ABSTRACT

Hercules T Maguma. COMPARISON OF TOLERANCE CHARACTERISTICS IN THE GUINEA PIG FOLLOWING CHRONIC *IN-VIVO* EXPOSURE TO OPIOID VERSUS CANNABINOID RECEPTOR AGONISTS. (under the guidance of David A. Taylor)

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Few studies have compared the nature of tolerance that develops following chronic *in vivo* opioid treatment with that which develops after chronic cannabinoid exposure in the same tissue and species. Based on similarities in signaling and overlapping neuroanatomical receptor localization the candidate determined whether the tolerance that develops to hypothermic, analgesic and inhibitory action on neurogenic contractions of the longitudinal muscle-myenteric plexus (LM/MP) in the guinea pig is qualitatively similar regardless of the agonist employed. Since previous *in vitro* drug exposure studies using the LM/MP model have reported bidirectional heterologous tolerance, it was hypothesized: 1) that *in vivo* exposure to either agonist would result in heterologous tolerance 2) that the type of tolerance could be used to define the underlying cellular mechanisms; 3) that homologous tolerance would employ a mechanism that involved receptor regulation; 4) that co-localization of opioid and cannabinoid receptors may provide a basis for some cross-tolerance between agonists; and 5) that the mechanisms that underlie the development of tolerance can vary among different tissues or models. Specific aim 1 assessed the effect of chronic cannabinoid or opioid exposure on the sensitivity of the LM/MP to inhibitory agonists (WIN-55,212-2-2, CADO or morphine) or an

excitatory agent (nicotine). Animals pretreated with morphine *in vivo* developed an increased responsiveness to nicotine and tolerance to all inhibitory agonists tested: the magnitude of rightward shift (i.e. ratio of mean IC_{50} values) or loss of sensitivity of the treated compared to the control group was 4.8-fold for DAMGO, 3.5-fold for CADO, and 5.2-fold for WIN-55,212-2. In contrast, *in vivo* WIN-55,212-2 pretreatment resulted in subsensitivity to WIN-55,212-2 **only** (factor of rightward shift of the IC_{50} values was 9.8) and reduced maximum responses to WIN-55,212-2 and DAMGO; no shift was observed in the dose response curves for DAMGO, CADO and nicotine. Specific aim 2 sought to determine the effect of chronic WIN-55,212-2 or morphine exposure on the levels of both mu-opioid receptor (MOR) and cannabinoid receptor 1 (CB_1) protein in homogenates of the LM/MP. WIN-55,212-2 treatment resulted in a selective reduction in CB_1 receptor protein levels by 35% while MOR levels remained unchanged whereas morphine exposure altered neither MOR nor CB_1 receptor protein levels. Specific aim 3 sought to determine the qualitative nature of tolerance that develops in analgesic (thermal and mechanical) and hypothermic models. Chronic morphine treatment resulted in heterologous tolerance to the thermal analgesic effect of morphine and WIN-55,212-2 but did not alter the sensitivity to the hypothermic effect of WIN-55,212-2. The nature of tolerance observed in the hot plate test corresponds closely to that observed in the LM/MP studies where chronic morphine treatment produced heterologous tolerance and WIN-55,212-2 pretreatment resulted in homologous tolerance. In contrast to the results in the LM/MP studies, WIN-55,212-2 pretreatment resulted in tolerance to the analgesic effect of morphine in the paw pressure model despite the fact that WIN-55,212-2 did not produce analgesia in this model. Unlike chronic treatment with WIN-55,212-2, chronic morphine treatment did not induce tolerance to the hypothermic effect of WIN-55,212-2. However, since only a very modest hypothermia was

observed in response to a morphine challenge, tolerance to this effect was difficult to assess and may not be pharmacologically relevant. For specific aim 4 the candidate explored the distribution of MOR and CB_1 receptor expressing neurons in the LM/MP and hypothalamus. Immunofluorescence assessment of the distribution of neurons expressing MOR and CB_1 receptors in the LM/MP revealed significant co-localization of receptors on myenteric plexus neurons thus raises the possibility of intracellular crosstalk between the two receptor systems. Furthermore, neither opioid nor cannabinoid treatment altered the density or distribution pattern of neurons expressing MOR or CB_1 receptors. Assessment of neurons expressing MOR and CB_1 receptors in the preoptic anterior hypothalamus revealed extensive co-localization suggesting possible interaction of the two receptor systems in the regulation of body temperature. In conclusion, the variable tolerance expression observed in different models affirms the notion that nature and potential cellular mechanisms of tolerance can vary depending on the model system, the drug, the species, and regimen used to establish the phenomenon. The data also suggest that multiple cellular effects may play a role in the induction of functional tolerance in different model systems.

COMPARISON OF TOLERANCE CHARACTERISTICS IN THE GUINEA PIG FOLLOWING CHRONIC *IN-VIVO* EXPOSURE TO OPIOID VERSUS CANNABINOID RECEPTOR AGONISTS.

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COMPARISON OF TOLERANCE CHARACTERISTICS IN THE GUINEA PIG

FOLLOWING CHRONIC *IN-VIVO* EXPOSURE TO OPIOID VERSUS CANNABINOID

RECEPTOR AGONISTS.

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2-AG	2-arachydonoylglycerol
CADO	2-chloroadenosine
AC	adenylyl cyclase
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid receptor type 1
CB_2	cannabinoid receptor type 2
CNS	central nervous system
CREB	cAMP response element binding
DAMGO	Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol
DOR	delta opioid receptor
ERK	extracellular signal regulated kinase
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
GAT-1	GABA transporter-1
GIRKs	G-protein-activated inwardly rectifying potassium channels
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
KOR	kappa opioid receptor
LM/MP	longitudinal muscle/myenteric plexus
MAGL	monoacyl-glycerol lipase

MOR	mu-opioid receptor
ORL_1	opioid receptor-like receptor 1
PAG	periaquaductal grey
PAGE	polyacrylamide gel electrophoresis
PKA	protein kinase A
PKC	protein kinase C
POAH	preoptic anterior hypothalamus
VTA	ventral tegmental area
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
β-ARK	beta-adrenergic receptor kinase

CHAPTER ONE: INTRODUCTION

I. General Introduction

The use of opioids, which dates back thousands of years, has evolved to its current state of conventional use in clinical settings over a long period of time, whereas the potential clinical uses of marijuana in clinical settings has only just begun to be realized in the past few decades. However, the recreational use of marijuana has been known for many years. The United Nations' World Drug Report of 2009 estimated that in 2007, between 143 and 190 million (~2.5% of the world population) worldwide used marijuana. Further evidence of the widespread use of this agent includes a 2007 National Survey on Drug Use and Health which revealed that 14.4 million Americans over 12 years of age had used marijuana at least once in the month preceding the survey. Regarding opioid use, a survey performed in 2008 by the National Institute on Drug Abuse (NIDA) estimated that 453,000 Americans over 12 years of age had used heroin once in the preceding year, proving that the illicit use of opioids is also relatively high.

Extensive studies on the opioid system have discovered a number of endogenous and exogenous ligands targeting specific receptor subtypes that provide some diversity with respect to the clinical uses of the agents and the endogenous regulation of responses to external stimuli. Opioids and related agents are clinically indicated for the treatment of pain, acute pulmonary edema, cough and diarrhea. Currently, cannabinoids are used clinically to treat muscle spasms in patients with multiple sclerosis, as anti-emetics in patients receiving chemotherapy for cancer, and to manage cachexia in HIV/AIDS patients. At least 66 phytocannabinoids have been isolated with Δ^9- tetrahydrocannabinol (Δ^9-THC), the main psychoactive alkaloid, serving as the prototypical compound. Extensive research in the last twenty years has led to the discovery of an

endogenous cannabinoid system that utilizes a number of different "cannabinoid-like" compounds which include arachidonoyl-ethanolamine (anandamide or AEA), 2-arachidonoyl glycerol (2-AG), virodhamine (O-arachidonoyl-ethanolamine (OAE), noladin ether (2-arachidonyl glyceryl ether) and N-arachidonoyl-dopamine (NADA) (Rodriguez de Fonseca et al., 2005). The discovery of two distinct cannabinoid receptors (i.e. CB_1 and CB_2), has spurred research into developing commercially viable agonists targeting these receptors e.g. dronabinol, nabilone and Sativex® (containing Δ^9- tetrahydrocannabinol and cannabidiol). The selective CB_1 receptor antagonist Rimonabant (SR141716) indicated for obesity and smoking cessation, has recently been withdrawn from the European market due to concerns over adverse effects, namely depression and suicidal ideation.

Chronic use of both cannabinoids and opioids is associated with the rapid development of tolerance which is defined by a progressive diminution of efficacy following prolonged drug exposure. This phenomenon results in the need for higher doses to achieve the same clinical benefit which increases the risk of adverse events including drug overdose, withdrawal effects and dependence. The extent of development of tolerance varies significantly depending on the pharmacological effect e.g. opioid tolerance develops quicker to its urinary retention and respiratory depression effects compared to its analgesic effects (Rang et al. 2007). In fact, tolerance to some effects of opioids e.g. constipation and miosis, does not occur in humans (Rang et al., 2007). Clinical studies evaluating tolerance to the analgesic effect of opioids have reported a reduction in tolerance by using agents like calcium channel blockers and the cholecystokinin antagonists (Santillan et al., 1994; Timar et al., 2005). In animal studies, calcium channel blockers (Smith et al., 1999), intrathecal magnesium and zinc (McCarthy et al., 1998; Larson et al., 2000), the phosphodiesterase inhibitor ibudilast (Ledeboer et al., 2007) or

cholecystokinin antagonists like proglumide (Watkins et al., 1984; Wong et al., 1996) have also been reported as promising remedies for analgesic tolerance observed following chronic opioid use. Tolerance to cannabinoids has not been widely reported in clinical settings and it has been suggested that extremely high doses may need to be consumed before tolerance can be observed (Flom et al., 1975; Jones et al., 1981).

Simultaneous or successive use of both opioids and cannabinoids has escalated over the last few years especially in terminally ill patients (e.g. HIV and cancer patients). Thus the potential interactions between these agents in terms of acute actions as well as the development of adverse effects should be of concern. Studies in animals have demonstrated rapid development of tolerance to these agents and possible cross-modulation of their pharmacological effects (Welch and Eads, 1999; Bass and Martin, 2000; Li et al., 2010). The interaction between opioids and cannabinoids stems from their similarities in cellular and physiological effects, and is likely influenced by the co-localization of their receptors (Salio et al., 2001). In spite of the large number of studies that have been performed, there are still conflicts, opposing data, and insufficient evidence to fully explain the tolerance phenomena observed following chronic treatment with these agents. This work described in this dissertation attempts to characterize the nature of the tolerance, look at the possible basis for the pharmacological interaction between these agents and describe the potential mechanisms associated with development of tolerance to the effects of these agents and to determine whether the mechanism is commonly shared or distinct.

II. Cannabinoid Pharmacology

1. Cannabinoid Receptor Subtypes

There are two major subtypes of cannabinoid receptors namely cannabinoid receptor subtype 1 (CB_1) and CB_2 receptor. The first CB_1 receptor cDNA was cloned using the rat brain (Matsuda et al., 1990). The CB_2 receptor which encodes fewer amino acids (360), displays a 48% amino acid sequence homology to the CB_1 receptor (Munro et al., 1993). The existence of a third cannabinoid receptor, the orphan receptor (GPR55), has been proposed based on its binding site sequence homology to classical cannabinoid receptors and the fact that it is activated by the cannabinoid receptor ligands O-1602 and abnormal cannabidiol (Slowe et al., 1999; Johns et al., 2007). In addition, the TRPV1 (transient receptor potential cation channel, subfamily V, member 1) or vanilloid receptor 1, a ligand-gated non-selective cation channel activated by anandamide (AEA) and N-arachidonoyl-dopamine (NAPA) has also been proposed to represent another cannabinoid receptor subtype (Huang et al., 2002).

2. Cannabinoid Receptor Distribution

A. Central nervous system distribution. The extent and pattern of CB_1 receptor distribution correlates with modulation of region-specific function associated with the effects of cannabinoids including alteration in memory, cognition, autonomic function and mood (Rodriguez de Fonseca et al., 2005). The hippocampus, cerebellum, caudate, substantia nigra, nucleus accumbens, globus pallidus and some olfactory regions demonstrate a high density of the CB_1 receptor. The amygdala, medial hypothalamus and solitary nucleus display a moderate density of CB_1 receptors whereas the thalamus and other parts of the brainstem e.g. nucleus of the solitary tract express low levels of CB_1 receptors. The regulation of pain by cannabinoid

receptor agonists directly correlates with CB_1 receptor expression in the periaquaductal gray, amygdala, raphe and the dorsal horn of the spinal cord where the receptor density is relatively low (Tsou et al., 1998; Farquhar-Smith et al., 2000). Although CB_1 receptors are predominantly found on the presynaptic terminals where they appear to regulate transmitter release, they have also been identified on post-synaptic structures and glia (Rodriguez et al., 2001). Central CB_2 receptor distribution and density is comparatively sparse with receptor expression mainly localized in the following regions: neurons of the piriform, orbital, visual, motor and auditory cortex (Svizenska et al., 2008). In addition, the anterior olfactory nucleus, periaquaductal grey, substantia nigra pars reticulata and the pyramidal neurons on the hippocampal allocortex also express a low levels of CB_2 receptors.

B. Peripheral nervous system distribution. In the enteric nervous system, both cholinergic and non-cholinergic sensorimotor submucosal neurons including those in the myenteric plexus innervating the longitudinal muscle express CB_1 receptors (Tyler et al., 2000; Adami et al., 2002). Activation of these enteric receptors attenuates the release of acetylcholine thus inhibiting smooth muscle contractility (Coutts and Pertwee, 1997). Intestinal contractility mediated through non-adrenergic non-cholinergic (NANC) neurons is also attenuated following CB_1 receptor activation (Izzo et al., 1998). Furthermore, activation of CB_1 receptors in the gastrointestinal tract inhibits intestinal and gastric fluid secretion (Pertwee, 2001). In the pelvic viscera, CB_1 receptors are expressed in the vas deferens, bladder and the uterine wall where they regulate smooth muscle contraction (Pertwee et al., 1992). Peripheral expression of CB_2 receptors is mostly restricted to the immune system cells including β-cells, natural killer cells and monocytes which are suspected to play an immunomodulatory role (Walter et al., 2003).

CB_2 receptor transcripts have also been found organs associated with the immune response including the spleen, thymus, tonsils and mast cells (Berdyshev, 2000; Sugiura and Waku, 2000).

3. Ligands Targeting the Cannabinoid Receptors

A. Endogenous ligands. Anandamide (AEA) was the first endogenous cannabinoid receptor ligand discovered and thereafter, 2-arachidonylglycerol (2-AG) was identified (Devane et al., 1992; Sugiura et al., 1995). 2-AG is a full agonist at both CB_1 and CB_2 receptors whereas anandamide acts as a partial agonist for both the CB_1 and CB_2 receptors although it has higher affinity for the latter (Hillard et al., 1999). The production of endocannabinoid neurotransmitters appears to be triggered by the stimulation of postjunctional neurotransmitter receptors (Giuffrida et al., 1999). Following release into the synaptic cleft, the ligand influxes back into the cells via an active and Na^+-independent transport mechanism (Beltramo et al., 1997). The sequestered 2-AG is eventually hydrolyzed by either monoacylglycerol lipase (MAGL) or fatty acid amide hydroxylase (FAAH) with the latter being the major player (Dinh et al., 2002).

Other recently discovered endocannabinoids include virodhamine (O-arachidonoyldopamine), which acts as a partial agonist at cannabinoid receptors (Porter et al., 2002; Walker et al., 2002); noladin ether (2-arachydonoyl glyceryl ether) which targets both CB_1 and CB_2 receptors but has higher affinity for the latter (Hanus et al., 2001); and N-arachidonoyldopamine which acts as a ligand at CB_1 and $TRPV_1$ receptors and elicits pharmacological responses associated with cannabinoid receptor activation including analgesia (Huang et al., 2002).

B. Exogenous ligands. The most widely used exogenous cannabinoids are the phytocannabinoids derived from *Cannabis sativa* (marijuana) with Δ^9-THC (Fig 1.1) being the

most thoroughly studied. There are four major classes of naturally occurring and synthetic exogenous cannabinoid ligands namely classical cannabinoids (e.g. Δ^9-THC, Δ^8-THC and HU-210), AC-bicyclic and ACD-tricyclic analogs (e.g. CP-55,940), aminoalkylindoles (e.g. WIN-55,212-2) and the diarylpyrazole compounds (e.g. SR141716 and AM521) which act as inverse agonists and/or antagonists at the cannabinoid receptors (Rinaldi-Carmona et al., 1994). In the current study we use WIN-55,212-2 which is highly lipid soluble and acts as a full agonist at both cannabinoid receptors though it has a two-fold higher affinity towards the CB_2 receptor.

4. Signal Transduction and Cellular Effects

Cannabinoid receptors are G protein coupled therefore ligand binding induces a conformational change that results in the interaction of the serpentine receptor with its cognate heterotrimeric G protein located within the cytosol. Following agonist activation of the cannabinoid receptor and shifts in conformation of the G protein, the GTP conjugated $G_{i/o}$-protein exchanges GTP for GDP then dissociates to form G_α–GTP and $G_{\beta\gamma}$ subunits which act as primary messengers for intracellular signaling.

Both CB_1 and CB_2 receptors are negatively coupled to adenylyl cyclase via the G_α subunit hence receptor activation reduces cytosolic cAMP levels in a pertussis toxin sensitive fashion (Howlett and Fleming, 1984; Howlett et al., 1986; Felder et al., 1992). The reduction in cAMP levels results in a decreased activation of cAMP dependent protein kinase A (PKA) activity hence attenuating PKA modulated downstream events. Ion channel activity is modulated either directly via the G protein $\beta\gamma$ subunits or indirectly using other secondary messengers like protein kinase A (PKA) (Childers and Deadwyler, 1996). CB_1 receptor agonists activate K^+ channels resulting in K^+ efflux thus hyperpolarization of the cell membrane potential. This

action is accomplished by direct activation of the G protein-activated inwardly rectifying K^+ channel (GIRK) via the $G_{\beta\gamma}$-protein subunit (Henry and Chavkin, 1995; Mackie et al., 1995). Activation of A-type-K^+ channels is inhibited by cAMP-dependent PKA hence the cannabinoid induced reduction in cAMP-dependent PKA activity also results in greater A-type-K^+ channel activity (Childers and Deadwyler, 1996). Cannabinoid receptor agonists indirectly inhibit L-type Ca^{2+} channels via the $G_{\beta\gamma}$ subunit (Gebremedhin et al., 1999). Other Ca^{2+} channels inhibited by cannabinoid receptors agonists include the N- and Q-type Ca^{2+} channels (Caulfield and Brown, 1992; Felder et al., 1993; Mackie et al., 1995). In a paradoxical fashion, calcium currents can also be activated following CB_1 receptor activation in a pertussis toxin and phospholipase C sensitive manner (Sugiura et al., 1996).

CB_1 receptor agonists have been reported to activate mitogen activated protein kinases (p38 and p42/p44) which results in increased early gene expression (Bouaboula et al., 1995; Reche et al., 1998; Liu et al., 2000). Anandamide has also been demonstrated to activate production of nitric oxide in the rat median eminence fragments and in human arterial endothelial cells (Prevot et al., 1998). Focal adhesion kinase (FAK), responsible for signal transduction events and integrating cytoskeleton changes like synaptic plasticity (neutric retraction), is also regulated by cannabinoid receptor agonists (Derkinderen et al., 1996).

5. Summary of Cannabinoid Actions

Endogenous cannabinoids are distributed throughout the body and cannabinoid receptors exist in tissues where endogenous cannabinoid innervations occurs as well as in tissues without any cognate cannabinoid innervations. The effects appear to be mediated by primarily 2 receptor subtypes (CB_1 and CB_2) with the majority of the pharmacological responses in the central and

peripheral nervous systems being mediated through the CB_1 receptor. The effects mediated by CB_2 receptors appear to focus more on the inflammatory processes and the immune system (Newton et al., 2009). The effects that are important from a clinical point of view include regulation of gastrointestinal motility, ataxia, appetite, reward, learning and memory, analgesia and body temperature.

The central nervous system effects of CB_1 receptor agonists include analgesia, appetite enhancement, modulation of muscle activity, and regulation of hormone and neurotransmitter release (Di Marzo et al., 2001; Fride and Shohami, 2002; Fride et al., 2003). Endocannabinoids in the brain regulate the release of acetylcholine, dopamine, GABA, histamine, serotonin, glutamate, prostaglandins, norepinephrine and opioid peptides thus indirectly affecting the physiological function of the organism. Cannabinoid-induced reduction of dopamine activity in brain areas involved in motor control and reward may explain its effect on motor co-ordination and addiction, respectively (Giuffrida et al., 2001; Gardner, 2002). Cannabinoids demonstrate effective analgesic activity in models of neuropathic pain and have also been shown to work synergistically with opioids in acute pain models (Welch and Eads, 1999). Neuropathic pain in humans generally responds poorly to opioid agents but cannabinoids have been shown to be effective in alleviating pain associated spinal cord injury, peripheral neuropathy, and nerve injury (Wilsey et al., 2008). A neuroprotective role for endocannabinoids in the traumatic brain injury animal model (Mechoulam et al., 2002) may be due to inhibition of metabotropic glutamate signaling that results in toxic Ca^{2+} influx or through an antioxidant effect that scavenges reactive oxygen species (Hampson et al., 2000; Grundy, 2002).

The cardiovascular effects of cannabinoids include tachycardia, increased cardiac output and elevated oxygen demand (Tashkin et al., 1977; Szabo et al., 2001). Other pharmacological

effects of cannabinoids include peripheral vasodilation, orthostatic hypotension, and reduced platelet aggregation (Lake et al., 1997). In the gastrointestinal tract, CB_1 receptor activation attenuates vagal drive leading to reduced gastric emptying and secretion, and decreased peristaltic activity (Coruzzi et al., 1999; McCallum et al., 1999). Coutts and Pertwee (1997) demonstrated that activation of presynaptic CB_1 receptors, predominantly located on cholinergic neurons in the myenteric plexus, results in the inhibition of release of acetylcholine from the myenteric 'S' neurons thus inhibiting peristaltic activity. Other pharmacological effects of cannabinoids mediated through CB_2 receptors include a reduction in sperm count (Hembree et al., 1978) and an interaction with the hypothalamic–pituitary-adrenal axis which influences a number of hormonal processes including hypothermia (Rawls et al., 2002).

III. Opioid Pharmacology

1. Opioid Receptor Subtypes

Several classes of opioid receptors have been identified that include the mu- (MOR), kappa- (KOR), delta- (DOR) opioid and nociceptin receptors (opioid like receptor [ORL-1]). Two MOR subtypes, MOR_1 and MOR_2, have been identified and characterized based upon variable biphasic binding characteristics and differential blockade of opioid effects by naloxonazine (Pasternak, 2005). Two delta receptor subtypes, DOR_1 and DOR_2, have been characterized by DOR-selective agonists and antagonists which display variable patterns of supraspinal analgesia (Traynor and Elliott, 1993). Another delta receptor classification postulates the existence of $delta_{cx}$ and $delta_{ncx}$ subtypes based on the hypothesis that the $delta_{cx}$ subtype is complexed with MOR (or perhaps KOR) whereas the $delta_{ncx}$ subtype exists independently (Traynor and Elliott, 1993). The existence of three subtypes of the kappa opioid receptor (KOR_1,

KOR_2 and KOR_3) has been proposed based on radioligand binding studies, but currently there is no categorical functional pharmacological evidence to fully support this idea (Devi, 2001). cDNA library screening has identified the opioid like receptor 1 (ORL_1) which has a high degree of homology to the classical receptor subtypes. The existence of other opioid receptor subtypes namely sigma (σ) and epsilon (ε) receptors has been proposed but there is either contradictory data or insufficient evidence to support the claims of physiological existence and function of these receptor subtypes (Contet et al., 2004).

2. Opioid Receptor Distribution

A. Central Distribution. The CNS comparatively expresses higher levels of opioid receptors than the peripheral nervous system. One or more of the mu-opioid receptor (MOR) subtypes are expressed to the highest levels in brain regions that include the cerebral cortex (especially laminae II and IV), thalamus, striosomes (striatum), periaquaductal gray and the substantia gelatinosa of the spinal cord whereas kappa opioid receptor subtypes (KOR) are highly expressed in the hypothalamus, periaquaductal gray, claustrum and the substantia gelatinosa of the spinal cord (Anand et al., 2010). The delta opioid receptor subtypes are distributed in the pontine nuclei, amygdala, the olfactory bulbs and deep cortex whereas ORL_1 expression has been identified in the cerebral cortex, amygdala, hippocampus, septal nuclei, habenula and the spinal cord. Regardless of the distribution, the cellular transduction pathway that mediates the effects of receptor activation is similar for all the receptors (Connor and Christie, 1999).

B. Peripheral Distribution. Expression of MOR and KOR in the myenteric plexus of the gut is well documented (Bagnol et al., 1997); activation of these receptors results in a reduction

of acetylcholine release from the myenteric ‘S’ neurons (Paton, 1957). Mu-opioid receptors (MOR) are located on the soma and reduce transmitter release by hyperpolarization-mediated reduction in excitability while kappa opioid receptors, located on the axon terminals, decrease acetylcholine release by inhibiting calcium influx into the nerve terminal (Kojima et al., 1994; Coutts and Pertwee, 1997). In addition, the small and large intestines also express DOR. Delta- and mu- opioid receptors have also been identified in the vas deferens (Marshall et al., 1979; Sheehan et al., 1986). Evaluation of testes, ovary, uterus, kidney and adrenal tissue suggests the presence of MOR in these tissues. Sparse populations of the DOR and KOR have also been reported to exist in these peripheral tissues as indicated in Table 1 (Wittert et al., 1996).

3. Opioid Receptor Ligands

A. Endogenous Ligands. Endogenous opioid ligands are derived from long-chained peptide precursor molecules. Pro-opiomelanocortin (POMC) is a precursor for opioid peptides like β-endorphin and the enkephalins, as well as non-opioid peptides like beta-melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH). β-endorphin displays high affinity for delta- and mu- opioid receptors but has weak affinity for the kappa opioid receptors (Corbett et al., 2006). Proenkephalin acts as a precursor for both Met- and Leu-enkephalin which are MOR agonists (Corbett et al., 2006). The derivative peptides cleaved from prodynorphin include dynorphin-A and dynorphin-B, α- and β-neoendorphin (Goldstein et al., 1981). Dynorphin A and B have a high affinity for KOR but also bind to MOR and DOR (Corbett et al., 2006). Pronociceptin acts as a precursor molecule for endomorphin-1 and -2, and nociceptin/orphanin-FQ, a ligand at ORL-1. Other active endogenous derivatives like dermorphin, and deltorphin I and II, have multiple precursors namely prodermorphin and

prodeltorphin. Dermophin is highly potent and selective for MOR whereas deltorphins are highly selective for DOR (Erspamer et al., 1989).

B. Exogenous Ligands. Opioids are naturally derived from the opium poppy plant (*Papaver somniferum*) and the structure of most synthetic analogs is derived from these naturally occurring ligands. The chemical classification groups the opioid ligands into four major classes namely phenanthrenes (e.g. morphine [Fig 1.2], codeine, hydromorphone, levorphanol, oxycodone, nalbuphine and hydrocodone), benzomorphans (e.g. pentazocine), phenylpiperidines (e.g. fentanyl, alfentanil, sulfentanil, and mepiridine) and diphenylheptanes (e.g. propoxyphene and methadone) (Trescot et al., 2008). Tramadol does not fit into any of the four groups stated above hence is classified as an atypical opioid.

The pharmacological classification of opioid ligands is based on the pharmacodynamic properties and defines the compounds as agonists, antagonists, and partial agonists or agonists-antagonists. Opioid receptor agonists include morphine, codeine, fentanyl, methadone and oxycodone. The partial agonist buprenorphine has a high affinity but low efficacy at the MOR and also acts as an antagonist at the KOR (Trescot et al., 2008). Most antagonists have high receptor affinity but are devoid of efficacy e.g. naloxone, naltrexone and nalmefene. Opioid agonist-antagonist agents e.g. pentazocine, nalbuphine and butopharnol, exhibit antagonistic effects on the MOR but also show agonist activity on the KOR.

4. Signal Transduction and Cellular Effects

As illustrated in Fig 1.3, opioid receptors are part of the GPCR superfamily which couple to pertussis toxin sensitive heterotrimeric $G_{i/o}$ proteins. Upon receptor activation the GTP conjugated heterotrimeric G protein exchanges GTP for GDP then dissociates to form G_{α}-GDP

and $G_{\beta\gamma}$ subunit complexes that are ultimately responsible for initiation of the downstream intracellular signaling. Opioid receptors are negatively coupled to adenylyl cyclase therefore activation lowers cytosolic cAMP concentrations resulting in the inhibition of a hyperpolarization-activated cation channel (Ih) also known as the pacemaker current thus decreasing neuronal excitability (Childers, 1991; Ingram and Williams, 1994). Inhibition of adenylyl cyclase activity is also associated with direct inhibition of neurotransmitter release in a PKA-dependent manner. Other pathways regulated by cAMP-dependent PKA including cell differentiation, ion channel conductivity and metabolism are also negatively regulated by opioid receptor activation (Krebs and Beavo, 1979; Schwartz and Rubin, 1983).

All opioid receptor subtypes have been shown to activate a variety of potassium channels, including the G protein-activated inwardly rectifying K^+ channel (GIRK) and BK calcium-sensitive potassium (Twitchell and Rane, 1993; Jan and Jan, 1997). Activation of opioid receptors has also been shown to inhibit voltage dependent conductance by dendrotoxin-sensitive and M-type channels (Madamba et al., 1999). Calcium channels inhibited following opioid receptor activation include L-, N-, P- and Q- type voltage dependent calcium channels (Samways and Henderson, 2006). In some cell types, receptor activation can also result in a paradoxical increase in calcium levels by releasing calcium from intracellular stores or enhancing its entry via a dihydropyridine-sensitive mechanism (Samways and Henderson, 2006).

Opioid receptor activation has been shown to attenuate neurotransmitter release. Activation of the MOR in the vas deferens and the LM/MP preparation results in the inhibition of ATP and acetylcholine release, respectively (Henderson et al., 1972; Kosterlitz and Waterfield, 1972; Coutts and Pertwee, 1997). Inhibition of neurotransmitter release has been attributed to the activation of potassium conductance and/or inhibition of calcium currents, or

inhibition of hyperpolarization-activated cation channel (Ih) (Ingram and Williams, 1994). In myenteric 'S' neurons, acetylcholine release is inhibited by both MOR and KOR activation; MOR-induced inhibition occurs via activation of K^+ currents at the level of the cell soma, while KOR activation inhibits the release through inhibition of Ca^{2+} currents. Other effects of opioid receptor activation include potentiation of NMDA currents via activation of PKC (Koyama and Akaike, 2008).

Opioid agonists have been shown to stimulate mitogen-activated protein kinase (MAPK) activity via different routes: one pathway involves $G_{\beta\gamma}$ subunit activation of phophatidyl-inositol 3-kinase (PI_3K) that eventually results in MAPK activation via a series of phosphorylation steps (Polakiewicz et al., 1998); a second method involves beta-arrestin which acts as an adaptor protein to bind both c-Src and the agonist-occupied receptor to form a mitogen signaling complex that is internalized (Ignatova et al., 1999). The internalized complex activates an extracellular signal regulated kinase (ERK), which is translocated into the nucleus and thus affects gene regulation through regulation of transcription factors such as cAMP response element binding (CREB) (Khokhlatchev et al., 1998).

5. Summary of Opioid Actions

The pharmacological effects of opioids are defined by the receptor type and its location. There is however, combined overlap of the effects amongst the receptors. Like MOR agonists, DOR ligands also stimulate supraspinal and spinal analgesia, and modulate release of hormone and neurotransmitter but lack some of the other pharmacological effects. Similarly, KOR activation results in supraspinal and spinal analgesia, as well as some of the psychosomatic effects and slowed gastrointestinal transit.

The central effects of opioid include regulation of sensory and affective components of pain. Euphoria that presents as a pleasant floating sensation is precipitated by opioid use. The sedative effect of opioids is employed clinically to supplement the action of hypnotic agents. At standard doses, morphine disrupts normal rapid eye movement and non-rapid eye movement (NREM) sleep patterns. Stimulation of MOR in the brainstem causes respiratory depression thus elevating alveolar pCO2 levels. Opioids also have antitussive and miotic properties. Direct stimulation of the chemoreceptor trigger zone results in nausea and vomiting especially with apormorphine. Other central effects of opioids include regulation of body temperature; the resultant effect depends on the receptor subtype activated e.g. selective activation of MOR in the anterior hypothalamus results in hyperthermia whereas KOR agonist activation induces hypothermia (Adler and Geller, 1987; Spencer et al., 1988). Central activation of MOR_1 results in supraspinal analgesia and physical dependence whereas the effects of MOR_2 activation include respiratory depression, miosis, euphoria, reduced gastrointestinal motility and physical dependence. Kappa opioid receptor activation results in spinal analgesia, sedation, miosis and inhibition of anti-diuretic hormone (ADH) release. The pharmacological effects observed following activation of the DOR include analgesia, antidepressant effects and physical dependence.

The cardiovascular effects of opioids are complex and ligand dependent. Most opioid agonists do not have a direct effect on the heart with meperidine being an exception since it causes tachycardia through its antimuscarinic action. Opioid-induced hypotension is probably due to peripheral and venous dilation attributed to central depression of vasomotor-stabilizing mechanisms and the release of histamine. Opioids also regulate gastrointestinal processes; in the myenteric plexus, activation of the MOR found on the soma of the ‘S’-type neurons results in

activation of a hyperpolarizing potassium current that leads to inhibition of acetylcholine release whereas activation of the KOR located on the axon terminal results in inhibition of calcium currents thus depress neuronal excitability (Cherubini and North, 1985; Kojima et al., 1994). Other gastrointestinal effects include contraction of the biliary smooth muscle. The sphincter of Oddi may contract resulting in biliary reflux (Rang et al. 2007). The renal effects of opioids include an increase in urethral, bladder, and sphincter smooth muscle tone, and a reduction in renal plasma flow. Other peripheral effects of opioids include uterine wall relaxation and histamine-induced pruritis.

Clinically, opioids are indicated for pain, congestive heart failure associated with pulmonary edema, elevated intracranial pressure, diarrhea, cough, shivering (meperidine) and in combination with other drugs for anesthesia. In spite of the large number of clinical uses, there are still forms of chronic pain that respond poorly to opioids. The development of alternative agents and protocols to the management of these types of pain has been somewhat of a shotgun approach with the selection of agents based upon speculation rather than evidence. Nevertheless, there are a number of agents that have been used either in place of or in conjunction with opioids to manage certain types of pain refractory to opioids e.g. valproate or carbamazepine or cannabinoids for neuropathic pain (Rang at al. 2007).

IV. The Phenomenon of Drug Tolerance

1. General Introduction

Tolerance occurs in response to chronic treatment when the pharmacological effects of a drug decrease such that larger doses are required to achieve the same response. Dosage adjustment potentially results in a greater incidence of adverse effects including drug overdose,

development of withdrawal and enhanced drug dependence. Tolerance and dependence to cannabinoids and opioids has been extensively studied and several animal models have been developed to test both phenomena. Multiple forms of tolerance, mediated via different sets of adaptation, are characterized by variable time courses for development (Bass and Martin, 2000; Li et al., 2010) and have been associated with reduced responsiveness of the test system to different agonists that is either homologous or heterologous. Homologous tolerance presents as limited alteration in responsiveness to specific agents using the same receptor or signaling pathway whereas heterologous tolerance exhibits as an altered responsiveness that extends to agents that do not utilize the same receptor or signaling pathways (Taylor and Fleming, 2001). The homologous form of diminished sensitivity has been shown to occur through a series of desensitization events that are precipitated by prolonged exposure to usually very high concentrations of an agonist. The latency to induce homologous desensitization varies from a few seconds to as long as days or weeks depending on the agonist, dose, species, and model used e.g. opioid induced homologous desensitization resulting from G protein uncoupling occurs within minutes (Law and Loh, 1999) whereas adenylyl cyclase (AC) upregulation as a form of adaptation may take hours to manifest (Nestler, 1993). In contrast, heterologous tolerance is usually associated with adaptive changes in responsiveness that develop over a longer time frame and decay over an even longer time course e.g. a reduction in the electrogenic function of the Na^+K^+ATPase (Fleming, 1999; Taylor and Fleming, 2001). While heterologous tolerance is certainly triggered by receptor-mediated events, the cellular impact extends to functions that are not tied to that receptor or signaling pathway.

It is generally believed that homologous tolerance is expressed faster than heterologous tolerance since it is associated with immediate receptor dependent changes (e.g. uncoupling of

the receptor from its cognate G protein, phosphorylation by GRKs, mobilization of beta-arrestins or changes in the adenylyl cyclase pathway) whereas heterologous tolerance is produced by non-receptor dependent changes like protein expression which can take days to weeks (Taylor and Fleming, 2001). The reversal of desensitization occurs relatively quickly following cessation of the drug exposure and depends on the length of agonist exposure, agonist used and the physiological/cellular effect being assessed. The time frame for recovery from heterologous tolerance is much longer and may last for weeks (Li et al., 2010).

2. Role of Phosphorylation in Desensitization

Early studies proposed the major mechanism implicated in desensitization to involve phosphorylation of the receptor (Stadel et al., 1983; Pitcher et al., 1998) by second messenger kinases like protein kinase C or protein kinase A (Benovic et al., 1985). However, later studies using PKC and PKA knockout animals identified beta-adrenergic receptor kinase (β-ARK), subsequently termed G protein-coupled receptor kinase subtype 2 (GRK2), as another kinase capable of phosphorylating the β_2-adrenergic receptor (Benovic et al., 1986). β_2-adrenergic receptor (β_2-AR) phosphorylation has been shown to occur through phosphorylation at two different regions; on serine residues (Ser262) in the 3rd intracellular loop by PKA and on the serine residues (Ser355 and Ser356) in the proximal COOH terminus by GRKs (Tran et al., 2004). The site of phosphorylation is dependent on the concentration of the agonist, with PKA activity triggered at low agonist concentrations and the GRK effect observed at higher agonist concentration (Tran et al., 2004). In addition, studies have shown that PKC can phosphorylate GRK2 thus enhancing the ability of the latter kinase to phosphorylate agonist activated GPCRs (Chuang et al., 1995; Krasel et al., 2001). PKA has also been implicated in the phosphorylation

of GRK-2 thus enhancing the latter's ability to phosphorylate the β_2-AR (Cong et al., 2001; Li et al., 2006).

In line with the concept of functional efficacy, the ability of a ligand to induce receptor phosphorylation appears to be ligand dependent. This could result from subtle variability in signaling between different ligands targeting the same receptor e.g. DAMGO to induces greater MOR internalization compared with morphine (Schulz et al., 2004). In human embryonic kidney (HEK) 293 cells expressing rat MOR_1 as well G protein-coupled inwardly rectifying potassium (GIRK) channel subunits, DAMGO was shown to induce greater MOR internalization in a GRK2-dependent fashion whereas morphine confers negligible internalization that is PKC dependent (Johnson et al., 2006). In the latter study, the desensitization by DAMGO was blocked by expression of a dominant negative mutant GRK2 whereas that induced by morphine was attenuated by PKC inhibitors.

3. The Role of Beta-Arrestins in Downregulation

Beta-arrestins have been demonstrated to be key players facilitating receptor desensitization (Lohse et al., 1990; Pitcher et al., 1992). Beta-arrestins have high affinity for the phosphorylated GPCRs and function by sterically hindering and uncoupling the receptor from its cognate G protein thus blocking the receptor-generated response (Gurevich and Gurevich, 2004). Fluorescence resonance energy transfer (FRET) analysis has illustrated beta-arrestin dissociation from the receptor upon agonist removal, even though the GPCR is still phosphorylated (Krasel et al., 2005). Beta-arrestin also directs the phosphorylated receptor as a GPCR/arrestin complex into clathrin coated pits. Upon internalization, the GPCR can either be dephosphorylated and recycled or degraded by lysosomes (Krupnick and Benovic, 1998). GRKs and arrestins also act

as multiprotein intracellular scaffolds, responsible for downstream activation of MAPK and other long-term cellular signaling pathways which may contribute to plasticity (DeWire et al., 2007; Ribas et al., 2007).

4. The Role of Changes in Protein Levels in Adaptive Heterologous Tolerance

Cellular tolerance may be associated with a decrease in receptor protein levels due to internalization that ultimately results in receptor degradation (Chavkin and Goldstein, 1984; Bohn et al., 2004). However, levels of other cellular proteins may also change as part of the key elements of adaptation that play a role in the development and maintenance of heterologous tolerance. The proteins that may be engaged in this process would likely be proteins that play a role in regulating general cell excitability since changes in responsiveness occur to agents that employ very different signaling pathways. Chronic morphine exposure has been reported to decrease the levels of the functional $alpha_3$ subunit isoform of the Na^+/K^+ ATPase responsible for the regulation of membrane potential (Biser et al., 2002). This has been proposed to account for a partial depolarization which results in increased supersensitivity to stimulatory agents and subsensitivity to multiple inhibitory agents (Meng et al., 1997; Taylor and Fleming, 2001; Li et al., 2010). Development of tolerance has also been associated with the alteration of specific AC isoforms involved in the long term adaptive phenomenon of cAMP superactivation (Rhee et al., 2000).

5. Functional Efficacy and Tolerance

The ability of different agonists targeting the same receptor to stimulate different signaling pathways has been explained by the concept of functional efficacy (Urban et al., 2007). The concept ascribes the ligand-specific effects to variable conformations of the GPCR conferred

by the agonist that results in activation of different sets of responses including the cellular processes responsible for desensitization (Perez and Karnik, 2005). Studies on the MOR demonstrate that DAMGO and etorphine both induce beta arrestin-GPCR interactions that lead to MOR internalization whereas exposure to morphine does not induce significant internalization (Keith et al., 1996; Zhang et al., 1998). It has been hypothesized that if the method of desensitization is agonist dependent, then resensitization and downregulation could also be agonist dependent. This potential was illustrated by Schulz et al. (2004) who discovered that morphine-induced phosphorylation and subsequent dephosphorylation and resensitization of the MOR occurs over a much slower timeframe than that associated with DAMGO exposure. However, the concept of the role of agonist efficacy in the development of functional tolerance is not universally accepted since studies with both opioids and cannabinoids have shown a lack of correlation between efficacy and the ability to induce desensitization (Clark et al., 1999). Other GPCRs that exhibit desensitization related functional selectivity include β_2-adrenergic receptor and 5-HT_{2C} receptors (Urban et al., 2007).

V. Development of Opioid Tolerance

1. The Role of Receptor Uncoupling and Phosphorylation in Tolerance

As with most G protein-coupled receptors (GPCR), opioid receptor function is regulated by several processes including phosphorylation primarily by GPCR kinase (GRK) (Appleyard et al., 1999) and secondary kinases like PKA and PKC (Smith et al., 2006). Upon agonist activation, the GPCR assumes a conformation that is receptive for phosphorylation by GRK, PKA or PKC (Smith et al., 2006). The PKC inhibitor, H7, and the PKA inhibitor, KT-5720, have been shown to reverse tolerance to the antinociceptive action of opioids (Narita et al., 1996;

Bernstein and Welch, 1997). Moreover, intrathecal administration of an anti-sense oligodeoxynucleotide to PKCα mRNA inhibited the development of tolerance to morphine (Hua et al., 2002). GRK phosphorylation of agonist-activated receptors results in beta-arrestin-induced uncoupling of the phosphorylated receptor thus resulting in receptor specific desensitization. Experiments assaying the coupling efficacy of MORs following chronic opioid exposure have illustrated functional uncoupling of the receptor that results in reduced GTPase activity, attenuated ability of opioids to stimulate G protein-activated inwardly rectifying K^+ channels (GIRK) and diminished regulation of calcium currents (Christie et al., 1987; Sim et al., 1996; Selley et al., 1997). The reduced coupling efficiency has also been attributed to beta-arrestin induced functional uncoupling of the receptors from their cognate G proteins or the loss of cell surface receptors (Chakrabarti et al., 1995a; Bohn et al., 2004).

2. The Role of Beta-Arrestins and Receptor Internalization in Tolerance

Beta-arrestin has been demonstrated to sterically hinder the interaction of an activated receptor with its cognate G protein. In addition, this protein also directs movement of the receptor to clathrin-coated pits in the cytosol thus initiating internalization, sequestration and trafficking of receptors (Gurevich and Gurevich, 2004). Phosphorylation of the opioid receptor has been shown to increase its affinity for beta-arrestin. The phosphorylated receptor/beta-arrestin complex is targeted for internalization through clathrin-coated pits and subsequently undergoes intracellular trafficking to subcellular compartments (e.g. lysosomes) where degradation or dephosphorylation occurs (Schulz et al., 2004). Receptor downregulation inevitably results in the loss of downstream signal transduction and is a key facet in the development of homologous tolerance (Chavkin and Goldstein, 1984; Chakrabarti et al., 1995a).

The extent of internalization and downregulation of MOR is ligand-dependent as DAMGO appears to have greater capacity to induce receptor downregulation when compared to morphine (Schulz et al., 2004; Johnson et al., 2006). Direct assessment of the density of the MOR on the cell surface has also shown downregulation in the brainstem following chronic treatment with morphine (Bernstein and Welch, 1998; Tao et al., 1998; Law and Loh, 1999). The involvement of beta-arrestin in the desensitization of opioid signaling is further supported by a study in which beta-arrestin 2 knockout mice exhibited enhanced, prolonged analgesia to morphine following chronic opioid exposure (Bohn et al., 1999). Although beta-arrestins are implicated in receptor internalization, they also act as scaffolding proteins for a wide spectrum of signaling molecules thus facilitating downstream signal transduction (DeWire et al., 2007).

3. Duality of G protein Signaling and Effect on AC in Tolerance

Previous studies have reported that adenylyl cyclase can be differentially regulated by the G_{α} and $G_{\beta\gamma}$ subunits, derived from the heterotrimeric Gi protein, with the latter enhancing and the former attenuating adenylyl cyclase activity (Wang and Gintzler, 1995; Sunahara et al., 1996; Wang and Gintzler, 1997). In acute opioid exposure, Gi_{α} activity dominates whereas chronic exposure shifts the balance towards $G_{\beta\gamma}$. The shift in signaling from G_{α} inhibitory to $G_{\beta\gamma}$ excitatory induced following sustained opioid exposure is further augmented by an increase in the synthesis of those AC isoforms (AC IV) stimulated by $G_{\beta\gamma}$ (Rivera and Gintzler, 1998). Furthermore phosphorylation of Gi_{β} subunit by PKC results in an increased potency of $G_{\beta\gamma}$ to stimulate AC II thus augments AC activity (Chakrabarti et al., 1998; Chakrabarti and Gintzler, 2003). This two pronged effect favoring activation of AC is triggered by prolonged opioid

exposure and results in a synergistic stimulation of AC thus shifting from G_{α} inhibitory to $G_{\beta\gamma}$ excitatory.

4. Pleiotropy of Opioid Receptors in Tolerance

As with most GPCR, opioid receptors interact with multiple G proteins including $Gi_{\alpha 3}$, $Gi_{\alpha 2}$, $Go_{\alpha 2}$ and also G_q (Chakrabarti et al., 1995b; Ho et al., 2003). Opioid receptor signaling has been reported to occur through both $G_{i\alpha}$ (inhibitory) and $G_{s\alpha}$ (excitatory), depending on the length of agonist exposure. Acute action of opioids is mediated through $G_{i\alpha}$ whereas chronic exposure to morphine and other opioid agonists appears to shift the signaling towards G_s resulting in increased production of cyclic AMP (Sunahara et al., 1996). G_s induced adenylyl cyclase activity raises cyclic AMP levels thus activating intracellular signaling pathways which affect neurotransmitter release and other intracellular pathways (Gintzler et al., 1987; Gintzler and Xu, 1991). Immunoprecipitation studies have also demonstrated an interaction of opioid receptors with $G_{s\alpha}$ whereas the interaction between the receptor and $G_{i\alpha}$ decreases following chronic exposure to opioids (Chakrabarti et al., 2005). Other data supporting opioid receptor coupling to G_s include the increased ability of opioids to enhance neurotransmitter release and AC activity in a cholera toxin sensitive manner following chronic morphine exposure (Wang and Gintzler, 1997). This G_s signaling combined with previously mentioned switch to $G_{i\beta\gamma}$- signaling following chronic opioid exposure contributes toward adenylyl cyclase superactivation which has been proposed to be a key facet in the development of opioid tolerance. These adaptations enable opioid tolerance mechanisms to be pliable and represent part of a much broader spectrum of adaptational events that may surround and play an integral role in the development of tolerance.

5. Changes in Membrane Potential in Opioid Tolerance

Electrophysiological assessment of LM/MP neurons from morphine tolerant animals have demonstrated a partial depolarization of the cell membrane potential (Leedham et al., 1992; Meng et al., 1997) that may account not only for the increased sensitivity to excitatory agents like K^+ ions, 5-hydroxytryptamine and nicotine but also the reduced responsiveness to inhibitory agents like morphine, CADO and clonidine (Schulz and Goldstein, 1973; Johnson et al., 1978; Taylor et al., 1988). Additional studies associated the partial depolarization to a reduced abundance of the Na^+/K^+-ATPase caused by decreased expression of its functional $alpha_3$ subunit isoform (Kong et al., 1997; Biser et al., 2002). The time course for reduction and recovery of $alpha_3$ subunit isoform protein has also been shown to parallel the time course for the development and recovery of heterologous tolerance following chronic morphine exposure (Li et al., 2010).

VI. Development of Cannabinoid Tolerance

1. Receptor Phosphorylation and Desensitization in Tolerance

As with most GPCR, desensitization of cannabinoid receptors involves GPCR kinase (GRK) induced phosphorylation and beta-arrestin initiated receptor decoupling and internalization (Jin et al., 1999; Kouznetsova et al., 2002). PKA and Src tyrosine kinase have also been implicated in the desensitization process since PKA or Src kinase inhibitors have been demonstrated to reverse the development of analgesic tolerance to cannabinoids following chronic WIN-55,212-2 exposure (Lee et al., 2003). Specific intracellular domains including S317, S426 and S430 have been identified to be critical for receptor desensitization since their

disruption results in decreased tolerance development (Garcia et al., 1998; Jin et al., 1999; Roche et al., 1999).

2. Involvement of Beta-Arrestins, Receptor Internalization and Downregulation

Cannabinoid receptor downregulation in the CNS is well documented, although the extent of the downregulation appears to be region dependent. The internalization of the receptors has been demonstrated in receptor-transfected cells (Hsieh et al., 1999) and, like the opioid receptors, it has been hypothesized that the agonist-activated cannabinoid receptor is targeted by beta-arrestin which directs the phosphorylated receptor as a GPCR/arrestin complex into clathrin coated pits. Upon internalization, the GPCR can either be dephosphorylated and recycled or degraded by lysosomes (Krupnick and Benovic, 1998; Schulz et al., 2004). Chronic treatment with the cannabinoid receptor agonists Δ^9-THC, CP-55,940 has also been shown to reduce CB_1 receptor levels (B_{max}) in the caudate putamen (Oviedo et al., 1993), striatum but not in the ventral mesencephalon (Rodriguez de Fonseca et al., 1994). As with opioid agonists, the ability of cannabinoids to desensitize receptors appears to be ligand dependent since anandamide, a poorly stable endocannabinoid, failed to elicit downregulation/desensitization whereas R-methanandamide, a more stable analog, induced it (Romero et al., 1999). Brain regions that show the highest magnitude of cannabinoid receptor downregulation include the cerebellum, caudate putamen, globus pallidus, substantia nigra, nucleus accumbens, amygdala, hypothalamus, thalamus and PAG: the basal ganglia output nuclei show a modest change in receptor number following chronic cannabinoid exposure (Sim-Selley and Martin, 2002).

A reduction in CB_1 receptor mRNA transcription has also been demonstrated in the caudate putamen following Δ^9-THC or CP-55940 exposure (Caberlotto et al., 2004). Importantly,

cannabinoid receptors appear to be more sensitive to downregulation in the brain compared to other $G_{i/o}$-coupled receptors, such as the MOR and 5-HT_{1A} receptors (Sim et al., 1996; Sim-Selley et al., 2000). Resensitization of an internalized receptor requires dephosphorylation by phosphatases and it has been proposed that endosomal acidification occurs for the internalized receptor to assume a conformation conducive for receptor dephosphorylation thus allowing receptor recycling (Hsieh et al., 1999).

3. Duality and Pleiotropy of Cannabinoid Signaling and AC Superactivation in Tolerance

As with chronic opioid exposure, persistent cannabinoid exposure has been demonstrated to result in increased adenylyl cyclase activity following antagonist-precipitated withdrawal (Fan et al., 1996; Rubino et al., 2000). Studies in CB_1-transfected cell lines have demonstrated the AC superactivation phenomenon. The cellular basis for the superactivation has not been fully defined, but it is suspected to be due to increased activation of G_s and decreased activation of the G_i-family of G proteins (Rhee et al., 2000). Moreover, studies in COS-7 cells transfected with CB_1 receptors show concentration-dependent selective superactivation of AC types I, III, V, VI, and VIII which increase cAMP levels. The cAMP increase is stimulated in part, by free $G_{\beta\gamma}$ subunit (Rhee et al., 2000).

4. Role of Protein Kinases in Tolerance

The cannabinoid induced reduction in cAMP-dependent PKA activity has been proposed to result in disinhibition of focal adhesion kinase (FAK) which in turn activates Src tyrosine kinase that ultimately results in the development of tolerance (Martin et al., 2004). In support of this idea is the data that shows inhibitors of Src tyrosine kinase to reverse analgesic tolerance induced following chronic Δ^9-THC exposure (Lee et al., 2003). Chronic cannabinoid exposure

also results in the uncoupling of $G_{i/o}$-proteins from the receptor resulting in disinhibition of AC and increased activity of c-AMP-dependent PKA (Martin et al., 2004). PKA activity has been proposed to be involved in the development of tolerance since inhibition of PKA results in attenuation of tolerance development (Lee et al., 2003). In contrast, inhibitors of PKC, PKG and phosphoinositide 3-kinase (PI3K) where shown to be ineffective in reversing Δ^9-THC induced tolerance to analgesia assessed using the tail flick assay (Lee et al., 2003). The onset, extent and duration of the development of cannabinoid tolerance has also been shown to be ligand dependent thus suggesting the existence of potentially distinct mechanisms of receptor regulation (Bass and Martin, 2000; Sim-Selley, 2003).

VI. Interaction of the Opioid and Cannabinoid Systems

1. Common Cellular Effects/Signaling Pathways

Significant similarities in the signal transduction events have been observed between opioid and cannabinoid systems. Both receptor families' systems are coupled to pertussis toxin sensitive heterotrimeric $G_{i/o}$ protein thus activation results in comparable downstream signaling pathways instigated by the Gi_{α} and $Gi_{\beta\gamma}$ subunits. The Gi_{α} subunit attenuates adenylyl cyclase activity thereby reducing cAMP production (Howlett et al., 1986; Childers, 1991). Other common cellular effects include inactivation of N, P/Q, and R-type Ca^{2+} channels (Rhim and Miller, 1994; Howlett et al., 2002); activation of the MAPK pathway (Bouaboula et al., 1995) and activation of G protein-activated inwardly rectifying potassium (GIRK) channels (McAllister et al., 1999). As with most GPCRs, chronic activation of both receptor systems results in phosphorylation of the receptor by GPCR kinases (GRK) and PKA (Kelly et al., 2008). Interaction between the phosphorylated receptor and beta-arrestins induces assembly of adapter

proteins responsible for internalization of the receptors through clathrin-coated pits (Krupnick and Benovic, 1998; Hsieh et al., 1999).

2. Shared Pharmacological Effects

The comparable anatomical distribution patterns between opioid and cannabinoid receptors may represent the basis for the similar pharmacological effects observed with agents targeting the receptors. Activation of KOR, MOR and CB_1 receptors in the gastrointestinal tract has been shown to reduce acetylcholine release thus attenuating intestinal peristalsis and increasing ileum transit time (Paton, 1957; Coutts and Pertwee, 1997). In the vas deferens, both MOR and CB_1 receptors have also been shown to reduce smooth muscle contractility (Hughes et al., 1975; Pertwee et al., 1992). Analgesic activity seen following CB_1 and opioid receptor activation is directly related to the presence of the cannabinoid and opioid receptors in the amygdala, dorsal horn, periaquaductal gray and raphe. Other common pharmacological effects include precipitation of hypothermia, sedation and euphoria, and the development of dependence.

3. Common Pharmacodynamic Substrates Underlying Tolerance Development

A. General overview. Like most GPCRs, both opioid and cannabinoid receptors' signal transduction are negatively regulated by receptor uncoupling, phosphorylation, internalization and degradation (Bailey and Connor, 2005). Upon activation, both receptor families are phosphorylated by G protein-coupled receptor kinases (GRKs) and subsequently bind to beta-arrestin proteins which inhibit additional signaling by impeding further coupling between the receptor and its cognate G protein (Ferguson, 2001; Connor et al., 2004). The nature of tolerance, whether homologous (i.e. reduced responsiveness limited to agonists employing the same

receptor or signaling pathway) or heterologous (i.e. alterations in responsiveness that extend to innate cellular properties that regulate global function such as cell excitability), is determined by the specific adaptive cellular changes that occur within the cell in response to chronic alterations in the environment such as chronic drug exposure. Homologous tolerance is mainly associated with receptor dependent modifications like receptor uncoupling from G proteins, receptor internalization or changes in cell signaling pathway and mostly occurs within hours/days after exposure whereas heterologous tolerance is often characterized by non-receptor dependent modifications that are non-specific in nature and expansive in character (e.g. membrane depolarization or changes in protein expression that regulate general cell function (Taylor and Fleming, 2001)). As outlined in previous sections, the adenylyl cyclase superactivation and reversal of response that is associated with tolerance is also observed with both receptor systems following chronic agonist exposure (Sunahara et al., 1996; Wang and Gintzler, 1997; Rhee et al., 2000). A number of other components of the cell have been proposed as being engaged in the adaptive process which has led to considerable controversy as no clear responsible cellular modification has been identified (Taylor and Fleming, 2001; Williams et al., 2001; Mizutani et al., 2005; Gintzler and Chakrabarti, 2008).

B. *In vitro* studies to define the cellular basis of tolerance. It should be pointed out at the onset that many of the *in vitro* studies employ expression systems or relatively acute exposure to high concentrations to effect the adaptive change. Therefore, it is not surprising that many of these studies have been evaluating the cellular mechanisms of desensitization as opposed to long term post-adaptive change that might occur as a result of extended exposure. *In vitro* studies using N18TG2 neuroblastoma cells co-expressing delta opioid and CB_1 receptors demonstrate that the opioid and cannabinoid receptors present in these cells are coupled to

different isoforms of Gi proteins or isoforms and that their signaling pathways converge only at the level of adenylyl cyclase as evidenced by the additive effect on the [^{35}S] GTPγS binding (Shapira et al., 1998).

Studies using cultured cells have reported conflicting data on whether heterologous or homologous tolerance is expressed following chronic exposure to these agents. Studies in HEK-293 cells co-transfected with DOR and CB_1 receptors demonstrate the development of heterologous tolerance following exposure to an opioid agonist (etorphine) whereas chronic cannabinoid agonist (desacetyllevonantradol [DALN]) exposure precipitates homologous desensitization (Shapira et al., 2003). The asymmetric expression of heterologous desensitization was also accompanied by asymmetric heterologous receptor downregulation expressed as an etorphine induced a reduction in cannabinoid receptor abundance; DALN on the other hand failed to reduce the level of DOR. In contrast, studies using the COS-7 cell line also transfected with DOR and CB_1 receptors resulted in heterologous desensitization and heterologous receptor downregulation following chronic exposure to either desacetyllevonantradol (DALN) or etorphine (Shapira et al., 2003). Moreover, studies using cultured splenocytes also showed heterologous desensitization to cAMP inhibition following chronic exposure to either opioids or cannabinoids (Massi et al., 2003). These interactions between opioids and cannabinoids suggest that the diverse interplay between the two receptor systems might be influenced by the cell/tissue/model system employed and the parameter(s) assessed. The differential results may be due to different levels of enzymes or isoforms involved in the adaptive desensitization; namely beta-arrestin, G protein-coupled receptor kinase (GRK), PKA, PKC and other kinases. A number of investigators have suggested various components of the cell signaling pathways as candidates

for the cellular locus of tolerance, including the protein kinases, adenylyl cyclases, transcription factors and tyrosine kinases (Shapira et al., 1998; Shapira et al., 2003).

5 hour exposure of excised guinea pig ileum to either an opioid or a cannabinoid *ex vivo* followed by assessment of the ability of various agents to inhibit neurogenic contractions in the longitudinal muscle/myenteric plexus (LM/MP) preparation, demonstrated the development of symmetrical heterologous tolerance (Basilico et al., 1999). However, no studies have been performed to determine whether the heterologous tolerance observed following *in vitro* opioid exposure in this LM/MP model can be extrapolated to an *in vivo* exposure model. Other studies using a similar approach have demonstrated that chronic *in vitro* exposure to cannabinoid agonists leads to a reduction in cannabinoid responsiveness (Pertwee et al., 1993; Guagnini et al., 2006). However, it should be noted that the effects of cannabinoid receptor agonists in the LM/MP model system do not reverse so the loss of responsiveness to opioids after exposure could reflect a general reduction in responsiveness of the system.

C. *In vivo* studies investigating the cellular basis of tolerance. Studies using intact animals have often reported conflicting results on the development of heterologous tolerance or changes in receptor population following chronic opioid or cannabinoid exposure. Heterologous tolerance, as evidenced by reduced responsiveness to the analgesic effect of Δ^9-THC, was observed in morphine tolerant mice (Thorat and Bhargava, 1994). On the other hand, studies using rats observed an opposite effect i.e. analgesic hypersensitivity to Δ^9-THC in morphine tolerant animals (Rubino et al., 1997). Furthermore, the effect of chronic opioid exposure on cannabinoid receptor population has provided varying results. Chronic morphine exposure has been reported to decrease CB_1 receptor levels in the rat hippocampus (Vigano et al., 2003). In contrast, other investigators have observed an increase in receptor number in the caudate

putamen and limbic structures (Gonzalez et al., 2002). These discordant results may have been influenced by differences in the species used, the use of different doses and the different brain regions assessed. Interestingly, Cichewicz and Welch (2003) demonstrated that co-administration of low doses of Δ^9-THC and opioid agonist morphine does not result in the development of analgesic tolerance but in fact maintains high antinociception to both agonists without changing the level of MOR, KOR and DOR. Synergistic analgesia following concurrent use of cannabinoid and opioid agonists has also been reported (Welch and Eads, 1999; Cichewicz, 2004). In contrast, Smith et al. (1994) reported symmetrical heterologous tolerance between Δ^9-THC and the KOR agonists U-50,488H and CI-977 in the mouse tail flick test following chronic treatment with a selective KOR (U-50,488H) agonist or Δ^9-THC.

The development of heterologous tolerance in the isolated LM/MP following chronic morphine exposure *in vivo* has been known for decades and was demonstrated to extend to both inhibitory agents and excitatory agents that did not employ the same signaling pathways (Goldstein and Schulz, 1973; Johnson et al., 1978; Taylor et al., 1988). The LM/MP neurons of morphine tolerant animals show a partial depolarization of the cell membrane potential (Leedham et al., 1991; Meng et al 1997) that has been proposed to account for the increased sensitivity to excitatory agents like K^+ ions, 5-hydroxytryptamine and nicotine and reduced responsiveness to inhibitory agents like morphine, CADO and clonidine (Johnson et al, 1978; Schulz and Goldstein, 1973). It was further suggested that the partial depolarization may be associated with the observed reduced function of the Na^+/K^+-ATPase caused by a decreased expression of the $alpha_3$ subunit isoform of the sodium pump (Kong et al., 1997; Biser et al., 2001). However, no data have been published to date on the effect of chronic *in vivo* cannabinoid exposure on the sensitivity of the LM/MP to excitatory agents like nicotine, K^+ and 5-

hydroxytryptamine. Such studies might be helpful in identifying the specific cellular mechanisms that need to be explored to define the cellular basis for tolerance.

D. Effect on the endogenous system. Cannabinoid agonists have been shown to independently influence the levels of endogenous opioid receptor ligands. Δ^9-THC and other endocannabinoids (except anandamide) have been shown to increase extracellular levels of dynorphin in the spinal cord thus enhancing KOR stimulation and analgesia (Mason et al., 1999; Welch and Eads, 1999). It has been proposed that cannabinoids may induce the release of endogenous opioids (Pugh et al., 1997; Welch and Eads, 1999; Houser et al., 2000). This notion is supported by studies which have demonstrated that opioid receptor antagonists can block cannabinoid-induced antinociception in the tail-flick, hot plate and paw pressure tests (Manzanares et al., 1999). Furthermore, chronic Δ^9-THC exposure has been shown to increase prodynorphin and proenkephalin gene expression in the rat spinal cord, and preopiomelanocortin gene expression in the arcuate nucleus (Corchero et al., 1997). An increase in proenkephalin mRNA in the rat ventro-medial nucleus of the hypothalamus and periaquaductal gray (PAG) has also been observed following chronic treatment with Δ^9-THC or methanandamide (Manzanares et al., 1998). Opioid exposure has also been reported to alter the levels of endocannabinoids; chronic morphine exposure reduced 2-arachidonoyl-glycerol (2-AG) levels without altering anandamide levels in several brain regions e.g. striatum, cortex, hippocampus, limbic area and hypothalamus (Vigano et al., 2003). These data point to a complex interplay between the opioid and cannabinoid systems which poses a challenge for dissecting specific pathways involved in the development of tolerance to these agents.

VII. Methods of Assessing Cannabinoid and Opioid Tolerance

1. Behavioral and Physiological Tests

The development of tolerance to cannabinoids and opioids has been evaluated by measuring the following physiological effects: blood pressure depression, miotic effect and respiratory depression efficacy. However, none of these systems have provided results as robust and reproducible as those that have been developed using the nociceptive, thermal regulatory and locomotor activities that are associated with the acute administration of both opioids and cannabinoids. These three systems have been utilized substantially in the assessment of not only the acute actions but also as models against which to detect the development of tolerance following chronic treatment.

A. Antinociception. This method assesses change in threshold required to elicit a response to noxious stimuli following a challenge dose of an analgesic agent. Several antinociceptive methods have been used e.g. tail flick, hot plate, paw pressure and radiant heat assays (Spina et al., 1998; McQuay, 1999). The tail flick test is generally regarded as a measure of spinal antinociception whereas the hot plate is thought to assess supraspinal analgesia (Manzanares, Corchero et al. 1999). The analgesic effect of opioids and cannabinoids is well documented in both acute and chronic models and the development of tolerance to the analgesic effect has been extensively studied using these model systems of antinociception (Spina et al., 1998; McQuay, 1999). Pain transmission appears to be regulated by opioid and cannabinoid receptor activation in the following regions: periaquaductal grey, amygdala, raphe and the dorsal horn (Rang et al., 2007).

B. Hypothermia. The hypothermic effect of opioids is receptor dependent and is affected by a variety of factors including ambient temperature, drug dose, level of restraint, and the

species or strain used (Baker and Meert, 2002). Morphine exposure generally results in hyperthermia at warmer ambient temperatures and hypothermia at cooler ambient temperatures (Rosow et al., 1980; Handler et al., 1994). The hypothermic efficacy of opioids is also agonist dependent with morphine exhibiting a very shallow dose-response relationship compared to that of loperamide (Baker and Meert, 2002). The nature of the response also appears to be receptor dependent since MOR stimulation appears to induce hyperthermia whereas KOR precipitates hypothermia (Xin L et al., 1997). Opioid receptors located in the preoptic hypothalamus and nucleus accumbens (Baldino et al., 1980; Tseng et al., 1980) have been implicated in the development of the hypothermia, though peripheral MOR stimulation by loperamide, a selective agonist with limited blood brain barrier penetration, also produces substantial hypothermia (Smith et al., 2006).

The hypothermic effect of cannabinoids has, in part, been attributed to the activation of CB_1 in the preoptic anterior hypothalamus (Sim-Selley, 2003). Stereotaxic injection of the selective CB_1 antagonist SR141617A into the preoptic anterior hypothalamus attenuates the hypothermic effect of WIN-55,212-2 in this brain region (Rawls et al., 2002). The development of tolerance to the hypothermic effect in cannabinoids is well documented in mice and rats and was one component of the cannabinoid tetrad that was originally proposed (Little et al., 1988; Rawls et al., 2002) to compare cannabinoid agonists. However, few studies have been performed characterizing the temperature regulation impact of cannabinoids and opioids using the guinea pig. Furthermore, the hypothermic response to cannabinoids appears to be much more robust and less variable than the hypothermic responses observed to opioids.

C. Catalepsy and hypomotility. The cataleptic effect of cannabinoid and opioids is dose dependent and is mostly observed at higher doses. Catalepsy can be assessed using the ring test

described by Pertwee (1972) which measures the ability of an animal to remain motionless after being placed on “the ring” which consists of horizontal wire ring of 5.5 cm diameter that is attached at a point on its circumference to the top of a 16 cm stainless steel tube held vertically. The development of tolerance to this cataleptic effect of cannabinoid agonists has been reported to occur within 9 days after chronic exposure in mice (Bass and Martin, 2000). Reduced locomotor activity following acute administration of both cannabinoids and opioids is well documented; the hypomotility response to a drug can be assessed by placing an animal into activity cages where animal movement is measured by recording the total number of photocell beam interruptions (Wise et al., 2007). The latency for the development of tolerance to the hypomotility effect of cannabinoids is 1.5 days after initiation of drug exposure (Bass and Martin, 2000). Tolerance to the hypomotility effect morphine (10mg/kg) has also been reported following prolonged opioid exposure (Timar et al., 2005).

2. Isolated Tissue and in vitro Tests

A. Longitudinal muscle/myenteric plexus (LM/MP) preparation. The guinea pig LM/MP model system has been shown to be reliable and robust for the assessment of tolerance and dependence to a number of agents following chronic drug treatment (Rezvani et al., 1983; Taylor et al., 1988; Johnson and Fleming, 1989; Taylor and Fleming, 2001). The model was first characterized as a useful method to assess the acute actions of opioids by Paton et al. (1965) and was then later used by Goldstein and his associates (Schulz and Goldstein, 1973) as a model system to assess the development of tolerance following chronic opioid treatment. The LM/MP preparation assesses the impact of chronic treatment on the ability of an agonist to inhibit electrically induced neurogenic contractions of the smooth muscle. In addition, this model

system is also easily employed to determine the ability of excitatory agents to induce a contraction and has been used to illustrate the development of supersensitivity to excitatory agents following chronic opioid treatment (Goldstein and Schulz, 1973; Johnson et al., 1978). Both cannabinoid and opioid agonists reduce intestinal peristaltic activity through activation of cannabinoid (CB_1) or opioid receptors (KOR and MOR), respectively. Studies have shown that activation of these receptors results in the inhibition of acetylcholine release from the myenteric 'S' neurons thus attenuating peristaltic activity (Coutts and Pertwee, 1997). The MOR and CB_1 receptors are found on the soma and reduce acetylcholine release by hyperpolarization and reduction in excitability while the KOR are located on the axon terminals and decrease acetylcholine release through inhibition of N-type voltage-sensitive calcium channels (Kojima et al., 1994). To date, few studies have been conducted using this model system to assess the impact of chronic cannabinoid treatment on the sensitivity to excitatory agents or to characterize the nature of the tolerance that develops following chronic cannabinoid treatment

B. Vas deferens model. The presence of MOR and CB_1 receptors in the mouse vas deferens has been used effectively to assess the acute effects of opioid and cannabinoid agonists (Christopoulos et al., 2001; Pertwee et al., 2002). Activation of these receptors inhibits the excitability of the noradrenergic neurons in the tissue that are responsible for the production of smooth muscle contraction (Hughes et al., 1975; Pertwee et al., 1992). MOR and CB_1 receptor agonists dose-dependently inhibit electrically induced neurogenic contractions of smooth muscles in these tissues (James et al., 1991). Thus, the mouse vas deferens has been employed as a model to assess the development of tolerance following chronic treatment by comparing the potency of agents to inhibit neurogenic contractions in a manner similar to that described for the ileum LM/MP preparation.

C. Cell line and expression system. HEK-293, COS-7 cell lines and *Xenopus oocytes* have been used to assess the development of tolerance to cannabinoids and opioids (Shapira et al., 2003). It should be pointed out that these systems require the transfection of receptors and/or the appropriate signaling partners in order to develop acute responsiveness to agonists which makes the system less realistic so that data generated from these studies needs to be interpreted carefully. Tolerance can be assessed by comparing the ability of agonists to open GIRK channels, activate Ca^{2+} channels or reduce cAMP levels before and following opioid and cannabinoid agonist exposure. Since the expression systems are usually exposed to agonists over shorter periods of time using higher concentrations, these chronic exposure studies may be more utilitarian in evaluating the mechanisms underlying the development of desensitization rather than "tolerance".

VIII. Purpose of Current Study

Data generated from studies investigating the characteristics and mechanisms of the development of opioid and cannabinoid tolerance have often been contradictory and ambiguous. This may be due, in part, to differences in the models used (tissues versus animal), the dosage regimen employed, and/or whether drug exposure is accomplished *in vivo* or *in vitro*. Therefore, in spite of the extensive investigation that has been done in this area, the precise nature and mechanism of the interactions between the opioid and cannabinoid systems and how those interactions impact upon the modifications in responsiveness following chronic treatment remains elusive. A clear consensus is yet to emerge regarding the mechanism(s) underlying the development of tolerance to opioids and cannabinoids individually and very few studies have evaluated how chronic exposure to agonists targeting one system affects sensitivity to agonists

targeting the other. Relatively few studies have compared the development of tolerance to the hypothermic, analgesic (antinociceptive) and ileum longitudinal muscle inhibitory effects of cannabinoids and opioids in the guinea pig. Advantages of the guinea pig include the fact that it exhibits closer anatomical, physiological, neurological and developmental similarities to the humans (Mansour et al., 1988; Bot et al., 1992). The guinea pig has also been used extensively as a model for studying gastro-intestinal and respiratory physiology, and assessment of opioid tolerance (Chavkin and Goldstein, 1984; Hunter et al., 1997; Gray et al., 2001).

Based on the overlapping neuroanatomical localization of receptors and the comparable cellular signal transduction pathways that are involved in the acute actions, the candidate hypothesized that bidirectional heterologous tolerance to the hypothermic, analgesic and gastrointestinal inhibitory effects of these agents will develop following chronic exposure to cannabinoid or opioid receptor agonists. The candidate developed an experimental plan to address the hypothesis that tolerance may overlap between opioids and cannabinoids. The experimental plan was based upon the following specific aims: *(1) To compare and contrast the effect of chronic in vivo cannabinoid or opioid exposure on the sensitivity of the LM/MP to excitatory agents like nicotine; (2) To determine if chronic cannabinoid/opioid exposure results in changes in the MOR and CB_1 receptor abundance; (3) To evaluate the distribution of MOR and CB_1 receptor expressing neurons in the terminal LM/MP and pre-optic hypothalamic area,; (4)To evaluate effect of chronic drug exposure on the development of tolerance to the analgesic and hypothermic effects of opioids and cannabinoids*.

Table 1.1 Peripheral and central distribution of mu-, delta, and kappa-opioid receptor

The table below shows the respective relative distribution of mu-, delta, and kappa-opioid receptor transcripts in peripheral and central tissues (Mansour et al., 1994; Wittert et al., 1996).

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	Mu-opioid receptor (μ)	Kappa-opioid receptor (κ)	Delta- opioid receptor (Δ)
CNS			
Spinal cord	high	high	moderate
Cerebral Cortex	high	high	high
Hypothalamus	high	high	high
Cerebellum	moderate	low	-
Thalamus	high	high	-
Raphe	high	high	low
PAG	high	high	low
Amygdala	high	low	high
PERIPHERY			
Stomach	low		
Small intestine	moderate	moderate	moderate
Large intestine	low	low	moderate
Liver	low	-	low
Adrenal	moderate	low	moderate
Kidney	moderate	low	low
Lung	low	low	low
Heart	-	Low/moderate	low
Endothelium	-	-	-
Spleen	high	Low/moderate	low
Synovium	-	-	-
Testis	moderate	low	moderate
Ovary	moderate	low	low
Uterus	moderate	low	low

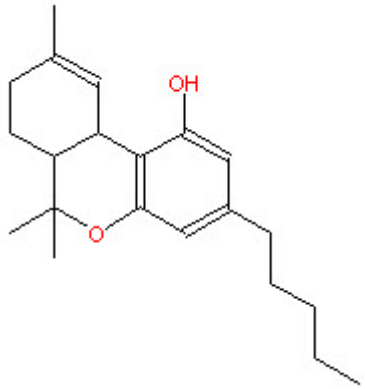
Table 1.2 Peripheral and central distribution of CB_1 and CB_2 receptors

The table below shows the respective relative distribution of CB_1 and CB_2 receptors in peripheral and central tissues (Mansour et al., 1994).

	Cannabinoid receptor 1 (CB_1)	Cannabinoid receptor 1 (CB_2)
CNS		
Hippocampus	high	low
Cerebellum	high	-
Substantia nigra	high	low
Nucleus accumbens	high	-
Caudate	high	-
Globus pallidus	high	-
Olfactory regions	high	low
Amygdala	moderate	-
Medial hypothalamus	moderate	-
Nucleus of the solitary tract	low	-
Thalamus	low	-
Brainstem	low	-
Amygdala	low	-
Raphe	low	-
Dorsal horn of the spinal cord	low	-
PAG	moderate	low
PERIPHERY		
Gastrointestinal tract	high	-
Vas deferens, bladder	present	-
Uterine wall	present	-
Spleen	-	present
Thymus	-	present
Tonsils	-	present
Mast cells	-	Present
B-cells, natural killer cells monocytes	-	present

Figure1.1 Chemical structures of the cannabinoid receptor ligands Δ^9-THC and WIN-55,212-2

Δ^9-THC



WIN-55,212-2

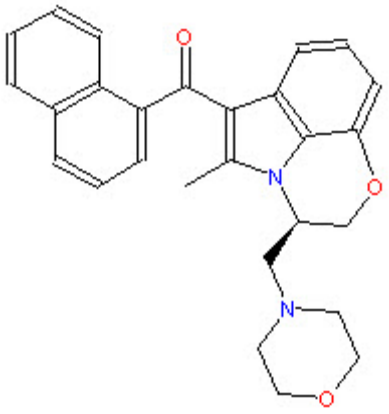


Figure 1.2 Chemical structure of the opioid receptor ligand: Morphine

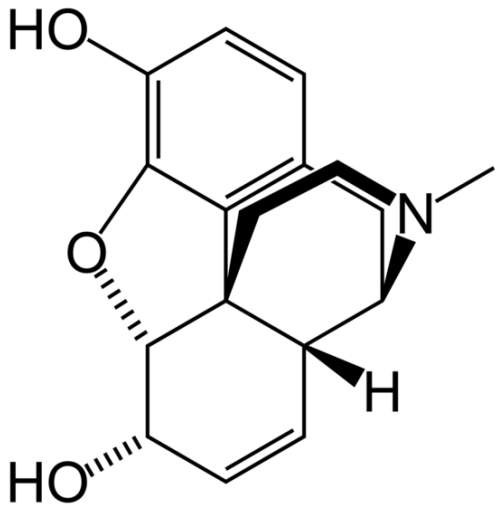
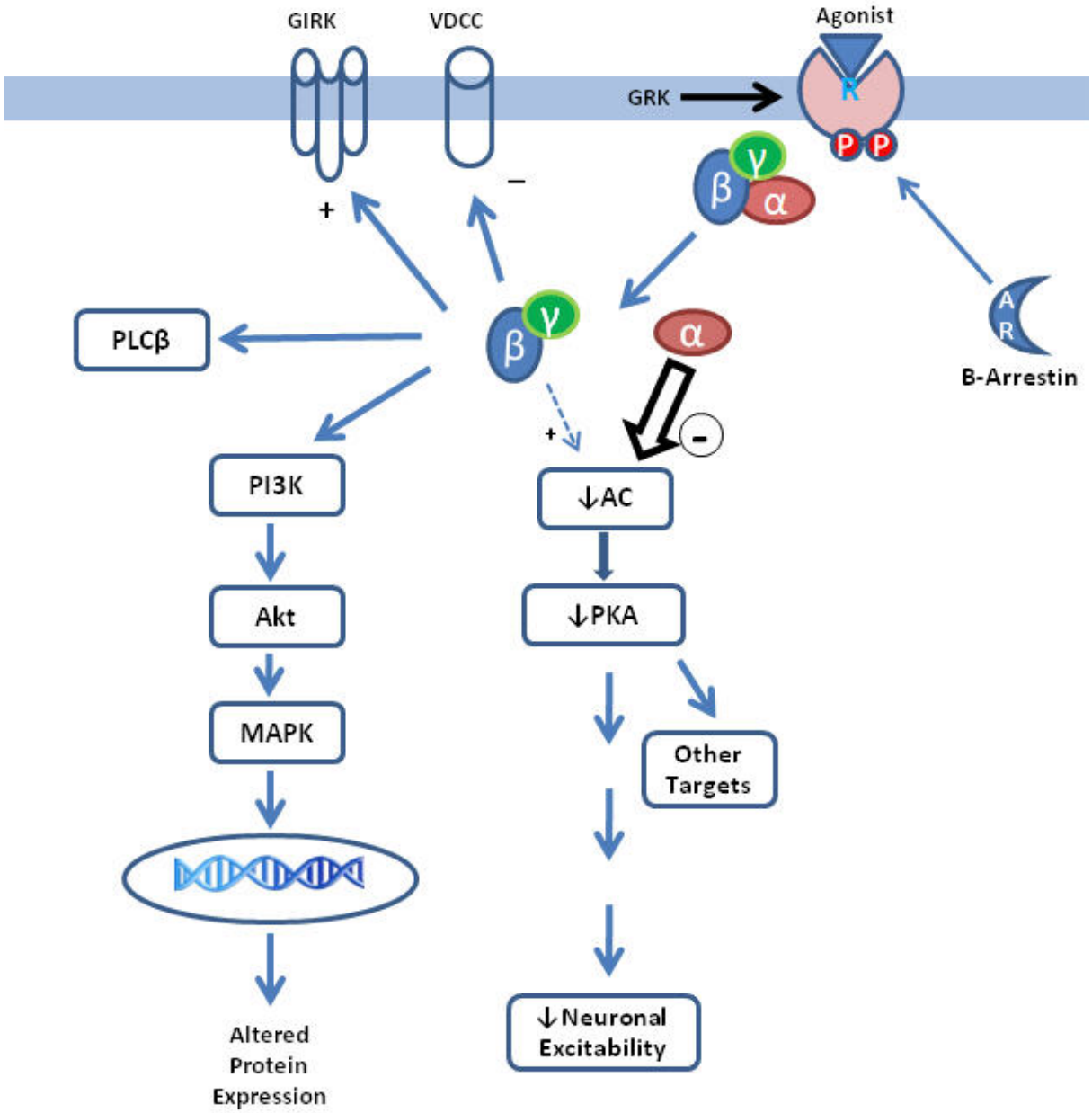

HO
N
O
H
HO

Figure 1.3 Common Signal Transduction Pathways Employed by Opioid and Cannabinoid Receptor Agonists

Common cellular signal transduction pathways activated following opioid (mu- kappa- and delta) or cannabinoid (CB_1 and CB_2) receptor activation.


GIRK
VDCC
Agonist
GRK
R
P
P
+
–
β
γ
α
PLCβ
A
R
B-Arrestin
+
-
PI3K
↓AC
Akt
↓PKA
MAPK
Other
Targets
Altered
Protein
Expression
↓Neuronal
Excitability

CHAPTER TWO: MATERIALS AND METHODS

I. Chemicals and Drugs

DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate), WIN-55,212-2 [(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone], morphine (morphine sulfate pentahydrate salt), nicotine (nicotine hydrogen tartrate) and CADO (2-chloroadenosine) were procured from Sigma-Aldrich Co. (St. Louis, MO). For organ bath studies, solutions of DAMGO, morphine and CADO were made by dissolving their respective salts in distilled water while the lipid soluble agent, WIN-55,212-2, was first dissolved in a vehicle containing DMSO/normal saline (1:9) from which serial dilutions were made using normal saline. Parenterally administered morphine was dissolved in normal saline whereas WIN-55,212-2 was dissolved in a vehicle consisting of normal saline and 10% DMSO (v:v).

II. Animals

Dunkin-Hartley guinea pigs (Charles-River labs; Raleigh, NC) of either sex weighing 200-450g were used in the study. The animals were housed two per cage with access to food and water *ad libitum*. The guinea pigs were kept in the animal facility for one week to permit acclimation prior to initiation of the treatment. All experimental procedures employing animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Brody School of Medicine at East Carolina University and were conducted in accordance with the guidelines for the humane use of animals in research (NIH "Public Health Service Policy on Humane Care and Use of Laboratory Animals" [revised 2002]). Every effort was made to

reduce the use of animals to the minimum number required to achieve sufficient statistical power.

III. Drug Exposure/Administration Schedules.

1. Morphine 7-day s.c. Administration Schedule

Previous studies have demonstrated that chronic exposure to morphine via pellet implantation and s.c. injection produces tolerance to opioids and other agents that is qualitatively and quantitatively similar (Li et al., 2010). The following dosing regimen was used for the morphine s.c 7-day exposure: day 1, 10mg/kg b.i.d.; day 2 and 3, 20mg/kg b.i.d.; days 4, 5 and 6, 40mg/kg b.i.d; and day 7, 80mg/kg b.i.d. The drug was injected 12-hourly (10:00 a.m. and 10:00 p.m.) and the animals were euthanized between 9 and 10 a.m. the following day after the last dose. This protocol was based on one previously employed in guinea pigs to produce dependence (Mizutani et al., 2005) and has been employed in previous studies in this laboratory (Li et al., 2010). The animals were weighed daily prior to dosing and the appropriate dose given subcutaneously. The animals were also examined daily for signs of discomfort. The control animals were injected with the drug-free vehicle in a similar fashion.

2. WIN-55,212-2 5-day i.p. Administration Schedule

WIN-55,212-2 was administered intraperitoneally at a dose of 6mg per 100g body weight once daily for 5 consecutive days. The drug was injected at 10:00 a.m. and the animals were euthanized between 9 and 10 a.m. the following day after the last dose. The drug regimen was adapted from a previously used regimen (Spina et al., 1998) and was modified based upon preliminary experiments which determined that this time period of treatment was necessary for tolerance to be induced. The animals were weighed prior to dosing and examined daily for signs

discomfort. The control animals were injected with the drug-free vehicle in a similar dosing schedule.

IV. Longitudinal Smooth Muscle-Myenteric Plexus (LM/MP) Model

1. Longitudinal Smooth Muscle-Myenteric Plexus (LM/MP) Preparations

The LM/MP from treated animals was removed and isolated as previously described by Taylor et al. (Taylor et al., 1988). Segments of the ileum were obtained from animals sacrificed by decapitation following isoflurane anesthesia. The abdomen was opened to expose the cecum. The 10 cm section of ileum closest to the cecum was removed and discarded, and 2-4 cm segments of ilea from the adjacent 10 cm of ileum were used to set the LM/MP preparations. The segments of ilea were threaded onto a glass rod and, using a cotton swab moistened with Krebs solution, the LM/MP carefully stripped tangentially from the point of mesenteric attachment until the muscle-nerve preparation was detached from the total area of the ileum (Fig 2.1).

The resulting sheet of LM/MP was tied at each end with a fine thread, passed through platinum-ring electrodes and placed in a 10 ml organ bath containing Krebs buffer solution. One thread was tied to a PowerLab force transducer and the other fixed to a tissue holder. A basal tension of 1.0 g was set and isometric tension generated by the muscle was recorded using the PowerLab/Chart 5 computer program (AD Instruments, Colorado Springs, CO) through a 4 channel power lab system using a 4 channel Quad bridge converter interface (AD instruments, Colorado Springs, CO).

The tissues were maintained at $37^{o}C$ in a physiological Krebs buffer solution bubbled continuously with a mixture of 95% O_2/5% CO_2 consisting of the following (in mM): NaCl (117), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25) and Dextrose (11.5).

Neurogenic contractions were elicited via electrical stimulation using supramaximal voltage delivered to the tissue through platinum-ring electrodes using a stimulation system consisting of a Grass S48 stimulator connected to the electrodes by a Med Lab Attenuator and Stimu-splitter. To ensure only nerve endings were stimulated, the following parameters were used: voltage (50V); impulse duration (<1msec); delay setting (zero); and frequency (0.1 Hz). Fig 2.2 shows a typical cumulative concentration response curve for 2-chloroadenosine on neurogenic contractions of the LM/MP.

2. Measurement of LM/MP Sensitivity to Inhibitory Agents

Following the equilibration period, the tissues were exposed to cumulatively increasing concentrations of the inhibitory drugs (final concentrations in the organ bath ranging between 1 nM and 10 µM). Three 5 min washes followed by three 15 min washes with drug free Kreb's solution were performed between concentration-response curves of different drugs permitted full recovery of the amplitude of neurogenic contractions. In each experiment, two LM/MP preparations from each test group of animals (i.e. placebo and drug-treated groups) were studied simultaneously and the responses of the tissues from the same animal averaged. The effect of each agonist on the amplitude of the neurogenic contractions was determined and calculated as percent inhibition from the original amplitude (Fig 2.3). Due to the highly lipophilic nature of the cannabinoid agonist, WIN-55,212-2, concentration-response curves for this agonist were always constructed last as neurogenic contractions following exposure to this agent did not recover in spite of numerous washes. Each of the other agonists was alternated in sequence to reduce the impact of the sequence upon the calculated IC_{50} value. Geometric mean IC_{50} values are calculated and used for comparison among treatment groups for each agonist as previously

described (Taylor et al., 1988). Computer assisted analysis of each concentration-response curve using Sigmaplot® software (SPSS Inc.) was employed to determine the IC_{50}.

3. Measurement of LM/MP Sensitivity to a Stimulatory Agent

The procedure outlined above was also used for experiments using nicotine with the only difference being the absence of electrical stimulation since nicotine elicited contractions by ganglionic stimulation of acetylcholine release. Nicotine (final bath concentrations ranging between 0.3 μM and 100 μM) was added in a non-cumulative manner with at least three 5 min washes performed before the next drug concentration was added. Responses were calculated in grams of tension or percent of the maximum contraction for that tissue. The values were used to determine the EC_{50} (i.e. concentration required to produce 50% of the maximum response) and to calculate the maximum tension produced by nicotine. Computer assisted analysis of each concentration-response curve using Sigmaplot® software (SPSS Inc.) was employed to determine the EC_{50}.

V. Receptor Protein Analysis

1. Tissue Homogenization

Western blotting was used to determine the MOR and CB_1 receptor protein levels in the LM/MP. The LM/MP tissue prepared as outlined earlier was immediately snap frozen in liquid nitrogen then stored at -80^{o}C pending homogenization at which time the tissue was thawed, weighed and placed in ice-cold protease inhibitor buffer (0.25 M sucrose, 10.0 mM EDTA, 4.08 mM phenylmethylsulfonyl fluoride, 1mM 4-aminobenzamidine and 1 mg/ml bacitracin) and homogenized using a glass homogenizer (PowerGen 125; Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 14,000 rpm for 5 seconds and the supernatant used for analysis.

2. Protein Concentration Determination

Spectrophotometric protein determination was performed using a Pierce® BCA protein assay kit (Pierce Biotechnology, Rockford, Illinois). Tissue homogenates or standard samples were mixed with Pierce® BCA reagent A/B solution and absorbance was measured in triplicate using the Synergy HT spectrophotometer (BiTek®, South Coast Metro, CA) at a wavelength of 562 nm. The sample absorbance was calculated and protein concentrations extrapolated from a standard curve generated with data from serially diluted pre-determined standard samples of bovine serum albumin.

3. Western Blotting

For each protein of interest, preliminary immunoblot experiments were performed to verify the specificity of the primary antibodies employed and to optimize conditions of the appropriate range of protein, the optimal primary and secondary antibody concentrations for the immunoblot procedure. The Western blotting procedure is similar to that previously described (Biser et al., 2002). Homogenates containing 10 µg total protein were loaded on 10% precast Tris-HCl Ready gels (Bio-Rad Laboratories, Inc., Hercules, CA) and size fractionated via electrophoresis at 110 V using a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were then transferred to nitrocellulose membranes presoaked in transfer buffer using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). After allowing the membrane to completely dry, it was prehybridized in pre-made Odyssey® blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 3 h. The membrane was washed with phosphate buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) containing Tween 20 (0.1%) three times for 15 min, and incubated overnight with the primary

antibody. The following primary antibodies were used; mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:60,000 – Millipore, Burlington, MA), rabbit anti-MOR (1:2000 – Millipore, Burlington, MA) and rabbit anti-CB_1 (1:200 – Cayman, Ann Arbor, MI). Following incubation, the blots were washed three times with PBS-T and incubated for 1 h with the appropriate secondary antibody obtained from Li-Cor® Biosciences (Lincoln, NE). The final blot was washed an additional three times in PBS to remove any excess secondary antibody before detection using the Odyssey® near-infrared imaging system (Li-Cor® Biosciences).

VI. Immunofluorescence Assessment

1. Tissue preparation

A. Longitudinal muscle/myenteric plexus preparation. Ileum segments (1 cm) excised from the terminal ileum were placed in cold PBS solution and cut longitudinally along the mesenteric border. The sheet of tissue was then pinned on a dissecting plate containing Sylgard® with the mucosal side facing up. The tissues were incubated and fixed overnight in 4% paraformaldehyde. Following the overnight incubation, the paraformaldehyde was removed by rinsing the tissue with PBS. The mucosal and circular layers were removed using fine forceps under a microscope and the LM/MP immersed and stored in PBS containing 0.1% sodium azide at 4^{o}C pending immunofluorescence probing.

B. Preparation of brain sections for near-infrared and immunofluorescence imaging.

Animal Perfusion and Tissue Fixing

Guinea pigs were first weighed then anesthetized with Ketamine (85 mg/ml)/Xyalazine (15 mg/ml) administered via intraperitoneal injection (0.10 ml/100 g) body weight. The

anesthetic effect was checked after 3 – 5 min to determine whether the animal was non-responsive to toe pinch. The procedure was performed by two people; one person held the animal in position and the other performed the dissection and perfusion. Once the animal had been positioned, an incision was made in the lower abdominal area then cut longitudinally along the midline towards the thoracic cavity to expose the heart. Once the thoracic cavity had been opened and heart exposed, an incision was made on the right atrium where the perfusate would exit. Cold PBS buffer was then transcardially perfused via the left ventricle using a 50 ml syringe with a 23G needle, until a clear perfusate exited of the right atrium. Once the PBS infusion was complete, a cold solution of 4% paraformaldehyde in PBS was injected into the left ventricle until the animal's extremities became stiff. Thereafter, the head was decapitated and the brain carefully extracted from the skull. The brain was then immersed in 4% paraformaldehyde and stored overnight at 4^{o}C. On the following day, the brain was transferred and stored in PBS containing 25% sucrose for at least 48 h pending sectioning.

Brain Sectioning

The sectioning of brain tissue was performed using the Leica VT1000S vibratome brain slicer (Leica Microsystems, Bannockburn, IL). First the brain was placed and aligned in the guinea pig brain matrix (Kent Scientific, Torrington, CT) where it was sliced in a coronal fashion at the -12.5 mm plane away from bregma to divide the cerebellum and the cerebrum (Fig 5.3). The cerebrum was further sliced into two sections by making a coronal section at the bregma as shown in Fig 5.3 The brain segments were then attached to the sample tray using super-glue and aligned to allow minimal movement of the blade during each sectioning pass. Sectioning of the brain was executed with the brain fully immersed in cold PBS. The resulting sections of approximately 40 μm thickness were immersed and stored in cold PBS containing 0.1% sodium

azide at 4^{o}C pending immunofluorescence probing. A total of 13 distinct regions of the cerebrum were sectioned (Fig 5.6).

2. Immunostaining Procedure

A. LM/MP preparation for immunofluorescence imaging. The CB_1 receptor was localized using a rabbit primary polyclonal antibody (Cayman, Ann Arbor, MI) directed against the C-terminus (1:50) and the secondary antibody was a donkey anti-rabbit FITC-conjugate (1:100 – Jackson Immunoresearch, Westgrove, PA). MOR was detected using a goat primary polyclonal antibody (Santa Cruz, Santa Cruz, CA) directed against the C-terminus (1:50) and the secondary antibody was a donkey anti-goat Cy5-conjugate (1:100 – Jackson Immunoresearch, Westgrove, PA). Prior to primary antibody incubation, the LM/MP tissues were blocked with donkey serum (10%) for 45 min. Dual-labeling experiments were performed with simultaneous incubation of antibodies targeting both MOR and CB_1 receptors. The tissues were incubated overnight using the primary antibodies at 4°C. Following the overnight incubation, the tissues were washed with phosphate buffered saline (PBS) and then incubated for 4 h at room temperature in a mixture containing both FITC- and Cy5- conjugated secondary antibodies. Negative control experiments excluded the primary antibodies and these revealed negligible faint labeling due to non-specific binding of the secondary antibodies (Fig. 2.2).

B. Coronal brain sections.

Coronal sections for near-infrared imaging

Initial studies involved attempting to outline general MOR and CB_1 receptor distribution and visualize the anatomical landmark structures in the 13 coronal sections of the cerebrum (Fig. 5.4). This was accomplished by tagging MOR and CB_1 receptors with near-infrared secondary

antibodies. Following coronal sectioning of the guinea pig brain at the forementioned coronal planar co-ordinates (Table 5.1); the brain slices were incubated with near-infrared antibodies targeting the MOR and CB_1 receptors. The CB_1 receptor was localized using a rabbit primary polyclonal antibody directed against the C-terminus (1:50 – Cayman, Ann Arbor, MI) and the secondary antibody used was a goat anti-rabbit (1:5000 – Li-Cor Biosciences. Lincoln, NE). MOR was detected using a goat primary polyclonal antibody directed against the C-terminus (1:50 – Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody used was a donkey anti-goat (1:15000 – Li-Cor Biosciences. Lincoln, NE). The brain sections were first incubated in 0.1% Triton X-100 in PBS for 25 min followed by three 10 min washes with PBS. Prior to primary antibody incubation, the LM/MP tissues were blocked with donkey serum (10%) for 45 min and rinsed using PBS. Thereafter, the tissues were incubated overnight in a cocktail of anti-CB_1 and anti-MOR primary antibodies in 2% BSA in PBS at 4°C. Following the overnight incubation, the tissues were washed with PBS and then incubated for 4 h in a mixture containing both secondary antibodies targeting MOR and CB_1 receptors. Following incubation with the respective secondary antibodies, the LM/MP tissues were mounted on slides, allowed to dry in total darkness, and then coverslipped pending imaging. Near infra-red imaging of these slices was performed using the Odyssey imaging system (Li-Cor Biosciences. Lincoln, NE.

Coronal section for immunofluorescence imaging

The method used to localize the MOR, CB_1 receptors and gonadotropin releasing hormone (GnRH) is identical to the one outlined above for the LM/MP. The concentrations of primary and secondary antibodies used for MOR and CB_1 receptors are identical to those used for the LM/MP (see APPENDIX F). In addition, GnRH was used as a marker protein for the preoptic anterior hypothalamic neurons where the hormone is exclusively expressed. GnRH was

localized using a mouse primary polyclonal antibody directed against the C-terminus (1:500 Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody used was a donkey anti-mouse Cy3-conjugate (1:500 – Jackson Immunoresearch, West Grove, PA). The experiment assessed GnRH distribution in coronal sections 7, 8, 9, 10, 11 and 12 since these are anatomically relevant sections based on the location of the hypothalamus. Only the brain slices that displayed GnRH immunofluorescence were used for later triple labeling studies targeting the MOR, CB_1 receptors and GnRH. Triple-labeling experiments were performed with simultaneous incubation of antibodies targeting GnRH (gonadotropin releasing hormone), MOR and CB_1 receptor populations. The tissues were incubated overnight using the primary antibodies at 4°C. Following the overnight incubation, the tissues are washed with phosphate buffered saline (PBS) and then incubated for 4 h at room temperature in a mixture containing FITC-, Cy5- and Cy3-conjugate secondary antibodies.

3. Image Acquisition and Processing

A. LM/MP preparation. A Zeiss® LSM 510 laser scanning confocal microscope and imaging system was used for image acquisition and processing. The donkey anti-rabbit Cy5-conjugated antibody was excited at 633 nm, whereas the donkey anti-goat FITC-conjugated antibody was excited at 488 nm. In dual-labeling experiments, a composite image targeting both the FITC- and Cy5- conjugates was scanned so that the image could be merged or separated and analyzed offline. Fig 2.2 shows prototypical immunofluorescence images of the LM/MP.

B. Coronal brain sections. Confocal imaging was performed as outlined above targeting FITC- (CB_1), Cy5- (MOR) and Cy3-conjugated secondary antibodies. The donkey-anti-rabbit Cy5-conjugate was excited at wavelength 633 nm, whereas the donkey anti-goat FITC- and

donkey anti-mouse Cy3-conjugates were excited at wavelengths 488 nm and 550 nm respectively. In triple labeling experiments, a composite image targeting the FITC-, Cy3- and Cy5-conjugates was scanned simultaneously and could be separately analyzed offline. Co-localization of MOR and CB_1 receptors was analyzed in the hypothalamic neurons where GnRH was observed.

4. Assessment of MOR and CB_1 Receptor-Immunopositive Cells

Qualitative analysis was used to determine the distribution pattern and extent of co-localization of MOR and CB_1 receptor positive neurons. Cells were considered to be immunopositive if they expressed visually detectable labeling. Immunopositive neurons with bright to faint labeling were analyzed, because the faint labeling could represent low protein expression in positively labeled cells. Zeiss® LSM software was used to capture the images and Image J® software was used to analyze the distribution and possible co-localization of MOR and CB_1 receptor-immunopositive neurons, and was also employed to determine the density of immunopositive neurons. The density of immunopositive neurons was assessed by counting the number of immunopositive neurons in a manually circumscribed region of the myenteric ganglia (i.e. number of neurons/area of circumscribed region). The area of the circumscribed region was computed using Image J® software whereas the number of visually detectable immunopositive neurons was counted manually. Neurons expressing both receptor proteins were counted individually and converted to a density based upon the circumscribed area evaluated. The relative expression of co-localized was expressed as a percentage of the total population of neurons possessing both receptor proteins versus those neurons expressing either MOR or CB_1 receptor proteins only.

VII. Behavioral Tests

1. Paw Withdrawal Test

All animals were acclimated to the observation room for 1 h prior to assessment. Baseline paw pressure on both hind paws was measured prior to injecting the vehicle or drug. The paw pressure test consisted of gently holding the body of the guinea pig while the hind paw was exposed to increasing mechanical pressure (Fig 2.4) using the Randall-Selitto analgesimeter model 2500 (IITC Life Science, Woodland Hills, CA) . The digital paw pressure applicator tip was used to exert a force on the paw in a related fashion to the Randall and Selitto test of mechanical nociception (Randall and Selitto, 1957). The pressure was manually applied to the plantar surface of the hind paw using a cone-shaped pusher with a rounded tip as illustrated in Fig. 2.4. The force (measured in grams) at which the guinea pig withdrew its hind paw was defined as the paw pressure threshold. A cut-off was set at 600 g to prevent tissue damage. Antinociception was then assessed using the minimum pressure values required to elicit a withdrawal at a specific time period after vehicle or test drug administration. The mean pressure (in grams) required to elicit the withdrawal response was determined ($\pm$standard error of the mean (S.E.M.)) at the following time intervals following drug or vehicle administration: 0 (baseline), 15, 30, 45, 60, 90 and 120 min. Antinociceptive activity was defined as an increase in the pressure required to elicit the withdrawal response. Tolerance to the antinociceptive effect of an agonist was defined as a reduction in the antinociceptive effect of the challenge dose such that the maximum amount of pressure required to elicit a paw withdrawal was decreased. Challenge doses used were determined based on preliminary studies done to assess the optimum dose required to produce an adequate and quantifiable analgesic response. The assessment intervals

were based on the latent time required to observe an effect, the approximate time of the maximum effect and the maximum duration of the effect.

2. Hot Plate Analgesia Test

The procedure used is similar to that previously described (Bannon and Malmberg, 2007). Guinea pigs were brought to the testing room and allowed to acclimatize for 1 h prior to testing. The response to a thermal stimulus was measured using a hot plate analgesia meter (IITC Life Science, Woodland Hills, CA). Based on preliminary studies, it was determined that optimum results would be achieved if surface of the hot plate was heated to a constant temperature of 50^{o}C. Once the animal was placed on the hot plate which is surrounded by a clear acrylic cage, a timer was activated and the latency to respond with either a paw lick or paw flick was measured by deactivating the timer when the response was observed. To prevent tissue damage, a cut-off point of 40 seconds was used if the guinea pig did not respond. Each animal was tested only once.

3. Hypothermic Testing

Assessment of rectal body temperature has been extensively used to monitor the acute actions of many drugs including the cannabinoids which produce a robust reduction in core temperature (Rawls et al., 2002). The method employed was adapted that used by Spina et al. (1998). All measurements were made in a quiet room at an ambient temperature of 25^{o}C. After a 1 h period of acclimatization in the test room, body temperature was measured with a digital rectal thermometer inserted to a constant depth of 2.5 cm. Following thermometer insertion, a 15 sec equilibration period was allowed to lapse before the temperature was recorded. The animal was gently held during temperature measurement but was unrestrained and allowed to move

freely at all other times. The rectal temperature was measured at the following time intervals post drug administration: 0 (baseline), 15, 30, 45, 60, 90 and 120 min. A baseline temperature was assessed twice prior to drug challenge. Though rectal temperature was measured in ^{o}F, these values were converted to ^{o}C in and results were expressed as the means $\pm$ S.E.M of the $^{\circ}$C change from baseline temperature.

VII. Data Analysis

1. LM/MP Studies

For the LM/MP organ bath experiments, the sensitivity of a group of tissues to an inhibitory agonist was determined by calculating the mean negative log of the concentration producing 50% inhibition of the electrically induced contraction (IC_{50} $\pm$ SEM). Percent inhibition was calculated using the mean contraction height at the maximum inhibition following addition of a given concentration of agonist, divided by the average contraction height 1 min before exposure to the initial dose of that agonist. Differences in sensitivity to a given agonist between two groups of tissues (drug-treated vs. vehicle treated groups) were determined by comparing the geometric mean IC_{50} values and the mean ratio of geometric mean IC_{50} values (calculated as the mean antilog of the difference in IC_{50} values between the two groups). For nicotine stimulation experiments, the EC_{50} (i.e. the concentration of drug required to produce a contraction magnitude equal to 50% of the maximum contraction obtained in that tissue) and the maximal isometric tension developed were determined and compared. Analysis of the immunoblotting results was performed by comparing the receptor protein to GAPDH protein intensity ratios (i.e. MOR or CB_1 receptor/GAPDH densitometric units) between the control and the test groups. Immunofluorescence images were analyzed to quantitatively determine the

density of neurons expressing MOR or CB_1 receptor protein. The density of immunopositive neuron co-expressing both MOR and CB_1 receptors was also assessed and further computed as a percent of MOR or CB_1 receptor expressing neurons Significant differences between the test and control groups were determined using unpaired Student's "t" test. Comparison of mean values between three or more groups was performed using one-way ANOVA followed by the appropriate post hoc test, usually Tukey's test, with the probability level of ≤ 0.05 accepted as significantly different.

2. Hypothermia and Analgesia Studies

Student's t-test for unpaired samples was used to analyze the maximum rectal temperature change and maximum analgesia thresholds. Statistical analysis was performed for each time point. p value ≤ 0.05 considered as a statistically significant difference.

Figure 2.1 Anatomy of the different layers of the ileum

Illustration depicting the layers of the ileum (Berne and Levi, 1988). The inner layer consists of the mucosal layer followed by the submucosal plexus and circular muscles. The outer layer comprises of the myenteric plexus and the longitudinal muscle.

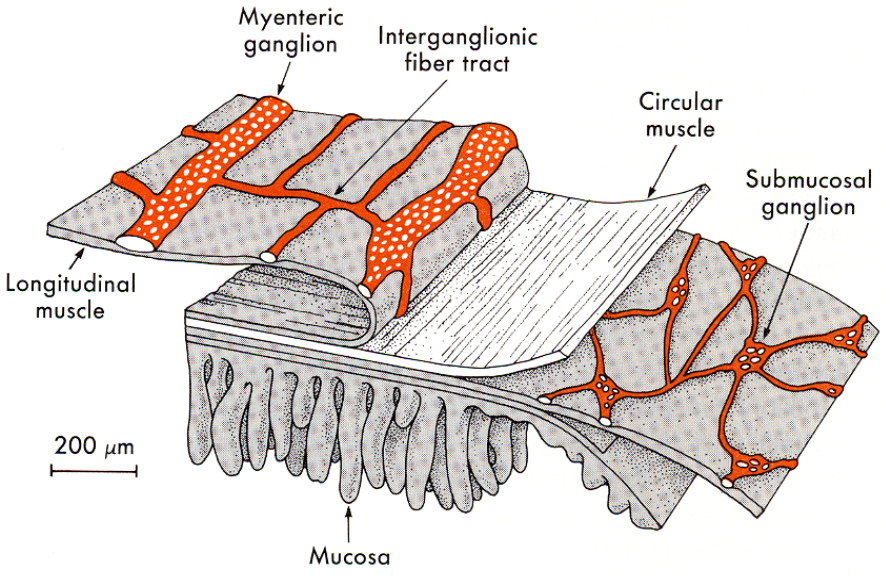
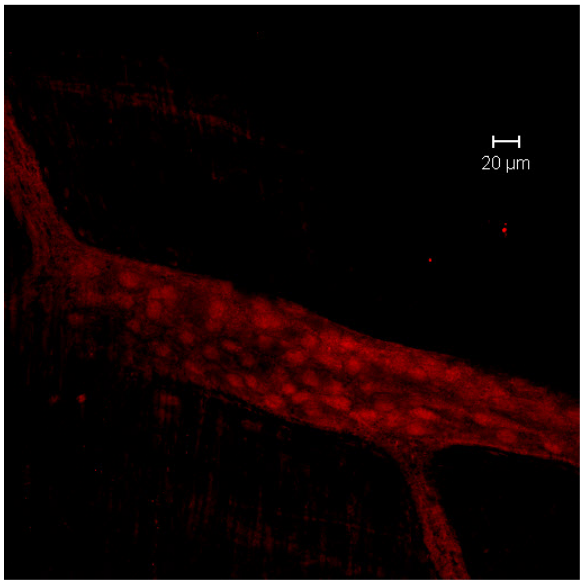

Myenteric ganglion
Interganglionic fiber tract
Circular muscle
Submucosal ganglion
Longitudinal muscle
200 μm
Mucosa

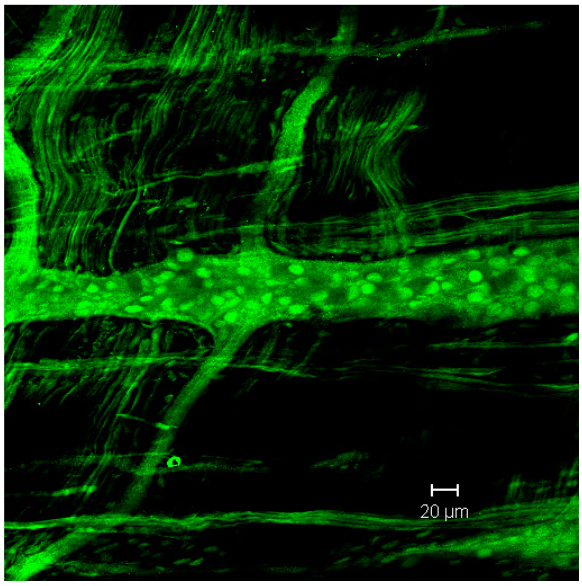
Figure 2.2 Typical immunofluorescence images depicting MOR or CB_1 receptor expressing neurons of the LM/MP stretch preparation:

A: MOR-immunopositive neurons **B**: CB_1 receptor-immunopositive neurons. **C**: negative control (primary antibodies excluded from the exposure)

A



B



C

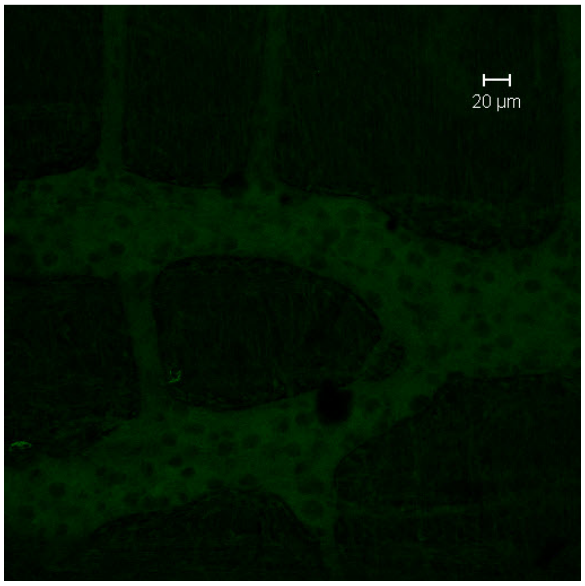


Figure 2.3 Typical LM/MP tracing illustrating concentration dependent reduction in neurogenic contractions following cumulative exposure to 2-chloroadenosine

Representative tracing showing the intrinsic efficacy of CADO on electrically-induced neurogenic contractions of the LM/MP preparation from the guinea pig ileum. The LM/MP tissue was exposed to cumulatively increasing concentrations of the inhibitory drug, 2-chloroadenosine (final organ bath concentrations ranging between 1nM and 10μM). The effect of 2-chloroadenosine on the amplitude of the neurogenic contractions was determined and calculated as percent inhibition from the original amplitude. The IC_{50} value was the concentration at which the tissue contraction falls to 50% of the initial amplitude.

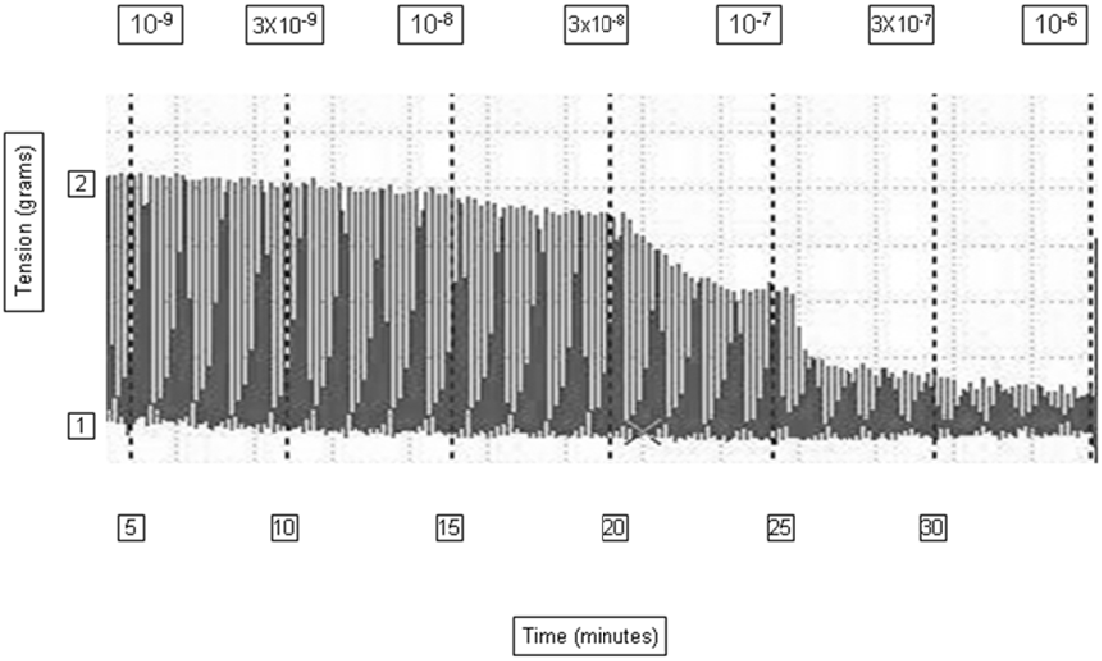
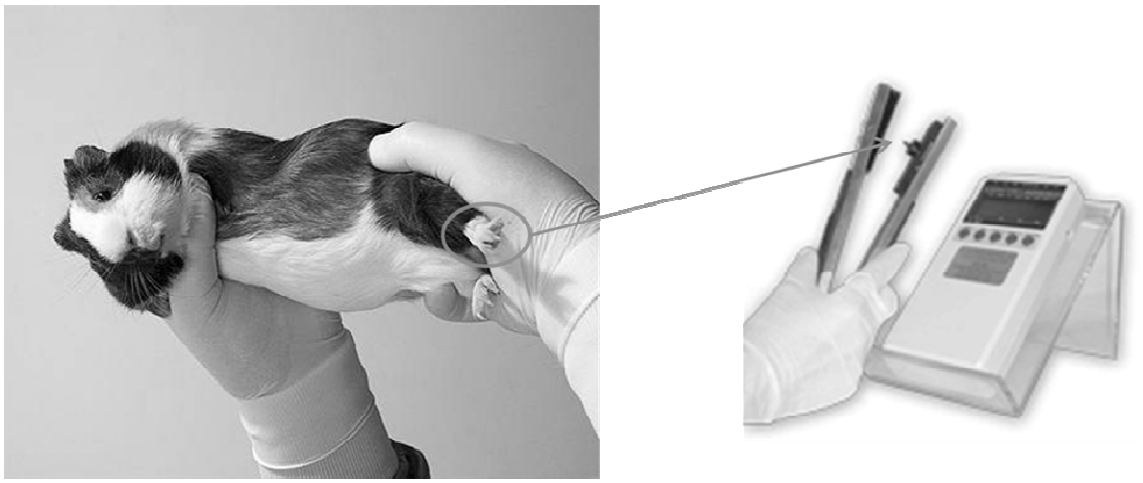

10-9
3X10-9
10-8
3x10-8
10-7
3X10-7
10-6
Tension (grams)
2
1
5
10
15
20
25
30
Time (minutes)

Figure 2.4 Method for mechanical analgesia assessment (paw pressure test)

A Randall-Selitto analgesimeter model 2500 (IITC Life Science, Woodland Hills, CA) used to assess the analgesic effects of agonists and the development of tolerance following chronic drug exposure. The digital paw pressure applicator tip is used to exert a force on the plantar surface of the hind paw in a fashion related to the Randall and Selitto test of mechanical nociception (Randall and Selitto, 1957).



CHAPTER THREE: COMPARISON OF THE CHARACTERISTICS OF TOLERANCE IN THE GUINEA PIG LONGITUDINAL MUSCLE/MYENTERIC PLEXUS PREPARATION AFTER CHRONIC *IN VIVO* EXPOSURE TO OPIOID VERSUS CANNABINOID RECEPTOR AGONISTS.

I. Abstract

Few studies have compared the nature of tolerance that develops following chronic opioid treatment with that which develops after chronic cannabinoid exposure in the same tissue and species. The degree and character of tolerance induced by 7 day exposure to morphine or 5 day exposure to the cannabinoid receptor agonist, WIN-55,212-2, was examined by comparing the ability of DAMGO (MOR selective agonist), CADO (non-selective adenosine receptor agonist) and WIN-55,212-2 (non-selective cannabinoid receptor agonist) to inhibit neurogenic contractions of the longitudinal muscle/myenteric plexus (LM/MP) preparation and the ability of nicotine to elicit contractions in the LM/MP. Chronic morphine treatment resulted in tolerance to all inhibitory agonists (rightward shift in IC_{50} values of 4-5-fold) and an increase in the responsiveness to the excitatory effect of nicotine while chronic WIN-55,212-2 exposure resulted in subsensitivity only to WIN-55,212-2. Chronic WIN-55,212-2 treatment significantly reduced CB_1 receptor but not MOR protein abundance while chronic morphine treatment did not change either receptor protein level. Thus, in contrast to the heterologous tolerance that develops after opioid treatment, tolerance in the LM/MP following chronic ***in vivo*** WIN-55,212-2 exposure is associated with the development of homologous tolerance that is accompanied by a decrease in CB_1 receptor abundance. The heterologous tolerance observed following chronic morphine exposure suggests that the two receptor systems share a number of sites for convergence of

action that could serve as the cellular basis of tolerance. The data also suggests that the cellular basis of tolerance differs between the two systems.

I. Introduction

While opioids have been used as analgesics, anti-diarrheals, antitussives and in palliative care for many years, the conventional therapeutic use of cannabinoids and related agents in the treatment of glaucoma, muscle spasticity, obesity, cachexia, nausea and possibly as immunomodulatory agents has only just begun to emerge. The use of both cannabinoids and opioids has been associated with the rapid emergence of tolerance (Bass and Martin, 2000; Alvarez et al., 2002) resulting in a need for higher doses to achieve the same pharmacological effects. Tolerance to common pharmacological effects like analgesia, hypomotility, hypothermia and inhibition of gastrointestinal motility is well documented with these two agents (Martin et al., 2004; Bailey and Connor, 2005). However, unlike the development of tolerance to the acute intestinal effects of opioids, there is little information from animal models regarding the development of tolerance to the acute gastrointestinal effects of cannabinoids *in vivo* (Pertwee et al., 1992; Basilico et al., 1999). Guagnini et al. (2006) have reported the development of tolerance in human intestinal tissue following *ex vivo* exposure to cannabinoids.

There is a growing body of evidence pointing toward convergence and commonality of activity and cell signaling pathways between the two receptor systems that may act in concert to alter many of the pharmacological effects of each including the development of tolerance. Common cellular effects include: coupling through $G_{i/o}$ proteins leading to inhibition of adenylyl cyclase (Howlett and Fleming, 1984); inactivation of N, P/Q, and R-type Ca^{2+} channels (Rhim and Miller, 1994; Howlett et al., 2002); activation of the MAPK pathway (Bouaboula et al.,

1995); and activation of G protein-activated inwardly rectifying potassium (GIRK) channels (McAllister et al., 1999). There is also evidence of comparable distribution of cannabinoid and opioid receptors within the ileum, vas deferens, caudate-putamen, dorsal hippocampus, substantia nigra and hypothalamus (Rosow et al., 1980; Ross et al., 1998; Rawls et al., 2002). Cellular co-localization of cannabinoid and opioid receptors in both the central nervous system and peripheral neurons (Salio et al., 2001)may contribute to the closely related physiological and clinical effects (Manzanares et al., 1999) including dependence and tolerance (Lichtman and Martin, 2005). The nature of tolerance, whether homologous (i.e. reduced responsiveness limited to agonists employing the same receptor or signaling pathway) or heterologous (i.e. adaptive alterations in responsiveness that extend to agents using different receptor and/or signaling pathways), can be characterized by the specificity of changes in responsiveness. The character of the tolerance also provides information regarding the most likely cellular and molecular adaptive changes that occur to account for the altered responsiveness. Homologous tolerance is mainly associated with receptor-dependent modifications such as receptor uncoupling from G proteins, receptor downregulation through internalization and degradation, or changes in the common components of the cell signaling pathway. In contrast to this type of tolerance that develops mostly within hours/days, the heterologous form of tolerance develops over much longer time periods (days/weeks) and is often characterized by non-receptor dependent modifications in cell function (Taylor and Fleming 2001).

Chronic exposure to opioids and cannabinoids has been shown to result in adaptive alterations in adenylyl cyclase and the enzyme's coupling to G proteins which may alter the responsiveness of agonists for other receptors coupled through $G_{i/o}$ proteins such as cannabinoids (Nestler, 1993; Rhee et al., 2000; Gintzler and Chakrabarti, 2006). Cannabinoid tolerance has

been suggested to be dependent on protein kinase A (PKA), Src kinase (Lee et al., 2003) and also nitric oxide (Spina et al., 1998) whereas development of opioid tolerance has been suggested to be dependent on protein kinase A (Nestler, 2001; Gintzler and Chakrabarti, 2008), protein kinase C, and is associated with a decrease in the abundance of the $alpha_3$ subunit of the sodium pump (Taylor and Fleming, 2001). Several laboratories have demonstrated development of heterologous tolerance in the LM/MP following chronic exposure to morphine (Johnson et al., 1978; Taylor et al., 1988). Electrophysiological assessment of the LM/MP neurons of morphine tolerant animals shows a partial depolarization of the cell membrane potential (Leedham et al., 1992; Meng et al., 1997) that accounts for the increased sensitivity to excitatory agents like K^+ ions, 5-hydroxytryptamine and nicotine and reduced responsiveness to inhibitory agents like morphine, CADO and clonidine (Schulz and Goldstein, 1973; Johnson et al., 1978). Later studies tied the partial depolarization to a reduction in the function of the Na^+/K^+-ATPase caused by decreased expression of its $alpha_3$ subunit isoform (Biser et al., 2002; Li et al., 2010). However, despite extensive research, no data have been published on the effect of chronic *in vivo* cannabinoid exposure on the sensitivity to excitatory agents like nicotine, K^+ and 5-hydroxytryptamine. Homologous tolerance among cannabinoid receptor agonists, namely Δ^9-THC, CP-55,940 and WIN-55,212-2 has been reported in the guinea pig ileum (Fan et al., 1994). Studies using *in vitro* exposure of the LM/MP preparation to opioid and cannabinoid agonists have reported the development of heterologous tolerance extending to both cannabinoid and opioid agonists (Pertwee et al., 1992; Guagnini et al., 2006) but no studies have assessed whether this type of change in response also occurs following *in vivo* exposure. Investigations using intact animals have reported conflicting and often discordant results on changes in receptor abundance following chronic opioid or cannabinoid exposure. Chronic opioid exposure has been

illustrated to decrease CB_1 receptor levels in the rat hippocampus (Vigano et al., 2003) or evoke an increase in the caudate putamen and limbic structures, whereas other labs have reported no significant change (Thorat and Bhargava, 1994). The varying results may have been influenced by differences in the species used, doses and brain regions assessed. However, no studies have assessed the effect of chronic *in vivo* exposure to either opioids or cannabinoids on both MOR and CB_1 receptor protein in the LM/MP.

The guinea pig LM/MP model is a reliable, robust *in vitro* model for evaluating the development of both tolerance and dependence (Rezvani et al., 1983; Johnson and Fleming, 1989). Activation of both cannabinoid (CB_1) and opioid (kappa- and mu-) receptors attenuates ileal intestinal peristaltic activity. CB_1 and mu-opioid receptors (MOR) are located on the soma and reduce transmitter release by hyperpolarization-mediated reduction in excitability while kappa opioid receptors, located on the axon terminals, decrease acetylcholine release by inhibiting calcium influx into the nerve terminal (Kojima et al., 1994; Coutts and Pertwee, 1997). The present study investigated the changes in LM/MP sensitivity to inhibitory agents (WIN-55,212-2 [non-selective cannabinoid receptor agonist], DAMGO [selective MOR agonist] and 2-chloroadenosine (CADO) [adenosine receptor agonist]) and an excitatory agent (nicotine) following chronic *in vivo* treatment with morphine or WIN-55,212-2. CADO was included because previous studies suggested that it hyperpolarized ‘S’ neurons and produced inhibition of neurogenic contractions through a mechanism different from that of morphine (Meng et al., 1997). Assessment and comparison of the effect of chronic *in-vivo* opioid versus cannabinoid exposure on LM/MP sensitivity to nicotine and CADO would also provide insight into the possible mechanisms that may contribute to the development of tolerance.

Studies using *in vitro* exposure of the LM/MP preparation to opioid and cannabinoid agonists have reported the development of heterologous tolerance extending to both cannabinoid and opioid agonists (Pertwee et al., 1992; Basilico et al., 1999; Guagnini et al., 2006) but no studies have assessed whether this type of change in response occurs following *in vivo* exposure or whether *in vivo* exposure to either agonist alters CB_1 receptor and/or MOR protein abundance in the LM/MP. Based upon previous reports on the development of symmetrical heterologous tolerance in the LM/MP *ex vitro* exposure model (Basilico et al., 1999), it was hypothesized that qualitatively similar tolerance will develop following *in vivo* drug exposure. Since previous studies have demonstrated that CB_1 receptor activation results in a significant degree of CB_1 receptor internalization (Sim et al., 1996; Sim-Selley et al., 2000) whereas morphine displays cellular desensitization with low MOR internalization (Johnson et al., 2006), we further hypothesize that the tolerance induced by CB_1 receptor activation will be associated with receptor level changes whereas chronic morphine exposure will not induce changes in receptor protein levels. In light of the controversies, conflicts and limited data comparing the development of tolerance following chronic *in vivo* opioid or cannabinoid exposure, the candidate set out to determine whether chronic *in vivo* exposure to opioids or cannabinoids would: (*1) result in the development of heterologous tolerance; (2) lead to an increase in responsiveness to nicotine; (3) produce changes in CB_1 receptor or MOR protein abundance.* The candidate predicts the development of symmetrical heterologous tolerance following chronic exposure to either opioid or cannabinoid receptor agonists. The tolerance induced by cannabinoid exposure is anticipated to involve changes in receptor abundance whereas opioid exposure will result in non-specific subsensitivity with no changes in receptor protein.

II. Experimental Protocol

1. Longitudinal smooth muscle/myenteric plexus (LM/MP) preparations

In the present study, guinea pigs were subjected chronic exposure to morphine or WIN-55,212-2 in an effort to induce the development of tolerance. The WIN-55,212-2 drug regimen was adopted from previously used regimens (Spina, Trovati et al. 1998) and was modified following preliminary experiments performed to determine when tolerance is induced The following dosing regimen was used for the morphine s.c 7-day exposure: day 1, 10mg/kg b.i.d.; day 2 and 3, 20mg/kg b.i.d.; days 4-6, 40mg/kg b.i.d; and day 7, 80mg/kg b.i.d (Table 3.1). This protocol was based on one previously employed in the guinea pig to produce dependence (Mizutani, Arvidsson et al. 2005). Morphine was injected 12-hourly (10:00 a.m. and 10:00 p.m.) and WIN -55,212-2 was administered intraperitoneally once daily (10:00 a.m.) for 5 days. The drugs were administered in the animal housing facility where guinea pigs were given free access to food and water. On the day of the experiment, following the chronic drug exposure, the guinea pigs were euthanized and the terminal ileum excised.

The organ bath assays were performed following chronic drug exposure (*in vivo* or *in vitro*). Assessment of tolerance to inhibitory agents (WIN-55,212-2, CADO and morphine) in the LM/MP preparation involved determination of cumulative doses of the drug required to inhibit neurogenic contraction by 50%. In addition the experiments also assessed the effect of chronic WIN-55,212-2 or morphine treatment on the sensitivity of the LM/MP preparation to nicotine. The values were used to determine the EC_{50} (i.e. concentration required to produce 50% of the maximum response) and to calculate the maximum tension produced by nicotine.

Statistical analysis of the data was done using the student t-test on the IC_{50} (concentration required to inhibit neurogenic twitches to 50% of initial) and EC_{50} (concentration required to

induce 50% of maximum LM/MP tissue contraction) values. Significant differences between the test and control groups were determined using unpaired Student's "t" test. Comparison of mean values between three or more groups was performed using one-way ANOVA followed by the appropriate post hoc test, usually Tukey's test, with the probability level of ≤ 0.05 accepted as significantly different..

2. Receptor Protein Analysis

The Western blotting procedure is similar to that previously described (Biser et al., 2002). Homogenates were loaded on 10% precast Tris-HCl Ready gels (Bio-Rad Laboratories, Inc., Hercules, CA) and size fractionated via electrophoresis at 110 V using a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were then transferred to nitrocellulose membranes presoaked in transfer buffer using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). After allowing the membrane to completely dry, it was prehybridized in pre-made Odyssey® blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 3 h. The membrane was washed with phosphate buffered saline (PBS) containing Tween 20 (0.1%) three times (15 min/wash) and incubated overnight with the primary antibody. The following primary antibodies were used; mouse anti-GAPDH (1:60,000), rabbit anti-MOR (1:2000) and rabbit anti-CB_1 (1:200). Following incubation, the blots were washed three times with PBS-T and incubated for 1 h with the appropriate secondary antibody obtained from Li-Cor® Biosciences (Lincoln, NE). The final blot was washed an additional three times in PBS to remove any excess secondary antibody before detection using the Odyssey® near-infrared imaging system (Li-Cor® Biosciences).

IV. Results

1. LM/MP organ bath assay

Treatment for 7-days with morphine resulted in the development of subsensitivity to 2-chloroadenosine (CADO), DAMGO and WIN-55,212-2, suggesting that the heterologous tolerance previously reported extends to cannabinoid agonists as well. As illustrated in Fig 3.2, concentration-response curves showed a significant rightward shift for all agonists and comparison of the calculated IC_{50} values of the morphine and vehicle treated groups revealed a significant reduction in responsiveness as indicated by the significantly lower IC_{50} values: DAMGO, 6.8 vs. 7.5 ($p \leq 0.05$); CADO, 6.6 vs. 7.1 ($p \leq 0.05$); WIN-55,212-2, 7.5 vs. 8.2 ($p \leq 0.05$). The calculated magnitude of rightward shift (i.e. ratio of mean IC_{50} values) or loss of sensitivity of the treated compared to the control group was 4.8-fold for DAMGO, 3.5-fold for CADO, and 5.2-fold for WIN-55,212-2. The calculated mean ratio of IC_{50} values (based on the determined geometric mean IC_{50} values for each group) for tissues obtained from animals that received morphine compared to the control group was not significantly different among the three agonists hence the degree of tolerance development was quantitatively comparable ($F(2, 5) = 1.07$, $p = 0.37$). In addition, analysis of the maximum inhibitory effect for each of the agonists between the control and morphine-treated groups did not show any statistically significant difference in the magnitude of the maximum inhibitory response for DAMGO and CADO. However, chronic treatment with morphine did lead to a significant reduction (23%; $p \leq 0.05$) in the maximum response to WIN-55,212-2.

As illustrated in the concentration-response curves presented in Fig 3.1, 5-day treatment with WIN-55,212-2 resulted in a reduction in sensitivity to WIN-55,212-2 only as shown by the

IC_{50} values for the WIN-55,212-2 and vehicle groups: DAMGO, 7.67 vs. 7.73 ($p > 0.05$); CADO, 7.2 vs. 7.3 ($p > 0.05$); WIN-55,212-2, 7.3 vs. 8.3 ($p \leq 0.05$). Data calculated from Fig 2 revealed that the magnitude of rightward shift in the concentration-response curve (i.e. ratio of mean IC_{50} values) was 1.3-fold for CADO, 1.1-fold for DAMGO and 9.8-fold for WIN-55,212-2. The rightward shift of the IC_{50} values was only significantly different for WIN-55,212-2, suggesting that homologous tolerance had developed in the LM/MP preparation following chronic *in vivo* cannabinoid treatment. Analysis of the maximum inhibitory effect revealed a statistically significant reduction (25%; $p \leq 0.05$) in the maximum inhibition of neurogenic contractions produced by WIN-55,212-2 in tissues obtained from animals chronically treated with WIN-55,212-2. Interestingly, a small but significant reduction (11%; $p \leq 0.05$) in the maximum inhibitory response to DAMGO was also observed in those same tissues obtained from animals chronically treated with cannabinoid agonist. In contrast, chronic cannabinoid treatment produced no significant change in the maximum inhibitory response obtained to CADO in the same tissues. The impact of chronic drug treatment on the response of the LM/MP to nicotine, illustrated in Fig 3.3, revealed no statistically significant difference in the nicotine EC_{50} values following either chronic morphine (treated, 5.32 vs. vehicle, 5.45; $p > 0.05$) or WIN-55,212-2 treatment (treated, 4.98 vs. vehicle, 5.34; $p > 0.05$). However, chronic morphine treatment (treated, 3.7 g vs. vehicle, 2.44 g; $p \leq 0.05$) but not WIN-55,212-2 treatment (treated, 2.73 g vs. vehicle, 2.68 g; $p > 0.05$) produced a significant increase the magnitude of the maximum contractions produced by nicotine (Table 3.2).

2. MOR and CB_1 receptor protein abundance assessment

In light of the fact that chronic agonist exposure often results in receptor downregulation, we assessed whether the treatments had any effect on receptor protein levels. Fig 3.4 shows results of Western blot analyses performed to assess possible changes in the MOR and CB_1 receptor protein abundance following chronic opioid or cannabinoid exposure. Quantitative analysis involved comparison of the specific receptor protein to GAPDH intensity ratios (MOR or CB_1 receptor/GAPDH densitometric units) between the control and the test groups. CB_1 receptor protein analysis showed a statistically significant reduction in receptor protein abundance in homogenates from tissues obtained from the WIN-55,212-2 treated group compared to the control (Fig 3.4A) as indicated by a 32% ($p \leq 0.05$) lower CB_1 receptor/GAPDH intensity ratio. In contrast, no statistically significant difference was observed in the MOR/GAPDH intensity ratios between the control and WIN-55,212-2 treated groups. As illustrated in Fig 3.4B, 7-day morphine treatment did not alter either MOR or CB_1 receptor protein abundance. For both treatments, no statistically significant difference was observed in the GAPDH levels between control and drug-treated tissue homogenates thus implying that the total cellular protein levels remained relatively constant.

V. Discussion

Based upon the similarity of cellular, physiological and pharmacological effects of cannabinoids and opioids, and the conflicting results regarding the character of tolerance that develops following chronic exposure, experiments were conducted to determine whether parenteral *in vivo* exposure to either opioids or cannabinoids results in the development of tolerance that is qualitatively similar. In order to assess potential cellular mechanisms, the candidate also sought

to determine the LM/MP sensitivity and responsiveness to excitatory agents like nicotine following chronic drug treatment. Since we hypothesized that chronic treatment with cannabinoids or opioids could evoke receptor-dependent changes in cells, we investigated the impact of chronic opioid or cannabinoid exposure on both the MOR and CB_1 receptor protein abundance. The present study found important qualitative similarities of the tolerance that develops following chronic 7-day morphine exposure with that previously reported in both *in vivo* and *in vitro* exposure studies (Taylor et al., 1988; Basilico et al., 1999; Li et al., 2010). However, the qualitative nature of the change in sensitivity to a limited number of agents observed following chronic WIN-55,212-2 treatment appears to contrast with that previously observed in morphine tolerant animals which exhibit subsensitivity to a variety LM/MP inhibitory agents and increased responsiveness to excitatory agents like nicotine (Schulz and Goldstein, 1973; Johnson et al., 1978). This difference in the character of the tolerance could have important implications for defining the potential mechanisms that underlie the development of the phenomenon in the guinea pig and could provide an avenue to explore the combinatorial use of these agents therapeutically.

The development of heterologous tolerance following chronic *in vivo* exposure to morphine is consistent with previously reported data that have shown tolerance to develop between 1 and 4 days of exposure, become maximal by day 7 and remain for several days beyond that (Taylor et al., 1988; Li et al., 2010). However, these are the first studies to examine the question of whether the heterologous subsensitivity observed following chronic *in vivo* opioid exposure extended to the cannabinoid receptor agonist, WIN-55,212-2, in the LM/MP model. The observed subsensitivity to WIN-55,212-2 substantiates previous studies using the LM/MP that revealed similar heterologous tolerance following chronic *in vitro* exposure to

opioids (Basilico et al., 1999). However, the change in responsiveness was accompanied by a significant reduction in the maximum response suggesting that there may also be a change in efficacy (Stephenson, 1956). Since opioids and cannabinoids employ comparable signaling pathways, shared components of these pathways offer some sites at which adaptive changes could occur. However, the basis for the difference in efficacy could also reside in the receptor reserve that is present for each receptor family. As demonstrated in these studies, chronic morphine treatment did not alter the abundance of either MOR or CB_1 receptor protein; this is consistent with the maintenance of the maximal inhibitory effect of DAMGO, and suggests that a non-receptor dependent adaptive mechanism is, at least in part, responsible for the development of tolerance. Furthermore, the inability of chronic morphine exposure to reduce MOR protein or the maximal effect of DAMGO is consistent with previous studies showing that morphine has a low capacity to induce MOR internalization and downregulation (Johnson et al., 2006) and supports the fact that the mu-opioid receptor reserve approaches ~90 % the LM/MP (Chavkin and Goldstein, 1984). The lab has previously proposed that the non-receptor dependent changes underlying the heterologous tolerance involve a partial depolarization of the resting membrane potential (Meng et al., 1997) secondary to a reduction in the level of the functional $alpha_3$ subunit of Na^+/ K^+-ATPase (Kong et al., 1997; Biser et al., 2002; Li et al., 2010). The alteration in the membrane potential ultimately accounts for the reduced sensitivity to inhibitory agents (e.g. DAMGO, CADO and WIN-55,212-2) and the enhanced responsiveness to excitatory agents like nicotine. The reduction in WIN-55,212-2 maximal response could also have been caused by a non-receptor mediated change since the cannabinoid system in the LM/MP appears to have a low functional reserve susceptible to chronic drug exposure (Basilico et al., 1999; Guagnini et al., 2006). The fact that CADO appears to inhibit neurogenic activity through a cellular mechanism

different from that of morphine (Meng et al., 1997) and WIN-55,212-2 (Coutts and Pertwee, 1997) suggests that the adaptation events precipitated by chronic morphine exposure must involve some alteration in basic cellular function.

In contrast to chronic opioid exposure, chronic *in vivo* cannabinoid exposure resulted in the development of homologous tolerance that was expressed as a loss of sensitivity to WIN-55,212-2 only as evidenced by a 9.8-fold rightward shift of the concentration-response curve. Interestingly, the maximum responses to both WIN-55,212-2 and DAMGO but not CADO were also significantly decreased. However, the reduction in maximal effect was significantly greater for WIN-55,212-2 than DAMGO and was associated with a selective reduction in the CB_1 receptor protein abundance that would account in part for the decrease in maxima. The fact that the percent reduction in CB_1 receptor protein levels was relatively proportionate to the reduction in maximal effect of WIN-55,212-2 (32% vs. 25% respectively) further reinforces the idea that the CB_1 receptor system in the LM/MP may possess a low functional receptor reserve as observed following *in vitro* exposure (Basilico et al., 1999; Guagnini et al., 2006). The reduction in CB_1 receptor protein abundance is consistent with previous studies which demonstrated robust cannabinoid receptor downregulation that occurs through beta-arrestin mediated desensitization, internalization and ultimately degradation (Gonzalez et al., 2005). The tolerance induced by chronic *in vivo* cannabinoid exposure mediated by CB_1 receptor activation may involve at least two components; one related exclusively to CB_1 receptor downregulation (receptor-dependent and specific) and another possibly through dual internalization of CB_1 receptors and MOR as heterodimers (receptor-dependent but non-specific). Studies have reported heterodimerization of MOR and CB_1 receptors resulting in signaling through common G proteins (Hojo et al., 2008). Receptor heterodimerization has also been reported to result in co-internalization of MOR and

other G protein-coupled receptors (e.g. somatostatin receptors) and has also been reported to result in receptor desensitization (Pfeiffer et al., 2003). Internalization or uncoupling of MOR/CB_1 receptor heterodimers following chronic CB_1 receptor activation would also result in as small a reduction in the apparent functional efficacy of MOR as the heterodimers contributed to the total receptor population. The possibility also exists that the lack of correlation between the change in responsiveness and receptor protein abundance might not be physiologically relevant.

The absence of increased responsiveness to the excitatory effect of nicotine after chronic cannabinoid exposure contrasts to the elevated responsiveness observed in morphine tolerant animals (Schulz and Goldstein, 1973; Johnson et al., 1978) and the enhanced maximum response observed in these studies. Since opioid-induced supersensitivity is thought to be associated with a partial depolarization of the resting cell membrane potential (Meng et al., 1997), this could imply that chronic cannabinoid treatment does not alter the cell resting membrane potential of neurons in the LM/MP model. Furthermore, it could signify that cannabinoid exposure may not alter Na^+/K^+ ATPase isoform expression or function, a proposed key facet in the maintenance of the resting membrane potential, as observed following chronic opioid exposure (Biser et al., 2002). Previous studies assessing the development of tolerance have reported conflicting data on the interactions between opioids and cannabinoids suggesting that the diverse interplay between the two receptor systems could be influenced by the cell/tissue/model system employed and the parameter(s) assessed. The differential results may be due to different levels of enzymes or isoforms involved in the adaptive desensitization; namely adenylyl cyclase, beta-arrestin, G protein-coupled receptor kinase (GRK), PKA, PKC and other kinases ((Bloom and Dewey, 1978; Hine, 1985; Smith et al., 1994; Thorat and Bhargava, 1994)

It is concluded that the type and nature of tolerance exhibited in the guinea pig LM/MP model following chronic *in vivo* WIN-55,212-2 treatment is qualitatively and mechanistically different from that observed following chronic morphine exposure despite the large number of similarities between the two systems. The data presented in this study provide support for the concept that the development of tolerance is a function of several converging influences and is subject to considerable variation that may impact upon the cellular processes that are employed to elicit the adaptive response (Taylor and Fleming, 2001). The fact that cannabinoid treatment produces tolerance that is primarily homologous in nature compared to the heterologous form of tolerance associated with chronic opioid exposure provides an important foundation upon which to develop mechanistic studies. Furthermore, the data raise the possibility that such differences in adaptive responses to these agents could be employed to lead to modification in the therapeutic management of patients with these agents.

Table 3.1 Schedule for morphine subcutaneous injection

Dose escalation schedule for parenteral (subcutaneous) administration of morphine for 7 days.

Day	Morphine dose (mg/kg body weight)	
	10:00 a.m.	10:00 p.m.
1	10	10
2	20	20
3	20	20
4	40	40
5	40	40
6	40	40
7	80	80

Table 3.2 Geometric mean IC_{50} and maximal efficacy values for the inhibitory effect of WIN-55,212-2, DAMGO and CADO following 7-day treatment with morphine or 5-day treatment with WIN-55,212-2.

The geometric mean IC_{50} values for agonists (the agonist concentration required to reduce the amplitude of the neurogenic twitch of the LM/MP to 50% of its initial value) are displayed as -log M (± S.E.M.). The maximal efficacy depicts the % inhibition at the highest agonist concentration. Statistically significant differences ($p \leq 0.05$) are identified by *. The N values for the experimental sets are between 6 and 13.

Experimental Treatment	Agonist								
	WIN-55,212-2			DAMGO			CADO		
	Control	Test	Mean Ratio of IC_{50}	Control	Test	Mean Ratio of IC_{50}	Control	Test	Mean Ratio of IC_{50}
Morphine vs. Control - IC_{50} (-log M ± S.E.M.)	8.23 (±0.18)	7.52 (±0.2)	5.16* (±2.0)	7.51 (±0.21)	6.83 (±0.2)	4.8* (±4.1)	7.12 (±0.13)	6.58 (±0.1)	3.5* (±0.9)
Maximal efficacy (% Inhibition)	97.32 (±4.3)	73.87* (±4.8)		86.55 (±5.6)	76.06 (±5.7)		95.87 (±2.7)	88.17 (±2.6)	
WIN-55,212 vs. Control - IC_{50} (-log M ± S.E.M.)	8.32 (±0.18)	7.33 (±0.15)	9.77* (±12.7)	7.73 (±0.08)	7.67 (±0.05)	1.1 (±0.41)	7.29 (±0.05)	7.17 (±0.05)	1.3 (±0.4)
Maximal efficacy (% Inhibition)	94.76 (±2.3)	70.37* (±3.7)		95.13 (±2.9)	84.29* (±3.4)		96.99 (±3.0)	95.76 (±2.9)	

Table 3.3 Geometric mean EC_{50} and maximum isometric tension values for nicotine following chronic treatment with either WIN-55,212-2 or morphine.

The geometric mean EC_{50} values for nicotine (the agonist concentration required to elicit the amplitude of the neurogenic twitch of the LM/MP to 50% of its maximal value) are displayed as -log M (± S.E.M.). Maximal efficacy depicts the maximum isometric tension attained in LM/MP. Statistically significant differences ($p \leq 0.05$) are identified by *. The N value for each experimental set is 4.

Experimental Treatment	Nicotine		
	Control	**Test**	**Mean Ratio of EC_{50}**
Morphine vs. Control - EC_{50} (-log M ± S.E.M.)	5.45 (±0.03)	5.32 (±0.1)	1.35 (±0.2)
Maximal efficacy (g)	2.43 (±0.1)	3.66 (±0.4)*	
WIN-55,21-2 vs. Control - EC_{50} (-log M ± S.E.M.)	5.34 (±0.10)	4.98 (±0.25)	2.3 (±2.8)
Maximal efficacy (g)	2.68 (±0.2)	2.73 (±0.3)	

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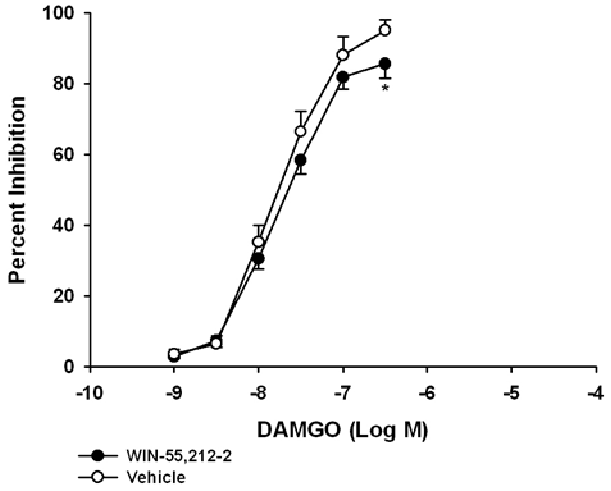
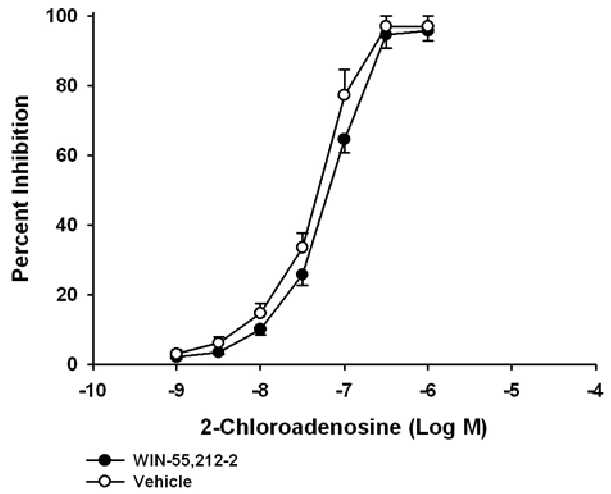
Figure 3.1 Mean concentration-response curves for DAMGO (A), CADO (B) and WIN-55,212-2 (C) in longitudinal muscle/myenteric plexus from control and guinea pigs chronically treated with WIN-55,212-2.

A significant rightward shift of the curve was observed for WIN-55,212-2 only. No significant rightward shift observed for DAMGO and CADO. Significant reduction in the maximum response was observed to both WIN-55,212-2 and DAMGO. Statistically significant differences ($p \leq 0.05$) are identified by *. The N values for the experimental sets are between 6 and 13.

A


Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
DAMGO (Log M)
WIN-55,212-2
Vehicle

B


Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
2-Chloroadenosine (Log M)
WIN-55,212-2
Vehicle

C

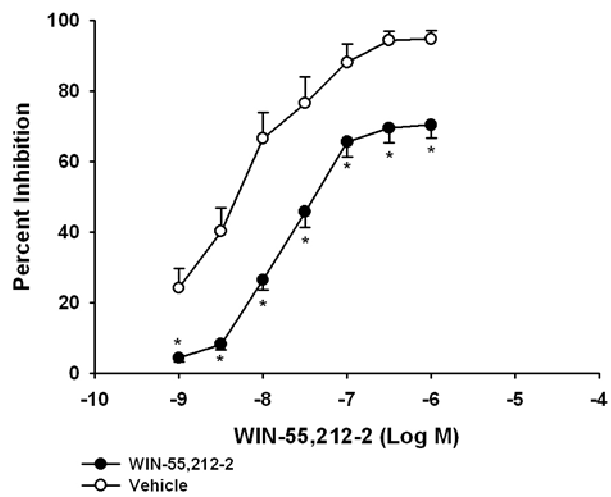
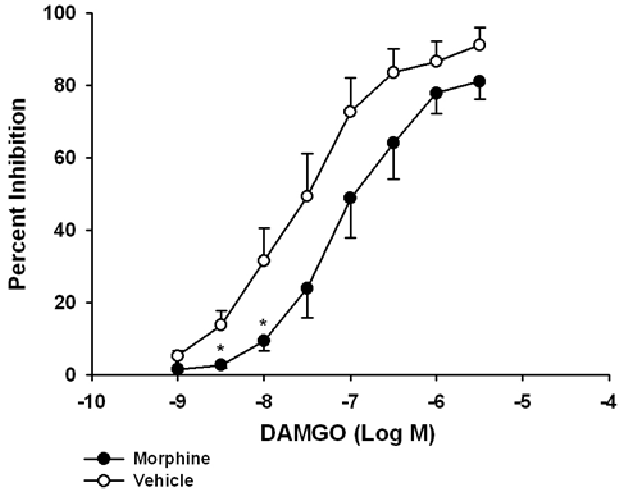

Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
WIN-55,212-2 (Log M)
WIN-55,212-2
Vehicle

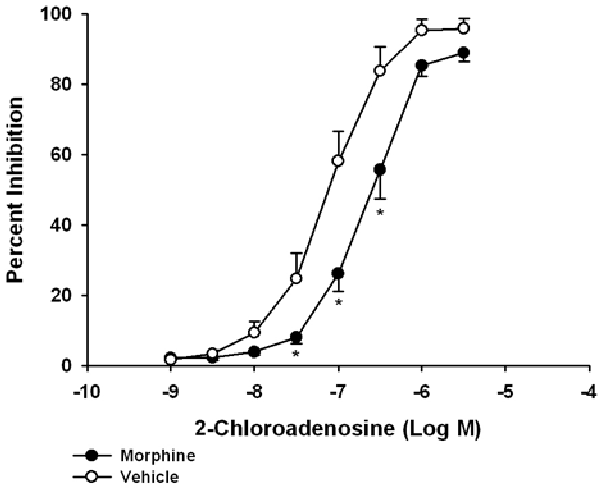
Figure 3.2 Mean concentration-response curves for DAMGO (A), CADO (B) and WIN-55,212-2 (C) in longitudinal muscle/myenteric plexus from control and guinea pigs chronically treated with morphine.

A significant rightward shift of the concentration-response curve was observed for DAMGO, CADO and WIN-55,212-2. The maximum response obtained to WIN-55,212-2 was significantly reduced. Statistically significant differences ($p \leq 0.05$) are identified by *. The N value for each experimental set is 8.

A


Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
DAMGO (Log M)
Morphine
Vehicle

B


Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
2-Chloroadenosine (Log M)
Morphine
Vehicle

C

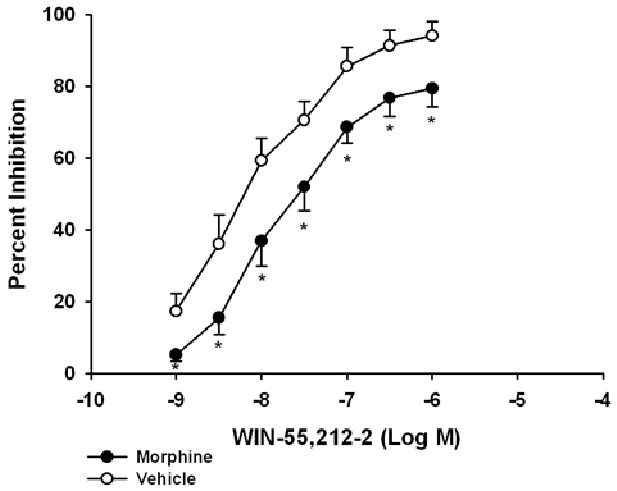
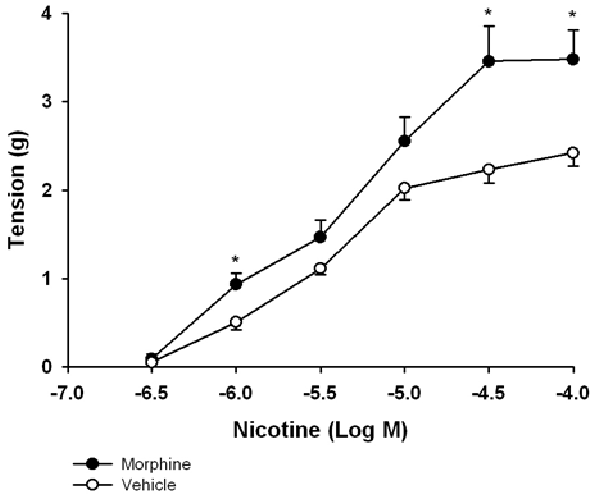

Percent Inhibition
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
WIN-55,212-2 (Log M)
Morphine
Vehicle

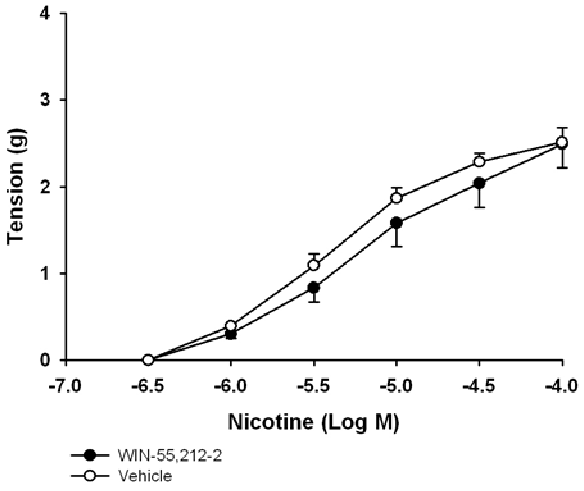
Figure 3.3 Mean concentration-response curves for nicotine in longitudinal muscle/myenteric plexus preparations obtained from morphine or WIN-55,212-2 pretreated guinea pigs and their respective controls.

No significant change in the EC_{50} values for nicotine was observed following chronic treatment with morphine (A) or WIN-55,212-2 (B). Fig. 3.3C shows a bar graph comparing the maximal tension values attained by nicotine stimulation in the control and test groups. A significant increase in nicotine maximal effect is observed in morphine-treated animals. Statistically significant differences ($p \leq 0.05$) are identified by *. The N value for each experimental set is 4.

A


Tension (g)
Nicotine (Log M)
Morphine
Vehicle

B


Tension (g)
Nicotine (Log M)
WIN-55,212-2
Vehicle

C

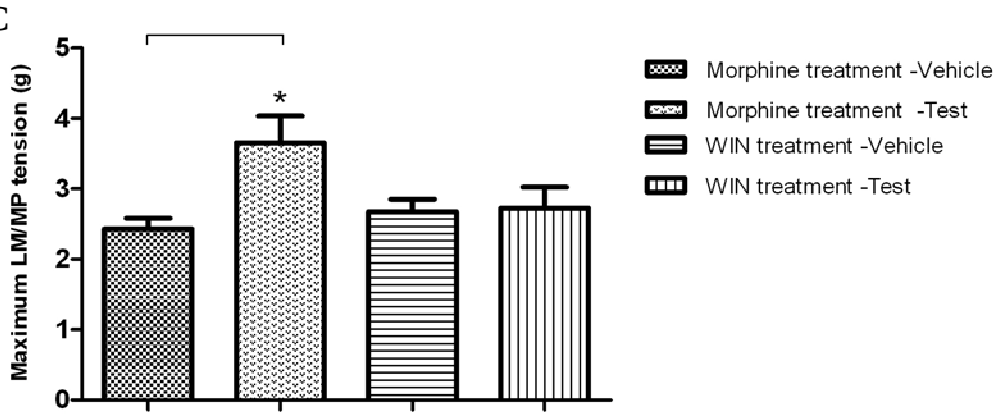
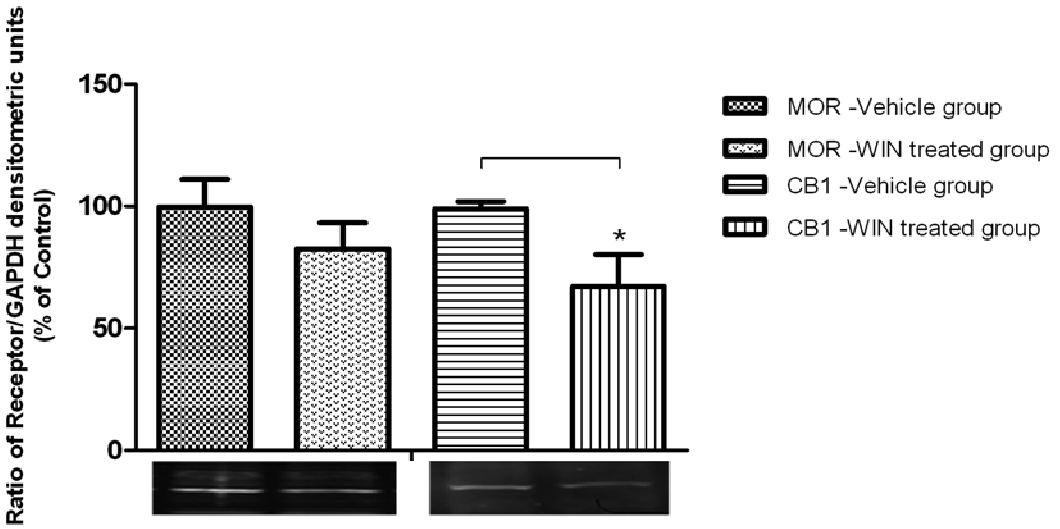

Maximum LM/MP tension (g)
Morphine treatment -Vehicle
Morphine treatment -Test
WIN treatment -Vehicle
WIN treatment -Test

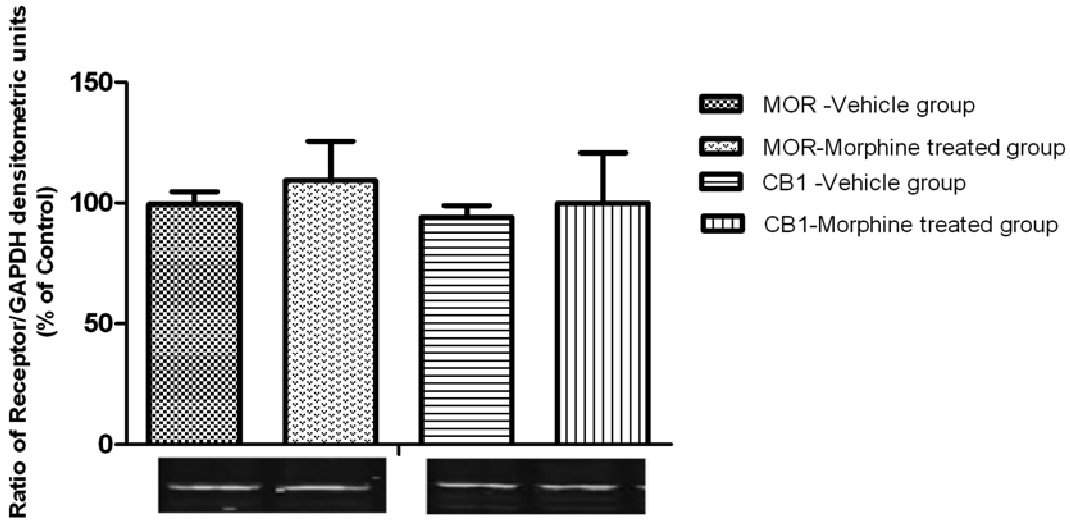
Figure 3.4 Comparison of MOR and CB_1 receptor protein levels following chronic morphine or WIN-55,212-2.

Fig 3.4A illustrates the ratio of the CB_1 receptor or MOR protein intensity to the GAPDH intensity from animals treated chronically with WIN-55,212-2. Fig. 3.4B illustrates the ratio of the MOR or CB_1 receptor protein intensity to the GAPDH intensity from animals treated chronically with morphine. Respective prototypical images of the immunoblot membrane showing the density of the MOR and CB_1 protein are shown below the bar graphs. $p^* \leq 0.05$ considered to be statistically significant. The N values for the experimental sets are between 4 and 6.

A


Ratio of Receptor/GAPDH densitometric units
(% of Control)
150
100
50
0
*
MOR -Vehicle group
MOR -WIN treated group
CB1 -Vehicle group
CB1 -WIN treated group

B


Ratio of Receptor/GAPDH densitometric units
(% of Control)
150
100
50
0
MOR -Vehicle group
MOR-Morphine treated group
CB1 -Vehicle group
CB1-Morphine treated group

CHAPTER FOUR: COMPARISON OF THE EXPRESSION OF TOLERANCE TO HYPOTHERMIA AND ANALGESIA IN THE GUINEA PIG FOLLOWING CHRONIC OPIOID VERSUS CANNABINOID EXPOSURE.

I. Abstract

The candidate previously reported that chronic *in vivo* treatment with morphine resulted in the development of heterologous tolerance in the guinea pig longitudinal muscle-myenteric plexus (LM/MP) whereas chronic WIN-55,212-2 exposure resulted in homologous tolerance. Few studies have compared tolerance that develops to the hypothermic and analgesic activity of opioids and cannabinoids with that observed in the guinea pig LM/MP model. Tolerance was induced by chronic morphine (7 days) or WIN-55,212-2 (5 days) exposure and assessed by determining the alteration in response to challenge doses of WIN-55,212-2 and morphine. Hypothermia was measured by a rectal thermometer while mechanical and thermal analgesia were assessed using the paw pressure and hot plate tests, respectively. The nature of tolerance observed in the hot plate test corresponds closely to that observed in the LM/MP studies (CHAPTER THREE) where morphine pretreatment produced heterologous tolerance and WIN-55,212-2 pretreatment resulted in homologous tolerance. In contrast to the results in the LM/MP studies, WIN-55,212-2 pretreatment produced tolerance to the analgesic effect of morphine in the paw pressure model despite the fact that it did not produce analgesia in this model. Unlike chronic treatment with WIN-55,212-2, chronic morphine treatment did not induce tolerance to the hypothermic effect of WIN-55,212-2. However, since only a very modest hypothermia was observed in response to a morphine challenge, tolerance to this effect was difficult to assess and may not be biologically relevant. The results suggest that the nature of tolerance that develops in

one model system cannot be inferred to another even in the same species since chronic morphine treatment produced heterologous tolerance in the hot plate test and homologous tolerance in the hypothermia model while chronic WIN-55,212-2 treatment elicited homologous tolerance in the LM/MP, hypothermia and hot plate models but heterologous tolerance in the paw pressure model. The present findings affirm the notion that the nature of tolerance depends on the model, drug, species, and regimen used. Our data also suggest that the analysis of tolerance using *in vivo* test systems involves complex neuronal interactions and that multiple cellular effects at various sites in the signal cascade may induce differential functional tolerance in different models.

II. Introduction

Common pharmacological effects observed with the use of opioid and cannabinoid agonists include the ability to induce hypothermia, sedation, hypotension, antinociception and inhibition of both intestinal motility and locomotor activity (Bloom and Dewey, 1978; Smith et al., 1994; Bass and Martin, 2000). These common effects are rooted in the similar cellular signaling events and comparable central and peripheral distribution of the opioid and cannabinoid receptors within the ileum, vas deferens, caudate-putamen, dorsal hippocampus, substantia nigra and hypothalamus (Rosow et al., 1980; Ross et al., 1998; Rawls et al., 2002). Common cellular signaling pathways between the two receptor systems include coupling through $G_{i/o}$ proteins leading to inhibition of adenylyl cyclase (Howlett and Fleming, 1984); inactivation of N, P/Q, and R-type Ca^{2+} channel (Rhim and Miller, 1994; Howlett et al., 2002); activation of the MAPK pathway (Bouaboula et al., 1995); and activation of G protein-activated inwardly rectifying potassium (GIRK) channels (McAllister et al., 1999). Cellular co-localization of cannabinoid and opioid receptors in both the central nervous system and peripheral neurons

(Salio et al., 2001) may contribute to the closely related physiological and clinical effects (Manzanares et al., 1999) including the development of dependence and tolerance (Lichtman and Martin, 2005).

Considerable numbers of the studies that have been undertaken to assess whether chronic opioid or cannabinoid exposure results in the development of tolerance to either agent have often reported conflicting and discordant results. The differential effects could be based on the fact that the analgesic effects of these agents follow from activation of their receptors in the one or more of the following brain areas associated with the pain pathway: periaquaductal gray, amygdala, raphe and the dorsal horn of the spinal cord (Tsou et al., 1998; Farquhar-Smith et al., 2000). Furthermore some studies have reported cellular co-localization of opioid and cannabinoid receptors in the superficial dorsal horn of the spinal cord and caudate-putamen (Hohmann et al., 1999; Rodriguez et al., 2001; Salio et al., 2001). In one study, morphine tolerant animals were reported to be hypersensitive to Δ^9-THC (Rubino et al., 1997) whereas in a separate study, morphine dependent animals exhibited a decrease in the analgesic effect of Δ^9-THC (Thorat and Bhargava, 1994). Furthermore, Cichewicz and Welch (2003) discovered that low doses of cannabinoids curtailed the development of morphine tolerance while preserving the levels of the three major opioid receptors. Though most behavioral studies have focused on analgesic tolerance, few studies have investigated the nature of tolerance that develops to the hypothermic effect of these agents (Rosow et al., 1980; Rawls et al., 2002). The basis for possible alteration in opioid and cannabinoid agonists' hypothermic potency could be related to the reported presence of both MOR and CB_1 receptors in the hypothalamus and the fact that activation of these of these receptors results in the regulation of body temperature. Furthermore, chronic cannabinoid exposure has been reported to regulate levels of MOR in the hypothalamus (Corchero et al.,

2004). Possible mechanisms that could be involved in development of tolerance include changes in receptor abundance or levels of the endogenous ligands. Previous studies assessing the effect of chronic drug administration on receptor abundance have often reported conflicting results; in one study chronic morphine exposure resulted in an increase in cannabinoid receptor density (Rubino et al., 1997) while in another the receptor density seemed not to be altered (Thorat and Bhargava, 1994). In addition, the change in cannabinoid receptor abundance following opioid exposure appears to be region dependent and varies amongst different species (Vigano et al., 2003; Gonzalez et al., 2005). The effects of cannabinoid pretreatment on opioid receptors has been reported to involve a time dependent increase in MOR density that was observed in several brain regions including the amygdala, thalamus and hypothalamus (Corchero et al., 2004). The interaction between the cannabinoid and opioid systems as evidenced by the cross-regulation of their endogenous ligands may also play a critical role in the qualitative nature of tolerance observed with these agents. Δ^9-THC and other endocannabinoids (except anandamide) have been shown to increase extracellular levels of dynorphin in the spinal cord thus enhancing KOR stimulation and analgesic (Mason et al., 1999; Welch and Eads, 1999). In contrast, the effect of opioid exposure on the endocannabinoid levels seems limited since chronic exposure to morphine did not affect the levels of anandamide or 2-AG in the striatum, cortex, hippocampus, limbic area or hypothalamus.

The assessment of mechanisms involved in the development tolerance in an *in vivo* model is complicated since it is dependent on complex neuronal network interactions involving multiple synaptic connections that may have different receptor population distribution. Thus, most studies are conducted *in vitro* using isolated tissues or cell lines. Most *in vivo* studies performed have used mice and rats: The current study used the guinea pig model whose

advantages include the fact that it exhibits closer anatomical, physiological, neurological and developmental similarities to the human that is well established thus making it an appropriate model for studying gastro-intestinal, respiratory and opioid tolerance (Chavkin and Goldstein, 1984; Hunter et al., 1997; Gray et al., 2001). Studies investigating the development tolerance to cannabinoids in the human and guinea pig ilea have reported comparable results thus further reinforcing the similarities between the two species and the utility of using the guinea pig as an animal model (Guagnini et al., 2006).

There is a growing body of evidence pointing toward convergence and commonality of activity and cell signaling pathways between the two receptor systems that may act in concert to alter many of the pharmacological effects of each including the development of tolerance (Bass and Martin, 2000). The effect of chronic cannabinoid or opioid pretreatment on hypothermic or analgesic efficacy of opioid and cannabinoid agonists is hard to define as it seems to be dependent on complex neuronal networking activated by these agonists. The goal of the present study was to evaluate the effect of chronic *in vivo* cannabinoid or opioid exposure on the expression of tolerance to the hypothermic effects or analgesic effects of both agents. Based on these similarities in signal transduction pathways, and comparable anatomical distribution and co-localization of their respective receptors, it was hypothesized that chronic treatment with either agonist would lead to the development of heterologous tolerance to the hypothermic and analgesic effects of these agents. Furthermore the studies investigated how the qualitative nature of tolerance to the analgesic and hypothermic effect of opioids and cannabinoids compares with that observed in the guinea pig LM/MP following chronic *in vivo* treatment.

III. Experimental Protocol

The present study investigated the development of tolerance to the analgesic and hypothermic effect of cannabinoid and opioid agents following chronic treatment with either agent. Drug pretreatment regimens employed were based on those previously used in other studies to induce tolerance or dependence (Mizutani et al., 2005; Spina et al., 1998) and by preliminary studies performed in the laboratory. The treatment regimens that were employed were identical those outlined previously (CHAPTER THREE) which were shown to induce the development of tolerance in the LM/MP model. Briefly, morphine was administered subcutaneously twice daily (10:00 a.m. and 10:00 p.m.) for 7 days in an escalating dose fashion whereas WIN-55,212-2 was administered intraperitoneally once daily (10:00 a.m.) for 5 days. Animals were tested between 9:00 and 10:00 am on the following day after administration of the last dose (12 h after the last dose of morphine and 24 hrs after the last dose of WIN-55,212-2)..

1. Analgesia Assessment

All animals were acclimated to the observation room for 1 h prior to the initiation of any assessment. Baseline mechanical (paw pressure) or thermal (hot plate) analgesia was measured prior to injecting the challenge drug. The Randall and Selitto test was used to assess mechanical nociception (Randall and Selitto, 1957). In this test, pressure was manually applied to the plantar surface of the hind paw using a cone-shaped pusher with a rounded tip. The force (measured in grams) at which the guinea pig withdrew its hind paw was defined as the paw pressure threshold. A cut-off was set at 600 g to prevent tissue damage. Mean response threshold was determined at the following time intervals after acute drug administration: 0 (baseline), 15, 30, 45, 60, 90 and 120 min. Tolerance was defined as a reduction in the antinociceptive effect (increased threshold

for withdrawal) of the challenge dose of agonist such that the maximum amount of pressure required to elicit a paw withdrawal was decreased. Challenge doses employed (morphine 10mg/kg or WIN-55,212-2 6mg/kg) were selected based on preliminary studies done to assess the optimum dose required to produce an adequate and quantifiable analgesic response.

The hot plate analgesia procedure used was similar to that previously described (Bannon and Malmberg, 2007). The response to a thermal stimulus was measured using a hot plate analgesia meter (IITC Life Science, Woodland Hills, CA) set at a constant temperature of 50^{o}C. Once an animal was placed on the hot plate, the timer was activated and the latency to respond with either a front paw lick or flick was determined. To prevent tissue damage, a 40 second cut-off time period was used at which the animal was removed from the hot plate and a time of 40 seconds recorded as the latency. Challenge doses (morphine 6mg/kg or WIN-55,212-2 3mg/kg) were determined based on preliminary studies done to assess the optimum dose required to produce an adequate and quantifiable analgesic response. The doses used in the hot plate test were lower than those used to assess mechanical analgesia since higher doses resulted in latency values beyond the 40 second cut off threshold.

2. Hypothermia Assessment

Assessment of body temperature using a rectal thermometer was performed in a quiet room at an ambient temperature of 25^{o}C. After a 1 h acclimatization period in the test room, body temperature was measured with a digital rectal thermometer inserted to a constant depth of 2.5 cm. Following thermometer insertion, a 15 sec equilibration period was allowed to lapse before the temperature was recorded. The rectal temperature was measured at the following time intervals post challenge drug administration: 0 (baseline), 15, 30, 45, 60, 90 and 120 min.

Baseline temperature was measured twice prior to drug challenge (morphine 10mg/kg or WIN-55,212-2 6mg/kg) and the results expressed as the means $\pm$ S.E.M of the ^{o}C change from baseline. Baseline temperature was also recorded to determine whether chronic treatment with either agonist had any impact upon core body temperature.

The Student's t-test (unpaired) was used to analyze the maximum rectal temperature change or analgesia threshold for mechanical and thermal tests. Analysis was also done at each time point. A p value ≤ 0.05 was considered to be statistically significantly different.

IV. Results

1. Assessment of acute analgesic response following chronic treatment

A. Paw pressure test. The analgesic effect of morphine (10mg/kg body weight) was determined using the paw pressure assessment method in control (vehicle treated) vs. drug-treated (7- day morphine or 5-day WIN-55,212-2 treated) guinea pigs. The analgesic effect of morphine was evident within 15-30 min and persisted throughout the observation period with the highest values of pressure required to elicit retraction ranging from 400 to up to 600g (Fig. 4.1.A). The maximum analgesic effect was observed between 45 and 60 min for both the vehicle and test groups. As illustrated in Fig. 4.1.A and B, significantly lower pressure was required to elicit a paw withdrawal in the test animals chronically treated with either cannabinoids (318 g vs. 557 g, $p \leq 0.05$) or opioids (217 g vs. 398 g, $p \leq 0.05$) compared to the vehicle groups following morphine challenge implying that tolerance had developed in response to the chronic treatment.

Results presented in Fig. 4.2.A and B illustrate the absence of any analgesic response to acute challenge with WIN-55,212-2 (6 mg/kg) in either vehicle- or drug-treated groups. In an effort to determine the basis for the lack of response, doses of WIN-55,212-2 up to 18mg/kg

were evaluated during the preliminary experiments without yielding any apparent analgesic effect. Therefore, the development of tolerance could not be assessed since no analgesic response to WIN-55,212-2 was observed even in naïve animals in this model of analgesia assessment.

B. Hot Plate Analgesia Test. The analgesic effect of morphine (6mg/kg body weight) and WIN-55,212-2 (3mg/kg body weight) was determined by hot plate assessment in vehicle treated controls vs. test (7-day morphine or 5-day WIN-55,212-2 treated) animals. The challenge doses of morphine and WIN-55,212-2 used were determined in preliminary experiments in which a dose dependent analgesic effect of both agents that occurred in the range of 1-10 mg/kg of morphine and 1-6 mg/kg of WIN-55,212-2 was observed. As illustrated in Fig. 4.3 and Fig. 4.4, comparison of the latency to thermal response (sec) between the morphine- and vehicle-treated groups shows the development of significant tolerance to the analgesic effect of morphine (9.0 vs. 23.5 seconds; $p \leq 0.05$) and WIN-55,212-2 (9.8 vs. 26.8 seconds; $p \leq 0.05$) as indicated by the substantial loss of latency to respond. Comparison of the latency to thermal response (sec) between WIN-55,212- and vehicle-treated groups shows the development of tolerance to the analgesic effect of WIN-55,212-2 (12 vs. 26.3 seconds; $p \leq 0.05$) but not to morphine challenge (22.3 vs. 36.0 seconds; $p > 0.05$).

2. The effect of chronic treatment on the hypothermic response

The hypothermic effect of morphine (10mg/kg body weight) and WIN-55,212-2 (6mg/kg) was determined by rectal body temperature assessment in vehicle treated controls vs. drug-treated (7-day morphine or 5-day WIN-55,212-2 treated) guinea pigs. The challenge doses used were based on preliminary studies performed which show a dose dependent reduction in rectal temperature with both agents. Body temperature was assessed at baseline (0 min), 15, 30,

45, 60, 90 and 120 min after challenge drug administration. Following chronic treatment with either cannabinoids or opioids, challenge with morphine or WIN-55,212-2 resulted in steadily increasing hypothermia at an ambient temperature of 25^{o}C that peaked around 90 min after acute exposure. The hypothermic effect of WIN-55,212-2 was considerably and statistically more pronounced than that observed with morphine (maximum change in temperature of 0.88 ± 0.23 °C for morphine versus 3.81± 0.17°C for WIN-55,212-2; $p \leq 0.05$).

As illustrated in Fig. 4.5A, comparison between morphine and vehicle treated animals shows the development of tolerance to the hypothermic effect of morphine (0.10 vs. -0.43°C; $p \leq 0.05$). In contrast, comparison between WIN-55,212-2 and vehicle treated animals (Fig 4.5B) shows that no tolerance develops to after the hypothermic effect of morphine challenge (0.34 vs. 1.3°C; $p > 0.05$). This lack of effect could have been influenced by the fact that morphine produced only modest hypothermia and/or by the wide variability in the hypothermia values. The hypothermic effect of morphine appears to develop slowly, and seemed to peak around 90 min for both groups. Fig. 4.6 illustrates the results of studies in which vehicle- and drug-treated animals were challenged with WIN-55,212-2. Challenge with WIN-55,212-2 (6 mg/kg) in animals chronically treated with morphine did not reveal the development of tolerance to the hypothermic effect (vehicle: -4.2 vs. test -3.8°C $p > 0.05$). The maximum hypothermic effect peaked at 90 min for both groups and by 120 min the body temperature had not recovered to baseline levels. The studies further assessed the hypothermic effect of lower challenge doses of WIN-55,212-2 (1mg/kg and 3mg/kg) following chronic exposure with morphine. The results depicted in Fig. 4.7 indicate that the development of tolerance was not apparent with both challenge doses, thereby reinforcing the results observed with WIN-55,212-2 (6 mg/kg) and suggesting that the highest dose was not supramaximal. Fig. 4.8 shows a dose-response curve

constructed by determining the maximal hypothermic effect of WIN-55,212-2 using the 1mg/kg, 3mg/kg and 6mg/kg challenge doses in control and morphine pretreated animals. The data indicate that the hypothermic effect of WIN-55,212-2 was dose-dependent and, furthermore, that no tolerance developed to that effect in animals treated chronically with morphine. In contrast, chronic WIN-55,212-2 treatment resulted in the development of tolerance to the acute hypothermic effect of WIN-55,212-2 (6 mg/kg) challenge (control: -3.79°C; test -2.49°C; $p \leq 0.05$) as shown in Fig. 4.6B. The maximum hypothermic effect was observed at 90 min for both groups and extended to at least 120 min which was chosen as the time period to stop the experiment. Preliminary studies in the laboratory demonstrated that acute WIN-55,212-2 exposure produces a long lasting hypothermic effect spanning up to 6 h.

V. Discussion

Complex interactions have been observed in the development of tolerance to the effects of opioids and cannabinoids especially in *in vivo* models where the complex neuronal network interactions are vast (Taylor and Fleming, 2001). It is for this reason that mechanistic studies on the development of tolerance are performed *in vitro* using isolated tissue or cell models (Johnson et al., 1978; Shapira et al., 2003; Li et al., 2006). The current study examined the development of tolerance using the *in vivo* approach cognizant of the complication presented by the complex neuronal network interaction in assessing and interpreting the development of tolerance to the effects of opioids and cannabinoids observed in the whole animal. Results from the present study clearly indicate that the interplay between the cannabinoid and opioid systems is very complicated and that development of tolerance to these effects may be dependent upon the model being assessed and the acute effects of the agonist in the model.

The current study reports the development of tolerance to the analgesic effect of morphine in the paw pressure model following pre-treatment with WIN-55,212-2 despite the fact that WIN-55,212-2 did not acutely induce analgesia in this model. This effect could have occurred through various pathways. One mechanism could involve an indirect intracellular interaction between the opioid and cannabinoid system whereby activation of CB_1 receptors results in non-specific adaptive desensitization through sequestration of common G proteins and making them unavailable to couple to other receptors (Vasquez and Lewis, 1999). This activity would result in a reduced potency of agonists whose receptors use the same G proteins and thus would result in the development of heterologous tolerance; however, the development of this effect would be dependent on the co-localization/co-expression of MOR and CB_1 receptors in the same neurons. Studies reporting co-localization of MOR and CB_1 receptors in regions responsible for pain regulation (e.g. the superficial dorsal horn of the spinal cord (Hohmann et al., 1999; Salio et al., 2001) and caudate putamen (Rodriguez et al., 2001)) seem to support this notion. Another possible pathway could involve a network interaction between the two receptor systems whereby the cannabinoid receptor input resides on neurons or brain regions upstream of the neurons engaged in the response such that its down-regulation would result in the reduced activation of the downstream endogenous opioid system hence tolerance to the analgesic effect of agonists targeting the latter would be evident. In support of this proposal are reports that show the attenuation of Δ^9-THC analgesia by the opioid antagonist, naloxone (Manzanares et al., 1999), and the fact that Δ^9-THC administration elevates pro-opiomelanocortin (POMC) levels in the hypothalamus, and increases the expression of preproenkephalin in the PAG, spinal cord and striatum (Corchero et al., 1997; Manzanares et al., 1998). Since cannabinoid receptor activation has been proposed to induce the release of enkephalins, hence performing a permissive or

synergistic role in opioid analgesia (Pugh et al., 1996; Pugh et al., 1997), the cannabinoid analgesic system may be perceived to be upstream of the opioid receptors such that downregulation of the system would negatively regulate the opioid system. Our results are consistent with this idea since chronic cannabinoid exposure results in the development of tolerance to opioid-induced analgesia in the paw pressure model (Thorat and Bhargava, 1994). It should be noted that WIN-55,212-2 did not elicit mechanical analgesia; this could imply that the paw pressure model may not be a sensitive enough model system to employ or that WIN-55,212-2 does not produce analgesia in the model hence tolerance could not be assessed. Studies in humans have also reported absence of analgesia to cannabinoids in similar pain models (Naef et al., 2003).

The development of tolerance to the thermal analgesic effect of WIN-55,212-2 following chronic morphine exposure appears to be related to the reduction in opioid signaling. Since cannabinoid analgesia is in part dependent on the release of enkephalins (Pugh et al., 1996; Pugh et al., 1997), it follows that opioid receptor desensitization (possibly due to receptor downregulation) following chronic morphine exposure may indirectly result in a decreased efficacy of cannabinoids (Manzanares et al., 1999). This would be consistent with the heterologous tolerance observed to the analgesic effect of WIN-55,212-2 following chronic opioid exposure and correlates with the tolerance observed in the LM/MP model. In contrast, chronic WIN-55,212-2 treatment did not result in tolerance to morphine challenge in the thermal analgesia model. The development of homologous tolerance in the thermal analgesia model following chronic WIN-55,212-2 pretreatment also supports the adaptive desensitization of the cannabinoid signaling and is consistent with WIN-55,212-2-induced CB_1 receptor-mediated desensitization and downregulation observed in the LM/MP (CHAPTER 3). It is important to

note that other studies have reported contrasting results in the form of enhanced analgesia (Welch and Eads, 1999); however, it should also be considered that the drug regimens and animal models used in these studies significantly differ from those used in the present study. The ability of chronic cannabinoid exposure to elicit tolerance to the analgesic effect of opioids appears to be dose-dependent since exposure to low non-analgesic doses of cannabinoids has been shown to maintain or augment analgesic sensitivity to opioids (Thorat and Bhargava, 1994; Welch and Eads, 1999). The difference between the nature of tolerance observed in the paw pressure or hot plate analgesia models following WIN-55,212-2 pretreatment could be a function of variance in the spinal and supraspinal analgesia pathways and the relative distribution and interaction between the activated opioid (mu- and kappa-) and cannabinoid receptors involved in analgesia. Differences in the analgesic pathways engaged are derived from the fact that the hot plate test more closely resembles a supraspinal response involving higher centers of the ascending and descending pathways including the PAG, the thalamus, the hypothalamus and the cerebral cortex, whereas the paw pressure is mainly a reflex under spinal control (Mansour et al., 1988; Kieffer, 1999). Differences in these pathways may have profound effects on the type of tolerance observed since the spinal response may only reflect interaction between receptors located on the first-order afferent fibers (A-delta or C type) and the dorsal root ganglia in the spinal cord whereas the supraspinal response additionally involves networks of nuclei in the higher centers (Almeida et al., 2004). The involvement of multiple nuclei in the expressed response to pain offers another level of complexity since such pathways frequently involve both excitatory and inhibitory pathways that may be differentially altered by the same agonist through the same receptor. Furthermore, permutations of possible interactions between opioid and cannabinoid receptors may also be vastly different since both kappa- and mu- opioid receptors

are involved in morphine-induced analgesia, and have been reported to interact and co-localize with the CB_1 receptors (Manzanares et al., 1999; Salio et al., 2001). The heterogeneity of first-order afferent neurons activated (C-type fibers versus A-delta type fibers) by different noxious stimuli (thermal vs. mechanical) should also be considered (Almeida et al., 2004). The levels and activity of opioid (mu- or kappa-) and CB_1 receptors may also vary between the afferent fibers. These factors may have a great influence on the input-output relationship to the spinal cord and could have profound impact on the type of tolerance observed in these two models. Tolerance to the acute analgesic effect of WIN-55,212-2 or morphine could have occurred through desensitization of signaling in the following brain areas where MOR and CB_1 receptors are expressed: amygdala; hypothalamus; cortex; PAG; or dorsal horn of the spinal cord (Rosow et al., 1980; Rodriguez et al., 2001; Salio et al., 2001; Rawls et al., 2002). Electron microscopy studies in rats have demonstrated co-localization of MOR and CB_1 receptors in the following areas involved in pain regulation: dorsal horn; and caudate-putamen (Rodriguez et al., 2001; Salio et al., 2001). Furthermore, studies in cells cotransfected with MOR and CB_1 receptors have demonstrated heterodimerization of these receptors (Hojo et al., 2008) suggesting a much closer interaction between the convergent signaling pathways if the receptors are expressed in the same neurons.

The development of tolerance to the hypothermic effect following chronic drug exposure appears to be a physiological adaptation dependent on whether an acute hypothermic effect is observed with the pretreatment agent. WIN-55,212-2 produced robust hypothermia supporting the well documented significant role for cannabinoid receptors in temperature regulation (Rawls et al., 2002). A desensitization of the CB_1 receptor signaling in the hypothalamus, consistent with

robust downregulation of CB_1 receptor-mediated effects (Oviedo et al., 1993; Rodriguez de Fonseca et al., 1994), could also result in the type of homologous tolerance as that which was observed in the current study. In contrast, the modest hypothermia observed in response to acute opioid treatment may be due to the fact that morphine activates both mu- and kappa- opioid receptors which produce opposing effects on core temperature with the latter producing hypothermia and the former hyperthermia (Rosow et al., 1980). The modest hypothermic effect produced by morphine may not have been sufficient to trigger a physiological adaptive response that would extend the reduced responsiveness to the hypothermic effect of WIN-55,212-2. This is consistent with previously published data that show that the use of minimally effective or sub-effective doses of cannabinoids and opioids does not induce tolerance (Welch and Eads, 1999) and reinforces the idea that the development of tolerance is an adaptive response of cells and tissues that is dependent upon chronic agonist activation of receptors. The candidate speculates that the absence of heterologous tolerance to WIN-55,212-2-induced hypothermia following chronic morphine exposure may be due to the fact that the slight reduction in body temperature may not facilitate a significant pharmacological stimulus to evoke adaptation responses in the temperature regulation pathway that possibly includes cannabinoid receptors (Rosow et al., 1980). WIN-55,212-2 pretreatment produced a robust reduction in body temperature that resulted in the development of tolerance to the cannabinoid hypothermic effect; this type of selective adaptational response correlates with previously reported data in the LM/MP model (CHAPTER THREE). However, it could not be ascertained whether WIN-55,212-2 pretreatment produced heterologous tolerance since acute morphine challenge elicited a modest hypothermic effect with high variability. It should be noted that hypothermia results from a complex interplay between the CNS and peripheral processes. Based upon the results obtained in this study, additional

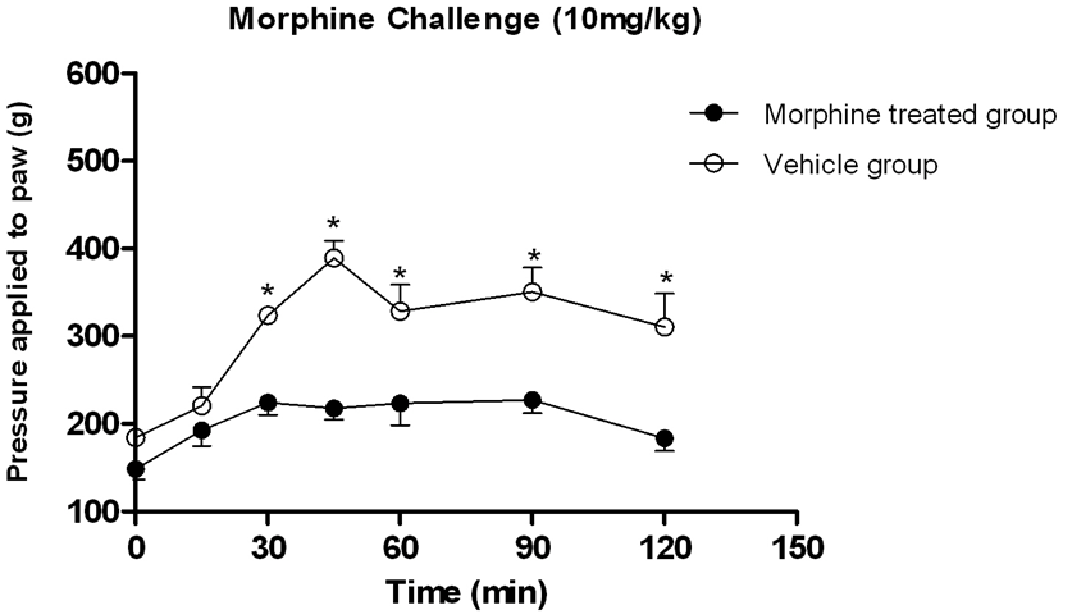
studies would be required to isolate the specific pathways that may be involved in the development of tolerance.

In light of the differences observed in the development of tolerance between opioids and cannabinoids, it is concluded that the analysis of the development of tolerance in intact animal models is complicated by the possible network interactions between the two receptor systems and their respective signaling pathways and the complex neural networks engaged in most outcomes being assessed at the level of the whole animal. Differences in the animal species can also make it difficult to interpret and compare results with those that have been obtained in other laboratories. Further studies are required to fully elucidate the receptor interactions and possible role that receptor co-localization between the endogenous opioid and cannabinoid networks may play in hypothermia and analgesia models that were employed as well as in the mechanism(s) responsible for the development of tolerance. In light of the results obtained in the hypothermia assessment, there is need to assess the distribution of the MOR and CB_1 receptor distribution in the hypothalamus to determine whether the possible co-localization of both receptor systems that has been reported in the hypothalamus may serve as a site through which the alteration in responsiveness is developed.

Figure 4.1 Effect of morphine challenge on mechanical analgesia following 7-day morphine or 5-day WIN-55,212-2 treatment

The analgesic effect of morphine (10mg/kg body weight) was determined by assessing the pressure required for paw retraction. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. Analgesia was measured at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set is 4.

A


Morphine Challenge (10mg/kg)
Pressure applied to paw (g)
600
500
400
300
200
100
0
30
60
90
120
150
Time (min)
Morphine treated group
Vehicle group
*
*
*
*
*
*

B

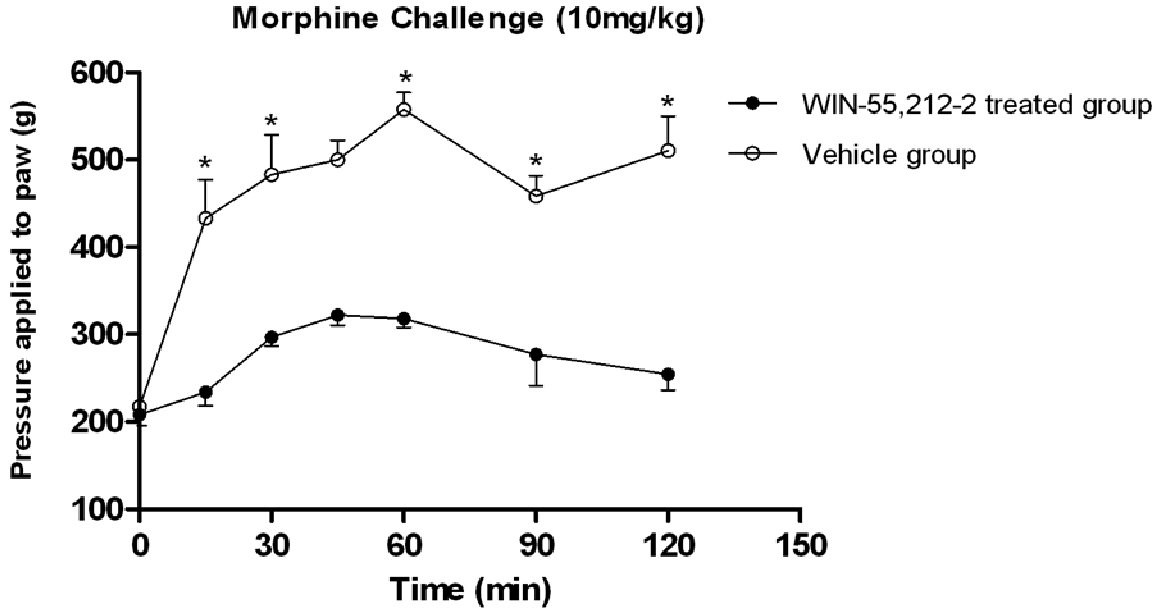
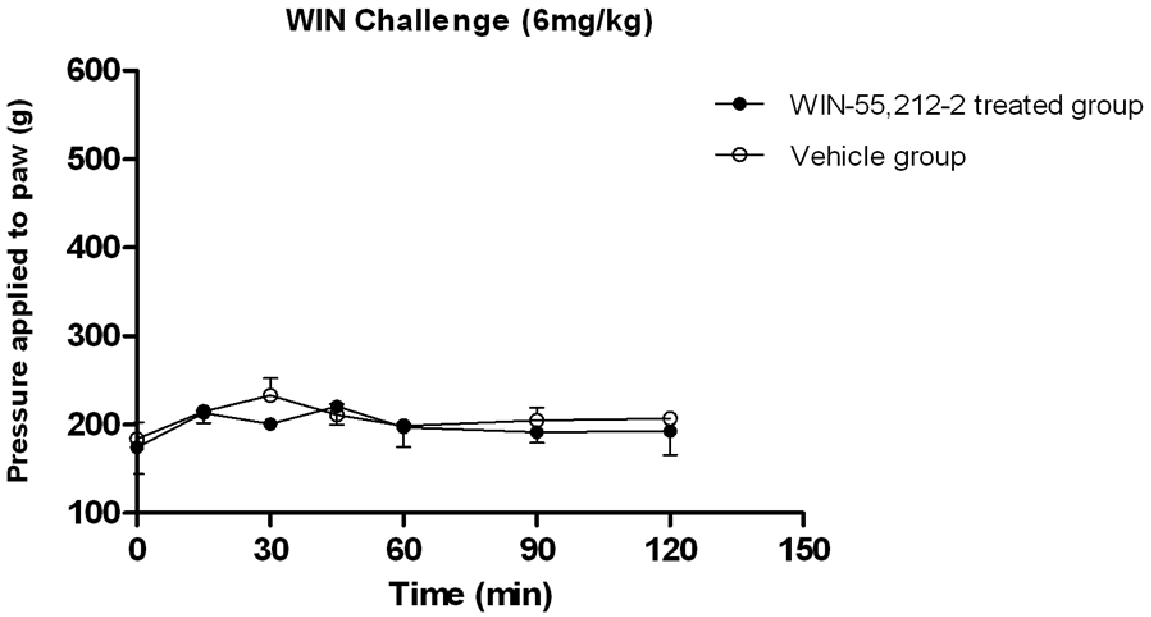

Morphine Challenge (10mg/kg)
Pressure applied to paw (g)
600
500
400
300
200
100
0
30
60
90
120
150
Time (min)
WIN-55,212-2 treated group
Vehicle group
*
*
*
*
*
*

Figure 4.2 Effect of WIN-55,212-2 challenge on mechanical analgesia following 7-day morphine or 5-day WIN-55,212-2 treatment

The analgesic effect of WIN-55,212-2 (6mg/kg body weight) was determined by measuring the pressure required to elicit paw retraction using the Randall-Selitto model system. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. Analgesia was measured at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set is 4.

A


WIN Challenge (6mg/kg)
Pressure applied to paw (g)
600
500
400
300
200
100
0
30
60
90
120
150
Time (min)
WIN-55,212-2 treated group
Vehicle group

B

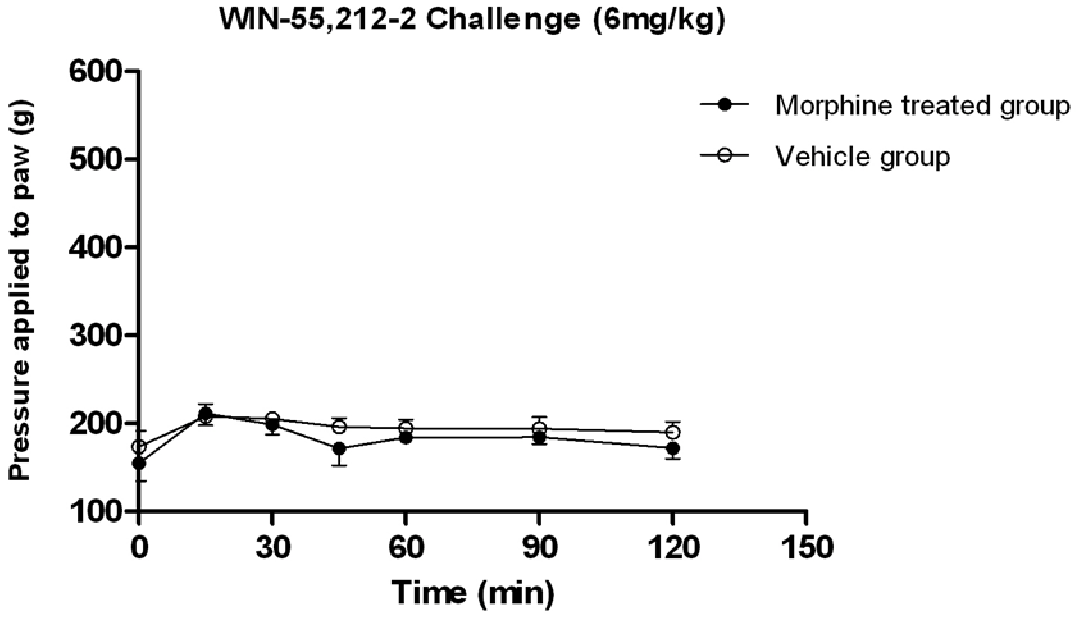
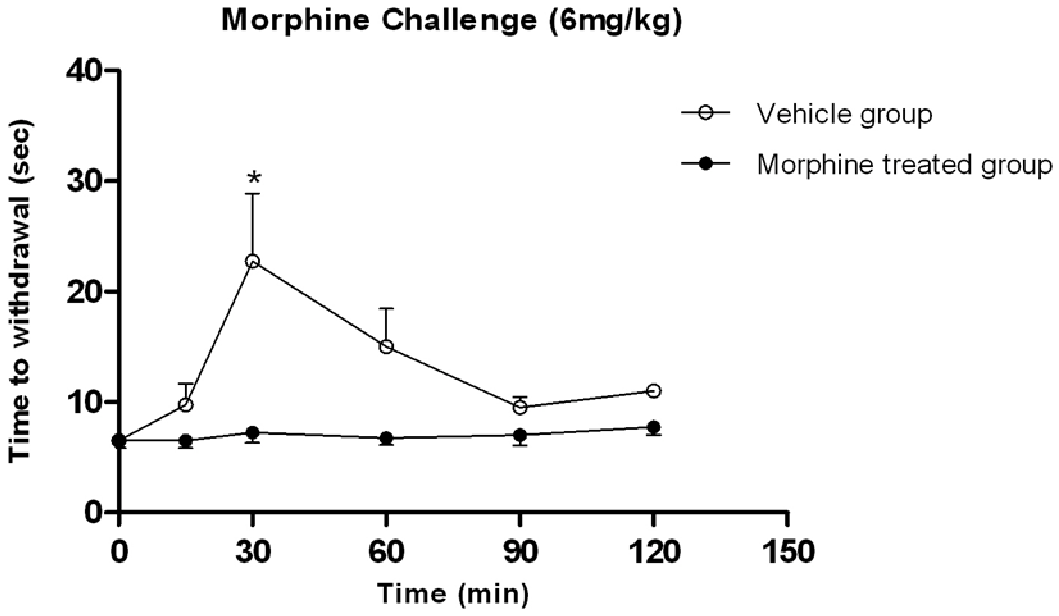

WIN-55,212-2 Challenge (6mg/kg)
Pressure applied to paw (g)
600
500
400
300
200
100
0
30
60
90
120
150
Time (min)
Morphine treated group
Vehicle group

Figure 4.3 Effect of morphine challenge on thermal analgesia following 7-day morphine or 5-day WIN-55,212-2 treatment

Assessment of the thermal analgesic effect of morphine (6mg/kg body weight) determined using the hot plate test. Time to withdrawal was determined manually by visual inspection of the animal movements. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. The level of analgesia was measured at baseline (0 min), 15, 30, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N values for each experimental set ranges from 4 – 6.

A


Morphine Challenge (6mg/kg)
Time to withdrawal (sec)
40
30
20
10
0
*
0
30
60
90
120
150
Time (min)
Vehicle group
Morphine treated group

B

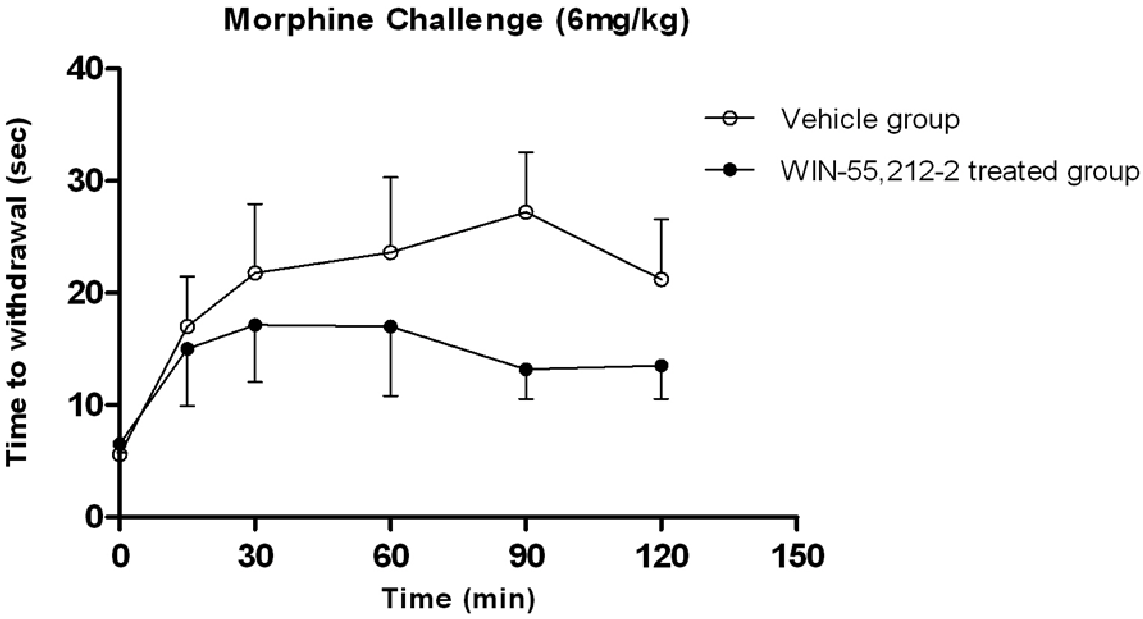
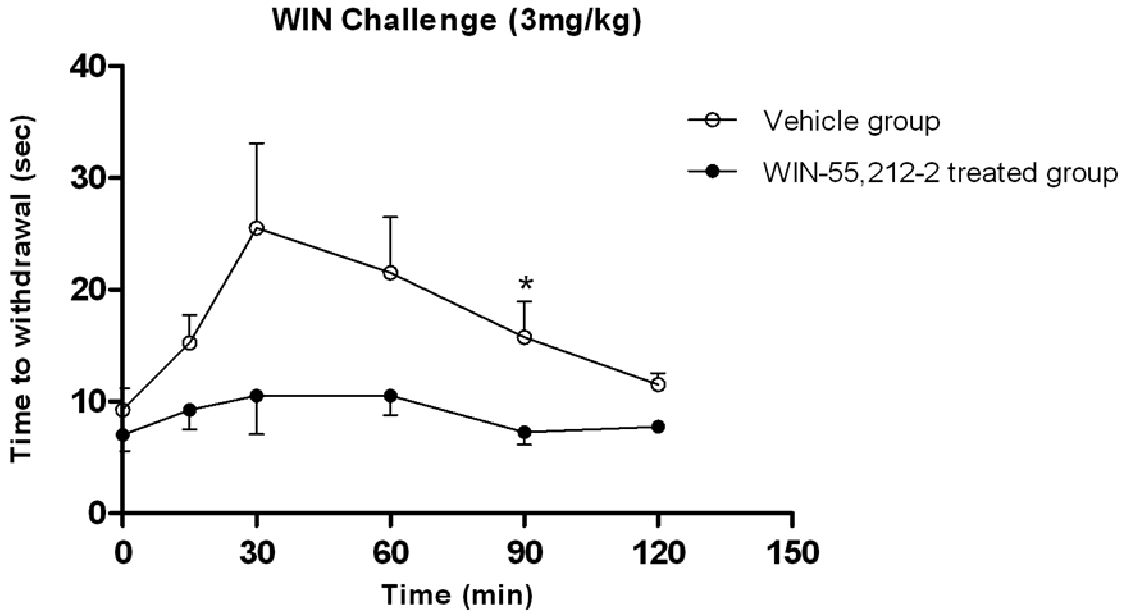

Morphine Challenge (6mg/kg)
Time to withdrawal (sec)
40
30
20
10
0
0
30
60
90
120
150
Time (min)
Vehicle group
WIN-55,212-2 treated group

Figure 4.4 Effect of WIN-55,212-2 challenge on thermal analgesia following 7-day morphine or 5-day WIN-55,212-2 treatment

Assessment of the thermal analgesic effect of WIN-55,212-2 (3mg/kg body weight) determined using the hot plate test. Time to withdrawal was determined manually by visual inspection of the animal movements. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. Analgesia was measured at baseline (0 min), 15, 30, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●)Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. A *p value $\leq$ 0.05 considered to be statistically significant. The N value for each experimental set is 4.

A


WIN Challenge (3mg/kg)
Time to withdrawal (sec)
40
30
20
10
0
0
30
60
90
120
150
Time (min)
*
Vehicle group
WIN-55,212-2 treated group

B

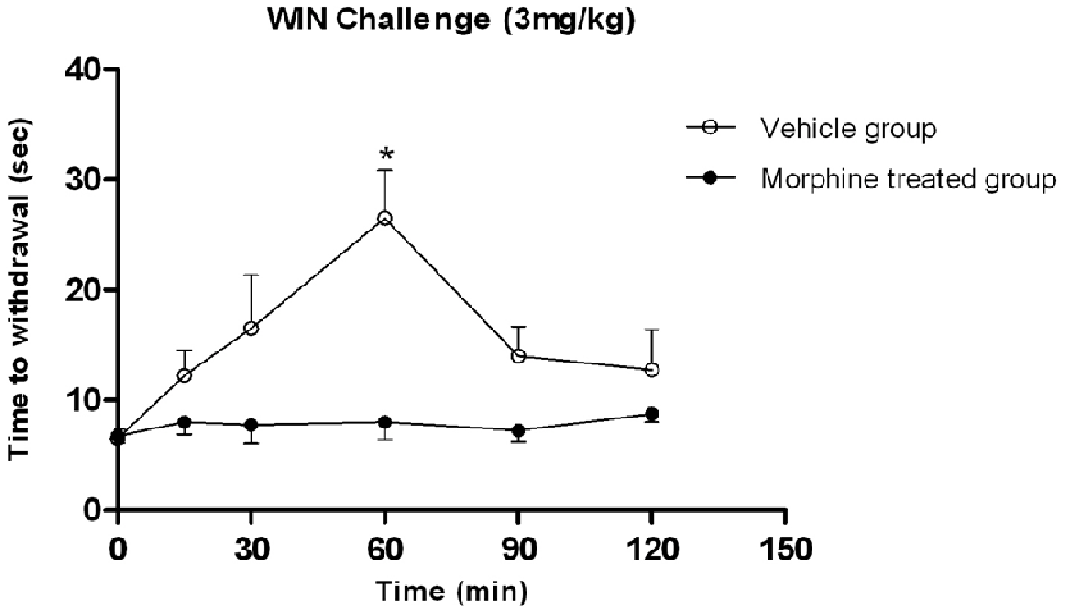
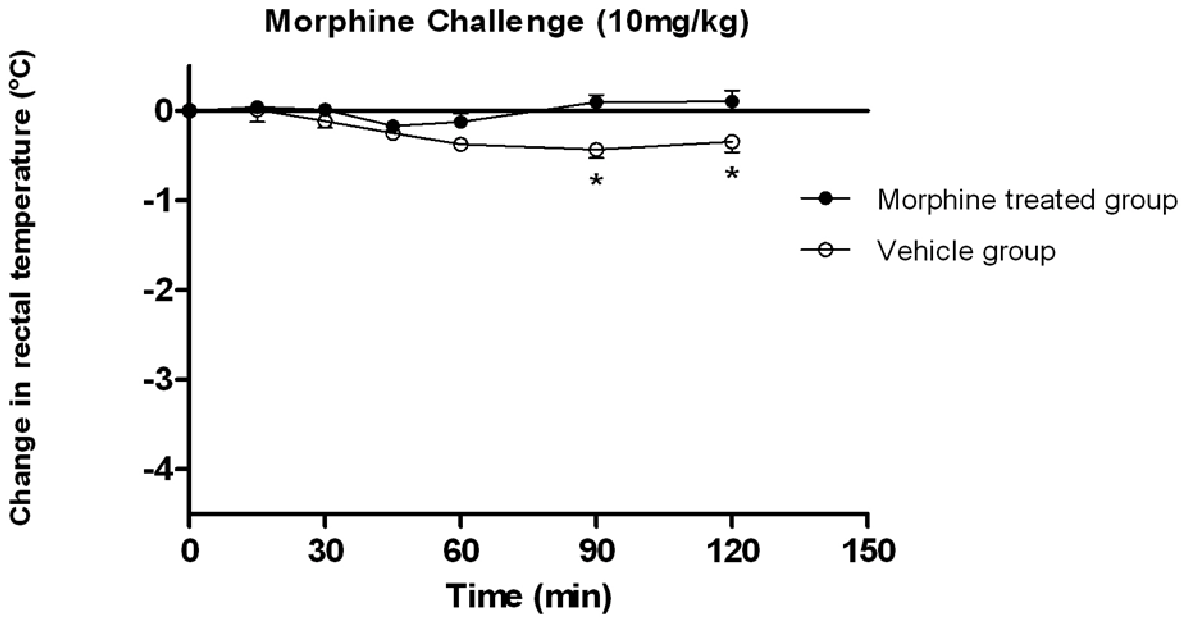

WIN Challenge (3mg/kg)
Time to withdrawal (sec)
40
30
20
10
0
0
30
60
90
120
150
Time (min)
*
Vehicle group
Morphine treated group

Figure 4.5 Effect of morphine challenge on rectal body temperature following 7-day morphine or 5-day WIN-55,212-2 treatment

Assessment of the hypothermic effect of morphine (10mg/kg body weight) determined by rectal body temperature measurement. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. The change in core temperature was assessed at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set is 4.

A


Morphine Challenge (10mg/kg)
Change in rectal temperature (°C)
0
-1
-2
-3
-4
0
30
60
90
120
150
Time (min)
*
*
Morphine treated group
Vehicle group

B

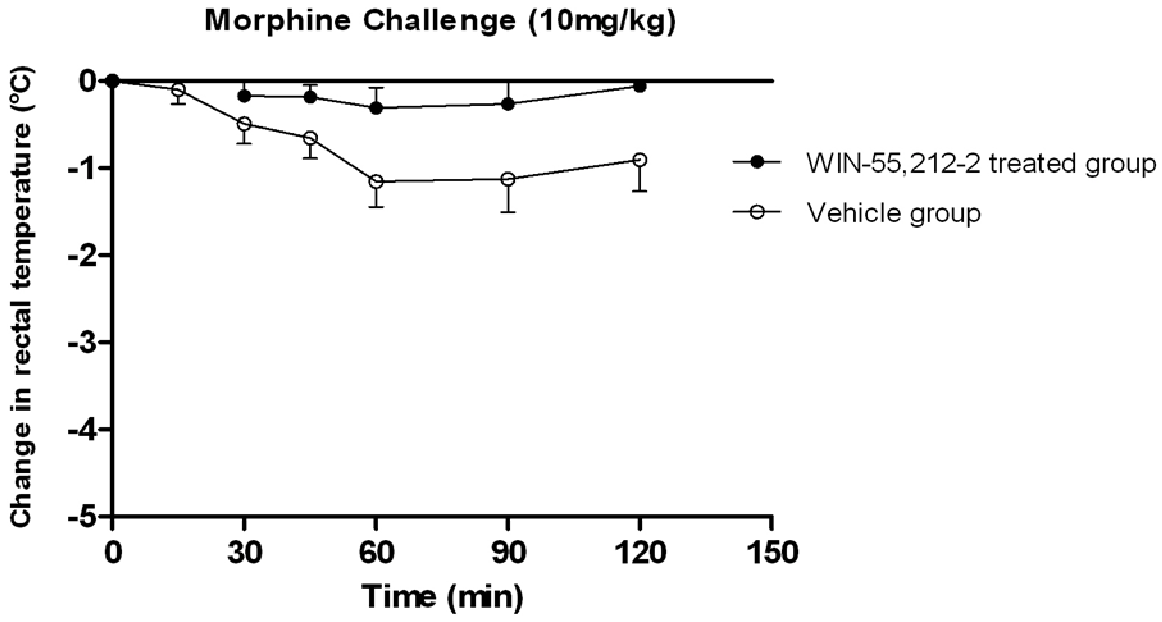
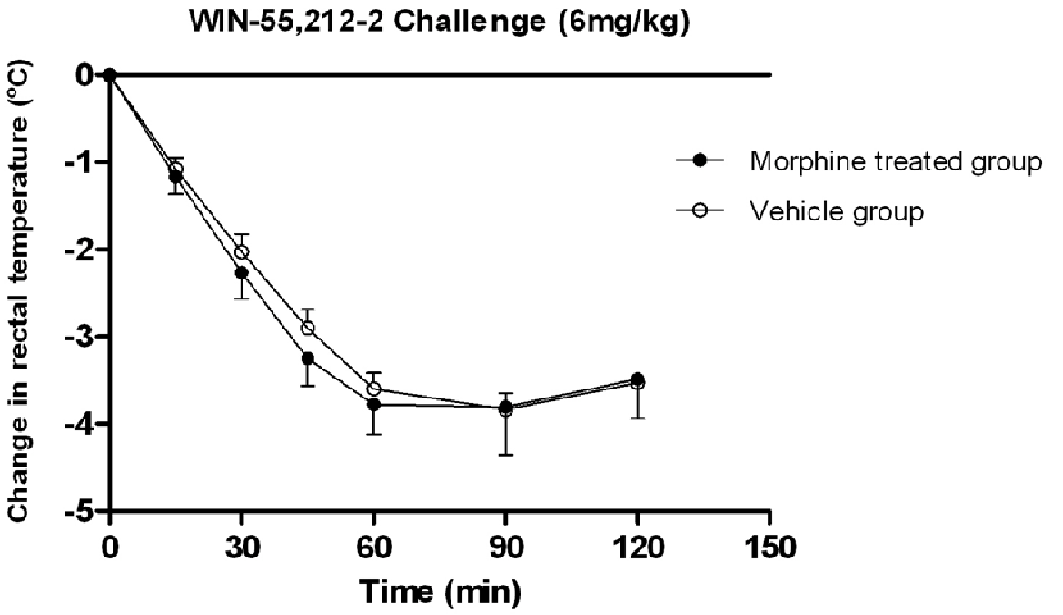

Morphine Challenge (10mg/kg)
Change in rectal temperature (°C)
0
-1
-2
-3
-4
-5
0
30
60
90
120
150
Time (min)
WIN-55,212-2 treated group
Vehicle group

Figure 4.6 Effect of WIN-55,212-2 challenge on rectal body temperature following 7-day morphine or 5-day WIN-55,212-2 treatment

Assessment of the hypothermic effect of WIN-55,212-2 (6mg/kg body weight) determined by rectal body temperature measurement. **A**: control vs. morphine (7 days) **B**: control vs. WIN-55,212-2 (5 days) treated animals. Hypothermia was assessed at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine or WIN-55,212-5 treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set is 4.

A


WIN-55,212-2 Challenge (6mg/kg)
Change in rectal temperature (°C)
0
-1
-2
-3
-4
-5
0
30
60
90
120
150
Time (min)
Morphine treated group
Vehicle group

B

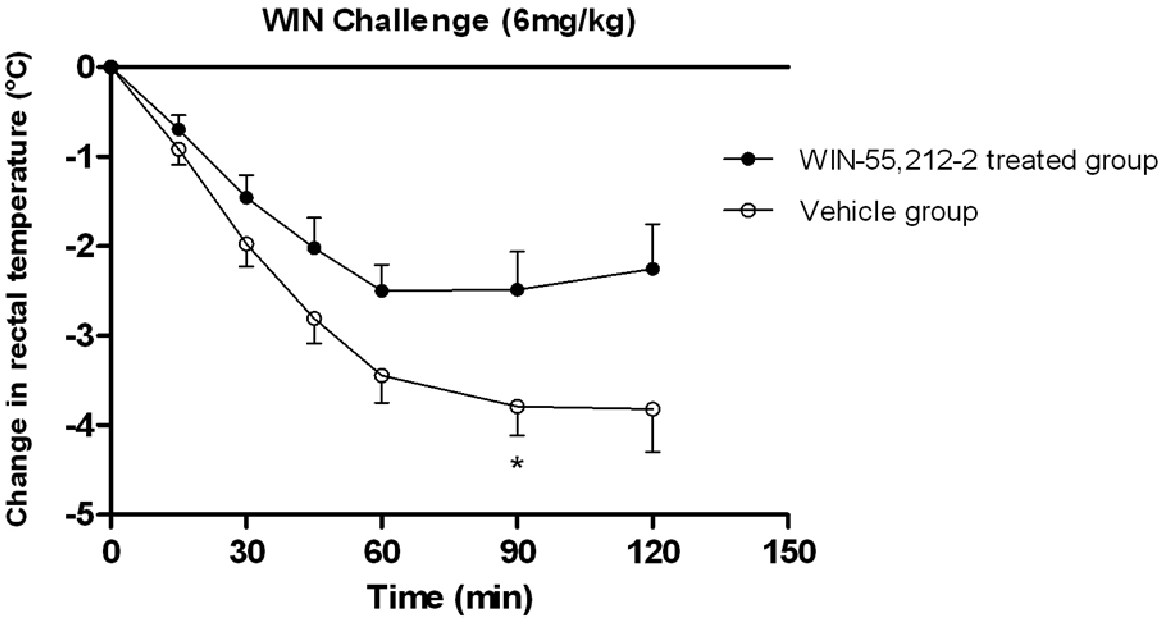
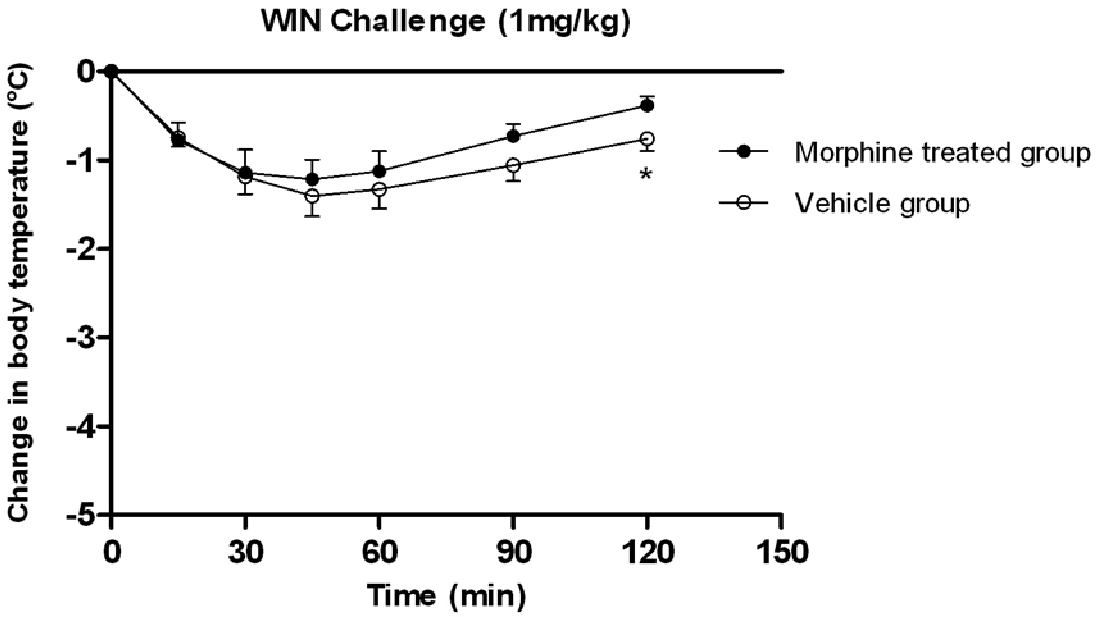

WIN Challenge (6mg/kg)
Change in rectal temperature (°C)
0
-1
-2
-3
-4
-5
0
30
60
90
120
150
Time (min)
*
WIN-55,212-2 treated group
Vehicle group

Figure 4.7 Effect of 7-day morphine treatment on the hypothermic effects of different doses of WIN-55,212-2 challenge.

Assessment of the hypothermic effect of WIN-55,212-2 (1mg/kg [**A**] or 3mg/kg [**B**] .body weight) determined by rectal body temperature measurement. Hypothermia was assessed at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M. (●) Test (morphine treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set ranges from 7 - 8.

A


WIN Challenge (1mg/kg)
Change in body temperature (°C)
0
-1
-2
-3
-4
-5
0
30
60
90
120
150
Time (min)
*
Morphine treated group
Vehicle group

B

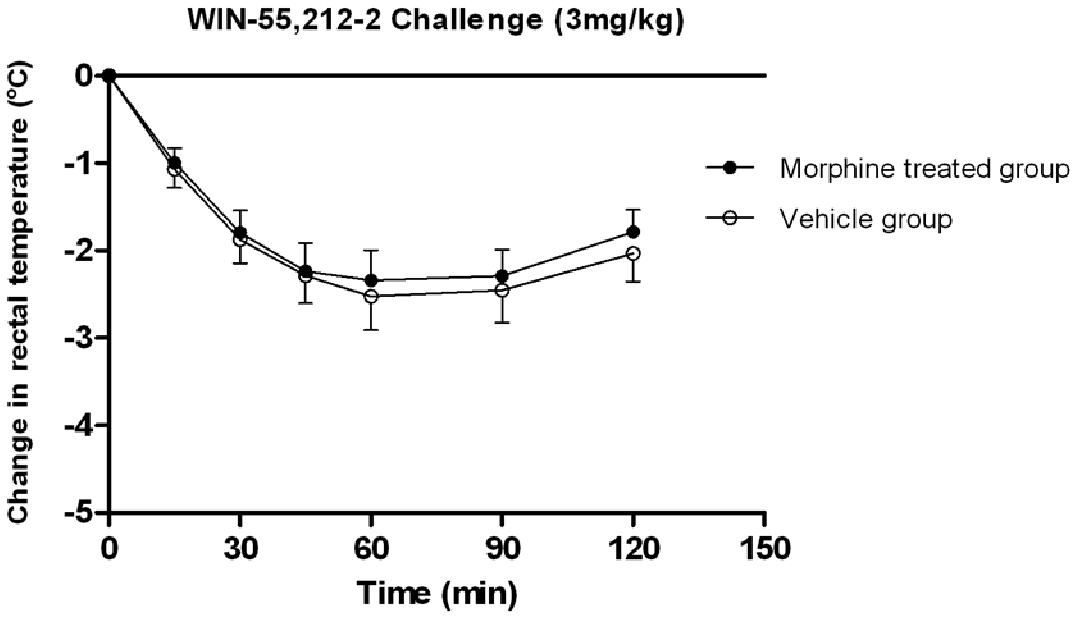
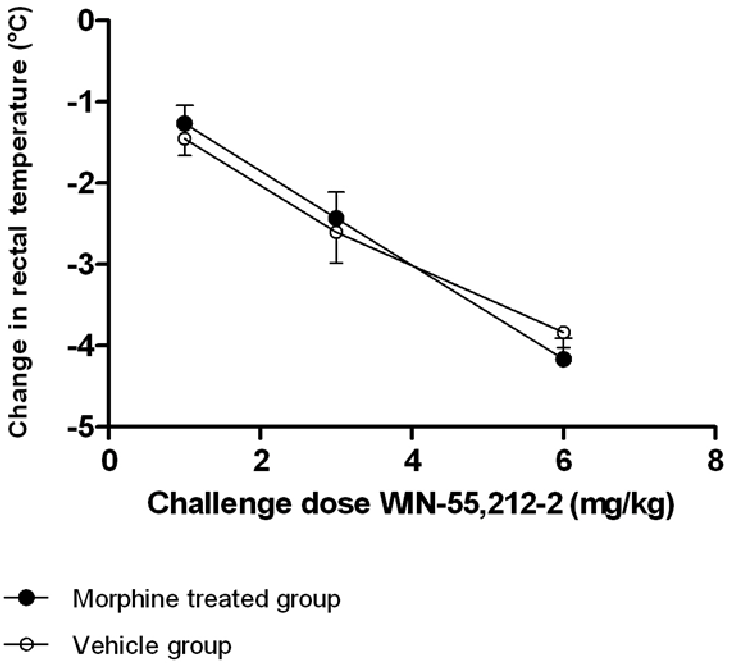

WIN-55,212-2 Challenge (3mg/kg)
Change in rectal temperature (°C)
0
-1
-2
-3
-4
-5
0
30
60
90
120
150
Time (min)
Morphine treated group
Vehicle group

Figure 4.8 Dose response curve of the maximal WIN-55,212-2 hypothermic effect following 7-day morphine treatment.

Comparison of the maximal hypothermic effect of WIN-55,212-2 challenge (1mg/kg, 3mg/kg and 6mg/kg) following 7-day morphine or vehicle treatment. Hypothermia was assessed at baseline (0 min), 15, 30, 45, 60, 90 and 120 min after challenge dose injection. Each point represents the mean ± S.E.M of measurements from at least 4 guinea pigs. (●) Test (morphine treated) group, (○) control (vehicle treated) group. *$p \leq 0.05$ considered to be statistically significant. The N value for each experimental set ranges form 4 - 8.


Change in rectal temperature (°C)
0
-1
-2
-3
-4
-5
0
2
4
6
8
Challenge dose WIN-55,212-2 (mg/kg)
Morphine treated group
Vehicle group

CHAPTER FIVE: ASSESSMENT OF MOR AND CB_1 RECEPTOR DISTRIBUTION IN THE LM/MP AND HYPOTHALAMUS FOLLOWING CHRONIC *IN VIVO* EXPOSURE TO OPIOID OR CANNABINOID RECEPTOR AGONISTS.

I. Abstract

Cellular co-localization or co-expression of receptors is considered to be one of the pre-requisites for intracellular interaction of signaling systems. Few studies to date have assessed the relative distribution and/or possible co-localization of CB_1 cannabinoid receptor and mu-opioid receptor (MOR) in either the LM/MP or hypothalamus and whether or not this characteristic could be related with the qualitative nature of the tolerance to the hypothermic or gastrointestinal inhibitory effects that are observed following chronic opioid or cannabinoid exposure. The candidate previously reported that chronic administration of morphine resulted in the development heterologous tolerance in the LM/MP model while chronic WIN-55,212-2 treatment not only evoked homologous tolerance associated with a 32% reduction in CB_1 receptor protein. Based on these data and the closely related gastrointestinal inhibitory effects observed with both opioids and cannabinoids, the candidate hypothesized that there is substantial co-localization of MOR and CB_1 receptors in the LM/MP. Furthermore since the hypothalamus is a common locus for the regulation of body temperature through region specific MOR and CB_1 receptor activation, also it was predicted that there would be extensive co-expression of these receptors in the hypothalamus. The present study used multiple-labeling immunofluorescence to examine the expression of MOR and CB_1 receptors in the LM/MP as well as the preoptic hypothalamic area, a key region responsible for regulation of body temperature. Results from the

study revealed a comparable distribution of MOR and CB_1 receptors in both myenteric and preoptic anterior hypothalamic neurons. In addition, the results demonstrated extensive co-localization of the receptors in both tissues. Up to 50% of the neurons expressing MOR or CB_1 receptor protein in the LM/MP co-expressed both receptors whereas up to 89% of hypothalamic neurons co-expressed MOR and CB_1 receptors. The receptor co-localization suggests that the two receptor systems share a number of sites for convergence of action that could serve as the cellular basis of tolerance which include receptor-dependent and/or receptor-independent but signaling pathway-dependent components.

II. Introduction

There is a growing body of evidence pointing toward convergence and commonality of activity between the MOR and the CB_1 receptor systems that may alter the cellular signaling pathways involved in producing many of the effects including the development of tolerance. There is also evidence for a similar neuroanatomical distribution of cannabinoid and opioid receptors within the ileum, vas deferens, caudate-putamen, dorsal hippocampus, substantia nigra and hypothalamus (Rosow et al., 1980; Ross et al., 1998; Rawls et al., 2002) that correlates with common pharmacological effects, namely hypothermia, hypomotility, catalepsy and decreased gastrointestinal motility. Furthermore, some studies have reported co-localization of these receptors in the superficial dorsal horn of the spinal cord (Hohmann et al., 1999; Salio et al., 2001) and caudate putamen (Rodriguez et al., 2001).

Our previous studies using the LM/MP model (CHAPTER THREE) revealed the development of heterologous tolerance following chronic opioid exposure that contrasted distinctly with the receptor-specific alteration in responsiveness and reduction in CB_1 receptor

protein levels following chronic cannabinoid exposure. Furthermore, previous studies assessing the development of tolerance to the hypothermic effects of opioids and cannabinoids (CHAPTER FOUR) seem to point to a possible interaction between the two systems since chronic WIN-55,212-2 treatment appears to induce the development of heterologous tolerance to the hypothermic effect of morphine, though chronic morphine treatment does not seem to affect the hypothermic potency of WIN-55,212-2. Based on these data and the reported correlation of neuroanatomical distribution and physiological effects between CB_1 and opioid receptors in the LM/MP and hypothalamus, it was hypothesized that there is significant co-localization of both receptor populations in LM/MP neurons that provides a common site of cellular interaction between the opioid and cannabinoid receptor system (Rawls et al., 2002). Cellular co-localization of cannabinoid and opioid receptors in the central nervous system and peripheral neurons (Salio et al., 2001) is suggested to contribute to the closely related physiological and clinical effects (Manzanares et al., 1999) including analgesia, hypothermia, gastrointestinal inhibitory activity and the development of dependence and tolerance (Lichtman and Martin, 2005). Both cannabinoid and opioid receptor agonists reduce intestinal peristaltic activity via cannabinoid (CB_1) and opioid (kappa- and mu-) receptor activation, respectively. Studies have shown that activation of these receptors results in the inhibition of release of acetylcholine from myenteric 'S' neurons that are the motor neurons to the longitudinal muscle resulting in decreased peristaltic activity (Coutts and Pertwee, 1997). MOR and CB_1 receptors are located on the soma and reduce transmitter release by hyperpolarization by opening GIRK channels while kappa opioid receptors are "presynaptic" on the axon terminals and decrease acetylcholine release by inhibiting calcium influx necessary for transmitter release (Kojima et al., 1994; Coutts and Pertwee, 1997). Approximately 15% of the myenteric 'S' motor neurons (Dogiel type I) are

reported to be cholinergic neurons and over 98% of these cholinergic neurons in the guinea pig have been reported to express CB_1 receptors (Coutts et al., 2002). In contrast, only 43% (mostly Dogiel type 1) of the cholinergic neurons express MOR (Ho et al., 2003). It is therefore reasonable to assume that co-localization of MOR and CB_1 receptors within the same neurons is likely to exist though the extent of co-localization is unknown.

The hypothermic effect of these agents is, at least in part, mediated via the hypothalamus (preoptic anterior hypothalamic area). Previous studies using mice and rats have shown the expression of MOR and CB_1 receptors within the anterior hypothalamic area (Rosow et al., 1980; Rawls et al., 2002; Zheng et al., 2005). Furthermore, direct microinjection of the CB_1 receptor agonist WIN-55,212-2 into the preoptic hypothalamic area of the rat has been associated with acute hypothermia that can be abolished by the cannabinoid receptor antagonist rimonabant (Rawls et al., 2002). Stimulation of opioid receptors has been shown to result in receptor-dependent regulation of body temperature; MOR selective agonists appear to induce hyperthermia whereas KOR agonists produce hypothermia (Adler and Geller, 1987; Spencer et al., 1988).

In the current study the candidate assess MOR and CB_1 receptor distribution in the LM/MP and the preoptic anterior hypothalamic area. The advantages of the guinea pig model include the fact that it exhibits closer anatomical, physiological, neurological and developmental similarities to the human which makes it an appropriate model for studying gastro-intestinal, respiratory and opioid tolerance (Chavkin and Goldstein, 1984; Hunter et al., 1997; Gray et al., 2001). In addition, the guinea pig does not exhibit an excitatory action to opioids and the distribution of opioid receptors in regions of the CNS more closely resembles that of human than

that observed in the rat (Mansour et al., 1988; Bot et al., 1992). However, one major drawback to using the guinea pig as a model for CNS studies is the lack of comprehensive information outlining specific anatomical co-ordinates for different regions of the brain. Only a handful of atlases have delineated specific regions of the guinea pig brain; most are too general and predate the current standards of comprehensive outlining of specific brain regions (Rapisarda and Bacchelli, 1977). The absence of specific stereotaxic co-ordinates has hampered its favorability as a model for assessing CNS effects; however, some studies have mapped co-ordinates for specific areas of the brain including the hypothalamus, brainstem, forebrain and midbrain (Luparello et al., 1964; Tindal, 1965; Voitenko and Marlinsky, 1993). Another drawback is that it is difficult to infer neuronal CNS projections in the guinea pig from those described for other species since inter-species variability between neurons of the same type has been observed (Livneh and Mizrahi, 2010).

In light of our previous studies that show a 32% reduction in CB_1 receptor protein following WIN-55,212-2 treatment (CHAPTER THREE), it was important to characterize the possible changes in distribution and localization of MOR and CB_1 receptor expression and investigate the possible presence and extent of co-localization of the receptor populations. This information will be critical in delineating the possible interactions between the two receptor systems in the regulation of body temperature and gastrointestinal motility. Based on our previous assessments in which WIN-55,212-2 pretreatment appears to induce tolerance to the hypothermic effect of morphine (CHAPTER FOUR) and the studies which have shown expression of MOR and CB_1 receptors in the hypothalamus (Gulledge et al., 2000; Zheng et al., 2005) and the fact that Δ^9-THC administration elevates pre-opiomelanocortin (POMC) levels in

the hypothalamus, (Corchero et al., 1997; Manzanares et al., 1998), it was also hypothesize extensive co-localization of these receptor populations will be evident in this region. The experimental plan was based upon the following specific aims: *1) To identify the distribution of MOR and CB_1 receptors in the LM/MP and in the pre-optic anterior hypothalamus (POAH) and evaluate the presence and extent of co-localization of these receptors in neurons in these regions. 2) To determine whether chronic drug exposure would modify the abundance and/or distribution of MOR and CB_1 receptor positive neurons in the terminal LM/MP and hypothalamus, and to assess whether the extent of receptor co-localization was altered.*

The unavailability of reliable stereotaxic coordinates made it imperative for us to devise a method for determining the location of the preoptic anterior hypothalamus. The current study employed antibodies targeting gonadotropin releasing hormone (GnRH), a hormone primarily expressed in the GnRH-secreting neurons mostly found in the preoptic anterior hypothalamus (POAH), to identify this region in coronal sections of the guinea pig brain. A previously published article outlining the stereotaxic co-ordinates of the hypothalamus was used as a basis to confirm and locate the preoptic anterior hypothalamus (POAH) (Luparello et al., 1964). The current study demonstrates the presence of robust co-localization of MOR and CB_1 receptors in both the LM/MP and POAH thereby providing further correlative evidence for the possible functional interaction between the MOR and CB_1 receptor systems that may relate to the mechanism by which tolerance develops.

III. Experimental Protocol

1. LM/MP Preparation

The LM/MP whole mount preparation was prepared as outlined earlier (CHAPTER 2) by obtaining segments of ileum that were flushed free of intestinal contents and opened along it mesenteric border, stretched and pinned in a chamber made of plexiglass containing Sylgard® to which the segments were pinned for dissection. The whole mounts were then fixed in 4% paraformaldehyde overnight prior to immunofluorescence probing with primary and secondary antibodies. Preliminary tests were performed to optimize the concentration of primary and secondary antibodies to be employed (APPENDIX D). The CB_1 receptor was localized using a rabbit primary polyclonal antibody directed against the C-terminus (1:50 – Cayman, Ann Arbor, MI) and the secondary antibody used was a donkey anti-rabbit FITC-conjugate (1:100 – Jackson Immunoresearch, West Grove, PA). MOR was detected using a goat primary polyclonal antibody directed against the C-terminus (1:50 – Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody used was a donkey anti-goat Cy5-conjugate (1:100 - Jackson Immunoresearch, West Grove, PA). LM/MP sections were incubated in 0.1% Triton X-100 in PBS for 25 min followed by three 10 min washes with PBS. Prior to primary antibody incubation, the LM/MP tissues were blocked with donkey serum (10%) for 45 min and rinsed using PBS. Thereafter, the tissues were incubated overnight in a cocktail of anti-CB_1 and anti-MOR primary antibodies in 2% BSA in PBS at 4°C. Following the overnight incubation, the tissues were washed with PBS and then incubated for 4 h in a mixture containing both Cy5- and FITC-conjugated secondary antibodies targeting MOR and CB_1 receptors, respectively. Negative control experiments excluded the primary antibodies and these revealed negligible faint labeling due to non-specific binding of the secondary antibodies (Fig. 2.2). Following incubation with the

respective secondary antibodies, the LM/MP tissues were mounted on slides, allowed to dry in total darkness, then cover slipped and retained until viewing.

2. Preparation of Brain Sections

Animals were anesthetized and transcardially perfused as outlined in CHAPTER TWO. In brief, animals were first anesthetized using Ketamine (85 mg/ml)/Xyalazine (15 mg/ml) administered via intraperitoneal injection (0.10 ml/100 g body weight) followed by opening of the chest cavity to expose the heart, Initial transcardial perfusion was performed using PBS until perfusate was clear and thereafter 4% paraformaldehyde was perfused until the animal extremities became stiff. Brains were then removed, postfixed in 4% paraformaldehyde for 24 h and later immersed in PBS solution containing 25% sucrose prior to sectioning. The preparation of brain slices and incubation of antibodies was performed as outlined in CHAPTER TWO. Sectioning of the brain was executed using the Leica VT1000S vibratome brain slicer (Leica Microsystems, Bannockburn, IL) with the brain fully immersed in cold PBS. The resulting sections of approximately 40 μm thickness were immersed and stored in cold PBS containing 0.1% sodium azide at 4^{o}C prior to immunofluorescence probing. Initial studies involved attempting to outline and visualize the anatomical landmark structures in 13 coronal sections of the cerebrum (Fig. 5.4). This was accomplished by tagging CB_1 receptor and MOR proteins with near-infrared secondary antibodies as outlined in CHAPTER 2. Near infra-red imaging of these slices was performed using the Odyssey imaging system (Li-Cor Biosciences. Lincoln, NE) in an effort to define the regions of greatest receptor density.

The first step in quantitative immunofluorescence imaging involved identifying the brain sections expressing GnRH, the preoptic anterior hypothalamic area marker. For these initial

studies, sections (7, 8, 9, 10, 11 and 12) were used since these were anatomically relevant sections where the hypothalamus is located based on the gross structure of the cerebrum. For subsequent experiments, only the brain slices that displayed GnRH immunofluorescence were used for triple labeling studies targeting the GnRH, CB_1 receptor and MOR proteins. The concentrations of primary and secondary antibodies used for CB_1 and MOR are identical to those used for the LM/MP (see APPENDIX F). In addition, GnRH was used as a marker protein for the preoptic anterior hypothalamic neurons where the hormone is exclusively expressed. GnRH was localized using a mouse primary polyclonal antibody directed against the C-terminus (1:500 Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody used was a donkey anti-mouse Cy3-conjugate (1:500 – Jackson Immunoresearch, West Grove, PA).

3. Confocal Imaging

A Zeiss® LSM 510 laser scanning confocal imaging system and microscope were used for image acquisition and processing of immunopositive neurons tagged by the FITC- (CB_1), Cy5- (MOR) and Cy3-conjugated secondary antibodies. In LM/MP dual-labeling experiments, a composite image targeting the FITC- and Cy5-conjugates was scanned simultaneously that could be merged or separated and analyzed offline. For triple labeling experiments (using FITC-, Cy3- and Cy5-conjugates), an initial scan was performed targeting the Cy3-GnRH fluorophore so as to identify the preoptic anterior hypothalamic area. Thereafter, a composite image targeting the FITC- and Cy5-conjugates was scanned simultaneously. All images from slides were captured using identical parameters for laser intensity, detector gain, pin hole size and amplifier offset. Co-localization in the brain tissues was only examined in brain areas where GnRH-positive neurons were detected.

4. Assessment of MOR and CB_1-Immunopositive neurons

A qualitative analysis of the scanned images was used to determine the distribution pattern and the extent of co-localization of MOR and CB_1 receptor-immunopositive neurons. Cells were considered to be immunopositive if they expressed visually detectable fluorescent labeling. Immunopositive neurons with bright to faint labeling were analyzed, because the faint labeling may represent low protein expression in positively labeled cells. The Zeiss® LSM software was used to analyze the distribution and co-localization of MOR and CB_1 receptor-immunopositive neurons and Image J® software was used to quantify the density of the neurons per defined unit area. The relative expression of co-localized was expressed as a percentage of the total population of neurons possessing both receptor proteins versus those neurons expressing either MOR or CB_1 receptor proteins only.

The density of immunopositive neurons was assessed by counting the number of immunopositive neurons in a manually circumscribed region of the myenteric ganglia (i.e. number of neurons/area of circumscribed region). The area of the circumscribed region was computed using Image J® software whereas the number of visually detectable immunopositive neurons was counted manually. Neurons expressing both receptor proteins were counted individually and converted to a density based upon the circumscribed area evaluated. The relative expression of co-localized was expressed as a percentage of the total population of neurons possessing both receptor proteins versus those neurons expressing either MOR or CB_1 receptor proteins only.

IV. Results

1. Assessment of MOR and CB_1 Receptor Protein Expressing Neurons in the LM/MP

Representative images from whole mounts of the guinea pig LM/MP showing CB_1 receptor and MOR-immunopositive neurons in the myenteric plexus are provided in Fig 5.1. The red immunofluorescence (Fig 5.1A) indicates MOR-expressing neurons while the green immunofluorescence (Fig 5.1B) depicts CB_1 receptor-expressing neurons. Neurons expressing both receptor populations appear orange-yellow (Fig 5.1C) in color. There are a number of important characteristics observed from these tissues. As indicated in Fig 5.1A and Fig 5.1B which depict MOR and CB_1 receptor-immunopositive-neurons, respectively, it appears that a significant proportion of myenteric neurons actually express both receptor populations simultaneously. It is also interesting to note that neurons exclusively expressing MOR protein seem to reside predominately in the periphery of the myenteric ganglion whereas neurons that exclusively express CB_1 receptors appear to congregate in the central part of the ganglion; this distribution pattern was consistent in drug-naïve, vehicle-treated, WIN-55,212-2- or morphine-treated animals. Finally, there appears to be some degree of co-localization as indicated by the high intensity of the orange-yellow color in Fig 5.1C. The co-localization observed (Fig 5.1C) between the MOR and CB_1 receptor positive neurons further suggests that the two receptors are co-expressed in some but not all myenteric neurons. The results in Fig 5.1D show a similar relative density of neurons expressing MOR or CB_1 receptors in the LM/MP as assessed using Image J® software (16.7 vs. 14.3 neurons/μm^2, respectively; $p \geq 0.05$). The density of neurons co-expressing both CB_1 and MOR receptors in drug-naïve animals was 2.9 neurons/μm^2 which translates to 17% and 20% of the total MOR or CB_1 receptor populations, respectively.

Comparison of vehicle or WIN-55,212-2 treated groups (Fig 5.2A) shows no statistically significant difference in the density of MOR-immunopositive ($p > 0.05$), CB_1-immunopositive neurons ($p > 0.05$) or neurons co-expressing both receptors. Similarly, chronic morphine treatment did not alter the relative density of MOR, CB_1-immunopositive neurons or neurons co-expressing both receptors. In tissues obtained from animals chronically treated with morphine (Fig 5.2A) or WIN-55,212-2 (Fig 5.2B) or respective drug-free vehicle solutions, the relative density of neurons expressing either CB_1 receptors ($F(4, 3) = 1.05$, $p = 0.45$) or MOR ($F(4, 3) = 1.43$, $p = 0.33$) was similar in magnitude to that observed in naïve animals (Fig 5.1D). Interestingly, in the preparations from vehicle and drug-treated animals, the relative percentage of neurons co-expressing both families of receptors appears to slightly increase from the 20% level seen in naïve animals to around 50% in the preparations from treated animals. Negative controls showed faintly labeled neurons, possibly due to non-specific binding, but the signal was not nearly as intense as that observed in the non-control test groups.

2. Assessment of MOR and CB_1 Receptors in Hypothalamus

Following slicing of guinea pig coronal brain slices at the outlined coronal co-ordinates (Table 5.1) the investigator incubated the slices with near-infrared antibodies targeting the MOR and CB_1 receptors. Qualitative analysis of these images show generalized distribution of the regions positive for the receptors. Anatomical outlines of major regions of the brain could be visualized including the cortex, hippocampus and ventricles; these were used as reference markers for other regions of the brain including the hypothalamus.

The first step in quantitative immunofluorescence imaging involved identifying the brain sections expressing GnRH, the preoptic anterior hypothalamic area marker. For these initial

studies, sections (7, 8, 9, 10, 11 and 12) were used since these were anatomically relevant sections where the hypothalamus is located based on the gross structure of the cerebrum. Initial assessment of the coronal brain sections 7, 8, 9 10 and 11 (Fig 5.3) of the cerebrum revealed that the expression of GnRH was only detected in coronal sections 9, 10 and 11 (Fig 5.5). The expression of GnRH was sparse in these regions with the greatest immunofluorescence observed in Coronal section 11 located -7.5 mm from bregma. Fig 5.5 shows extensive co-localization of the MOR and CB_1 receptors in the preoptic hypothalamic area in coronal section 11. Assessment of the hypothalamic area expressing GnRH revealed the presence of robust immunoreactivity or expression of MOR and CB_1 receptor protein (Fig 5.5). The area where GnRH was expressed correlates with the location of the hypothalamus that has been reported in previous atlases (Luparello et al., 1964). These data are consistent with previously reported data which reported extensive CB_1 receptor expression in the POAH (Rawls et al., 2002). Immunofluorescence images of the cortex show no expression of the GnRH; however, expression of the MOR and CB_1 receptors can be seen in this region (Fig 5.6).

As indicated in Fig 5.5B and Fig 5.5C which depict MOR and CB_1 receptor-immunopositive-neurons, respectively, there appears to be a significant degree of co-localization in the POAH as indicated by the high intensity of the orange-yellow color in Fig 5.5D. Comparison of vehicle or WIN-55,212-2 treated groups (Fig 5.7B) shows no statistically significant difference the density of MOR-immunopositive (4.9 vs. 3.9; $p > 0.05$), CB_1-immunopositive neurons (4.1 vs. 3.9; $p > 0.05$) or neurons co-expressing both receptors (2.6 vs. 2.9; $p > 0.05$). Similarly, chronic morphine treatment did not alter the relative density of MOR (3.9 vs. 3.2; $p > 0.05$), CB_1-immunopositive neurons (4.2 vs. 4.0; $p > 0.05$) or neurons co-expressing both receptors (2.5 vs. 2.9; $p > 0.05$). Up to 89% of neurons in the POAH co-

expressed MOR and CB_1 receptors suggesting that there is substantial co-localization of these receptor populations in this region.

V. Discussion

The rapid development of subsensitivity to pharmacological effects of opioid and cannabinoid agonists like analgesia, hypomotility, hypothermia and inhibition of gastrointestinal motility following the chronic use of opioids and cannabinoids is well documented (Martin et al., 2004; Bailey and Connor, 2005). There is increasing emphasis on the common anatomical distribution and shared cell signaling pathways between opioid and cannabinoid receptor systems as potential overlapping sites for producing the pharmacological effects of each. Common cellular effects include inhibition of adenylyl cyclase (Howlett and Fleming, 1984), inactivation of Ca^{2+} channels (Rhim and Miller, 1994; Howlett et al., 2002), activation of the MAPK pathway (Bouaboula et al., 1995), and activation of G protein-activated inwardly rectifying potassium (GIRK) channels (McAllister et al., 1999). Similar distribution of opioid and cannabinoid receptors in the central nervous system (Rosow et al., 1980; Ross et al., 1998; Rawls et al., 2002) and their cellular co-localization (Salio et al., 2001) may also contribute to the comparable physiological and clinical effects (Manzanares et al., 1999) including dependence and tolerance (Lichtman and Martin, 2005). Cellular co-localization of cannabinoid and opioid receptors in central neurons (e.g. the superficial dorsal horn of the spinal cord and the caudate-putamen) has also been reported (Hohmann et al., 1999; Rodriguez et al., 2001; Salio et al., 2001). In this study the candidate attempted to determine the distribution of neurons expressing MOR in relation to those expressing CB_1 receptors in the LM/MP and preoptic anterior hypothalamic area (POAH) in an effort to determine whether any distribution or alteration in receptor properties might be

associated with the development of tolerance that was observed in the previous studies (CHAPTER THREE and FOUR).

Our immunofluorescence data support the possibility of substantial co-localization of MOR and CB_1 receptors since up to 50% of CB_1 receptor-immunopositive neurons also expressed MOR in LM/MP preparations from treated animals and even greater percentages of neurons expressed both receptor populations in the POAH. However further studies would need to be performed to verify whether the co-localization reflected mere direct physical presence and interaction in the same neurons or whether the cellular co-expression represented the presence of heterodimerization between the receptor populations. The observed reduction in CB_1 receptor protein following chronic WIN-55-212-2 treatment does not seem to alter the relative distribution of CB_1-, MOR- or CB_1/MOR co-expressing neurons and is further reinforced by the similar pattern of distribution that was observed in drug-naïve, vehicle- and morphine-treated groups. The absence of changes in density of neurons expressing CB_1 receptors following WIN-55,212-2 treatment in the presence of a significant reduction in total CB_1 receptor protein observed in western blot studies could be due to a modest but uniform reduction in total CB_1 receptor protein that does not substantially eliminate the receptor from individual neurons and, therefore, would not alter the density of CB_1-expressing neurons. Further studies would be required to determine whether the mean integrated CB_1 receptor immunofluorescence intensity levels per neuron was decreased and whether it corresponds to the observed reduction in CB_1 receptor protein levels. While the fact that the expression of both receptors on the same neuron does not automatically translate into heterodimers, the observed CB_1/MOR co-localization suggests a common neuronal expression that may facilitate convergence of the two receptor signaling pathways involved in regulating acetylcholine release. Approximately 15% of the

myenteric ‘S’ motor neurons (Dogiel type I) are reported to be cholinergic neurons; over 98% of these cholinergic neurons in the guinea pig have been reported to express CB_1 receptors (Coutts et al., 2002) whereas 43% (mostly Dogiel type 1) express MOR (Ho et al., 2003). It is therefore reasonable to assume that co-localization of MOR and CB_1 receptors in the LM/MP is likely as demonstrated by these studies which show up to 50% of the MOR and CB_1 neurons co-expressing both receptors in the LM/MP. The larger relative density of co-localized neurons in vehicle and drug-treated animals compared to drug-naïve animals could represent the impact of treatment or could reflect the mere fact that different samples or different areas of evaluation of the stretch preparations were employed.

Assessment of the hypothalamic area expressing GnRH revealed the presence of robust immunoreactivity or expression of MOR and CB_1 receptor protein (Fig 5.5). The area where GnRH was expressed correlates with the location of the hypothalamus that has been reported in previous atlases (Luparello et al., 1964). These data are also consistent with previously reported data which reported extensive CB_1 receptor expression in the POAH (Rawls et al., 2002). The presence of MOR and CB_1 receptor positive neurons in the preoptic anterior hypothalamus (POAH) is consistent with effect of agents targeting these receptors in this brain region on temperature regulation (Rosow et al., 1980; Rawls et al., 2002) and is consistent with the opioid-induced analgesia and modulation of hormone release (Rang et al. 2007). The observed co-localization of MOR and CB_1 receptors points to a complex interplay between the two receptor populations in the regulation of body temperature since MOR activation has been shown to induce hyperthermia while CB_1 receptor activation results in hypothermia (Rosow et al., 1980; Adler and Geller, 1987; Rawls et al., 2002). Our results outlined in CHAPTER FOUR show that chronic WIN-55,212-2 treatment appears to induce the development of tolerance to the

hypothermic effect of morphine therefore suggests that there is an interaction between the two receptor systems in this particular pharmacological response. In contrast, morphine pretreatment does not appear to affect the potency of WIN-55,212-2 to produce hypothermia. This asymmetrical development of tolerance suggests the possibility of downstream-upstream interactions between the two receptor systems. Cannabinoid receptor agonists also interact with the hypothalamic–pituitary-adrenal axis which regulates body temperature through a number of hormonal processes (for review see Murphy 2002).

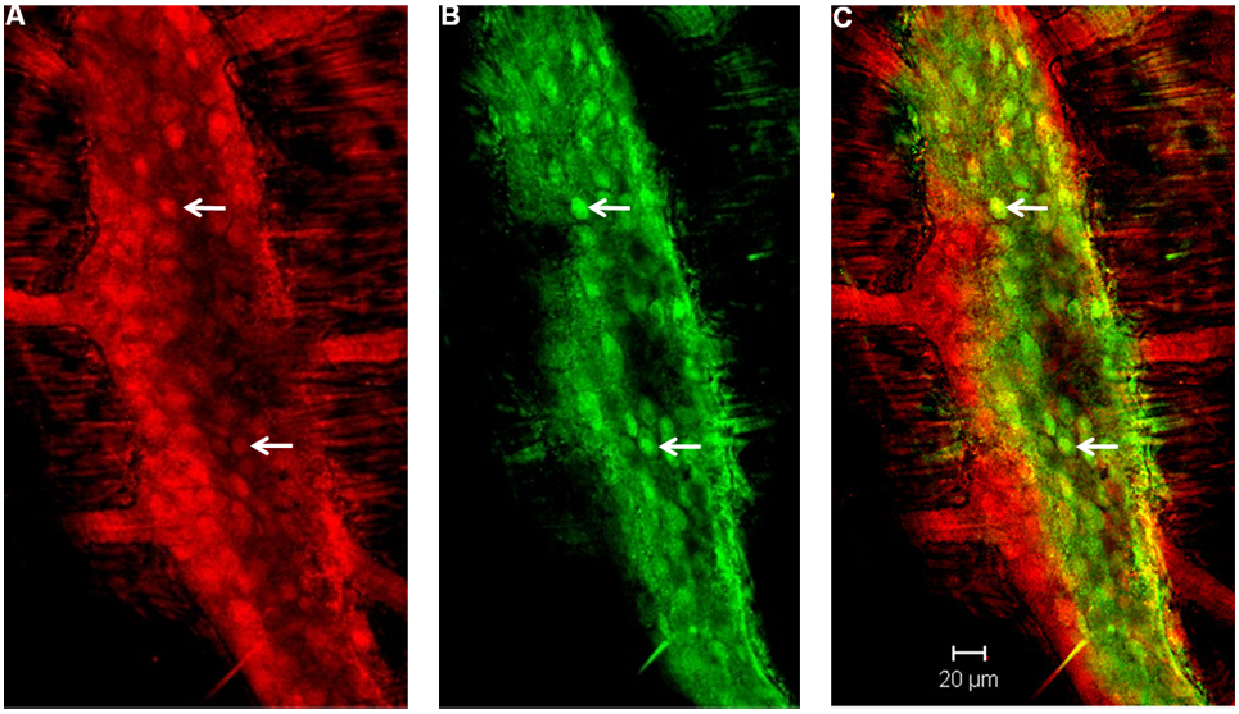
The interaction of co-localized receptors may involve heterodimerization; studies in co-transfected cells have reported heterodimerization of MOR and CB_1 receptors (Hojo et al., 2008) but no reports have been made using animal tissues. Heterodimerization of GPCRs, in general, has been shown to stimulate complex novel signaling pathways that are foreign to each of the individual receptors that can alter the potency of agonists targeting these receptors or instigate several other effects including co-internalization (Pfeiffer et al., 2003; Milligan et al., 2006). Internalization of MOR with other G protein-coupled receptors as heterodimers has also been reported to result in receptor desensitization (Pfeiffer et al., 2003). The previously reported heterodimerization of the MOR and CB_1 receptors (Hojo et al., 2008) in baby hamster kidney (BHK) cells co-expressing both receptors may provide a locus whose activation could result in internalization or uncoupling of CB_1/MOR heterodimers as an adaptive response following chronic agonist exposure which could have a significant impact on the nature of the tolerance that develops. This possibility is especially important when considering the fact that both systems have comparable physiological and pharmacological effects and the fact that their respective receptors have been identified to be co-localized in the same neurons. However, there is need to perform further studies to ascertain whether the interaction between the MOR and CB_1

receptors is actually heterodimeric. Possible experimental techniques that can be employed to evaluate this possibility include co-immunoprecipitation, fluorescence resonance energy transfer (FRET) or high resolution electron microscopy.

In summary, the presence of MOR and CB_1 receptors in the LM/MP and hypothalamus in high abundance demonstrate the importance of endocannabinoid and opioid systems in these tissues. The substantial MOR and CB_1 receptor co-localization analysis in the LM/MP and hypothalamus suggests that the two receptor systems share a number of sites including cellular localization where convergence of action could serve as the cellular basis of tolerance for both the receptor-dependent and/or receptor independent cell signaling pathway-dependent components. The fact that the two receptor populations exist separately as well as together potentially as heterodimers suggests that the expression of tolerance may reflect the additive effect of receptor-dependent and –independent components and that the relative ability of the agonist to activate specific intracellular adaptive processes may involve the ability to interact distinctly with heterodimeric receptors versus homomeric co-localized receptors.

Figure 5.1 Representative images from whole mounts of the guinea pig ileum showing immunofluorescence in neurons expressing MOR and CB_1 receptors in myenteric ganglia.

Fig. 5.1A shows MOR-immunopositive neurons (red) only whereas Fig. 5.1B illustrates CB_1 receptor-immunopositive neurons (green) only. Fig. 5.1C shows a merged image depicting neurons in the myenteric plexus that are immunopositive for both MOR and CB_1 receptors. The graph displayed in Fig. 5.1D shows a comparison of the density of MOR and CB_1 receptor-immunopositive neurons (number of immunopositive neurons per unit area) in the myenteric plexus of drug-naïve animals. The graph also illustrates the density of neurons co-expressing MOR and CB_1 receptors. Each bar represents the mean $\pm$ S.E.M of measurement of tissue sections obtained from 3 guinea pigs. $p^* \leq 0.05$ considered to be statistically significant.


A
B
C
20 µm

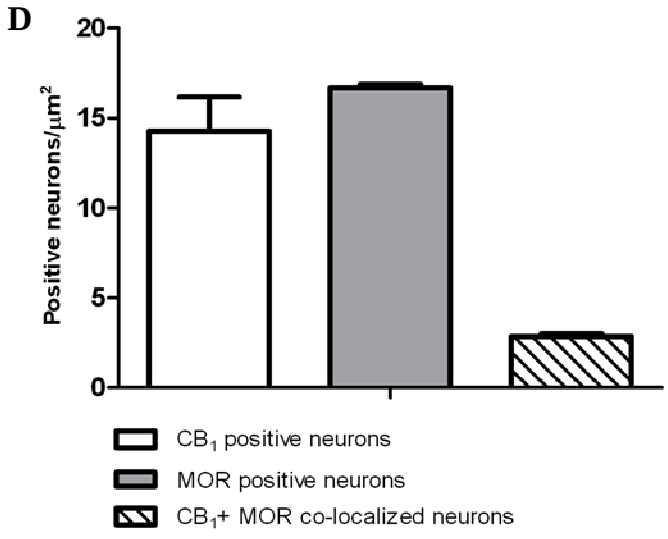
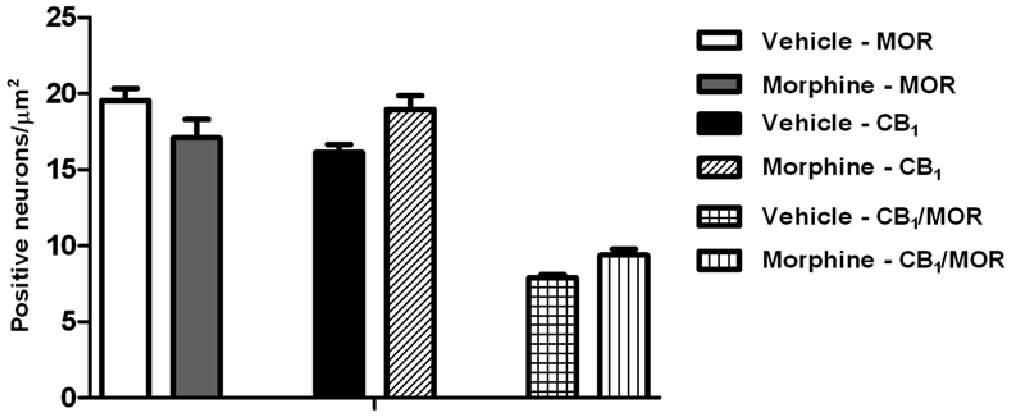

D
Positive neurons/µm²
20
15
10
5
0
CB1 positive neurons
MOR positive neurons
CB1+ MOR co-localized neurons

Figure 5.2 Comparison of the density of MOR and CB_1 receptor-immunopositive neurons in the myenteric plexus in vehicle- and drug-treated animals.

Fig 5.2A shows a comparison of the density of neurons expressing MOR or CB_1 receptors, and the density of neurons co-expressing both MOR and CB_1 receptors in the myenteric plexus samples from vehicle and morphine treated animals. Fig 5.2B compares the same parameters between tissues obtained from vehicle- and WIN-55,212-2-treated animals. Each bar represents the mean $\pm$ S.E.M of measurement in tissues obtained from 3 guinea pigs. $p^* \leq 0.05$ considered to be statistically significant.

A


Positive neurons/μm²
25
20
15
10
5
0
Vehicle - MOR
Morphine - MOR
Vehicle - CB1
Morphine - CB1
Vehicle - CB1/MOR
Morphine - CB1/MOR

B

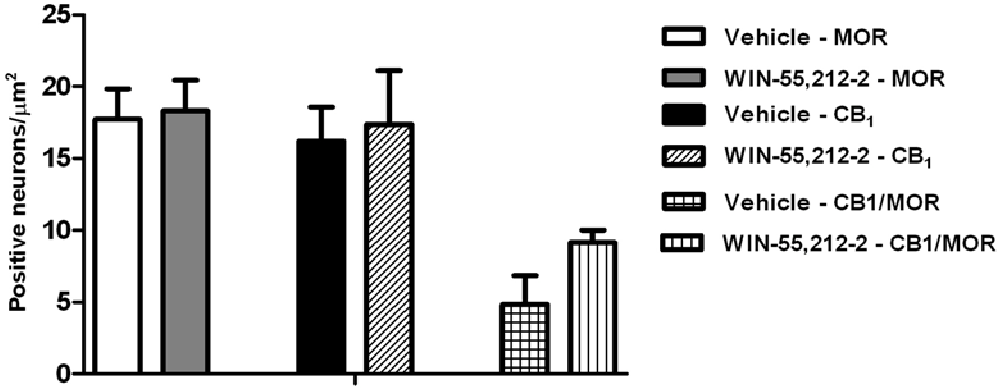

Positive neurons/μm²
25
20
15
10
5
0
Vehicle - MOR
WIN-55,212-2 - MOR
Vehicle - CB1
WIN-55,212-2 - CB1
Vehicle - CB1/MOR
WIN-55,212-2 - CB1/MOR

Figure 5.3 Picture of the guinea pig brain.

The guinea pig brain in preparation for serial coronal sectioning to identify areas of interest for evaluation of receptor localization and distribution. Sections were obtained and numbered sequentially from rostral to caudal.

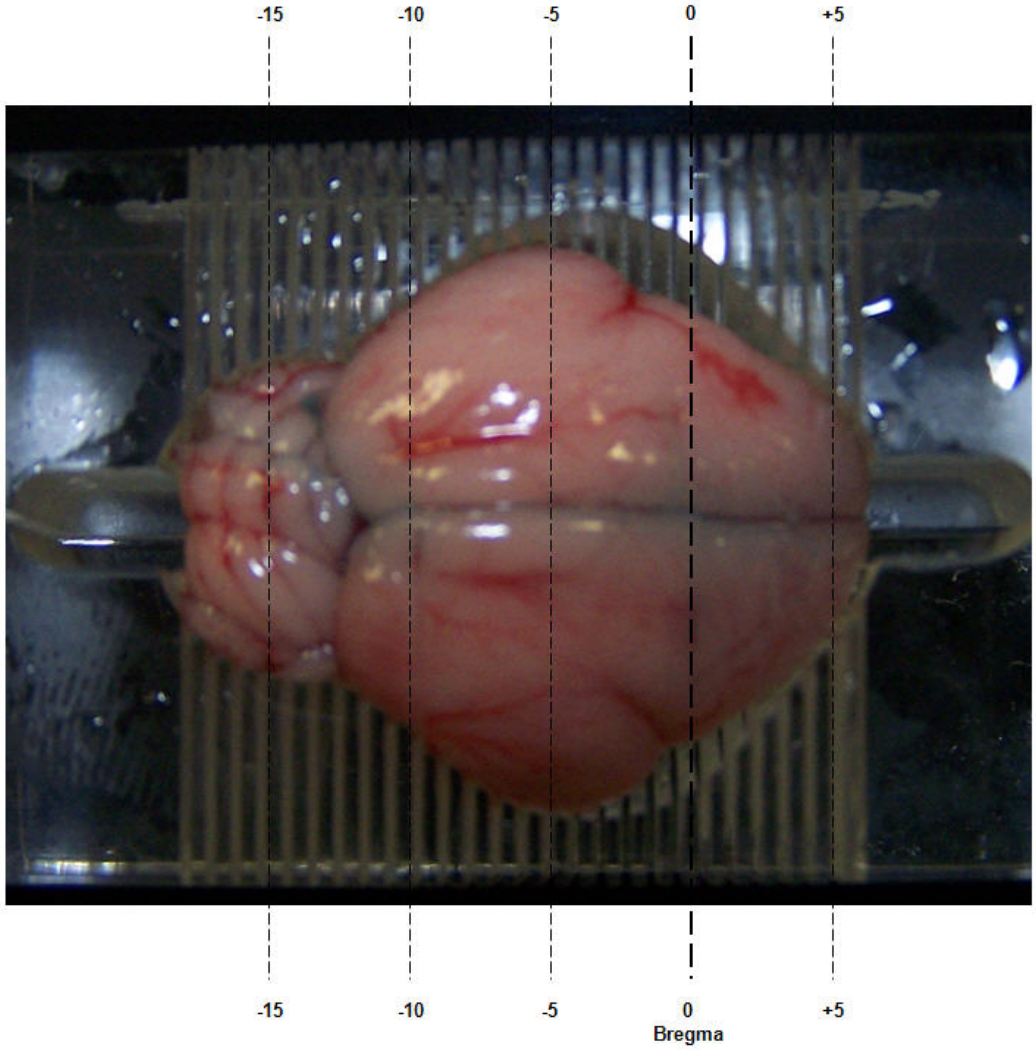
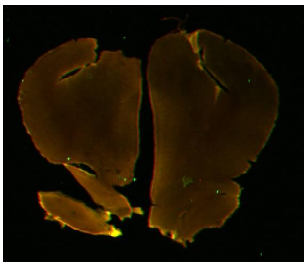

-15
-10
-5
0
+5
-15
-10
-5
0
+5
Bregma

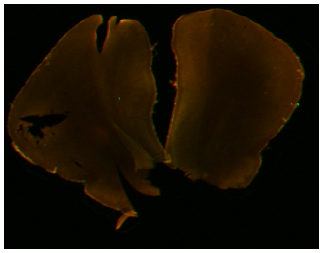
Figure 5.4 Coronal sections of the cerebrum

Representative coronal sections representing serial sections 1 - 13 of the guinea pig brain following incubation with infra-red antibodies targeting MOR and CB_1 receptors. Sections were obtained and numbered sequentially from rostral to caudal.

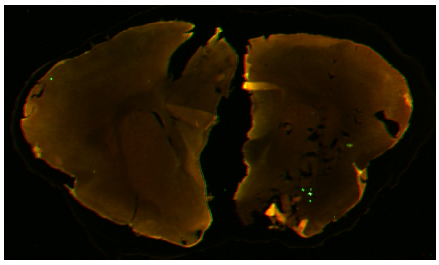
1



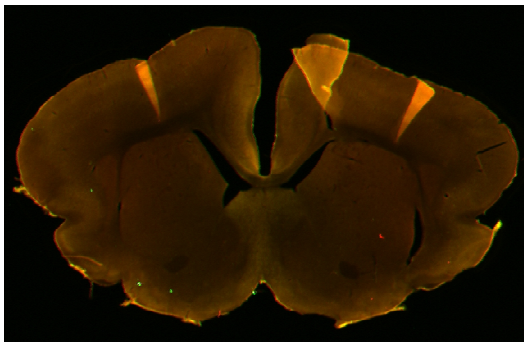
2



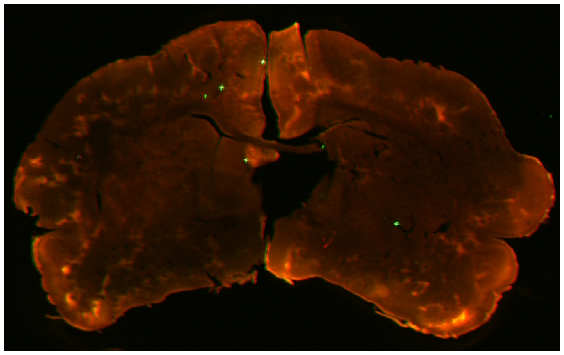
3



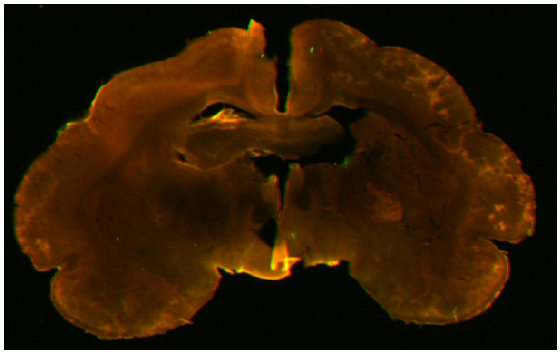
4



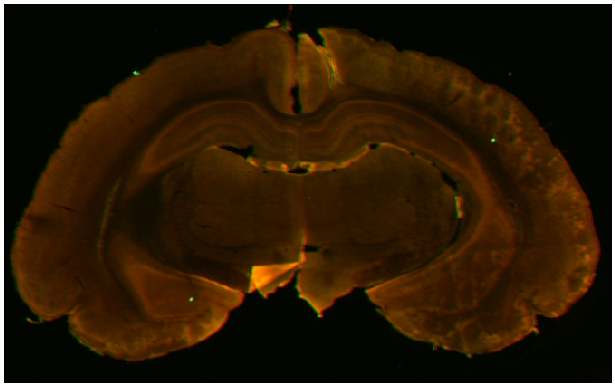
5



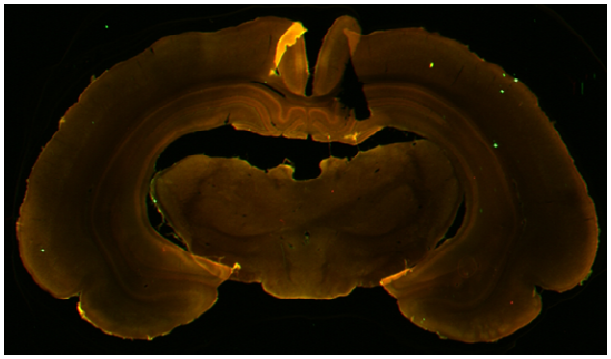
6



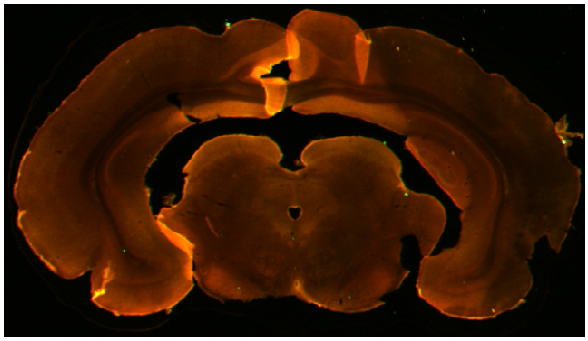
7



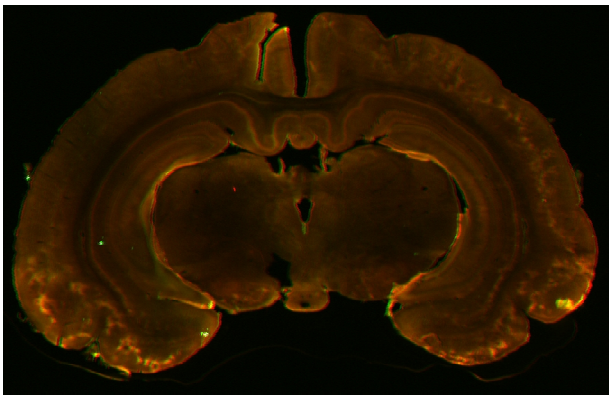
8



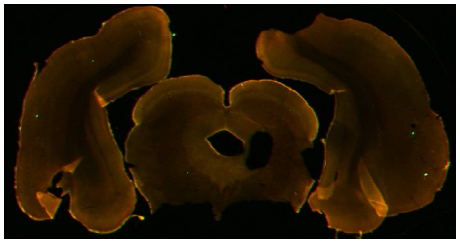
11



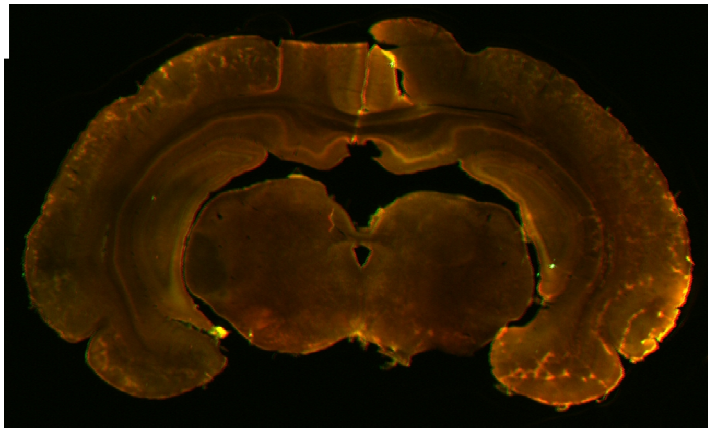
9



12



10



13

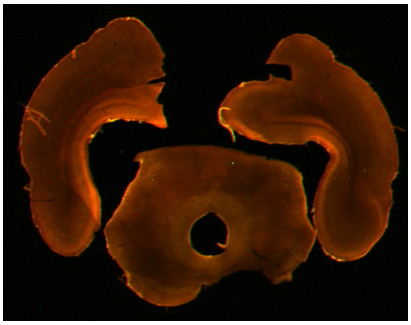


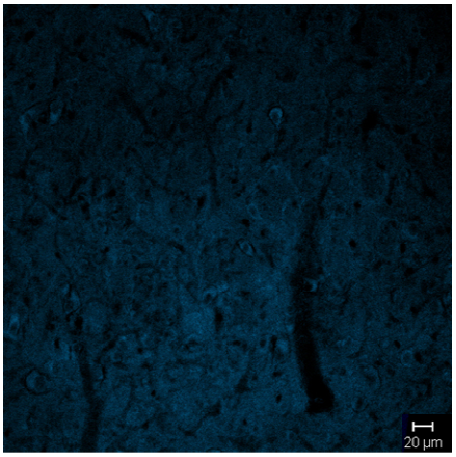
Table 5.1 Stereotaxic co-ordinates of coronal sections relative to bregma.

Coronal section	Position Relative To Bregma
Section 1	**+5**
Section 2	**+4**
Section 3	**+3**
Section 4	**+2.5**
Section 5	**+1**
Section 6	**0**
Section 7	**-2**
Section 8	**-3**
Section 9	**-4.5**
Section 10	**-6**
Section 11	**-7.5**
Section 12	**-9**
Section 13	**-11**

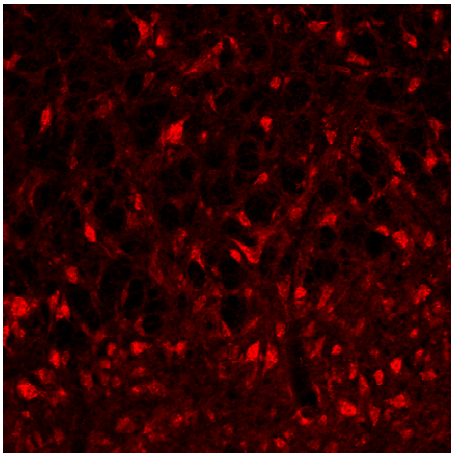
Figure 5.5 Representative images of guinea pig brain sections (serial sections 9, 10 and 11) showing immunofluorescence in neurons expressing MOR, CB_1 receptors and GnRH presumed to be in the hypothalamus.

A: GnRH-immunopositive neurons (blue). **B**: MOR-immunopositive neurons (red). **C** CB_1 receptor-immunopositive neurons (green). **D**: merged image depicting both CB_1 receptor and MOR-immunopositive neurons illustrating the significant co-localization between the two immunofluorescence markers. **E**: merged image depicting immunopositive neurons expressing MOR, CB_1 receptors and GnRH simultaneously.

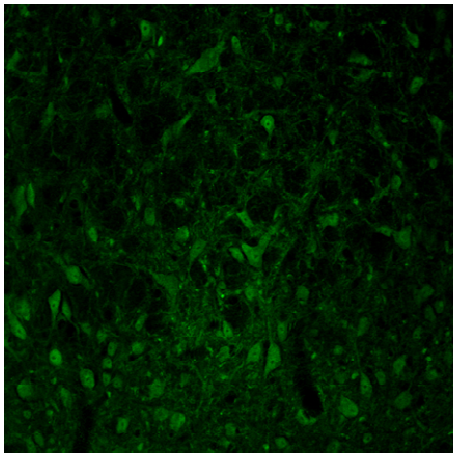
A. GnRH



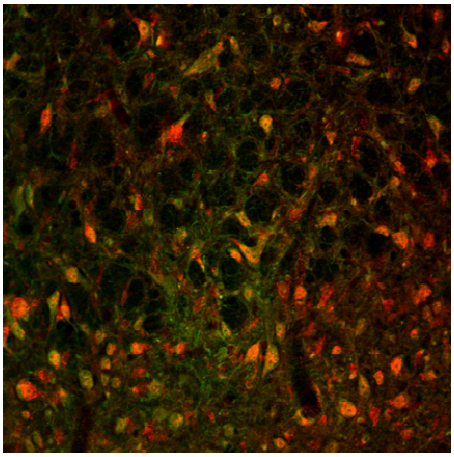
B. MOR



C. CB_1



D. MOR/CB_1



E. MOR/CB_1/GnRH

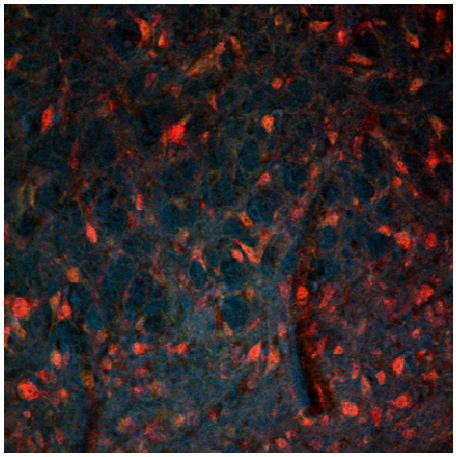


Figure 5.6 Representative images from brain sections showing immunofluorescence in neurons expressing MOR, CB_1 receptors and GnRH in the outer cerebral cortex.

A: MOR-immunopositive neurons (red). **B**: CB_1 receptor-immunopositive neurons (green). **C**: GnRH-immunopositive neurons (blue). **D**: merged image depicting both CB_1 receptor and MOR-immunopositive neurons. Note the significant co-localization between the two immunofluorescence markers and the absence of any reactive product for GnRH-immunopositive cells in the cerebral cortex.

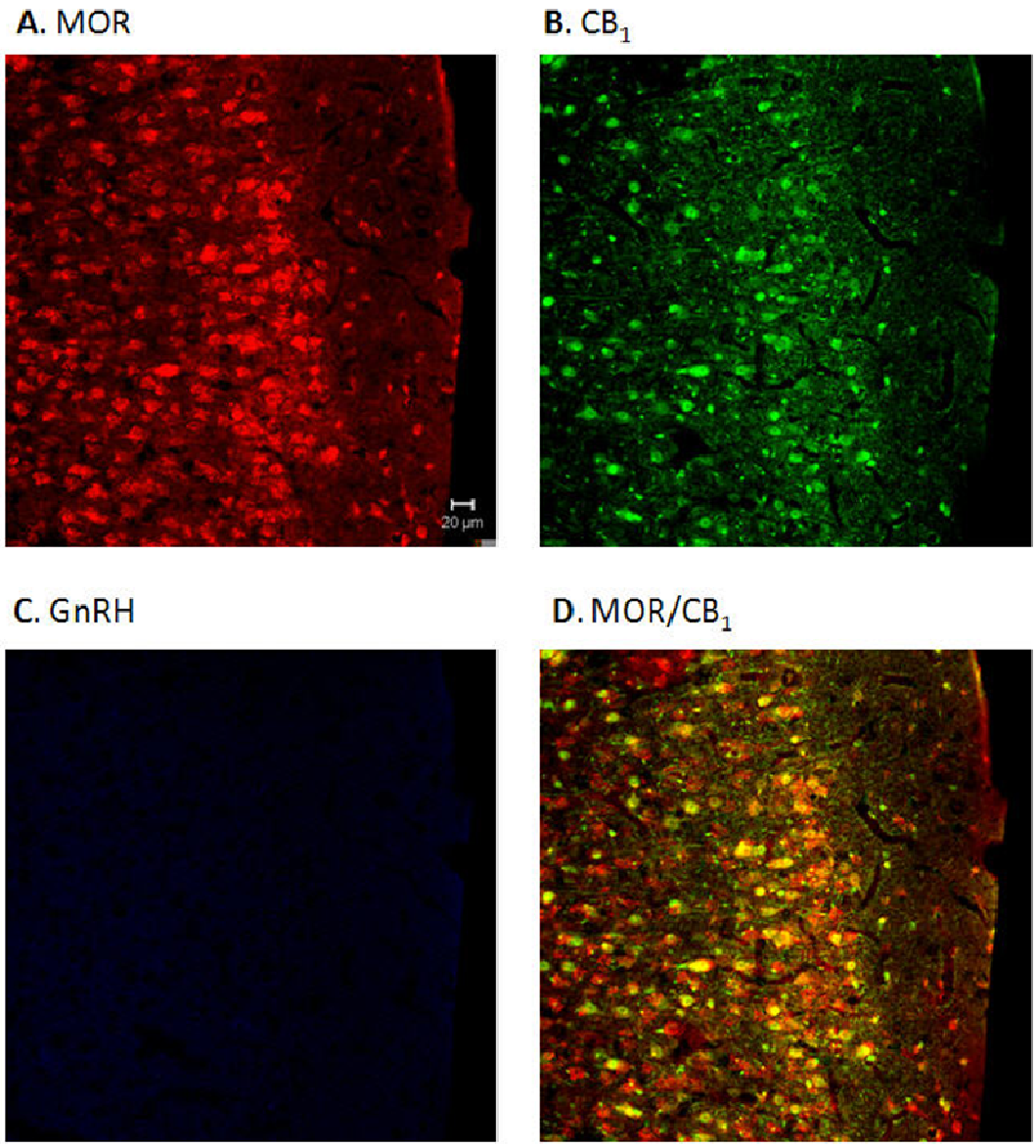
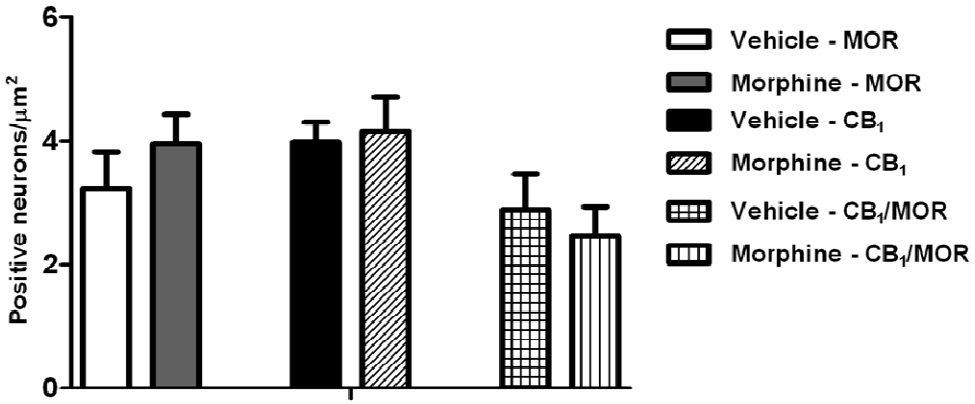

A. MOR
B. CB1
20 µm
C. GnRH
D. MOR/CB1

Figure 5.7 Comparison of the density of MOR and CB_1 receptor-immunopositive neurons in the preoptic anterior hypothalamus of vehicle and drug-treated animals.

Fig 5.7A shows a comparison of the density of neurons expressing MOR or CB_1 receptors, and the density of neurons co-expressing both MOR and CB_1 receptors in the preoptic anterior hypothalamus in vehicle and morphine treated animals. Fig 5.7B compares the same parameters between vehicle and WIN-55,212-2 treated animals. Each bar represents the mean $\pm$ S.E.M of measurement in tissues obtained from 3 guinea pigs. A *p value $\leq$ 0.05 considered to be statistically significant.

A


Positive neurons/μm²
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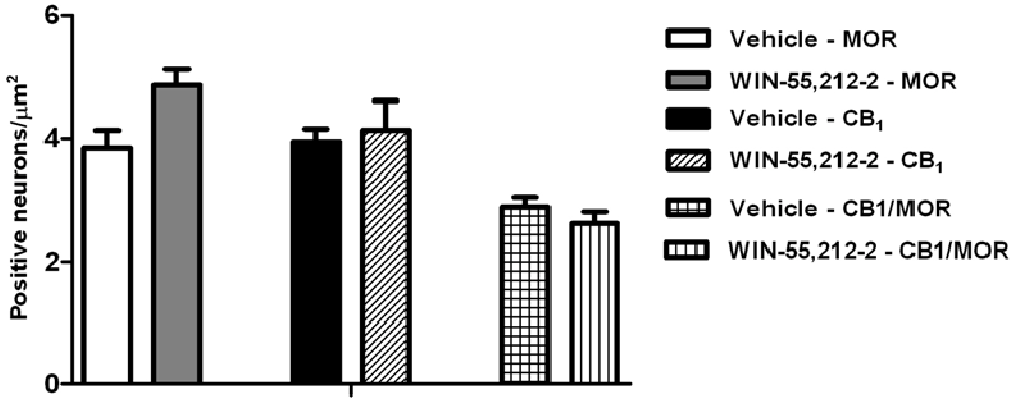
4
2
0
Vehicle - MOR
Morphine - MOR
Vehicle - CB1
Morphine - CB1
Vehicle - CB1/MOR
Morphine - CB1/MOR

B


Positive neurons/μm²
6
4
2
0
Vehicle - MOR
WIN-55,212-2 - MOR
Vehicle - CB1
WIN-55,212-2 - CB1
Vehicle - CB1/MOR
WIN-55,212-2 - CB1/MOR

CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

The development of tolerance following chronic treatment with drugs is a common consequence of a number of drugs from several different classes. The nature of tolerance, whether homologous or heterologous, can be an important component of the expression of the altered phenotype that can be characterized by determining the nature of the specificity of changes in responsiveness. The character of the tolerance that develops also provides important information regarding the most likely cellular and molecular adaptive changes that occur to account for the altered responsiveness. Homologous tolerance, which generally occurs within min/hours, is mainly associated with receptor-dependent modifications such as functional uncoupling of the agonist-occupied receptor from G proteins, receptor downregulation through internalization and degradation, or changes in the cellular components specific to the signaling pathway that is activated. In contrast, heterologous tolerance develops over much longer time periods (days/weeks) and is often characterized by non-receptor dependent modifications in global cell function (Taylor and Fleming, 2001). Chronic exposure to opioids and cannabinoids has been shown to result in adaptive changes in responsiveness that have been attributed to alterations in adenylyl cyclase and/or the coupling of the enzyme to the cognate G proteins ($G_{i/o}$ proteins) which are responsible for mediating the action of the activated receptor(s) (Nestler, 1993; Rhee et al., 2000; Gintzler and Chakrabarti, 2008). Cannabinoid tolerance has been suggested to be dependent on changes in protein kinase A (PKA), Src kinase (Lee et al., 2003) and also nitric oxide (Spina et al., 1998) whereas development of opioid tolerance appears to be dependent on changes in protein kinase A (Nestler, 2001; Gintzler and Chakrabarti, 2008), protein kinase C, or the level of the $alpha_3$ subunit of the sodium pump (Taylor and Fleming, 2001). Beta-arrestin mediated downregulation of opioid or cannabinoid receptors following

prolonged exposure to the respective agonists has also been proposed as a key facet in the development of tolerance though changes in receptor abundance are usually associated with the rapidly developing form of tolerance. The development of tolerance to cannabinoids and opioids is particularly intriguing because of the speed with which it manifests.

The remarkable similarities between the neuroanatomical distribution of the MOR and CB_1 receptor systems and the comparable physiological and cellular signaling pathways, suspected to manifest in synergism of action or heterologous desensitization depending on the model of drug exposure, has led us to propose the possibility of a significant interaction between agonists targeting the two receptor systems with respect to the development of tolerance. The development of heterologous tolerance to these agents would prompt the need to use higher doses to achieve the same pharmacological effect, hence potentially resulting in the expression of a complex myriad of adverse events especially in terminally ill patients (e.g. cancer patients using morphine for pain and dronabinol for nausea). This is especially apparent for biological effects where tolerance is not observed e.g. constipating and miotic effects of opioids. Several methods have been proposed to combat the development of tolerance including the use of low sub-effective doses of synergistic drugs targeting different pathways (Welch and Eads, 1999) or instituting drug holidays to allow upregulation of receptors to regain pretreatment levels. Clinical studies evaluating tolerance to the analgesic effect of opioids have reported attenuation of tolerance upon concurrent use of agents like calcium channel blockers and cholecystokinin antagonists (Santillan et al., 1994; McCleane, 2003). In animal studies, the phosphodiesterase inhibitor ibudilast (Ledeboer et al., 2007), or the intrathecal administration of magnesium and zinc (McCarthy et al., 1998; Larson et al., 2000) have also been reported as promising remedies for overcoming opioid tolerance (McCarthy et al., 1998; Larson et al., 2000).

Research on the possible mechanisms of tolerance associated with sequential or concurrent use of cannabinoids and opioids is important to establish possible drug interactions that may be involved with chronic use of these drugs. Based on the similarities between opioid and cannabinoid distribution and signaling systems, and previous *in vitro* drug exposure studies using the LM/MP model that have reported bidirectional heterologous tolerance (Basilico et al., 1999) it was hypothesized: *1) that in vivo drug exposure would also result in bidirectional heterologous tolerance; 2) that the qualitative characteristics of the tolerance expressed could be used to define the cellular mechanisms most appropriate to explore mechanistically; 3) that if homologous tolerance was displayed, it would employ a mechanism that involved receptor regulation; 4) that co-localization of opioid and cannabinoid receptors to the same neurons could provide a basis for some cross-tolerance between agonists; and 5) that the mechanisms that underlie the development of tolerance need not be the same for every tissue, drug or model system studied.*

Complex interactions have been observed in tissue or cell models used for assessing the development of tolerance to the effects of opioids and cannabinoids (Shapira et al., 2003; Johnson et al., 2006). The task of dissecting the cellular pathways involved is more challenging in *in vivo* models where complex neuronal interactions are vast and may contribute to the concurrent development of both receptor-dependent and –independent forms of tolerance (Taylor and Fleming, 2001). The fact that *in vivo* systems may involve multiple sequential sites at which the same receptor may exert an action provides even greater complexity when considered within the context of the question of whether the development of tolerance at one site in a signaling cascade would modify the development of tolerance at another site in the same cascade that uses the same receptor. The current studies assessed the development of tolerance after chronic *in*

vivo treatment cognizant of the intricate neuronal interactions that exist and may be altered especially in the hypothermia and analgesia models. Though previous studies have used *in vitro* drug exposure models (Basilico et al., 1999; Guagnini et al., 2006), the current study employed *in vivo* exposure regimens which are more physiologically relevant as they account for the complex interaction between the peripheral and central nervous systems in regulating the physiological effects assessed namely hypothermia, analgesia and gastrointestinal inhibition. The *in vivo* drug exposure is also clinically relevant since it mimics the therapeutic administration of these agents. In these studies the guinea pig was chosen as a model on the basis of a number of advantages that the animal model system offers. These include the fact that this species exhibits closer anatomical, physiological, neurological and developmental similarities to the human thus making it an appropriate model system for studying gastrointestinal, respiratory and opioid tolerance (Chavkin and Goldstein, 1984; Hunter et al., 1997; Gray et al., 2001). In addition, the guinea pig does not exhibit an excitatory action to opioids and the distribution of opioid receptors in regions of the CNS more closely resembles that of the human than that observed in the rat (Mansour et al., 1988; Bot et al., 1992). Use of the LM/MP isolated nerve-smooth muscle model also provides a model system that has been used for decades to assess the development of tolerance in an *in vitro* system following *in vivo* treatment. Furthermore, this particular model may be able to provide greater insight into possible neuronal interactions in the CNS since similar neurotransmitters are found in both the enteric and central nervous systems (Rang et al. 2007).

Data generated from studies investigating the characteristics and mechanisms of the development of opioid and cannabinoid tolerance have often been contradictory and ambiguous. This confusion may be due, in part, to differences in the species and models used for analysis,

the dosage regimen employed to induce the change in responsiveness, and/or whether drug exposure is accomplished *in vivo* or *in vitro*. Heterologous tolerance, as evidenced by reduced responsiveness to the analgesic effect of Δ^9-THC, was observed in morphine tolerant mice (Thorat and Bhargava, 1994). On the other hand, studies using rats observed an opposite effect (i.e. analgesic hypersensitivity) to Δ^9-THC in morphine tolerant animals (Rubino et al., 1997). Furthermore, the effect of chronic opioid exposure on the cannabinoid receptor population has also provided varying results with decreases in CB_1 receptor levels observed in the rat hippocampus (Vigano et al., 2003) and an increase in receptor levels in the caudate putamen and limbic structures (Gonzalez et al., 2005). Therefore, in spite of the extensive investigation that has been done, the precise nature and mechanism of the interactions between the opioid and cannabinoid systems and how those interactions impact upon the modifications in responsiveness following chronic treatment remains elusive. Relatively few studies have compared the development of tolerance to the hypothermic, antinociceptive and the inhibitory effects of cannabinoids and opioids on ileum longitudinal muscle in the guinea pig. Furthermore, no studies have explored possible opioid and cannabinoid receptor downregulation or co-localization of MOR and CB_1 receptors in the LM/MP and how such receptor localization may impact upon the nature of tolerance observed in this animal model. In light of the controversies, conflicts and limited data comparing the development of tolerance following chronic *in vivo* opioid or cannabinoid exposure, an experimental plan was developed to address the hypothesis that tolerance would appear similar between opioids and cannabinoids. The experimental plan was based upon the following specific aims: (*1) To determine whether chronic in vivo exposure to opioids or cannabinoids would result in the development of heterologous tolerance that extended to both agents; (2) To compare and contrast the effect of chronic in vivo cannabinoid*

or opioid exposure on the sensitivity of the LM/MP to excitatory agents like nicotine as a possible clue towards cellular mechanisms; (3) To determine if chronic cannabinoid/opioid exposure results in changes in the MOR and CB_1 receptor population; 4) To evaluate the anatomical distribution of MOR and CB_1 receptor-positive neurons in the terminal LM/MP and hypothalamus, and to assess possible receptor co-localization; 5)To evaluate the effect of chronic drug exposure on the development of tolerance to the analgesic and hypothermic effects of opioids and cannabinoids. Results from the present study clearly indicate that the interaction between the endocannabinoid system and the opioid system is complex and shows the importance of the neural context in which receptor activation occurs. The nature of tolerance induced by chronic treatment appears to be dependent on the model being employed as well as the model being assessed.

The first section of the current study (CHAPTER THREE) evaluated the development of tolerance in an *in vitro* LM/MP model whereas the second part focused on the phenomenon as it developed in *in vivo* assessment models (hypothermia and analgesia). The impact of chronic *in vivo* opioid or cannabinoid pretreatment on the sensitivity of the LM/MP to inhibitory and excitatory agents was evaluated. In addition, the effect of agonist exposure on MOR and CB_1 receptor protein abundance and possible co-localization MOR and CB_1 receptors was also determined. Behavioral assessment of the development of tolerance compared the effect of chronic agonist exposure on the development of tolerance to the analgesic and hypothermic effect of challenge doses of morphine and WIN-55,212-2. As an extension of the *in vivo* assessment, the studies evaluated the distribution of MOR and CB_1 receptors in the preoptic anterior hypothalamus (POAH), a key area involved in the regulation of body temperature.

The *in vitro* LM/MP model was employed determine whether prolonged parenteral treatment with either opioids or cannabinoids results in the development of tolerance that is qualitatively similar. Chronic drug exposure involved 7 twice daily injections of morphine or 5 daily injections of the cannabinoid receptor agonist, WIN-55,212-2, and tolerance was examined and confirmed by comparing the ability of DAMGO, 2-chloroadenosine (CADO) and WIN-55,212-2 to inhibit neurogenic contractions of the longitudinal muscle/myenteric plexus preparation (LM/MP). To investigate the potential cellular mechanisms involved in the development of tolerance, the studies assessed the sensitivity of the LM/MP to the neuroexcitatory substance, nicotine, and also evaluated the impact of cannabinoid or opioid pretreatment on MOR or CB_1 receptor abundance. Chronic morphine treatment resulted in the development of heterologous tolerance that extended to inhibitory agonists working through activation of cannabinoid, opioid and adenosine receptors and increased the responsiveness of the LM/MP to the neuroexcitatory effect of nicotine. In contrast, chronic WIN-55,212-2 treatment resulted in subsensitivity only to WIN-55,212-2 and a reduction in maximum response to both WIN-55,212-2 and DAMGO without any change in responsiveness to CADO. As demonstrated in these studies, chronic morphine treatment did not alter the abundance of either MOR or CB_1 receptor protein; this observation is consistent with the maintenance of the maximal inhibitory effect of DAMGO, and suggests that a non-receptor dependent adaptive mechanism is, at least in part, responsible for the development of tolerance after chronic morphine exposure. The fact that CADO appears to inhibit neurogenic activity through a receptor-mediated effect with a cellular mechanism different from that of morphine (Meng et al., 1997) suggests that the adaptation events induced by chronic morphine exposure must involve some alteration in basic cellular function. The laboratory has proposed that the non-receptor

dependent changes underlying the heterologous tolerance involve a partial depolarization of the resting membrane potential (Meng et al., 1997) secondary to a reduction in the level of the functional alpha$_3$ subunit of Na^+/ K^+-ATPase (Kong et al., 1997; Biser et al., 2002; Maguma et al., 2010) which accounts for the reduced sensitivity to inhibitory agents (e.g. DAMGO, CADO and WIN-55,212-2) and the enhanced responsiveness to excitatory agents such as nicotine (see Fig 6.1 for proposed pathways of opioid tolerance in the LM/MP). The reduction in WIN-55,212-2 maximal response could also have been caused by a non-receptor mediated change since the cannabinoid system in the LM/MP appears to have a low functional reserve that may be susceptible to chronic drug exposure (Basilico et al., 1999; Guagnini et al., 2006). Since chronic WIN-55,212-2 pretreatment resulted in a reduction in CB_1 receptor protein that was relatively proportionate to the reduction in the maximal effect of WIN-55,212-2, it can be proposed that the mechanism by which tolerance is induced by WIN-55-212-2 is receptor-dependent and that the CB_1 receptor system in the LM/MP may possess a low functional receptor reserve as was suggested following *in vitro* exposure (Basilico et al., 1999; Guagnini et al., 2006). The reduction in CB_1 receptor levels is also consistent with previous studies which demonstrated robust cannabinoid receptor downregulation that occurs through beta-arrestin mediated desensitization, internalization and ultimately degradation (Gonzalez et al., 2005) (see Fig 6.2 for proposed pathways of cannabinoid tolerance in the LM/MP). The WIN-55,212-2-induced CB_1 receptor downregulation may involve at least two components; one related exclusively to CB_1 receptor downregulation that would be receptor-dependent and specific to cannabinoid agonists and the second one due to dual internalization of CB_1 receptors that are also coupled to MOR as heterodimers that would be receptor-dependent but not specific to cannabinoid receptor agonist (Pfeiffer et al., 2003; Hojo et al., 2008). The fact that chronic cannabinoid treatment produces

tolerance that is primarily homologous in nature compared to the heterologous form of tolerance associated with chronic opioid exposure provided an important foundation upon which to develop mechanistic studies. The MOR and CB_1 receptor co-localization analysis in the LM/MP suggests that the two receptor systems share a number of sites for convergence of action that could serve as the common cellular basis of tolerance which include receptor-dependent and/or signaling pathway-dependent components. The fact that the two receptor populations exist separately as well as together potentially as heterodimers suggests that the expression of tolerance may reflect the additive effect of receptor-dependent and –independent components and the relative ability of the agonist to activate specific intracellular adaptive processes.

In the *in vivo* behavioral experiments, the development of tolerance to the analgesic and hypothermic effects of opioids or cannabinoids was investigated following chronic *in vivo* drug exposure. Since these studies were performed after the LM/MP studies, one of the specific aims was to assess whether the results would corroborate and correlate with those observed in the LM/MP model. These studies presented several unanticipated challenges as the experimental results began to accumulate. While chronic morphine treatment produced heterologous tolerance to the analgesic effects of morphine and WIN-55,212-2 in the hot plate analgesia tests, it could not be assessed whether morphine pretreatment produced heterologous tolerance in the paw pressure model since no analgesia was observed following WIN-55,212-2 challenge. In contrast, chronic WIN-55,212-2 pretreatment resulted in the development of apparent homologous tolerance in the thermal analgesia test since morphine challenge did not show a significant difference between the control and test groups. Interestingly, chronic WIN-55,212-2 pretreatment also produced tolerance to morphine-induced mechanical analgesia despite the fact that WIN-55,212-2 did not produce analgesia in this model. The development of heterologous

tolerance to the analgesic effect (thermal) of morphine or WIN-55,212-2 following pre-treatment with an opioid could have occurred through a variety of mechanisms. One involves a direct intracellular interaction whereby activation of either MOR or CB_1 receptors results in a non-specific adaptive desensitization through sequestration of $G_{i/o}$ proteins thereby making them unavailable to couple to any other receptors using the common pool of $G_{i/o}$ proteins (Vasquez and Lewis, 1999) (Vasquez and Lewis, 1999). Thus, deactivation of both MOR and CB_1 receptor-mediated downstream signaling leads to the development of heterologous tolerance (i.e. reduced responsiveness to agents utilizing different receptor populations but similar signaling pathways). For this mechanism to be operative, however, it would be dependent on the co-localization of MOR and CB_1 receptors in the same neurons. Co-localization of CB_1 receptors and MOR reported in regions responsible for pain regulation such as the superficial dorsal horn of the spinal cord (Hohmann et al., 1999; Salio et al., 2001) and caudate putamen (Rodriguez et al., 2001) seem to support this notion. Another possible scenario involves a network interaction between the two receptor systems whereby the cannabinoid input mediating the response resides upstream of the site of action of opioid receptor activation such that down-regulation of CB_1 receptors results in the reduced activation of the downstream opioid system thereby leading to the development of tolerance to the mechanical analgesic effect of morphine. In support of this idea are reports that show the attenuation of Δ^9-THC analgesia by the selective opioid antagonist, naloxone (Manzanares et al., 1999) and the fact that Δ^9-THC administration elevates pre-opiomelanocortin (POMC) levels in the hypothalamus (Corchero et al., 1997; Manzanares et al., 1998), and increases the expression of preproenkephalin in the PAG, spinal cord and striatum.

Unlike chronic WIN-55,212-2 treatment, chronic morphine treatment did not induce tolerance to the hypothermic effect of WIN-55,212-2. However, the magnitude of the

hypothermic effect of morphine in the guinea pig was not very robust making clear interpretation of the development of tolerance to the response somewhat difficult. Confocal immunofluorescence evaluation of the densities of neurons expressing MOR and CB_1 receptors in the preoptic anterior hypothalamus (POAH), a key area responsible for the regulation of body temperature, revealed extensive co-localization of the two receptor populations suggesting possible convergence and interaction between the two receptor systems which establishes a platform for receptor-dependent and/or receptor-independent but signaling pathway-dependent interactions in the regulation of body temperature. The modest hypothermic effect produced by morphine may not have been sufficient to trigger a pharmacological adaptive response that would extend the reduced responsiveness to the hypothermic effect of WIN-55,212-2. This is consistent with previously published data that show that the use of minimally effective or sub-effective doses of cannabinoids and opioids does not induce tolerance (Welch and Eads, 1999) and reinforces the idea that the development of tolerance is an adaptive response of cells and tissues to chronic agonist activation of receptors. In addition, the lack of tolerance to the hypothermic response to WIN-55,212-2 could be due to the ability of morphine to act at multiple sites to modulate body temperature in both hypo- and hyperthermic fashions. Acute morphine administration is associated with either hypothermic or hyperthermic responses depending on the ambient temperature or receptor subtype activated. This suggests that the action of morphine may be mediated through multiple sites which may or may not involve only the hypothalamus.

In summary, comparison of our behavioral findings with the LM/MP data, show that nature of tolerance in one model cannot be inferred to another even in the same species as evidenced by the observation that morphine pretreatment produces heterologous tolerance in the hot plate and LM/MP models and homologous tolerance in the hypothermic model while chronic

WIN-55,212-2 treatment elicits homologous tolerance in the LM/MP and hot plate but heterologous tolerance in the paw pressure model. The *in vitro* LM/MP data are most qualitatively explained by the production of modification of cell excitability following chronic morphine treatment (Taylor and Fleming, 2001; Li et al., 2010) and receptor downregulation following chronic cannabinoid receptor agonist treatment (CHAPTER 3). In contrast, the data obtained using *in vivo* analysis suggests that the development of the phenomenon of tolerance involves complex neuronal interactions and multiple cellular effects that may induce differential functional tolerance in different models. Other studies have also reported the development of both symmetrical (Hine, 1985; Thorat and Bhargava, 1994; Shapira et al., 2003) and asymmetrical (Bloom and Dewey, 1978; Smith et al., 1994) interactions between opioids and cannabinoids suggesting that the diverse interplay between the two receptor systems might be influenced by the cell/tissue/model system being employed and the parameter(s) being assessed. Such an interaction is particularly dependent upon the ability of the agonists to adequately activate the receptors in appropriate regions to promote the stimulation of the cellular processes that are responsible for the development of tolerance. The differential results may be due to different levels of enzymes or enzyme isoforms involved in the adaptive desensitization process; namely beta-arrestin, G protein-coupled receptor kinase (GRK), PKA, PKC and other kinases (Bloom and Dewey, 1978; Hine, 1985; Smith et al., 1994; Shapira et al., 2003). A number of investigators have suggested various components of the cell signaling pathways as candidates for the cellular locus of tolerance, including the protein kinases, adenylyl cyclases, transcription factors and tyrosine kinases (Shapira et al., 1998; Shapira et al., 2003).

I. Future Directions

In light of the differences observed in the development of tolerance between opioids and cannabinoids in the various models, it can be concluded that the development of tolerance in intact animal models is challenging to interpret because it is complicated by the possibility of intracellular or neuronal network interactions between the two receptor systems at multiple sites and levels. These interactions could occur separately, in sequence or in tandem creating intricate interactions *in vivo*. Hence, it is important to consider the neural context in which receptor activation occurs in the interpretation of the results obtained. Differences in the animal species also make it difficult to compare results that have been compiled in other laboratories. In addition, the inability to observe an alteration in responsiveness empirically depends upon the ability of an agonist to acutely produce a response through the same network that is employed by the agonist used for chronic treatment.

The development of heterologous tolerance in the analgesia model points toward possible interactions of the opioid and cannabinoid systems in the different regions responsible for regulation of pain namely the PAG, dorsal horn of the spinal cord, raphe and the hypothalamus (Tsou et al., 1998; Farquhar-Smith et al., 2000). Since studies have demonstrated the existence of both receptor families in these regions, it would be worthwhile to conduct a series of experiments to ascertain whether co-localization and/or heterodimerization of opioid and cannabinoid receptors can be observed in these regions. Such information would be critical in evaluating the possible cellular interactions between these systems since the presence of heterodimers could produce changes in responsiveness that appear to be non-selective. The assessment of analgesia and hypothermia used a non specific agonist (morphine) which targets both MOR and KOR. The fact that both of these receptors are involved in the regulation of pain and body temperature

(Rosow et al., 1980) makes it challenging to interpret the interaction between the CB_1 and the two opioid receptors. This is particularly significant with respect to the temperature responses since MOR and KOR produce opposing changes on body temperature. A closer examination using selective agonists targeting the MOR (e.g. DAMGO) or KOR (e.g. niravoline, U50,488H) as part of the treatment regimens and/or acute challenge drugs could potentially delineate specific effects of the targeted receptor subtypes and how they interact with the cannabinoid system. It would also be important to establish the ambient temperature at which a significant and consistent hypothermic response is obtained. Subcutaneous and intraperitoneal drug administration used in the study did not target specific regions or tissues hence the *in vivo* results were due to activation of a combination of both peripheral and central inputs. In order to determine the specific regions of the CNS involved in the development of tolerance to the hypothermic or analgesic effects, there may be need to consider stereotaxic microinjection into specific regions of the brain; however, prior to those types of studies in the guinea pig, there is a more important need to develop a comprehensive guinea pig stereotaxic co-ordinates for the relevant areas.

In our previous discussion (CHAPTER THREE), the candidate speculated that since cannabinoid treatment does not change the sensitivity of the LM/MP to nicotine, then it might not alter the resting membrane potential as has been observed with chronic morphine treatment. It may be worthwhile to pursue electrophysiological studies to explore this notion. Such studies would clarify the involvement of a change in membrane potential on the development of tolerance. To investigate potential cellular pathways involved in the development of tolerance, it may be important to explore the impact of other key players (e.g. kinases) proposed to be involved; *in vitro* exposure studies using opioid or cannabinoid agonists in the presence of

inhibitors of PKA, PKC, PKG or Src kinases could be vital in ascertaining the involvement of kinases in the development of tolerance in the LM/MP model.

The effect of chronic *in vivo* cannabinoid treatment on CB_1 receptor protein levels in the LM/MP preparation was considerably different from that observed following chronic morphine exposure. These differences might be due to subtle variability in the signaling or coupling pathways that are involved, as has been shown to occur with ligands that employ similar signaling pathways (e.g. DAMGO induces greater MOR internalization and the ensuing desensitization is GRK2-dependent whereas morphine confers negligible internalization and desensitization is PKC dependent (Johnson et al., 2006)). It has been proposed that this difference in spectrum of actions could be due to differences in the receptor configuration attained upon receptor activation, which determines the differential downstream effects (Violin and Lefkowitz, 2007). It can be speculated that this could occur between the MOR and CB_1 receptor systems investigated as demonstrated in previous studies (Swaminath et al., 2004; Rochais et al., 2007). The role of beta-arrestin in internalization efficacy, development tolerance and downstream signaling efficacy could also be affected by the phenomenon known as ligand-dependent signaling bias. Different receptor conformations may induce differential assembly of adaptor and scaffolding protein entities that determine the nature and mechanism of the tolerance that is expressed. The configuration attained by the CB_1 receptor following WIN-55,212-2 activation might only be conducive to triggering selective signaling events that precipitate CB_1 receptor-targeted homologous tolerance like receptor phosphorylation, internalization and degradation, whereas that conformation induced by morphine-induced activation of MOR induces differential pathways that result in heterologous tolerance like differential protein expression (e.g. reduction in $alpha_3$ subunit levels of the Na^+/K^+ ATPase) or a change in

membrane potential which alters cell excitability. The ligand residence time at the receptor might also contribute to the difference. WIN-55,212-2 is a highly lipophilic compound that is known to have a high receptor residence or occupancy time whereas the residence time for morphine at the MOR is considerably shorter and thus may modify different intracellular processes which will result in different characteristics of tolerance.

The data presented in this study provide support for the concept that the development of tolerance is a function of several converging influences and is subject to considerable variation that may impact upon the cellular processes that are employed to elicit the adaptive response. Delineation of specific mechanisms involved in the interaction between cannabinoid and opioid systems will represent an important advance in the knowledge of the functional interaction in these two systems. Furthermore, the data raise the possibility that such differences in adaptive responses to these agents could potentially be employed to lead to modification in the therapeutic management of patients with these agents.

Figure 6.1 Proposed mechanisms involved in the development of tolerance in the longitudinal muscle/myenteric plexus following cannabinoid exposure

Proposed mechanism of cannabinoid induced homologous tolerance in the guinea pig LM/MP following chronic exposure to WIN-55,212-2. Treatment results in receptor dependent adaptive changes in the form of exclusive CB_1 receptor downregulation and CB_1/MOR heterodimer internalization.

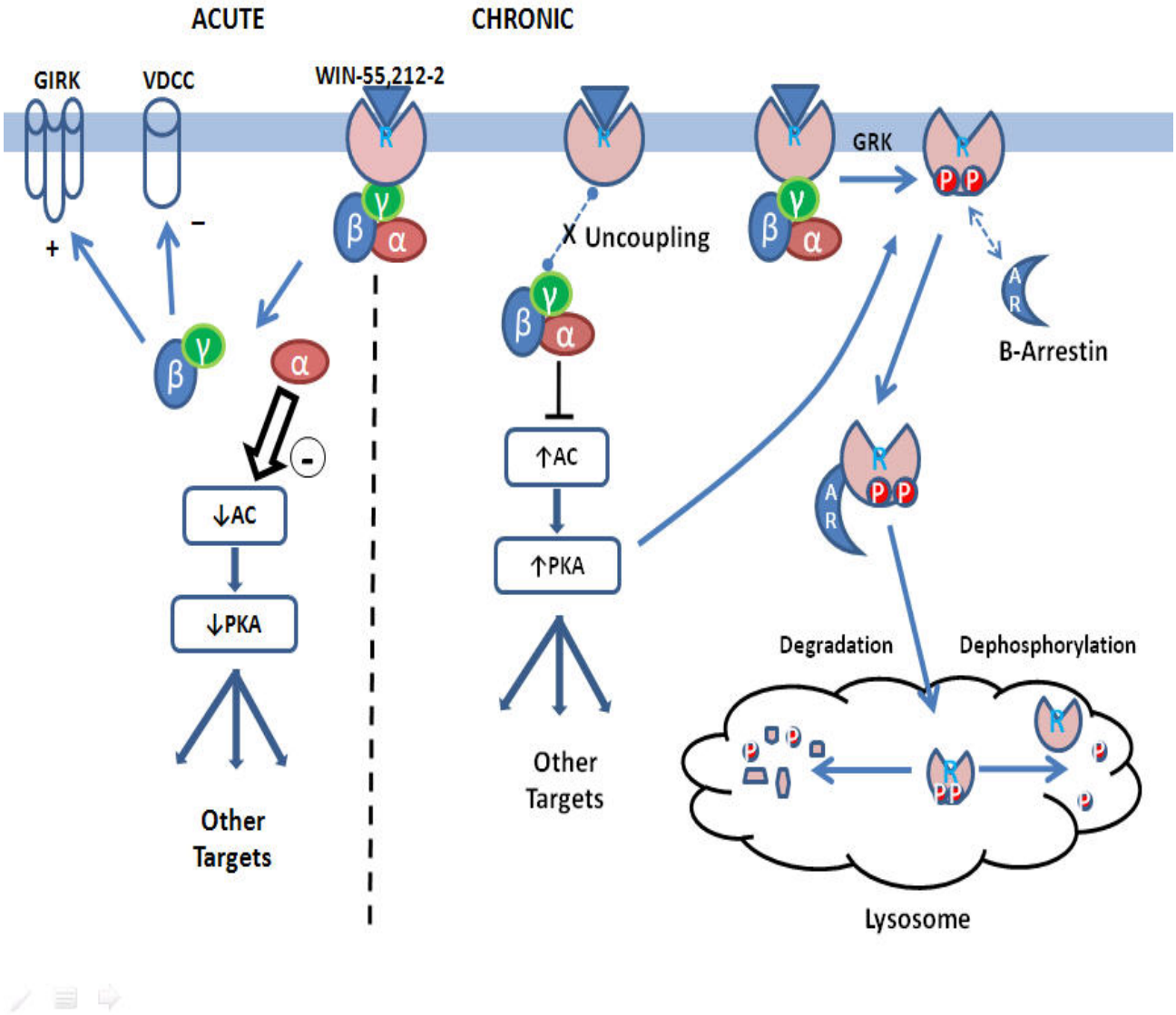
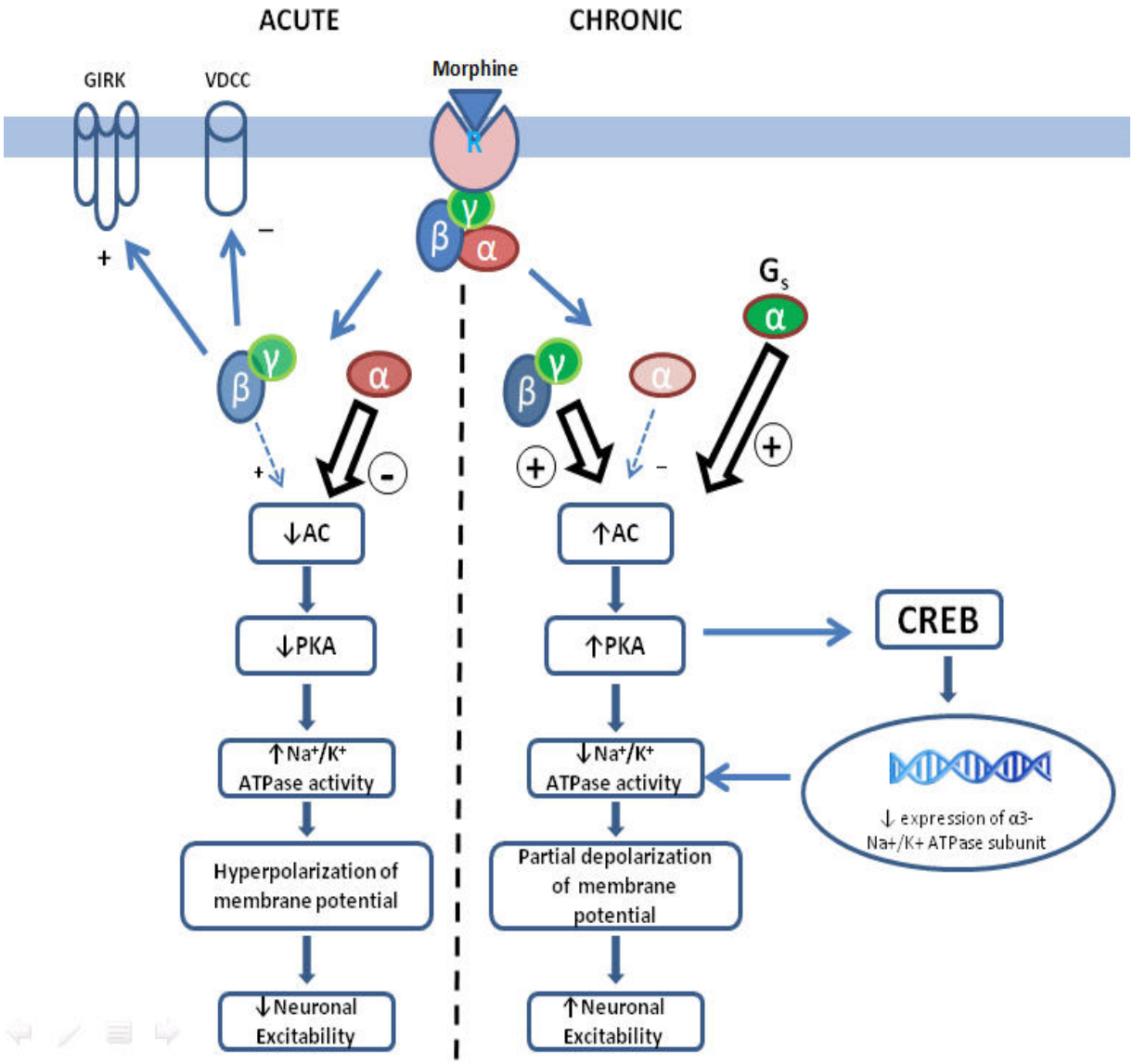

ACUTE
CHRONIC
GIRK
VDCC
WIN-55,212-2
GRK
X Uncoupling
B-Arrestin
↓AC
↓PKA
↑AC
↑PKA
Other Targets
Other Targets
Degradation
Dephosphorylation
Lysosome

Figure 6.2 Proposed pathways for the development of opioid tolerance in the longitudinal muscle/myenteric plexus

Proposed mechanism of opioid induced heterologous tolerance in the guinea pig LM/MP following chronic exposure to morphine. Treatment elicits global non- receptor dependent adaptive changes (e.g. attenuation of sodium pump activity) that results in decreased sensitivity to inhibitory agonists e.g. DAMGO and WIN-55,212-2and increased sensitivity to excitatory agent like nicotine. A negligible reduction in MOR is expected since morphine exhibits a low MOR internalization efficacy.


ACUTE
CHRONIC
GIRK
VDCC
Morphine
R
γ
β
α
G_s
↓AC
↓PKA
↑Na^+/K^+ ATPase activity
Hyperpolarization of membrane potential
↓Neuronal Excitability
↑AC
↑PKA
CREB
↓Na^+/K^+ ATPase activity
↓ expression of α3-Na+/K+ ATPase subunit
Partial depolarization of membrane potential
↑Neuronal Excitability

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APPENDIX A: ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University.

1907-2007 CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

January 27, 2010

David Taylor, Ph.D.
Department Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Taylor:

Your Animal Use Protocol entitled, "Cell Signaling and Tolerance to Drugs," (AUP #W201b) was reviewed by this institution's Animal Care and Use Committee on 1/25/10. The following action was taken by the Committee:

"Approved as submitted"

Comments for consideration:

1. Please consider using the tissues from the animals from the behavioral studies for the histological studies. As written, they are two separate groups.

2. Remember to contact Dale Aycock at 744-2997 prior to biohazard use.

3. There was some uncertainty as to how the fixed drug content of the pellets would allow dosing on a per gram body weight basis. As a pharmacologist, we assume you have an approach for this issue.

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

APPENDIX B: PROTEASE INHIBITOR BUFFER

Protease Inhibitor buffer (PIB)

PMSF (Phenylmethyl Sulfonyl Fluoride)

35.6 mg into 1 ml of isopropanol (concentration = 204mM), this is more concentrated than Sigma recommends but it will get into solution by heating it at 55^{o}C for 5 min.

Bacitracin

50mg added to 500µl of dH_2O then add an additional 500µl to of dH_2O to bring it to 1ml; this can be heated at 55^{o}C for 10-15 min. Store the remainder at <0^{o}C

4-Aminobenzamide

50.4mg into 1ml of dH_2O (concentration = 242 mM)

EDTA

46.53g into 500ml of dH_2O for a concentration of 250mM, pH to 8.0

Working solution (make up daily for use)

0.5ml PMSF for a final concentration of 4.08mM
500µl Bacitracin for a final concentration of 1mg/ml
100µl 4-Aminobenzamide for a final concentration of 1mM
2.14g Sucrose for a final concentration of 0.25M
1ml EDTA for a final concentration of 10mM
Bring up to 25ml with dH_2O

APPENDIX C: WESTERN BLOT BUFFER SOLUTIONS

Western Blot Buffer solutions

10X Running buffer
- 30.28 Tris-Base
- 150.14g Glycine
- 10.00g Sodium Dodecyl Sulfate (SDS)
- pH to 8.6 and adjust volume to 1000ml
- Dilute 1:10 in dH_2O for working solution

Transfer Buffer
- 2.9 Glycine
- 5.8Tris-Base
- 0.37 g SDS
- 200 ml Methanol
- pH to 8.3 and adjust volume to 1000ml.

10X Phosphate Buffered Saline (PBS)
- 80.00g NaCl
- 2.00g K Cl
- 14.40g Na_2HPO_4
- 2.40g KH_2PO_4
- pH to 7.4 and adjust volume to 1000ml

Phosphate Buffered Saline – 0.1% Tween 20 (PBS-T)
- 100ml 10X PBS
- 1.0ml Tween 20
- Bring to 1000ml with dH_2O

Sample buffer (Laemmli, 1970)
- 295.5mg Tris-HCl (pH 6.8)
- 0.6g SDS
- 3.0g Glycerol
- 0.0003g Bromophenol Blue
- Bring to 28.5ml – this is a stock solution

Working solution:
- 950µl of Sample Buffer Stock
- 50µl of β-mercaptoethanol (**β**ME)

APPENDIX D: IMMUNOFLUORESCENCE BUFFER SOLUTIONS

Immunofluorescence buffer solutions

Solutions for

10X Phosphate Buffered Saline (PBS)
80.00g NaCl
2.00g K Cl
14.40g Na_2HPO_4
2.40g KH_2PO_4
pH to 7.4 and adjust volume to 1000ml

4% Para-formaldehyde in 1X PBS (Fixative solution)
100ml 10X PBS (heated to 45 - 60^oC)
40g Para-formaldehyde
Bring to 1000ml with dH_2O

Phosphate Buffered Saline – 0.1% Sodium Azide
100ml 10X PBS
1.0g Sodium Azide
Bring to 1000ml with dH_2O

APPENDIX E: ANTIBODIES USED FOR WESTERN IMMUNOBLOTTING

Antibodies used for Western Immunoblotting

Primary Antibodies

Protein of Interest:	Cannabinoid receptor subtype 1 (Cat. # 10006590)
Host	Rabbit
Dilution	1:200
Antibody Source	Cayman, Ann Arbor, MI (Cat. # 10006590)

Protein of Interest:	mu-Opioid Receptor (MOR) (Cat # AB5511)
Host	Rabbit
Dilution	1:2000
Antibody Source	Millipore, Burlington, MA

Protein of Interest:	GAPDH (Cat. # MAB 374)
Host	Mouse
Dilution	1:60 000
Antibody Source	Millipore, Burlington, MA

Secondary Antibodies

Secondary Antibody:	Donkey anti-rabbit IRDye 800
Dilution	1:20 000 (for CB_1 receptor)
Antibody Source	Li-Cor Biosciences (Lincoln, NE)

Secondary Antibody:	Donkey anti-rabbit IRDye 800
Dilution	1:60 000 (for MOR)
Antibody Source	Li-Cor Biosciences (Lincoln, NE)

Secondary Antibody:	Donkey anti-mouse IRDye 800
Dilution	1:60 000 (for GAPDH)
Antibody Source	Li-Cor Biosciences (Lincoln, NE)

APPENDIX F: ANTIBODIES USED FOR IMMUNOFLUORESCENCE

Antibodies used for Immunofluorescence

Primary Antibodies

Protein of Interest:	Cannabinoid receptor subtype 1 (Cat. # 10006590)
Host	Rabbit
Dilution	1:50
Antibody Source	Cayman (Ann Arbor, MI)

Protein of Interest:	mu-Opioid Receptor (Cat. # Sc-7488)
Host	Goat
Dilution	1:50
Antibody Source	Santa Cruz Biotechnology (Santa Cruz, CA)

Protein of Interest:	Gonadotropin Releasing Hormone (Cat # sc-32292)
Host	Mouse
Dilution	1:500
Antibody Source	Santa Cruz Biotechnology (Santa Cruz, CA)

Secondary Antibodies

Secondary Antibody:	Donkey anti-rabbit FITC-conjugate (Cat. #711-095-152)
Dilution	1:100 (for CB_1 receptor)
Antibody Source	Jackson Immunoresearch (West Grove, PA)

Secondary Antibody:	Donkey anti-goat Cy5-conjugate (7Cat. # 705-175-003)
Dilution	1:100 (for MOR)
Antibody Source	Jackson Immunoresearch (West Grove, PA)

Secondary Antibody:	Donkey anti-mouse Cy3-conjugate (Cat. # 715-165-150)
Dilution	1:500 (for GnRH)
Antibody Source	Jackson Immunoresearch (West Grove, PA)