin test is still debatable.

This experiment suggests that ultra-low dose opioid antagonists do not enhance morphine-induced analgesia in the formalin test of pain. Furthermore, although ultra-low dose antagonists prevent the development of morphine-induced tolerance in the tail-flick test (Powell et al. 2002; Wang et al. 2005), this tolerance attenuation effect did not extend to the formalin test of pain. Further research should be directed toward testing this combination treatment in other tests of chronic and inflammatory pain.

Chapter 5. General Discussion

This thesis demonstrates that the ultra-low dose phenomenon, previously identified in the opioid receptor system, is also a property of the cannabinoid receptor system. Experiment 1 revealed that ultra-low doses of an opioid receptor antagonist enhance cannabinoid receptor agonist-induced antinociception (Paquette & Olmstead, 2005). In experiment 2, ultra-low doses of a cannabinoid antagonist enhanced the duration of cannabinoid agonist-induced antinociception, and attenuated the development of cannabinoid agonist-induced antinociceptive tolerance. Biochemical studies revealed a potential mechanism of cannabinoid agonist-induced tolerance, and demonstrated that ultra-low dose cannabinoid antagonist treatment attenuated this process (Paquette, Wang, Bakshi, & Olmstead, in press). This research also demonstrates that the opioid receptor ultra-low dose phenomenon does not extend to an animal model of persistent inflammatory pain. Experiment 3 showed that ultra-low doses of an opioid antagonist had no significant effect on opioid agonist-induced analgesia in the formalin test, either in drug-naïve animals or in animals subjected to daily drug treatments for the 6 days prior to testing. Together, these studies suggest that the ultra-low dose phenomenon may be a common mechanism in G-protein coupled receptor systems. At the same time, the changes in receptor signaling cascades induced by these treatments may not always be manifested in behaviour.

In line with previous studies in the opioid system, ultra-low dose cannabinoid antagonists prevented a CB1R agonist-induced G-protein coupling switch. Despite the information this provides on the mechanisms underlying the ultra-low dose phenomenon, many questions remain unanswered. For instance, is a CB1R G-protein signaling cascade

involved with increasing CB1R coupling to Gs proteins and, if so, which of the two G-proteins activated by CB1Rs are responsible: Gi or Gs? Why do ultra-low dose antagonists prevent this switch? Is it because these ligands preferentially bind to the receptors coupling to Gs proteins, as suggested by Crain and Shen (1992)? If so, how do binding affinities of CB1 or opioid receptors differ when they are coupling to Gs or Gi proteins? Or, are ultra-low-dose antagonists binding a site downstream of the receptor and G proteins, such as the synaptic scaffolding protein, and resulting in conformational changes to enhance Gi coupling? Do the existing receptors change in order to switch coupling between G-protein subtypes? Or, are the Gs-coupling receptors a completely different population of cannabinoid receptors from those that couple to Gi proteins? These questions must be addressed in future biochemical studies in order to gain a full understanding of the ultra-low dose phenomenon.

If receptor changes underlie the ultra-low dose phenomenon, the process may be explained by receptor dimerization, defined as one receptor joining with another. Receptor dimerization can change receptor signaling: for instance, the CB1R and the D2 receptor can form a heterodimer and activate Gs proteins instead of their usual activation of Gi proteins (Kearn et al. 2005). Receptor dimers can form in the absence of agonists, and this process may contribute to constitutive activity of G-protein coupled receptors (Kroeger, Hanyaloglu, Seeber, Miles, & Eidne, 2001). Because CB1Rs exhibit constitutive activity, and rimonabant inhibits this activity (Landsman, Burkey, Consroe, Roeske, & Yamamura, 1997; Pan, Ikeda, & Lewis, 1998), ultra-low dose rimonabant may occlude the site responsible for CB1R homo- or heterodimer formation, thereby

attenuating the G-protein coupling switch and the development of tolerance. Future research needs to be directed at testing these hypotheses.

Ultra-low dose effects appear to involve alterations in receptor coupling, so it is interesting that ultra-low dose treatments do not extend to all behaviours influenced by activation of these receptors. That is, morphine-induced antinociception is enhanced and tolerance is attenuated by ultra-low dose opioid antagonists in the tail-flick test (Crain & Shen, 1995; Powell et al. 2002), but ultra-low dose antagonist treatment appears to have no effect on morphine-induced analgesia or analgesic tolerance in the formalin test of tonic inflammatory pain. Furthermore, ultra-low dose opioid antagonists prolong the rewarding properties of opioid agonists (Powell et al. 2002), but appear to have no effect on opioid agonist-induced catalepsy (K. Tuerke, unpublished findings). Thus, the G-protein switching properties of opioid receptors may be restricted to particular brain nuclei. More specifically, it may be the case that opioid receptor G-protein switching is more likely to occur in the spinal cord and nucleus accumbens, accounting for the effects on antinociception and reward, but less likely to occur in the substantia nigra and anterior cingulate, explaining the lack of effects on catalepsy and inflammatory pain models. Unfortunately, these behavioural discrepancies have yet to be examined in the cannabinoid receptor system, as the ultra-low dose cannabinoid effects have only been tested in the tail-flick test.

One important finding of this thesis is that the ultra-low dose phenomenon is not specific to the opioid receptor system. We can speculate, therefore, that ultra-low dose effects are a generalized principle that applies to many G-protein coupled receptors. In support of this hypothesis, there is evidence that other receptor systems appear to exhibit

ultra-low dose effects, although these have not been tested specifically. For instance, serotonin 1A receptors (5HT-1A) typically activate Gi proteins but, in the presence of a Gi protein coupling inhibitor, they activate Gs signaling (Malmberg & Strange, 2000). Likewise, dopamine D2-type receptors, which typically activate inhibitory-type G-proteins, can also activate stimulatory-type G-proteins (Glass & Felder, 1997; Kearns et al. 2005; Obadiah et al. 1999). Moreover, haloperidol, a dopamine D2 receptor antagonist, inhibits D1 receptor agonist-induced adenylate cyclase activity at high doses (10^{-4} M and higher) but has excitatory effects at ultra-low doses (as low as 10^{-8} M) (Saller & Salma, 1986). In hippocampal preparations in which serotonergic afferents have been lesioned, administration of an α -1 adrenergic agonist produces increased cAMP formation at ultra-low doses (as low as 5×10^{-7} M) and decreased formation at higher doses (5×10^{-4} M and higher; Consolo et al. 1988). Interestingly, neurons subjected to long-term immersion in ultra-low dose glutamate solutions, as low as 10^{-30} M, show neuroprotective effects when they are later immersed in an excitotoxic (25 μ M) glutamate solution (Jonas, Lin, & Tortella, 2001). Similar to the effects in the opioid system (Crain & Shen, 2001; Shen & Crain, 1989, 1990a, b, 2001), these low dose effects are approximately 1000+ fold lower than the typically-employed doses of these agents. At the same time, there are many other examples of biphasic dose response effects in which the doses that produce opposing effects differ only by a factor of 10 to 100 fold. For instance, some serotonergic agonists produce increased aggression, catalepsy, and decreased brain stimulation reward thresholds at 'low' doses, but produce decreased aggression, catalepsy, and increased brain stimulation reward thresholds with higher doses (10-100 fold higher; Calabrese, 2001; Harrison & Markou, 2001; Hennig, 1980).

Similarly, low doses of γ -aminobutyric acid (GABA) receptor agonists attenuate haloperidol-induced catalepsy but higher doses (10 fold higher) potentiate this effect; the opposite effect occurs with GABAergic antagonists (Richardson & Richardson, 1982; Worms & Lloyd, 1978; Worms & Lloyd, 1980). Biphasic dose response curves have also been reported for prostaglandin, adenosine, nitric oxide, estrogen, and androgen receptors, to name a few (see the special issue: McClellan, 2001 for reviews). Although these biphasic effects are typically explained by receptor interactions and non-selective drug effects, some of the effects could be explained by receptors coupling to multiple G-proteins.

The ultra-low dose phenomenon is reminiscent of homeopathic principles. Homeopathy (Greek for “similar suffering”) is the practice of treating symptoms with highly diluted agents that would induce the same symptoms if the agent were given in a much higher dose. Thus, if a patient reports having a headache and nausea, a homeopathic specialist could find an herb that causes headaches and nausea, and treat the patient with a highly diluted solution of this herb. This is similar to the ultra-low dose research demonstrating that an antagonist, which would normally block the effects of an agonists at high doses, will enhance the properties of the agonist when given in highly diluted concentrations (Crain & Shen, 1995; Paquette et al. in press; Powell et al. 2002), and that morphine, which typically has antinociceptive properties, will produce pronociceptive effects when given in ultra-low doses (Kayser et al. 1987; Crain & Shen, 2001). Although the homeopathic remedies have been criticized as being nothing more than placebo treatments, a meta-analysis on studies investigating homeopathy concluded that the placebo effect is not entirely responsible for these effects (Linde et al. 1997).

Studies on homeopathy, however, typically use poor scientific methodology, for instance not having proper control groups, and rarely seek mechanistic explanations for their effects (Jonas, Anderson, Crawford, & Lyons, 2001). The ultra-low dose research is different from traditional homeopathic research because it uses proper control groups and seeks mechanistic explanations for the results (Chindalore et al. 2005; Paquette et al. in press; Powell et al. 2002; Wang & Burns, 2006; Wang et al. 2005; Webster et al. 2006). Although much of the knowledge about homeopathic remedies is anecdotal, the ultra-low dose research may provide mechanistic evidence that some homeopathic remedies are producing effects through pharmacological mechanisms.

Ultra-low dose research may also contribute to our understanding of low-level environmental contamination. One contaminate that has recently received abundant media coverage is bisphenol-A, a byproduct of epoxy resins and polycarbonate plastics. Many plastic and metal food containers leach bisphenol-A into our food (Kang, Kondo, & Katayama, 2006). The problem is that bisphenol-A mimics the effects of estrogen (Krishnan, Stathis, Permuth, Tokes, & Feldman, 1993). Europeans consume about 400 ng/kg/day of bisphenol-A, but some animal studies show adverse effects with doses as low as 25 ng/kg/day (European Commission, 2002; Chitra, Latchoumycandane, & Mathur, 2003; Munoz-de-Toro et al. 2005), although not all researchers agree and suggest that adverse effects primarily occur at near toxic doses (Haighton et al. 2002; Kamrin, 2007; Tyl et al. 2002). Despite these dosing arguments, a greater understanding of the ultra-low dose phenomenon could help to clarify the changes that occur with chronic ingestion of ultra-low dose bisphenol-A. In an extreme example, just like Crain and Shen (1989) used ultra-low doses of an antagonist to prevent the stimulatory effects

of ultra-low dose morphine, impregnating ultra-low doses of antagonists into the plastics could be investigated as a means to resolve the health issues associated with bisphenol-A leaching.

Though the latter parts of this discussion may be overextending the applicability of the ultra-low dose effect, the point is that this effect could have far reaching implications. Of course, there is still much to be learned about the effects of ultra-low doses and the mechanism responsible for the G-protein coupling switch. To date, there is one new ultra-low dose combination therapeutic, Oxytrex, which is in clinical trials for osteoarthritis; no doubt others will soon follow. Future research should be directed at finding other receptor types which show the ultra-low dose effect and/or the chronic agonist-induced G-protein coupling switch. Ultimately, a better understanding of the mechanisms of these processes will hopefully lead to new drugs with fewer side effects, a pharmaceutical magic bullet.

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Appendix I

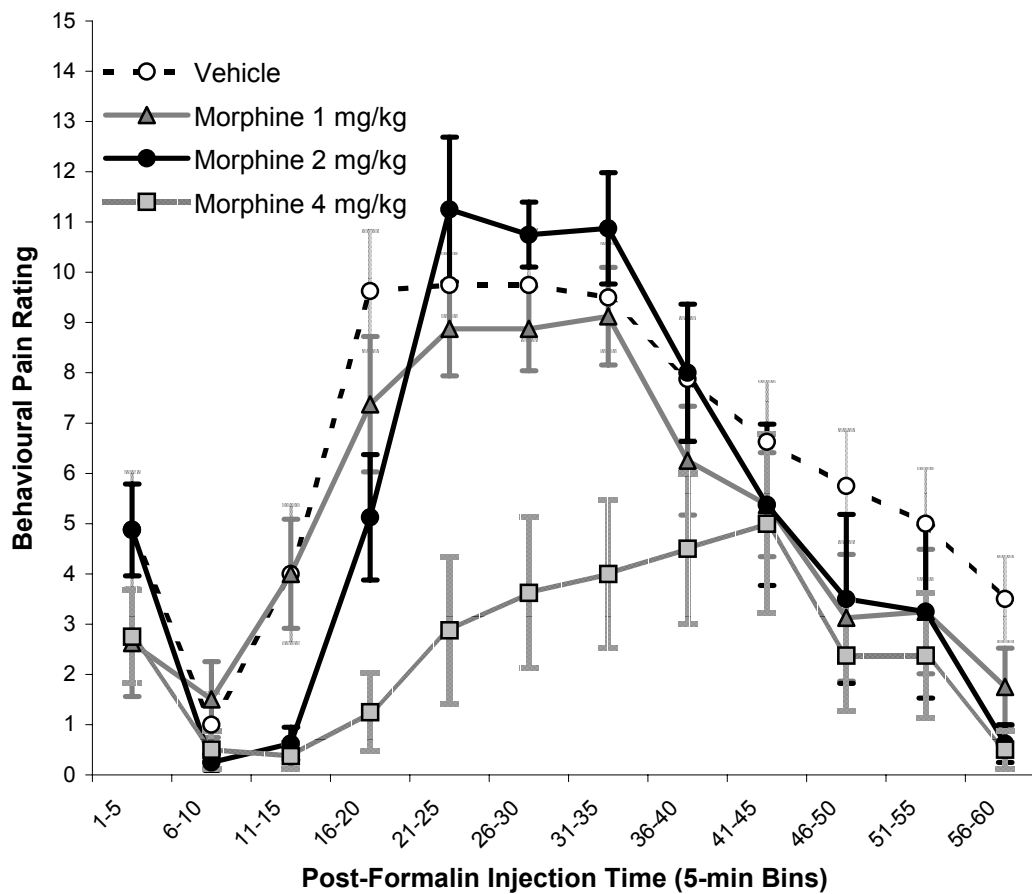


Figure A1. Formalin test time course data from the morphine dose response (see figure 4.1) as represented in 5-min bins. Behavioural pain ratings for each animal were added across each 5-min bin. Points represent the mean (\pm SEM) for each drug group. Animals received a drug injection 30 min prior to a 2% formalin injection in the hind left paw.

Appendix II

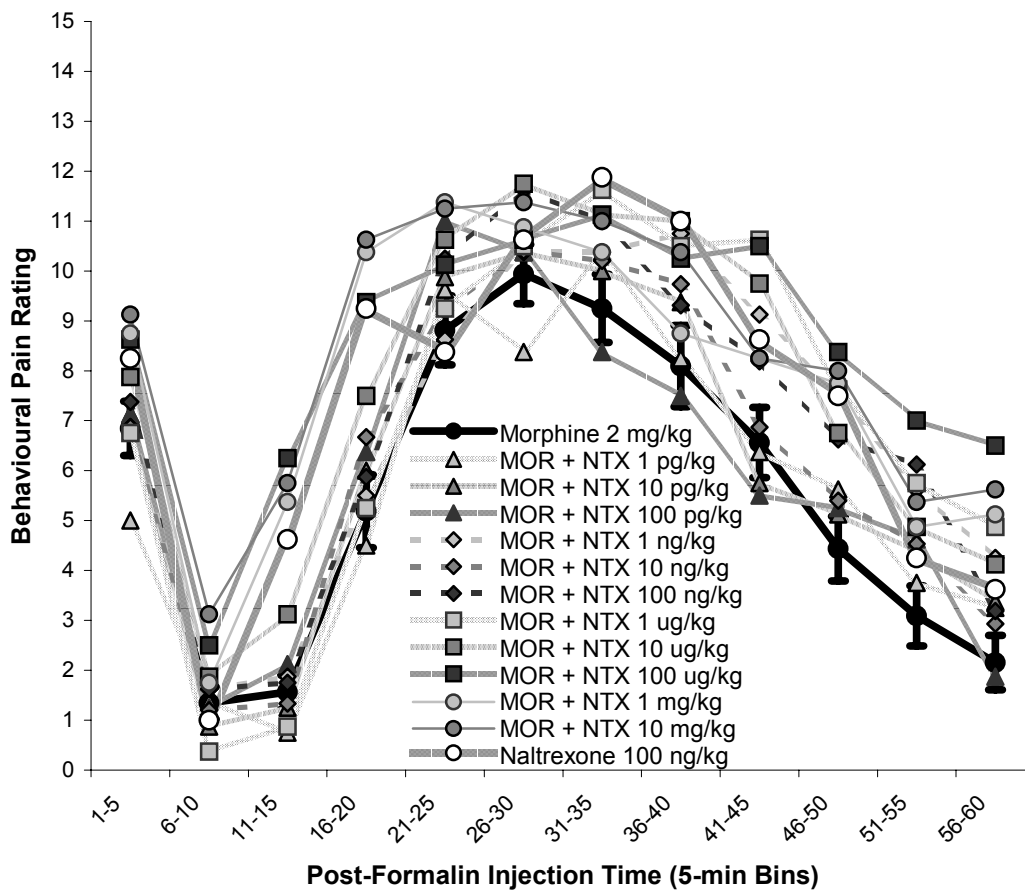


Figure A2. Formalin test time course data from the morphine (2 mg/kg) plus naltrexone (NTX) dose response (see figure 4.2) as represented in 5-min bins. Behavioural pain ratings for each animal were added across each 5-min bin. Points represent the mean (\pm SEM for the morphine 10 mg/kg alone group) for each drug group. Animals received a drug injection 30 min prior to a 2% formalin injection in the hind left paw.

Appendix III

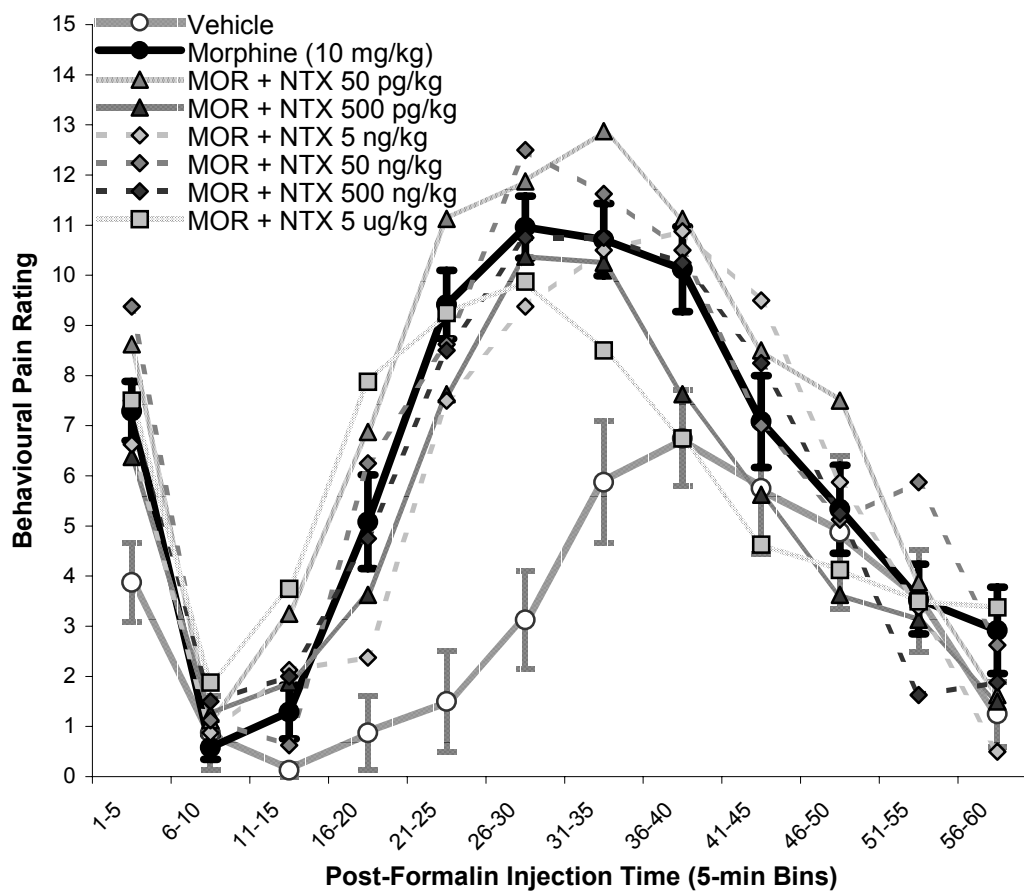


Figure A3. Formalin test time course data from the sub-chronic morphine (10 mg/kg) plus naltrexone (NTX) dose response (see figure 4.4) groups as represented in 5-min bins. Behavioural pain ratings for each animal were added across each 5-min bin. Points represent the mean (\pm SEM for the morphine 10 mg/kg alone and vehicle groups) for each drug group. All animals had a drug history (see figure legend) that involved one injection/day for 6 days. On day 7, all animals received a 10-mg/kg morphine injection 30 min prior to a 2% formalin injection in the hind left paw.

Appendix IV

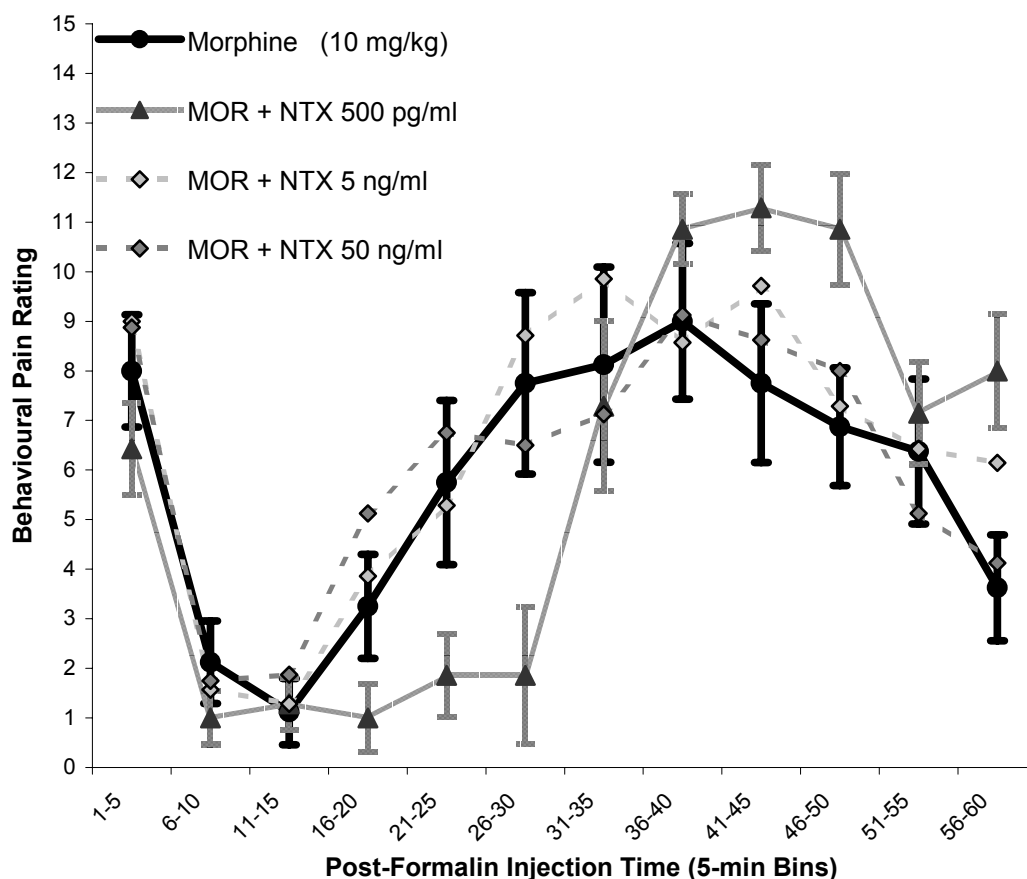


Figure A4. Formalin test time course data from the sub-chronic morphine (10 mg/kg) plus naltrexone (NTX) dose response (see figure 4.6) groups as represented in 5-min bins. Behavioural pain ratings for each animal were added across each 5-min bin. Points represent the mean (\pm SEM for the morphine 10 mg/kg alone and morphine 10mg.kg + naltrexone 500 pg/kg groups) for each drug group. All animals had a drug history (see figure legend) that involved one injection/day for 6 days. On day 7, animals received the same injection they have been getting for the previous 6 days 30 min prior to a 2% formalin injection in the hind left paw.