The Pennsylvania State University

The Graduate School

College of Medicine

AGONIST-SPECIFIC MECHANISMS OF CANNABINOID TOLERANCE IN PRECLINICAL MODELS OF ACUTE AND CHRONIC PAIN

A Dissertation in

Biomedical Sciences

by

Caitlin M. Nealon

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

May 2018

The dissertation of Caitlin M. Nealon was reviewed and approved* by the following:

Daniel J. Morgan
Assistant Professor of Anesthesiology and Perioperative Medicine
Dissertation Advisor
Chair of Committee

John Ellis
Professor of Psychiatry and Pharmacology

Patricia Sue Grigson-Kennedy
Professor of Neural and Behavioral Sciences

Ira J. Ropson
Associate Professor of Biochemistry and Molecular Biology

Ralph L. Keil
Chair of Biomedical Sciences Graduate Program
Associate Member of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School

Abstract

Cannabinoids demonstrate substantial potential as pain therapeutics, particularly for treatment of types of chronic pain that do not currently have effective therapies. One barrier to their use is that tolerance develops after prolonged treatment, where more drug is required to achieve previous effects. Tolerance develops as a result of changes in receptor signaling, including receptor desensitization. Desensitization is caused by phosphorylation of cannabinoid receptor 1 (CB_1) by a G protein-coupled receptor kinase (GRK), and subsequent association of the receptor with arrestin. We have previously found that tolerance to delta-9-tetrahydrocannabinol (Δ^9-THC) is mediated by two distinct signaling mechanisms. Mice expressing a mutant form of CB_1, in which the serine residues at two putative phosphorylation sites necessary for desensitization are replaced by non-phosphorylatable alanine (S426A/S430A), display reduced tolerance to the antinociceptive and hypothermic effects of Δ^9-THC. However, we have found that tolerance to the effects of Δ^9-THC on nociceptive pain is only prevented by subsequent inhibition of activation of c-Jun N-terminal kinase (JNK) in desensitization-resistant S426A/S430A mice. The objective of this study is to understand the mechanisms of tolerance to WIN55,212-2 and CP55,940, both synthetic, high potency cannabinoid agonists. We found that tolerance to CP55,940 was not significantly altered in S426A/S430A mice in either thermal (tail flick) or inflammatory (formalin test) antinociceptive pain assays or in a model of chronic cisplatin-induced neuropathic pain. In contrast, tolerance to WIN55,212-2 was significantly delayed (although not abolished) in these tests in desensitization-resistant mice. Interestingly, we observed contrasting effects of inhibiting JNK signaling using the JNK antagonist SP600125 on cannabinoid tolerance. Disruption of JNK signaling, which consistently delays development of antinociceptive tolerance to Δ^9-THC, actually accelerated tolerance to the antinociceptive effects of CP55,940. Tolerance to the antinociceptive effects of WIN55,212-2 was unaffected

by SP600125. Collectively, these findings show that tolerance to cannabinoid agonists develops through different mechanisms, where GRK- and βarrestin2-mediated desensitization of CB_1 may be the predominant mechanism responsible for tolerance to the antinociceptive effects of WIN55,212-2 while JNK signaling contributes to tolerance to CP55,940, and raises the possibility of complex agonist-specific mechanisms of tolerance for different cannabinoid agonists. A greater understanding of the mechanisms responsible for these effects is important for the development of better cannabinoid-based therapies, particularly for more effective pain management therapies.

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List of Abbreviations

ACEA	Arachidonyl-2'-chloroethylamide
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBD	Cannabidiol
CNS	Central nervous system
CP55,940	(1α,2β)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl-cyclohexyl]phenyl
CPS	Composite pain score
DIO	Diet-induced obesity
ERK	Extracellular signal-related kinase
FAAH	Fatty acid amino hydrolase
GASP-1	G protein-coupled receptor-associated sorting protein 1
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HED	High-energy diet
HIV	Human immunodeficiency syndrome
HU-210	1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol
i.c.v.	Intracerebroventricular injection
i.p.	Intraperitoneal injection
i.t.	Intrathecal injection
JNK	c-Jun N-terminal kinase
MAGL	Monoacylglycerol lipase

MAPK	Mitogen-activated protein kinase
MOR	μ-opioid receptor
NSAID	Nonsteroidal anti-inflammatory drug
PAG	Periaqueductal grey
PKA	Protein kinase A
PKCε	Protein kinase c epsilon type
RVM	Rostroventromedial medulla
s.c.	Subcutaneous injection
SEM	Standard error of the mean
SP6	SP600125
SR141716A	Rimonabant; N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
TD-NMR	Time-domain nuclear magnetic resonance spectroscopy
WIN55,212-2	R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl-methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-mapthalenyl)methanone mesylate
WT	Wild-type mouse
2-AG	2-arachidonoylglycerol
[^{35}S]-GTPyS	[^{35}S] labeled guanosine 5'-O-[gamma-thio]triphosphate
Δ^9-THC	(-)-*trans*-delta-9-tetrahydrocannabinol
%MPE	Percent maximal possible effect

Acknowledgements

I would like to first thank my dissertation advisor, Dr. Daniel Morgan for taking me into his lab as his very first graduate student. His support and guidance has provided me with fantastic opportunities to grow as a scientist over the last several years. Some advisors are not very enthusiastic about training students who have no intentions to remain in academia, and I am *extremely* thankful that he has provided an environment that was supportive of my outside endeavors and where I never felt the need to hide my career goals.

I would also like to thank some of the current and former members of the Morgan Lab for their support, inspiration, and encouragement over the last several years. We have a lot of fun the in the Morgan Lab while we take on science, and a lot of that is due to the fantastic people who work in it. Particular thanks are due to Mike Zee for his constant support and for acting as a sounding board to for many of my more ridiculous ideas, to Diana Sepulveda for her encouragement and willingness to take on any task, and to David Hale for brightening up the lab and providing the most essential perspectives on life. I would also like to thank the National Institutes of Health for their support of grants DA036385 (DJM) and DA037355 (DJM) and the Pennsylvania Department of Health using Tobacco CURE Funds for their support of grant 4100057673 (DJM).

I owe huge thanks to my parents for believing in me and being a source of never-ending support. They've encouraged me in every step I've taken along this path and I don't know that I will ever find a way to express all of my gratitude for what they've done for me. I'm really fortunate in that I ended up doing my PhD close to home and have been able to lean on both my parents and my extended family for advice, support, and I appreciate all they have done to help keep me grounded and make sure I succeed.

Finally, I have to thank my lab-mate Dr. Angela Henderson-Redmond, who has been the most fantastic mentor, teacher, and friend I could have hoped to find. Her advice has never failed me[1], and I owe an alarming number of my successes directly to her guidance. I am positive that without her I would not have made it past my third year. Thank you, Angela, for giving me essential professional guidance, for your constant encouragement of my plans, for teaching me nearly everything I know about statistics, for the laughter and mouse songs, and for putting up with my terrible puns about chickens. I look forward to being the fun (and in the future, corrupting) Auntie Caitlin to your children for many years to come.

[1] Professional and scientific advice. Relationship advice was removed from the data set prior to evaluation.

Chapter 1

Introduction

1.1. Pain

1.1.1 Prevalence and cost of pain

Pain, both acute and chronic, is a significant public health problem and one of the most substantial burdens on human health and welfare (International Association for the Study of Pain (IASP), 2012). More than 100 million adults in the United States suffer from chronic pain (Bair et al., 2008; Dzau and Pizzo, 2014; Johannes et al., 2010; Nahin, 2015), more than the number of patients who suffer from cancer, diabetes, and heart disease combined (Centers for Disease Control and Prevention, 2002; Gatchel et al., 2014; U.S. Department of Health and Human Services, 2006). Outside of the United States, the burden of pain and its treatment is even greater. Approximately 80% of the global population has either no access or inadequate access to treatment for pain which is moderate or severe (Lohman et al., 2010). Chronic pain affects approximately 1 in 5 people in well-resourced countries (Blyth et al., 2001; Breivik et al., 2006; Nahin, 2015; Tsang et al., 2008), but the rates of pain double (2 in 5 people) in countries with limited resources (Tsang et al., 2008). There is also a growing understanding of the high rates of pediatric pain and the connection between untreated pain in childhood and persistent pain in adulthood (Fearon and Hotopf, 2001; Stanford et al., 2008). The costs of pain are not insignificant. Pain is the cause of more than 50 million days of lost work-time each year (Stewart et al., 2003), and the total cost of pain, both directly through treatment and indirectly through lost productivity and quality of life, was estimated to be between $560-$635 billion dollars in 2012 (Gaskin and Richard, 2012).

Among the major chronic diseases (cardiovascular, lung, and kidney), pain is associated with the worst quality of life (Lynch, 2011). Chronic pain is associated with decreases in functioning

and quality of life (Bair et al., 2008; Gatchel et al., 2014). Decreased mood is also observed in conjunction with chronic pain; the risk of suicide doubles in patients who suffer from chronic pain (TANG and CRANE, 2006). Advances in the treatment of acute illnesses (cardiovascular disease, human immunodeficiency syndrome (HIV), neoplasms) have led to increases in patient survival. It has been observed that survival "increases the quality of life, but decreases the quantity of life" (Lynch and Campbell, 2011) due to the presence of persistent pain, caused by either continued illness or tissue damage as a result of the disease cure (Deandrea et al., 2008; McGillion et al., 2012; Phillips et al., 2010).

1.1.2 Current standards of pain treatment

Pain treatment leaves much to be desired and adequate treatments for chronic pain remain a critical unmet need. In a study of 46,000 patients with chronic pain, it was found that nearly 40% of patients reported inadequate pain control (Breivik et al., 2006). Patients in this study relied on a variety of pain management therapies, 50% used over-the-counter pain medication, 60% used prescription pain medication, and 70% used therapies which were not pharmacological (e.g. massage or physical therapy). Another study found that nearly one in two cancer patients report insufficient pain management (Deandrea et al., 2008). The obvious deficit in chronic pain management approaches suggests that multi-disciplinary approaches to pain management are necessary to address this unmet clinical need (Shah et al., 2017).

It is not just pain that presents a significant public health problem. The treatment of pain (however insufficient) has also creased a second public health problem- opioid abuse and overdose. Currently, more than 2% of the adult population is prescribed opioid painkillers (Deyo et al., 2011), and there is growing evidence that widespread adaptation of prescription opioids for the treatment of chronic non-cancer pain has been a key driver of the opioid epidemic (Kolodny et al., 2015). Prescription opioid painkillers are effective in the short-term and for treatment of acute pain but show decreasing benefit to the patient throughout continuing therapy

(Krashin et al., 2013; Levy et al., 2015; Volkow and McLellan, 2016). Prescription opioid painkillers are frequently prescribed to patients early in chronic non-cancer pain treatment despite serious risks (Levy et al., 2015; Yoast et al., 2001). Ever-increasing doses of opioids are required for pain relief in chronic pain patients with minimal improvement in pain symptoms (M. a. Bachhuber et al., 2014; Bloodworth, 2006; Chou et al., 2014; Potter and Marino, 2013). This extended excessive use increases community access to habit-forming drugs which can contribute to opioid overdose and lead to dependence and addiction (Bloodworth, 2006; Centers for Disease Control and Prevention, 2012; Okie, 2010; Rudd et al., 2016). Most clinical studies of opioid analgesics are not of sufficient duration to evaluate long-term analgesic efficacy or potential for dependence and addiction (Dowell et al., 2016; Furlan et al., 2006; Shah et al., 2017). Some studies have reported that opioids have no efficacy in chronic pain (Arnér and Meyerson, 1988), or do not vary significantly in efficacy from nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of neuropathic pain (Furlan et al., 2006). Chronic opioid analgesic treatment has also been correlated with increased pain severity and decreased quality of life patients in diagnosed with chronic non-cancer pain (Eriksen et al., 2006; Sjøgren et al., 2010). While other clinical studies have reported substantial efficacy of opioid analgesics for treatment of chronic pain, many show efficacy only in treatment of specific pain conditions, which limits the widespread application of the findings (Ballantyne and Shin, 2008). Opioid analgesics are a significant and costly problem to which alternative therapies are required (Gatchel et al., 2014).

1.2 Cannabinoids and the endocannabinoid system

1.2.1 *Cannabis sativa* and Δ^9-tetrahydrocannabinol (Δ^9-THC)

Marijuana (*Cannabis*) has long been utilized as a therapeutic. The earliest reports of therapeutic use of marijuana date back to approximately 2600 BC (Mechoulam, 1986), and there are reports of its widespread use throughout Asia as an analgesic and anti-inflammatory

agent well into the 1800's (O'Shaughnessy, 1843). The primary psychoactive component of marijuana is (-) trans-delta-9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC was originally isolated from *Cannabis sativa* in 1964 (Gaoni and Mechoulam, 1964), and it is from this plant that the class of cannabinoid agonists and receptors derive their name. More than 60 other cannabinoids have been isolated from *Cannabis sativa*, but Δ^9-THC remains the primary component responsible for the psychoactive and mood-altering effects of the plant (Mechoulam and Parker, 2013).

Currently, Δ^9-THC and other plant-derived cannabinoids are classified as Schedule I drugs in the United States (21 U.S.C. § 812), defined as a drug that has "a high potential for abuse…no currently accepted medical use in treatment in the United States… [and] a lack of accepted safety for use of the drug or other substance under medical supervision" (*Controlled Substances Act*, n.d.). Petitions for the rescheduling of marijuana to allow expanded legal access for use of its therapeutic effects have not been successful (Marhsall, 2001). Despite this, legal access to and use of medical cannabis (including medical marijuana and plant extracts) has expanded dramatically in recent years following establishment of these programs in individual states (M. A. Bachhuber et al., 2014; National Conference of State Legislators, 2018). In 2009 federal prosecutors were advised not to prioritize prosecution of individuals who use medical cannabis in compliance with the laws of the state (Ogden, 2009). This has served to relax the restrictions on access to medical cannabis and increase its therapeutic use.

1.2.2 The endocannabinoid system

Patients who utilize medical marijuana for therapeutic treatment are taking advantage of the effects of Δ^9-THC on the endocannabinoid system in the brain. The endocannabinoid system is primarily comprised of two receptor subtypes, the cannabinoid receptor 1 (CB_1) and the cannabinoid receptor 2 (CB_2). As one of the most highly expressed G protein-coupled receptors (GPCRs) in the brain, CB_1 has the potential to have a major role in modulating activity in the

central nervous system (CNS) (Devane et al., 1988; Herkenham et al., 1990). Indeed, CB_1 has been implicated in a wide range of physiological responses, including thermoregulation (Wenger and Moldrich, 2002), energy balance (Cardinal et al., 2012), neurogenesis (Jin et al., 2004), movement and memory (Jansen et al., 1992), and immune activity (Kaminski, 1998). The CB_2 receptor is found at much lower levels in the CNS than CB_1 and is primarily expressed in immune tissues (Childers et al., 1994; Munro et al., 1993). CB_1 and CB_2 share 44% homology, mainly in driven by homology in transmembrane regions (Matsuda et al., 1990; Munro et al., 1993).

The CB_1 receptor was identified in rat brains as a specific guanine nucleotide-sensitive high affinity binding site (Devane et al., 1988) which was later cloned and confirmed to be a seven-transmembrane GPCR (Matsuda et al., 1990). This confirmed previous studies which observed that cannabinoid agonists inhibited cyclic adenosine monophosphate (cAMP) in manner which was pertussis toxin sensitive (Howlett, 1985; Howlett et al., 1986), suggesting that CB_1 predominately couples to $G\alpha_{i/o}$ (Childers et al., 1994; Howlett et al., 1986). When activated, CB_1 also causes inhibition of voltage-gated Ca^{+2} channels (Mackie and Hille, 1992; Mackie and Lai, 1995) and activation of both inwardly-rectifying potassium channels (Guo and Ikeda, 2004; Mackie and Lai, 1995) and mitogen-activated protein kinases (MAPKs) (Bouaboula et al., 1995; Tanya L. Daigle et al., 2008), including c-Jun N-terminal kinase (JNK) (Delgado-Peraza et al., 2016; Rueda et al., 2000a).

CB_1 is expressed throughout the CNS with the highest density found in the cortex, basal ganglia, hippocampus, and cerebellum (De Jesús et al., 2006; Herkenham et al., 1990; Jansen et al., 1992). Regional variations in CB_1 signaling have been identified, particularly in signal amplification (Breivogel et al., 1997). In one study, CB_1 coupling efficiency, which measures the number of G proteins activated by the receptor, varied significantly by brain region, with the highest signal amplification in the hypothalamus and the lowest in the cerebellum and

hippocampus (Breivogel et al., 1997). Interestingly, the regions with poor efficiency had the highest amount of CB_1 expression. Although CB_1 has significantly less efficiency than either the mu- or delta- opioid receptors (MOR, DOR; average activation of 3 G proteins/receptor versus 20 G proteins/receptor) (Laura J. Sim et al., 1996), the wide array of different behavioral effects of cannabinoids are likely driven by regional variations in coupling with signal transduction systems and not by different receptor expression levels.

The endocannabinoid system also includes endogenous cannabinoid agonists, including N-arachidonoyl-ethanolamide (anandamide) (Devane et al., 1992; Mechoulam et al., 1995) and 2-arachidonoylgylcerol (2-AG) (Sugiura and Waku, 2000). Endocannabinoid agonists are unique among neurotransmitters in that they are lipophilic arachidonic acid derivatives that are synthesized on-demand and are not stored in vesicles (Devane et al., 1992; Mechoulam et al., 1995). Typically, endogenous agonists are considered to be full agonists at their receptor, but anandamide is among the few endogenous agonists which does not fully activate their receptor (Breivogel et al., 1998; Mackie et al., 1993). Endogenous cannabinoid agonists play a role in activity-dependent synaptic transmission, where they control neuronal firing via retrograde inhibitory signaling (Diana et al., 2002). Endocannabinoid signaling is rapidly terminated in the synapse by enzymatic hydrolysis (Cravatt et al., 2001, 1996; Deutsch and Chin, 1993; Karlsson et al., 1997). Both anandamide and 2-AG are hydrolyzed by fatty acid amino hydrolase (FAAH) (Cravatt et al., 2001, 1996; Deutsch and Chin, 1993), while hydrolysis of 2-AG is also performed by monoacylglycerol lipase (MAGL) (Karlsson et al., 1997; Long et al., 2009; Tornqvist and Belfrage, 1976). Inhibition of endogenous cannabinoid hydrolysis by FAAH (Russo et al., 2007) or MAGL (Ghosh et al., 2013) using small molecule inhibitors produces significant antinociception in mouse pain models. In addition, mice with a genetic deletion of FAAH are more sensitive to the effects of exogenously administered anandamide and demonstrate increase endocannabinoid signaling (Cravatt et al., 2001).

1.2.3 Exogenous cannabinoid agonists

Exogenous cannabinoid agonists exhibit substantial structural diversity (Pertwee, 2008), which may lead to distinct differences in the responses activated by their association with CB_1. The prototypical cannabinoid agonist, Δ^9-THC exerts its effects through both the CB_1 and CB_2 receptors. Δ^9-THC is a classical cannabinoid agonist, with a tricyclic dibenzopyran structure (Gaoni and Mechoulam, 1964; Howlett, 2002). Δ^9-THC is a low-efficacy partial agonist at the CB_1 receptor that stimulates [^{35}S]-GTPγS binding at only 20% of the stimulation of other synthetic cannabinoid agonists (Breivogel et al., 1998; Burkey et al., 1997; Laura J. Sim et al., 1996).

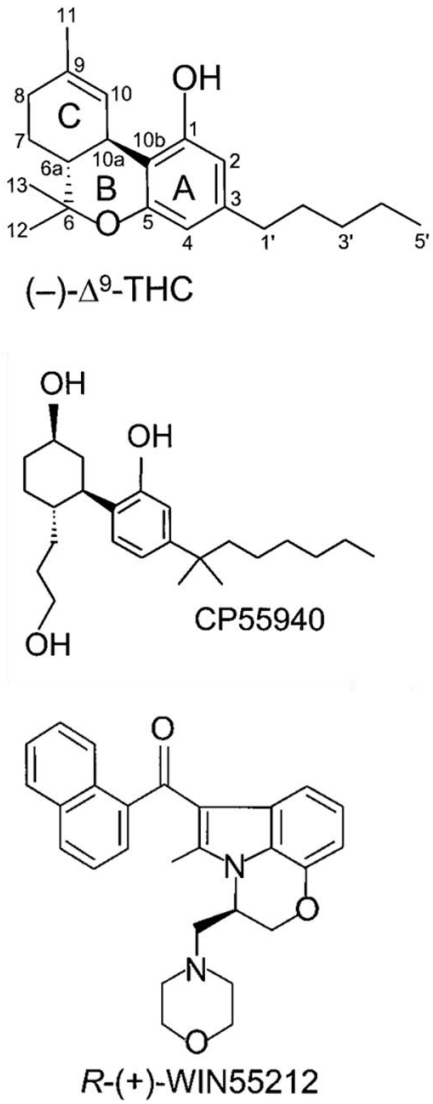

Figure 1.1. Structures of Δ9-THC, CP55,940, and WIN55,212-2. Adapted from (Howlett, 2002).

The cannabinoids R-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl0methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-mapthalenyl)methanone mesylate (WIN55,212-2) and (1α,2β)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl0cyclohexyl]phenyl (CP55,940) are synthetic cannabinoids that are structurally very different from each other (Bell et al., 1991; Devane et al., 1988; Pacheco et al., 1991). CP55,940 is a non-classical cannabinoid which was used to characterize the CB_1 receptor (Devane et al., 1988; M Herkenham et al., 1991; M. Herkenham et al., 1991). It is a bicyclic analog of Δ^9-THC which lacks the dihydropyran ring of Δ^9-THC and is highly potent (Compton et al., 1992b; Itagaki et al., 2005; Little et al., 1988), unlike other bicyclic cannabinoids like cannabidiol, which loses pharmacologic activity as

a result of conversion from Δ^9-THC (Melvin et al., 1993). CP55,940 binds to both CB_1 and CB_2 with similar affinity and is a full agonist for both receptor subtypes with maximal effects that are equivalent to or greater than other cannabinoid agonists (MacLennan et al., 1998; Pacheco et al., 1991; Sacerdote et al., 2000; Thomas et al., 1998). CP55,940 produced full cannabimimetic effects in tetrad tests (Section 1.2.4.4) and is 10-50x more potent than Δ^9-THC in those tests (Compton et al., 1992b; Darmani et al., 2003; Melvin et al., 1993)

WIN55,212-2 is an aminoalkylindole that is structurally distinct from both classical (Δ^9-THC) and non-classical (CP55,940) cannabinoids. Originally developed as a pravadoline analog with reduced function as a nonsteroidal anti-inflammatory drug (Bell et al., 1991; Pacheco et al., 1991), WIN55,212-2 binds to both CB_1 and CB_2 despite significant structural differences (Bouaboula et al., 1995; Tao and Abood, 1998) and substitutes for other cannabinoid agonists in discriminative stimulus tests (Järbe et al., 2010; J L Wiley et al., 1995; J. L. Wiley et al., 1995). WIN55,212-2 has high relative activity at CB_1 and is a full agonist of the CB_1 receptor as measured by [^{35}S]-GTPγS binding (Breivogel et al., 1998; Burkey et al., 1997; Evans et al., 1994; Lauckner et al., 2005; Laura J. Sim et al., 1996). There is evidence that WIN55,212-2 binds to CB_1 in a manner which is different from classical and non-classical cannabinoids, but WIN55,212-2 is still sensitive to displacement by other cannabinoid agonists (Georgieva et al., 2008; Petitet et al., 1996; Tao and Abood, 1998). The behavioral effects of WIN55,212-2 in the tetrad test resemble the effects caused by treatment with Δ^9-THC in both mice and Wistar rats (Compton et al., 1992a; Sim-Selley and Martin, 2002). However, the Compton *et. al* study also found that Δ^9-THC was not antagonized by inactive structural isomers of WIN55,212-2, suggesting that structural restrictions for cannabimimetic activity apply to compounds of substantially different structure from classical cannabinoid agonists.

1.2.4 Therapeutic applications of cannabinoid drugs

1.2.4.1 Medical marijuana

Access to and use of medical marijuana, or medical cannabis, has grown substantially since 1996, when a system for access to medical cannabis was passed by ballot measure in California (Cal. Health & Safety § 11362.5). As of February 2018, 29 states and the District of Columbia have enacted legislation to allow access to public medical cannabis programs either through use of products containing Δ^9-THC or limited-access marijuana products (high-cannabidiol (CBD), low Δ^9-THC) (National Conference of State Legislators, 2018). As this dissertation is submitted toward the completion of a degree from the Pennsylvania State University, it is important to consider the medical marijuana laws that have been implemented in Pennsylvania. The Medical Marijuana Act allows for the use of oil extracts containing Δ^9-THC for the treatment of any of 17 "serious medical conditions" (35 Pa. Cons. Stat. §§ 10231.101 through 10231.2110). Of particular interest to the topic at hand is the indication for treatment of "[s]evere chronic or intractable pain of neuropathic origin or severe chronic or intractable pain in which conventional therapeutic intervention and opiate therapy is contraindicated or ineffective."

Medical cannabis has a wide range of potential applications. A common use of medical cannabis is for the treatment of pain (Abrams et al., 2007; Ellis et al., 2009; Lynch, 2011; Whiting et al., 2016), although the drug is also used by patients to treat pain in states that have not established medical cannabis programs. Medical cannabis produces analgesic effects in patients experiencing chronic non-cancer pain, including pain caused by multiple sclerosis and HIV-associated sensory neuropathy, which is a type of neuropathic pain that is notoriously resistant to other established approaches to treatment (Abrams et al., 2007; Ellis et al., 2009; Gatchel et al., 2014; Hill, 2015; Whiting et al., 2016). However, other studies have indicated that medical cannabis produces only a modest effect on chronic non-cancer pain (Lynch and Campbell, 2011). Patients who are currently undergoing treatment with prescription opioid analgesics also use medical cannabis as an adjuvant therapy. Patients with chronic pain who use cannabis report significant improvements in pain severity and quality of life over patients

who do not use cannabis, but do not show differences in opioid usage (Shah et al., 2017). There is growing evidence that medical cannabis-based interventions show potential to substitute for prescription opioid analgesics and may prevent opioid overdose (Lucas, 2017; Lucas and Walsh, 2017), however adequate clinical trials to investigate these uses of medical cannabis are severely lacking.

Use of medical cannabis has increased alongside access, as marijuana use prevalence has increased since 1997 (Chu, 2014). Despite increased use and consumption of medical cannabis, rates of negative health consequences associated with use have not increased (Shi, 2017). Implementation of medical marijuana policies have not led to increases in substance abuse- or marijuana-related hospitalizations (Pacula et al., 2015; Shi, 2017). In addition, rates of suicide and overall crime rates have also not increased with expanded access to medical marijuana (Anderson and Rees, 2014; Morris et al., 2014).

1.2.4.2 Currently available cannabinoid-based therapeutics

There are currently two cannabinoid-based therapeutics which are approved for use in the United States (U.S. Food and Drug Administration, 2017), dronabinol, a synthetic Δ^9-THC that is not plant-derived (schedule III), and nabilone, a synthetic Δ^9-THC analogue (schedule II). Both were initially approved in 1985 for treatment of chemotherapy-induced nausea and vomiting and anorexia and weight loss in patients diagnosed with acquired immunodeficiency syndrome (AIDS) (GW Pharmaceuticals, 1985; U.S. Food and Drug Administration, 2006). Clinical studies with dronabinol have shown efficacy for treatment of pain, either alone or as an adjuvant therapeutic in combination with opioid analgesics (Narang et al., 2008; Svendsen et al., 2004). Similarly, clinical studies with nabilone have demonstrated significant analgesic effects of the drug in treatment of spinal, spasticity-related, and fibromyalgia-related pain (Berlach et al., 2006; Skrabek et al., 2008; Wissel et al., 2010). Despite this, there is not a cannabinoid-based drug that is currently approved for treatment of pain in the United States. Sativex, a drug

comprised of plant-derived Δ^9-THC and CBD, remains under approval in the United States for treatment of pain caused by spasticity in patients diagnosed with multiple sclerosis (GW Pharmaceuticals, 2016).

1.2.4.3 Medical cannabis and prescription opioid analgesics

Medical cannabis presents a viable alternative to the use of prescription opioid analgesics, and has been proposed as a strategy to curb prescription opioid abuse and overdose (Choo et al., 2016; Shi, 2017). In particular, the lack of fatal overdose observed in medical cannabis use (Calabria et al., 2010; Hall, 2015) and the potential for medical cannabis use as an adjuvant therapy to allow use of lower doses of opioid analgesics and with fewer negative side effects and reduced withdrawal (Abrams et al., 2007; Lynch and Clark, 2003; Scavone et al., 2013). Establishment of medical cannabis policies lead to reductions in prescription of opioid analgesics (Bradford and Bradford, 2017, 2016). Some studies report no effects of combination therapy (medical cannabis and prescription opioid analgesics) on responses in experimental pain tests, but these studies were performed in healthy volunteers and not in patients experiencing chronic pain (Naef et al., 2003; Roberts et al., 2006).

Despite the dearth of controlled, long-duration clinical studies of interactions between medical cannabis and prescription opioid analgesics, there is growing evidence for the recommendation of medical cannabis as a tool both to treat pain and manage the burgeoning opioid abuse epidemic. Rates of opioid overdose mortality decrease significantly in states that have established medical cannabis policies when compared to states without access to medical cannabis (M. a. Bachhuber et al., 2014; Pardo, 2017; Shi, 2017) . Rates of mortality from other conditions (e.g. cardiovascular disease) do not change following passage of medical cannabis laws, suggesting that medical cannabis access only affects patients using prescription opioid analgesics (M. a. Bachhuber et al., 2014). Medical cannabis policies are also associated with reductions in rates of hospitalizations related to the use of prescription opioid analgesics (23%

reduction) and admissions for opioid substance abuse (28%) (Powell et al., 2015; Shi, 2017). Positive tests for opioids (all opioids, not just prescription analgesics) in drivers fatally injured in automotive accidents dropped following implementation of medical cannabis laws (Kim et al., 2016). There appears to be a lag in the effects of establishment of medical cannabis laws and the effects on prescription opioid painkiller hospitalization and mortality, but the positive correlations between medical cannabis access and decreases in prescription opioid painkiller hospitalizations increase over time (Powell et al., 2015; Shi, 2017). While the goal of establishment of medical cannabis policies is to “assist patients suffering from certain serious medical conditions” (Pennsylvania Department of Health, n.d.), there appears to be an unintended but positive effect of these policies on rates of mortality and hospitalization related to use of prescription opioid analgesics (Shi, 2017), although current research is not yet identified a conclusive relationship between these two phenomena (Hurd et al., 2015).

1.2.4.4 Barriers to clinical use of cannabinoid therapeutics

Cannabinoid-based therapeutics have shown efficacy in clinical studies for treatment of chronic and neuropathic pain, particularly as adjuvant therapies to opioid treatment (Whiting et al., 2016). Despite the potential of cannabinoids as clinical analgesics, there are limitations to their therapeutic use, including dependence (Degenhardt et al., 2013), tolerance (D’Souza et al., 2008), and adverse side effects (Hall, 2015; Whiting et al., 2016). Some studies have indicated that medical cannabis only produces moderate analgesic effects which are counteracted by the high potential for serious harm after treatment, although there is substantial disagreement about this interpretation (Campbell et al., 2001; Lynch and Campbell, 2011; Martín-Sánchez et al., 2009). A greater understanding of the mechanisms responsible for these adverse effects is important for the development of better cannabinoid-based therapies.

While cannabis presents a lower risk of development of dependence than other drugs of abuse, there is still a large risk for development of cannabis use disorder following prolonged use

(Budney and Moore, 2002). The Diagnostic and Statistical Manual of Mental Disorders identifies cannabis use disorder as "[a] problematic pattern of cannabis use leading to clinically significant impairment or distress" (American Psychiatric Association, 2013). Increases in cannabis use disorder have been observed in many states following establishment of medical cannabis programs (Cerdá et al., 2012; Hasin et al., 2017). However, the effects of medical cannabis programs on recreational use have yet to be established (Pacula et al., 2015; Wen et al., 2015), although an increased risk for recreational cannabis use and treatment referrals for cannabis dependence have been associated with the establishment of cannabis dispensaries (Wen et al., 2015). Other studies have indicated that medical cannabis programs are associated with fewer admissions to substance abuse treatment programs (Pacula et al., 2015), although patients who report cannabis use during establishment of chronic pain therapy demonstrate higher risks for adverse outcomes related to substance use (Shah et al., 2017).

Overall, there remains a critical need for comprehensive, long-duration clinical studies to better evaluate the potential efficacy of medical cannabis, both alone and in combination with prescription opioid analgesics. In particular, clinical studies of the efficacy of medical cannabis in the treatment of pain are lacking (Campbell et al., 2001). Broader, controlled, extended-duration (longer than six weeks) clinical studies are necessary to monitor the analgesic effects of medical cannabis, particularly as adoption of medical cannabis use increases.

1.2.5 Cannabinoid modulation of pain

Cannabinoid-mediated antinociception has both spinal and supraspinal components. The antinociceptive effects of cannabinoid agonists are exerted via activation of descending inhibitory analgesic pathways and ascending primary afferent nociceptors (Lichtman et al., 1996; Martin et al., 1998). Intracerebroventricular (i.c.v.) injection and microinjection into the posterior ventrolateral periaqueductal grey (PAG) of CP55,940 and Δ^9-THC produced significant antinociception in Sprague-Dawley rats assessed in the tail flick test (Lichtman et al., 1996).

CP55,940-induced antinociception was pertussis-toxin sensitive, suggesting that these effects were $G_{i/o}$ protein mediated. Further studies also identified the rostral ventromedial medulla (RVM) in cannabinoid-mediated antinociception (Martin et al., 1998), where microinjection of WIN55,212-2 and HU-210 led to significant inhibition of tail flick antinociception in rats that was reverse by administration of SR141716, a CB_1 antagonist. Both the PAG and RVM are part of the descending analgesic pathway to the dorsal horn of the spinal cord that suppress nociceptive signaling from peripheral nociceptors to the brain (Fields et al., 1991; Millan, 2002). Intrathecal (i.t.) injection of SR141716A (Rinaldi-Carmona et al., 1994) produces thermal hyperalgesia in mice (Richardson et al., 1997). Injection (i.t.) of levonantradol, a high efficacy cannabinoid agonist, also produces antinociception in rats (Yaksh, 1981). Spinal transection reduced but did not eliminate the antinociceptive effects of Δ^9-THC or CP55,940, suggesting that cannabinoids mediate pain in both spinal and supraspinal locations (Lichtman and Martin, 1991).

The effects of cannabinoid agonists are routinely measured by evaluating their tetrad effects (Little et al., 1988). The cannabinoid tetrad of behavioral tests measures spontaneous activity (via open field activity), analgesia (via tail flick or hot plate tests), hypothermia, and catalepsy (bar or ring test). Cannabimimetic activity of novel agonists is determined by modulation of behavioral responses in all four tests (Howlett, 2002). The tetrad tests demonstrate a substantial advantage in that the effects elicited by a single dose of drug on all four tests can be measured within a consistent time frame, which reduces the number of animals needed to determine drug effects and allows in-animal controls between different tests (Little et al., 1988). The antinociceptive effects of cannabinoids have been demonstrated in a wide range of preclinical models of acute (Lichtman and Martin, 1991; Morgan et al., 2014), chronic neuropathic (Deng et al., 2015; Fox et al., 2001; Kohli et al., 2010), and inflammatory (Vincent et al., 2016; Walker et al., 1999) pain.

1.3 Tolerance to cannabinoids

Prolonged administration of a GPCR agonist leads to reduced receptor response (Lohse et al., 1989), which is caused by desensitization and downregulation of receptors (Gainetdinov et al., 2004). Tolerance has been demonstrated in a wide variety of neuronal systems, including the cannabinoid system (Lefkowitz, 1980; Lichtman and Martin, 2005). Sustained administration of cannabinoid agonists leads to the development of tolerance to the effects of cannabinoid agonists, including their antinociceptive effects (Abood and Martin, 1992). This development of tolerance does not involve changes in the pharmacokinetics of cannabinoid agonists (Dewey, 1986). Chronic treatment with Δ^9-THC produces a reduction in CB_1 activity, gene expression, and behavioral responses to drug (Breivogel et al., 1999; Kittler et al., 2000; McMillan et al., 1971; Morgan et al., 2014; Sim-Selley and Martin, 2002). Chronic administration of Δ^9-THC in mice leads to reductions in WIN55,212-2-stimulated [^{35}S]GTPγS binding (Breivogel et al., 1999). This loss of CB_1 activity develops after three days of chronic Δ^9-THC administration and is correlated with reduced levels of CB_1 in the cerebellum, hippocampus, caudate-putamen, and globus pallidus. In addition, changes in gene expression are also observed following chronic Δ^9-THC treatment which affect genes involved in regulating metabolism, signal transduction, protein folding, and glial differentiation in the hippocampus (Kittler et al., 2000).

Cross-tolerance between cannabinoid agonists has been reported (Fan et al., 1994; Fride, 1995; L. Hruba et al., 2012; McMahon, 2011; Singh et al., 2011). Chronic administration of Δ^9-THC induces partial tolerance to the effects of WIN55,212-2 and CP55,940 in mice (Fan et al., 1994). Studies of discriminative stimulus effects of cannabinoid agonists in rhesus monkeys observed that chronic Δ^9-THC was able to reduce responding rates to a range of synthetic cannabinoid agonists, including CP55,940, WIN55,212-2, JWH-013, and JWH-073 (Lenka Hruba et al., 2012; McMahon, 2011). These studies concluded that cannabinoid agonist efficacy is inversely correlated with the potential to induce cross-tolerance to other cannabinoid

agonists. However, a different study found that chronic administration of Δ^9-THC did not alter the effects of CP55,940 on operant responses in mice (Singh et al., 2011), although these differences could be due to species-specific variations in cannabinoid discrimination.

1.3.1 Mechanisms of tolerance

1.3.1.1 Receptor Desensitization

Prolonged exposure to agonist activation leads to tolerance; receptor desensitization has been implicated as one possible underlying mechanism (Martin et al., 2004; Morgan et al., 2014). The classical model of tolerance is through receptor desensitization (Gainetdinov et al., 2004; Lefkowitz, 1998). After prolonged activation, GPCRs can become phosphorylated at serine or threonine residues by G protein-coupled receptor kinases (GRKs), typically in either the third intracellular loop or the C-terminal tail of the receptor (Bouvier et al., 1988; Eason and Liggett, 1993; Hoffman et al., 1994). Phosphorylation allows an inhibitory protein (β-arrestin1 (Lohse et al., 1990) or β-arrestin2 (Attramadal et al., 1992)) to bind the receptor, which uncouples the receptor from its cognate G proteins and leads to homologous, activation-dependent, receptor-specific desensitization marked by decreases in receptor-activated G proteins or other signal transduction effectors (Kovoor et al., 1997; Lohse et al., 1992; Pei et al., 1995; Pippig et al., 1993; Zhang et al., 1998; L. Zhang et al., 1996). This system facilitates internalization and recycling of re-sensitized receptors (Hsieh et al., 1999; J. Zhang et al., 1996). GRK-mediated phosphorylation has been implicated in the desensitization of adrenergic (Benovic et al., 1987; Lohse et al., 1992; Pippig et al., 1993), opioid (Appleyard et al., 1999; Bohn et al., 1999; Kovoor et al., 1997; Zhang et al., 1998), and cannabinoid receptors (Tanya L Daigle et al., 2008; Jin et al., 1999).

Disruption of this pathway alters nociceptive responses to opioid agonists. β-arrestin2 knockout mice demonstrate greater sensitivity and prolonged response to the antinociceptive effects of acute administration of morphine (Bohn et al., 1999). These mice are also slower to develop

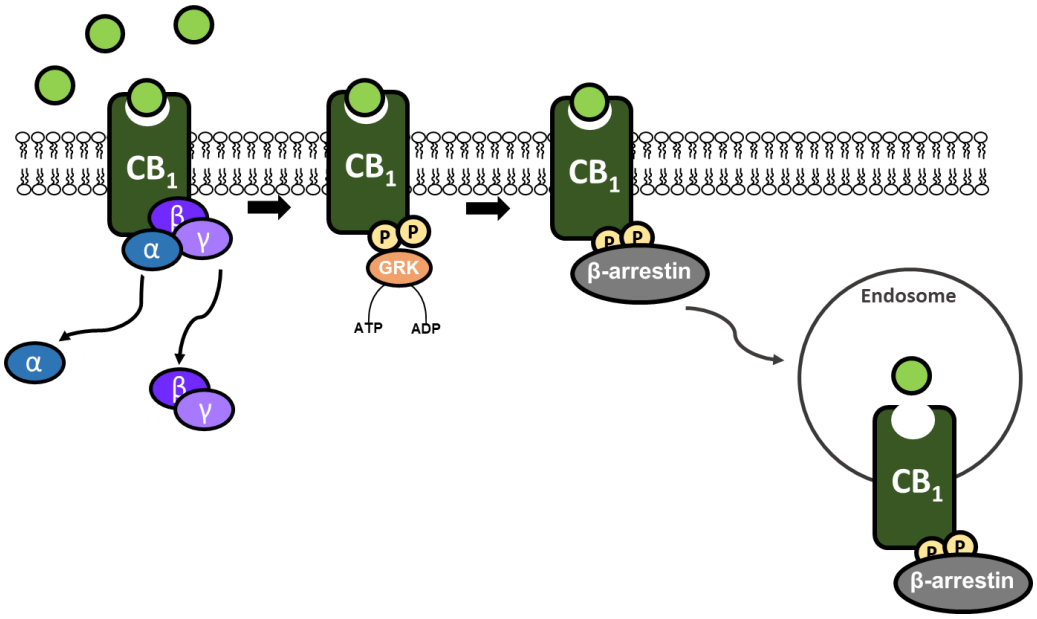

Figure 1.2. Role of GRK and β-arrestin2 in desensitization of CB_1 signaling. Activation of CB_1 leads to G protein-mediated signaling. However, sustained activation of CB_1 leads to phosphorylation of the receptor by GRK. This phosphorylation allows β-arrestin to bind to the receptor, where the arrestin protein blocks coupling of the G protein with the receptor, causing receptor desensitization. Desensitized CB_1 is endocytosed for either degradation or recycling to the plasma membrane.

tolerance to the antinociceptive effects of morphine in both the hot plate and tail flick tests but do not show alterations in morphine dependence (Bohn et al., 2002, 2000). GRK3 knockout mice also demonstrate attenuated antinociceptive tolerance following chronic administration of both morphine and fentanyl (Terman et al., 2004). Together, these studies demonstrate a clear role for GRK3/β-arrestin2-medidated desensitization of MOR in modulation of responsiveness of pain pathways in the CNS.

CB_1 desensitization was first reported in brains of rats chronically administered either Δ^9-THC or WIN55,212-2 (L J Sim et al., 1996). Reduced CB_1 activity was observed in brains of rats treated with chronic Δ^9-THC. However, acute Δ^9-THC administration did not affect WIN55,212-2-induced [^{35}S]-GTPγS binding, suggesting that the desensitization observed was a result of chronic Δ^9-THC administration. Other studies have also observed these effects of chronic Δ^9-THC administration on CB_1 desensitization (Breivogel et al., 2003, 1999). Chronic

administration of either WIN55,212-2 or CP55,940 has also been demonstrated to induce CB_1 desensitization in both tissue sections and brain homogenates (T Rubino et al., 2000; Selley et al., 2004; Sim-Selley and Martin, 2002) Interestingly, another study observed increases in CB_1 mRNA expression levels without any changes in [^{35}S]GTPγS binding after chronic Δ^9-THC administration, although this difference in receptor activity after chronic Δ^9-THC is likely a result of variations in Δ^9-THC treatment paradigms to induce tolerance .

1.3.1.2 Receptor downregulation

Downregulation of CB_1 is an important mechanism that contributes to the development of tolerance (Fan et al., 1996; Martini et al., 2007; T Rubino et al., 2000; T. Rubino et al., 2000; Sim-Selley, 2003; Tappe-Theodor et al., 2007). Chronic administration of either Δ^9-THC or CP55,940 leads to reduced binding of [^{3}H]CP55,940 to CB_1 by 50% or more in most regions of the rat brain (Oviedo et al., 1993). This decrease in [^{3}H]CP55,940 was driven by decrease binding affinity (K_d) in acutely treated animals and by decreases in levels of CB_1 in the brain (b_{max}) in animals that were chronically treated with either Δ^9-THC or CP55,940. Interestingly, chronic treatment with cannabidiol did not affect either CB_1 binding affinity or levels of CB_1 protein. Regional variations in CB_1 have been identified downregulation following chronic agonist treatment (De Fonseca et al., 1994; Fan et al., 1996; T. Rubino et al., 2000; Sim-Selley and Martin, 2002), where CB_1 downregulation shows regional variation which is consistent for different cannabinoid agonists (De Fonseca et al., 1994; Sim-Selley and Martin, 2002) or varies between cannabinoid agonists (Fan et al., 1996; T. Rubino et al., 2000; Wu et al., 2008).

1.3.1.3 GRK/β-arrestin2-mediated receptor desensitization and tolerance to cannabinoid agonists

The role of both GRKs and β-arrestin2 in receptor desensitization were initially established in related neuronal systems (Freedman and Lefkowitz, 1996); however, roles for both proteins in CB_1 desensitization and cannabinoid tolerance have been identified. Phosphorylation of CB_1 by

GRK leads to desensitization (Gainetdinov et al., 2004) and endocytosis (Wu et al., 2008). Expression of a dominant-negative form of GRK blocked WIN55,212-2-induced desensitization of inhibition of voltage-gated Ca^{+2} channels in hippocampal neurons (Kouznetsova et al., 2002). β-arrestin has also been found to be an essential component of the scaffolding necessary to bring the components of the extracellular signal-related kinase (ERK) signaling pathway into proximity to begin signaling (DeWire et al., 2008). Knockdown of β-arrestin1 attenuates CB_1-mediated ERK activation, while knockdown of β-arrestin2 does not change ERK signaling but significantly disrupts CB_1 internalization (Ahn et al., 2013; Delgado-Peraza et al., 2016; Flores-Otero et al., 2014). *In vivo* studies have found that mice lacking β-arrestin2 are more sensitive to the antinociceptive and hypothermic effects of Δ^9-THC (Breivogel et al., 2008). These mice do not show differences in CB_1 receptor density after Δ^9-THC treatment, but also did not vary in their responses to several other full- and partial- cannabinoid agonists, including CP55,940. β-arrestin2 knockout mice also demonstrate attenuated tolerance to the antinociceptive effects of Δ^9-THC after chronic treatment, along with reduced CB_1 desensitization and downregulation (Nguyen et al., 2012).

1.3.2 S426A/S430A Mutation and CB_1 desensitization and tolerance

After exposure to cannabinoid agonists, β-arrestin2 is rapidly recruited to the plasma membrane (T. Daigle et al., 2008). Agonist-bound CB_1 can be phosphorylated on the intracellular tail of CB_1 to provide high-affinity targets for subsequent interactions with an arrestin protein, such as β-arrestin2 (DeWire et al., 2008; Moore et al., 2007). A role for β-arrestin2 in CB_1 desensitization was identified in studies using β-arrestin2 overexpression in xenopus oocytes measuring modulation of GIRK activation (Jin et al., 1999) and expression of a dominant negative β-arrestin2 mutant in cultured hippocampal neurons, measuring modulation of voltage-gated Ca^{+2} channels (Kouznetsova et al., 2002). The intracellular tail of CB_1 contains two distinct β-arrestin binding sites, one at serine residues 426 and 430 which is responsible for

desensitization of G-protein signaling (Jin et al., 1999) and another at the extreme carboxy terminus of the receptor that mediates CB_1 internalization (Hsieh et al., 1999). Mutation of these putative phosphorylation sites (S426A/S430A) leads to decreased desensitization of CB_1 (T. Daigle et al., 2008; Delgado-Peraza et al., 2016; Hsieh et al., 1999; Jin et al., 1999; Morgan et al., 2014) but does not alter internalization of the receptor (Hsieh et al., 1999; Jin et al., 1999). Interestingly, the S426A/S430A mutant CB_1 receptor recruits β-arrestin2 with similar kinetics as wild-type CB_1, suggesting the residues involved in CB_1 desensitization are not required for β-arrestin recruitment (T. Daigle et al., 2008), however this may be masked by large amounts of β-arrestin2 which are recruited to the membrane for scaffolding functions as part of signaling that are much larger than the amounts of β-arrestin2 recruited for desensitization (Lohman et al., 2010; Shenoy and Lefkowitz, 2005). Cells expressing S426A/S430A mutant CB_1 show prolonged activation of ERK 1/2 (pERK) following exposure to cannabinoid agonists (T. Daigle et al., 2008), indicating that decreases in ERK1/2 observed after chronic administration of cannabinoid agonists (Derkinderen et al., 2003; Valjent et al., 2001) is likely a result of phosphorylation of CB_1 at these residues and subsequent disruption of CB_1 desensitization. Interestingly, the S426A/S430A mutant CB_1 receptor initiates different downstream signaling than wild-type CB_1. ERK1/2 activation in HEK 293 cells expressing wild-type CB_1 is abolished by exposure to pertussis toxin while ERK1/2 activation is eliminated by β-arrestin1 knockdown in cells expressing S426A/S430A mutant CB_1, indicating that ERK1/2 signaling via wild-type CB_1 is G protein mediated while the S426A/S430A mutant receptor induces signaling via β-arrestin1 (Delgado-Peraza et al., 2016). We have previously demonstrated that mice expressing this desensitization-resistant form of CB_1 are more sensitive to the acute effects of Δ^9-THC and are also slower to develop tolerance to its antinociceptive effects (Morgan et al., 2014). S426A/S430A mutant mice also develop greater dependence to Δ^9-THC, as demonstrated by increased precipitated-withdrawal behaviors following treatment with a CB_1 antagonist.

1.3.3 MAP kinases as a potential alternative signaling pathway

Signaling through CB_1 is G protein mediated, but other non-classical mechanisms have been proposed which may also contribute, including signaling through mitogen-activated protein kinases (MAPKs) (Bouaboula et al., 1995; Rinaldi-Carmona and Duigou, 1998). Signaling via c-Jun N-terminal kinase (JNK) has been shown to play a role in tolerance to opioid agonists (Melief et al., 2010). Pretreatment with a small molecule JNK inhibitor (SP600125) to disrupt activation of JNK signaling has been implicated in the development of tolerance to both the antinociceptive and antiallodynic effects of morphine via MOR (Hervera et al., 2012; Marcus et al., 2015; Melief et al., 2010; Yuill et al., 2017). Deletion of JNK2 eliminated desensitization of MOR-stimulated [^{35}S]-GTPγS binding. Chronic morphine treatment also led to increases in activated JNK (phospho-JNK) in both transfected cells and isolated rat embryonic cortical neurons (Cao et al., 2013; Kuhar et al., 2015; Melief et al., 2010). JNK has also been demonstrated to regulate inactivation of both the mu and kappa opioid and dopamine D2 receptors through regulation of palmitoylation of $G\alpha_i$ by peroxiredoxin 6 and subsequent regulation of tolerance to opioid agonists (Schattauer et al., 2017). Multiple studies have found that signaling via CB_1 leads to activation of JNK (Bosier et al., 2008; Delgado-Peraza et al., 2016; Downer et al., 2003; Rueda et al., 2000b). Activation of JNK by CB_1 may be mediated through β-arrestin1. Activation by WIN55,212-2 leads to JNK activation through both wild-type CB_1 and the S426A/S430A mutant CB_1 receptor that is β-arrestin1-dependent (Delgado-Peraza et al., 2016).

1.4 Biased agonism and functional selectivity

Cannabinoid receptor agonists, like many other GPCR agonists, are able to selectively activate different intracellular signaling responses, a concept known as biased agonism or functional selectivity (Bosier et al., 2010). Unlike traditional pharmacology concepts like intrinsic efficacy, where the effects of ligands are categorized by affinity of the ligand for the receptor and the efficacy of the ligand to activate a response through the receptor that produces a response

which is consistent wherever the receptor is expressed (KENAKIN, 1997; Urban et al., 2006), biased agonism posits that GPCRs have multiple potential active conformations which each have a distinct affinity for activation of secondary messengers (Hudson et al., 2010; Priestley et al., 2017; Urban et al., 2006). This allows distinct agonists to regulate multiple signaling pathways through the same receptor, a hallmark of GPCR signaling, through careful discrimination between activation of intracellular effector proteins (Diez-alarcia et al., 2016; Kenakin, 2007; Priestley et al., 2017). Biased agonism has been demonstrated in a range of neuronal systems, including the adrenergic (Hausdorff et al., 1991; Tran et al., 2004), opioid (Audet et al., 2012; Melief et al., 2010), and cannabinoid (Diez-alarcia et al., 2016; Laprairie et al., 2014) systems.

1.4.1 Biased agonism in cannabinoid signaling

Differences in CB_1 desensitization and downregulation may be driven by agonist biases (Flores-Otero et al., 2014; Wu et al., 2008). Cannabinoid agonists have been shown to induce agonist-specific receptor dwell times in clathrin-coated pits, where WIN55,212-2 induced short endocytic dwell times while binding to 2-AG produced significantly prolonged CB_1 dwell times (Flores-Otero et al., 2014). In this study, both agonists induced CB_1 desensitization at similar rates. The differences in endocytic pit dwell time may have important implications for the ability of major signaling proteins (G protein, β-arrestins, etc.) to scaffold and induce signaling and may provide a potential mechanism for how agonist biases develop. Another study also observed that rates of CB_1 internalization in rat cortical neurons are agonist-dependent, where CP55,940 and WIN55,212-2 induced much more rapid CB_1 internalization than Δ^9-THC (Wu et al., 2008).

The Wu study also observed that endocytic potential of a cannabinoid agonist was negatively correlated with the ability of an agonist to induce CB_1 desensitization (Wu et al., 2008). WIN55,212-2 and CP55,940 were found to cause CB_1 desensitization at a much slower rate than Δ^9-THC, likely as a result of faster receptor internalization and subsequent recycling and

recovery of desensitized receptors. Other studies in mice have observed that after cessation of drug administration, desensitized CB_1 receptors recover sensitivity at a faster rate following chronic administration of WIN55,212-2 than chronic administration of Δ^9-THC (Sim-selley et al., 2006). Both Δ^9-THC and WIN55,212-2 have been shown to induce agonist-specific regional patterns of CB_1 desensitization and downregulation in mouse brains (Sim-Selley and Martin, 2002).

There is ample evidence of agonist biases in activation of downstream signaling mediated by CB_1. Agonist-stimulated G protein activation has been used to assess agonist-specific differences (Selley et al., 1997; Laura J. Sim et al., 1996). Different CB_1 agonists have been shown to induce different levels of $G\alpha_{i/o}$ activation, where HU-210 produced maximal activation of both $G\alpha_i$ and $G\alpha_0$, WIN55,212-2 produced maximal activation of $G\alpha_i$ but not $G\alpha_o$, and Δ^9-THC did not fully activate either $G\alpha_i$ or $G\alpha_o$ (Glass and Northup, 1999). Another study observed that Δ^9-THC, WIN55,212-2, and Arachidonyl-2'-chloroethylamide (ACEA) each produced specific patterns of Gα subunit activation in the cortex in mice (Diez-alarcia et al., 2016).

Agonist biases were also observed in CB_1-induced cAMP activation following the development of tolerance, where chronic Δ^9-THC treatment increased both levels of cAMP and cAMP-dependent protein kinase A (PKA) activity in regions of the brain where CB_1 downregulation was observed (striatum, cortex, and cerebellum) (T Rubino et al., 2000). However, following chronic treatment with CP55,940 no changes in either cAMP levels or PKA activity were observed, despite downregulation of CB_1 in the striatum, cortex, and cerebellum (T. Rubino et al., 2000). Interestingly, in a study of acute cannabinoid agonist effects on CB_1 signaling in CHO cells, Khajehali *et. al* found that CP55,940 and WIN55,212-2 produced agonist-specific effects on cAMP inhibition, where CP55,940 binding biased CB_1 toward cAMP inhibition over phosphorylation of ERK1/2 while WIN55,212-2 treatment produced no bias for activation of either signaling pathway (Khajehali et al., 2015). The differences in these results may arise

from either the different experimental systems or duration of drug treatment, but these studies all identify an agonist-specific role of activation of downstream CB_1 signaling. Studies in striatal neurons demonstrated that Δ^9-THC and CP55,940 increase association of CB_1 and β-arrestin2 more than WIN55,212-2 (Laprairie et al., 2014). It has also been reported that WIN55,212-2 and CP55,940 differentially activate ERK1/2 phosphorylation and JNK activation (Bosier et al., 2008). Biases in CB_1 receptor endocytosis have also been reported. CP55,940 was found to induce CB_1 internalization while WIN55,212-2 did not (Atwood et al., 2012). In addition, 2-AG and CP55,940 have been shown to initiate induction of receptor endocytosis in an agonist-specific manner in cells expressing CB_2 (Shoemaker et al., 2005)

Agonist biases for signaling via CB_2 have also been reported. 2-AG and CP55,940 induce differential activation of G protein-mediated signaling pathways in cells expressing CB_2 (Shoemaker et al., 2005). Δ^9-THC and CP55,940 have also been shown to induce agonist-specific activation of downstream signaling through $G\alpha_i$ following CB_2 activation (Dhopeshwarkar and Mackie, 2016). That study also observed agonist biases in β-arrestin2 recruitment to CB_2.

1.4.2 *In vivo* studies of cannabinoid agonist biases

Agonist biases have also been observed using *in vivo* models to study the effects of cannabinoid agonists. Differences between exogenous cannabinoid agonists have also been reported. One study found that Wistar rats administered HU-210 and CP55,940 demonstrated significant catalepsy and hypolocomotion, but despite similar distributions of the two agonists in the brain, tyrosine hydroxylase expression in the striatum was only upregulated following treatment with HU-210, suggesting that cannabinoid agonists of similar efficacy and chemotype can produce different effects on protein expression in pathways that are responsible for mediating reward (Bosier et al., 2012). Studies of cannabinoid effects on brain stimulation and reward observed that administration of WIN55,212-2 and CP55,940 were able to restore heroin-

seeking behavior in rats following long-term extinction, while Δ^9-THC injection did not affect heroin-seeking responses (Fattore et al., 2003). Cannabinoid agonist biases were also observed in β-arrestin2 knockout mice (Breivogel et al., 2008). β-arrestin2 knockout mice demonstrated increased antinociceptive responses to acute administration of Δ^9-THC, but responses to CP55,940 did not vary between wild-type and β-arrestin2 knockout mice (Breivogel et al., 1999). A different study found that after 15 days of treatment with either Δ^9-THC or WIN55,212-2, levels of CB_1 desensitization were agonist-dependent (Sim-Selley and Martin, 2002). Chronic Δ^9-THC treatment produced significant increases in CB_1 desensitization compared to WIN55,212-2 treatment in the caudate-putamen, cortex, hippocampus, amygdala, and cerebellum. Interestingly, these changes in desensitization levels were not accompanied by equivalent differences in CB_1 downregulation. Agonist biases in tolerance development have also been reported. The antinociceptive effects of acute CP55,940 were attenuated in β-arrestin1 knockout mice, while the effects of acute administration of Δ^9-THC were not altered following β-arrestin1 knockout (Breivogel and Vaghela, 2015). Interestingly, no differences in the development of tolerance to either agonist were observed in β-arrestin1 knockout mice.

1.5 Aims

There is good evidence to support a role for GRK/β-arrestin2-mediated desensitization as a potential mechanism for the development of tolerance to cannabinoid agonists (Morgan et al., 2014; Nguyen et al., 2012). However, whether biased agonism in the activation of this pathway plays a role in development of cannabinoid tolerance has not been widely investigated. Therefore, we utilized models of acute nociceptive pain (tail-flick, formalin test) and chronic neuropathic pain to investigate biased agonism in the development of tolerance to the synthetic, high-potency, full cannabinoid agonists WIN55,212-2 and CP55,940 in S426A/S430A mutant mice resistant to GRK/β-arrestin2-mediated desensitization of CB_1. There are a range of studies which focus on agonist biases that report contradictory findings (Diez-alarcia et al.,

2016), and therefore we aimed to study tolerance to the effects of WIN55,212-2 and CP55,940 under the same experimental conditions.

The overall aim of the studies included in this dissertation is to better understand the mechanisms responsible for the development to the effects of different cannabinoid agonists on pain, with the goal of further elucidating the agonist-specific features of tolerance. Our overall hypothesis is that synthetic, high-potency cannabinoid agonists can produce tolerance their behavioral effects through specific (and different) mechanisms. Specifically, we addressed the following hypotheses: 1) GRK/β-arrestin2-mediated desensitization of CB_1 is responsible for the development of antinociceptive tolerance to synthetic cannabinoid agonists. 2) Tolerance to synthetic cannabinoid agonists is mediated by similar mechanisms in different types of pain. 3) JNK signaling contributes to the development of antinociceptive tolerance to the effects of some cannabinoid agonists.

Chapter 2

Materials and Methods

2.1 Subjects and Drugs for Behavioral Assays

2.1.1 Ethics Statement

All animal experiments and animal care procedures were conducted with approval from the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (IACUC; protocol 46334) All animal care procedures were carried out in accordance with the Guidelines of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 2011).

2.1.2 Subjects

Male wild-type and S426A/S430A mutant mice on a C57BL/6J background were obtained from an in-house breeding colony. Experimentally naïve 8-12-week-old littermate-controlled mice were used for all studies. The generation of S426A/S430A mutant mice has been previously described (Morgan et al., 2014). After weaning, mice were group housed on a standard 12:12h light/dark cycle (lights on at 07:00, lights off at 19:00). Mice were provided with *ad libitum* access to water and standard rodent chow (Teklad 2018, Envigo, Indianapolis, IN). All testing conditions were optimized to minimize environmental effects on responses in behavioral tests.

2.1.3 Drugs

Δ^9-THC was obtained from the National Institute of Drug Abuse (NIDA) Drug Supply (Bethesda, MD). WIN55,212-2, CP55,940, and SP600125 were purchased from Cayman Chemical (Ann Arbor, MI). Cisplatin was purchased from Tocris Bioscience (Minneapolis, MN). Formaldehyde and sodium bicarbonate were purchased from Fisher Scientific (Pittsburgh, PA).

2.1.4 Drug preparation and administration

Control (vehicle) mice were administered the appropriate vehicle for the drug of interest in each experiment. Mice were treated with either vehicle containing 0.9% sterile saline and 5% Cremaphor EL (Sigma-Aldrich, St Louis, MO) (18:1 vehicle v/v) or vehicle containing 0.9% sterile saline, 5% Cremaphor EL, and 5% ethanol (18:1:1 vehicle v/v). To account for the behavioral effects of ethanol, drugs which were not prepared in solution containing ethanol were prepared using 18:1:1 vehicle. CP55,940 and Δ^9-THC were prepared in ethanol and subsequently diluted 20-fold in 18:1 vehicle. When necessary, additional ethanol was added to the injection preparation to achieve a final concentration of 5% ethanol. WIN55,212-2 and SP600125 were prepared in DMSO and subsequently diluted in 18:1:1 vehicle and ethanol. The total amount of DMSO contained in all WIN55,212-2 injections (and corresponding vehicle injections) was 4% v/v. Δ^9-THC, CP55,940, WIN55,212-2, and SP600125 were all administered via intraperitoneal injection (i.p.) in a single volume of 10 ml/kg of body weight.

Aqueous formalin solution (2.5% v/v) was prepared by diluting one volume of formaldehyde (37%) in 15 parts of water. Formalin was administered in a 10uL volume into the plantar surface of a single hind paw using a 0.5 mL syringe with a 28½ gauge needle (Becton Dickson, Franklin Lakes, NJ).

Sodium bicarbonate and cisplatin were dissolved in 0.9% sterile saline for administration. Cisplatin (5 mg/kg) was administered via i.p. injection in a single volume of 10 mg/kg body weight. To prevent renal damage as a result of cisplatin treatment, mice were also administered 1 mL sodium bicarbonate (4%) via subcutaneous (s.c.) injection. Mice were administered cisplatin i.p. and immediately administered sodium bicarbonate s.c. (Guindon et al., 2014).

2.2 Behavioral Assays

2.2.1 Tail Flick

The tail flick test measures reflexive pain responses which are primarily spinally mediated. Tail flick antinociception was measured using a Columbus Instruments TF-1 tail flick analgesia meter (Columbus, OH). The radiant heat source (light bulb) was set to an intensity level of five. A test duration cutoff was observed to avoid tissue damage. Mice were allowed to remain in the test for up to six seconds for baseline measurements in untreated animals or ten seconds in drug treated animals. Mice were assayed for tail flick responses immediately prior to drug administration to determine baseline responses and one hour following all administrations of drug. The maximal percent efficacy (%MPE) was calculated from the pre- and post-drug responses, where %MPE = [(post-drug latency – pre-drug latency) / (10 – pre-drug latency)] * 100.

2.2.2 Hot Plate

The hot plate assay measures thermal nociceptive pain responses which are mediated through both spinal and supraspinal mechanisms. Mice were assayed for antinociception using a Columbus Instruments hot plate analgesia meter set to 55°C (Columbus, OH). A test duration cutoff was again observed to limit any potential tissue damage. Mice were allowed to remain in the test for up to 15 seconds for baseline measurements in untreated animals or 30 seconds in animals administered drug. Similar to the tail flick test, mice were assayed for nociceptive responses immediately prior to and one hour following each drug administration. The %MPE of drug was calculated as previously described for the tail flick test (sec. 2.2.1), where %MPE = [(post-drug latency – pre-drug latency) / (30 – pre-drug latency)].

2.2.3 Measures of hypothermia

Hypothermia was measured by determining body temperature with a mouse rectal thermometer (RET-1 probe, Physitemp Instruments, Clifton, NJ). Body temperature measurements were recorded immediately prior to the initial administration of drug and one hour following each

administration of drug. Mice were also assayed for body temperature for up to five hours following drug administration. Temperature values were converted to % change in body temperature, where % change = [(post-injection temp − pre-injection temp) / (pre-injection temp)] * 100.

2.2.4 Catalepsy

Catalepsy, a measure of behavioral immobility following drug administration, was determined using the bar test. Mice were placed in a chamber containing a metal rod 0.5 cm in diameter elevated 4 cm above the bottom of the chamber. Mice were placed with their front paws resting on the bar and hind paws on the floor of the chamber. The total amount of time (s) spent motionless with at least one front paw on the raised bar was recorded during the one-minute test period. Catalepsy was assayed one hour following each drug administration. The %MPE of drug was calculated as previously described for the tail flick test (sec. 2.2.1), where %MPE = [(post-drug catalepsy – pre-drug catalepsy) / (60 – pre-drug catalepsy).

2.1.9 Formalin Test

Inflammatory pain responses were assessed using the formalin test, which produces a characteristic, transient biphasic pattern of pain behaviors (Tjølsen et al., 1992). Mice were administered drug or appropriate vehicle and assayed for pain.

Mice were acclimated in a Plexiglass chamber (5”x5”x5”) on an elevated transparent platform for 20 minutes prior to testing. Prior to and during testing, mice were observed using a mirror underneath the platform placed at a 45° angle to allow constant observation of the paws of the mouse. In addition, mice were recorded using a high-definition camera (Logitech, Newark, CA) placed underneath the platform. Following acclimation, mice were administered formalin (2.5%) via intraplantar injection (Section 2.1.4) and returned to the observation chamber to begin behavioral recording immediately following formalin administration.

Nociceptive behavior was measured for 60 min and quantified within twelve 5 min time bins throughout that observation period. Quantified behaviors included the three following pain responses: the injected paw has little weight placed on it (0); the injected paw is held above the surface of the platform (1); or the injected paw is bitten, shook, or licked (2). The amount of time the mouse spent engaged in each category was recorded and weighted using the composite pain score-weighted scores technique (CPS-WST_{0-2}), which produced composite pain scores (CPS) between 0 (no pain behavior) and 2 (continuous pain behavior) for each 5 min bin (Guindon et al., 2011; Marcus et al., 2015; Watson et al., 1997). The area under the curve (AUC) was calculated for the acute phase (0-15 min; Phase 1) and the inflammatory phase (15-60min, Phase II).

2.2.5 Chronic Neuropathic Pain

Neuropathy was induced following treatment with cisplatin, a common chemotherapeutic agent, and was measured by assaying mice for mechanical allodynia using an electronic von Frey anesthesiometer equipped with a semi-flexible polypropylene super-tip (IITC Life Science Inc, Woodland Hills, CA). Prior to cisplatin treatment, mice were administered the appropriate vehicle and assayed in the von Frey test to determine baseline allodynic responses. One week following the final cisplatin treatment, mice were again assayed for allodynic responses to confirm persistent chronic pain states. Mice were administered drug every day during the testing period and were assayed for antiallodynic effects of drug on alternating days (1, 3, 5, 7, etc.). Allodynia was assayed on alternating days of drug administration to minimize any potential learning behaviors.

2.3 Measurements of Tolerance

2.3.1 Dose-Response Shifts

Male wild-type and S426A/S430A mutant mice were assessed for antinociceptive (hot plate and tail flick), hypothermic, and cataleptic responses to vehicle and cumulative doses (Falenski et al., 2010) of 0.3, 1, 3, 10, and 30 mg/kg WIN55,212-2 or 0.01, 0.03, 0.1, 0.3, and 1 mg/kg CP55,940, or 1, 3, 10, 30, and 100 mg/kg Δ^9-THC. Following baseline assessments of nociceptive responses, all vehicle and drug measurements were taken one hour apart, or 60 minutes following each injection. Mice were administered 0.3, 0.7, 2, 7, and 20 mg/kg WIN55,212-2, 0.01, 0.02, 0.07, 0.2, and 0.7 mg/kg CP55,940, or 1, 2, 7, 20, and 70 mg/kg Δ^9-THC to complete cumulative dosing.

Following assessment of cumulative dose responses in drug naïve mice, tolerance to the antinociceptive, hypothermic, and cataleptic effects of WIN55,212-2 and CP55,940 was induced by administering either 10 mg/kg WIN55,212-22, 0.3 mg/kg CP55,940, or 30 mg/kg Δ^9-THC (i.p.) once daily for six consecutive days. A second (post) cumulative dose response was performed on the seventh day as described above.

2.3.2 Daily Tolerance to Cannabinoid Agonists

Tolerance to the antinociceptive (hot plate, tail flick), hypothermic, and cataleptic effects of daily injections of WIN55,212-2 and CP55,940 was assessed. Wild-type and S426A/S430A mutant mice were injected once daily (i.p.) with either 10 mg/kg WIN55,212-2 for 20 consecutive days or 0.3 mg/g CP55,940 for 15 consecutive days. Tail flick and hot plate antinociception, catalepsy, and hypothermia were measured each day prior to and 60 minutes following drug administration and %MPE was calculated as described above (Section 2.2).

2.3.3 Assessing Tolerance in a Model of Inflammatory Pain

Tolerance to the effects of once-daily injections of 3 mg/kg WIN55,212-2 and 0.3 mg/kg CP55,940 were assessed in male wild-type and S426A/S430A mutant mice. Mice were administered drug via i.p. injection once daily for prior to assessment in the formalin test on the

final day of the dosing period (e.g. 6 days of chronic administration and assessment of pain behaviors in the formalin test one hour following drug administration on day 7). Mice were assessed for inflammatory pain response following 1, 7, 14, and 21 consecutive days of administration of 3 mg/kg WIN55,212-2 and following 7, 14, and 21 consecutive days of administration of 0.3 mg/kg CP55,940. Mice were not assessed for inflammatory pain responses to 0.3 mg/kg CP55,940 on the first day of drug administration due to excessive catalepsy that made it impossible to assess pain behavior.

Sensitivity to WIN55,212-2 in the formalin test was assessed in a dose response. Drug naïve mice were administered 2, 2.5, 3, or 10 mg/kg WIN55,212-2 one hour prior to formalin (2.5%) administration.

2.3.4 von Frey in Chronic Neuropathic Pain Model

Tolerance to the effects of once daily treatment with 3 mg/kg WIN55,212-2 or 0.3 mg/kg CP55,940 was assessed in male wild-type and S426A/S430A mice. Mice were administered drug via i.p injection once daily for up to 25 (WIN55,212-2) or 19 (CP55,940) days. Mice were assayed for allodynic responses on alternating days (days 1, 3, 5, 7, etc.).

2.3.5 Data Analysis

Data were analyzed using Prism 5 (GraphPad, La Jolla, CA). All analyses involving formalin testing were run as two-way between measures analysis of variance (ANOVAs) with genotype as one factor and day of treatment/number of injections as the second factor. The once-daily tolerance studies and daily cisplatin studies were assessed as two-way mixed ANOVAs where genotype was the between subjects factor and time/number of injections served as the repeated measure. For all dose response curves, both a between subjects and mixed ANOVA were run to determine differences in (1) acute sensitivity to each drug dose as a function of genotype (with dose and genotype serving as the between subjects factors) and (2) differences in

sensitivity as a function of prolonged drug treatment (with time as the between subjects factor and dose as the within subjects factor). Additionally, nonlinear regression analyses were performed for each dose response curve to calculate ED_{50} values which were used to assess shifts in dose response curves. For all tests, Bonferroni post hoc analyses were performed where appropriate, and for all analyses, significance was set at $p<0.05$.

Chapter 3

Inhibition of GRK/β-arrestin2-mediated CB_1 desensitization produces cannabinoid agonist-specific effects on development of tolerance.

Adapted from: **Nealon, CM**; Hale, DE; Henderson-Redmond, AN; Morgan, DJ. Tolerance to WIN55,212-2 but not CP55,940 is significantly delayed in desensitization-resistant S426A/S430A mice. Submitted to *Neuropharmacology* Feb 2018.

3.1 Rationale

There is good evidence to support a role for GRK/β-arrestin2-mediated desensitization as a potential mechanism for the development of tolerance to cannabinoid agonists (Morgan et al., 2014; Nguyen et al., 2012). Studies of acute cannabinoid sensitivity in β-arrestin2 knockout mice observed agonist-specific responses, where β-arrestin2 knockout mice were more sensitive to Δ^9-THC but not to CP55,940 (Breivogel et al., 2008). We have reported a similar response in S426A/S430A mutant mice, finding elevated sensitivity to the acute effects of Δ^9-THC and attenuated antinociceptive tolerance following chronic administration of Δ^9-THC (Morgan et al., 2014). However, studies of cannabinoid tolerance in these mice only investigated tolerance to Δ^9-THC (Nguyen et al., 2012). Whether biased agonism at this pathway plays a role in cannabinoid tolerance has not been widely investigated. In addition, we observed that mice expressing the S426A/S430A mutant CB_1 receptor demonstrate decreased desensitization in the hippocampus, spinal cord, and PAG compared to wild-type mice after chronic administration of Δ^9-THC (Morgan et al., 2014). Agonist-specific differences in CB_1 desensitization have previously been reported, where treatment with Δ^9-THC induces greater desensitization of CB_1 than either WIN55,212-2 or CP55,940 (Fan et al., 1996).

Therefore, we utilized models of acute nociceptive pain (hot plate, tail flick, formalin test), hypothermia, catalepsy, and chronic neuropathic pain to investigate agonist differences in the development of tolerance to the synthetic, high-potency, full cannabinoid agonists WIN55,212-2

and CP55,940 in S426A/S430A mutant mice resistant to GRK/β-arrestin2-mediated desensitization of CB_1.

We chose to assess WIN55,212-2 and CP55,940 in four different pain assays in order to determine whether CB_1 desensitization contributes to tolerance to these drugs for the treatment of different types of pain (thermal nociceptive, inflammatory, and chronic neuropathic). Assessment of the effects of disruption of GRK/β-arrestin2-mediated CB_1 desensitization in multiple pain models provides a comprehensive evaluation of the role of this pathway in pain and is necessary to identify potential agonist biases by eliminating the potential for region-specific differences in cannabinoid signaling.

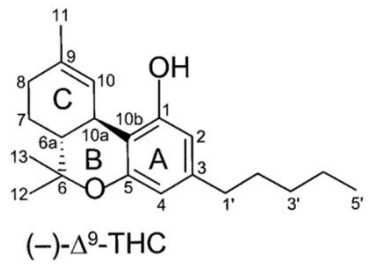

Figure 3.1. Structure of Δ^9-THC. Adapted from (Howlett, 2002).

3.2 Δ^9-THC

3.2.1 Shifts in Δ^9-THC dose response curves

3.2.1.1 Hot plate antinociceptive tolerance

In order to determine the effects of chronic administration of 30 mg/kg Δ^9-THC on antinociceptive responses in the hot plate assay, wild-type and S426A/S430A mutant mice were tested for Δ^9-THC-induced nociception. No significant differences in either baseline latencies or latencies after saline treatment were observed. Acute Δ^9-THC treatment (0, 1, 3, 10, 30, and 100 mg/kg) produced dose-dependent antinociception in the hot plate test ($F_{4,92}$=45.98, p<0.0001; Figure 3.1 A). Wild type and S426A/S430A mutant mice demonstrated a main effect of genotype on antinociceptive effects of Δ^9-THC in the hot plate test ($F_{1,23}$=2.47, p<0.0001), however a significant interaction (genotype x dose) was not observed

	Δ^9-THC Hot Plate	
	WT	S426A/S430A
Pre drug	14.71 (9.018-24.00)	8.847 (5.880-13.31
Post drug	299.9 (130.7-687.8)	333.2 (140.1-800.4)
F statistic	57.95 (p<0.0001)	113.2 (p<0.0001)

Table 3.1. Calculated ED50 values (mg/kg) from hot plate tests following treatment with Δ^9-THC. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-14 mice were tested for each group. Data were analyzed using F tests.

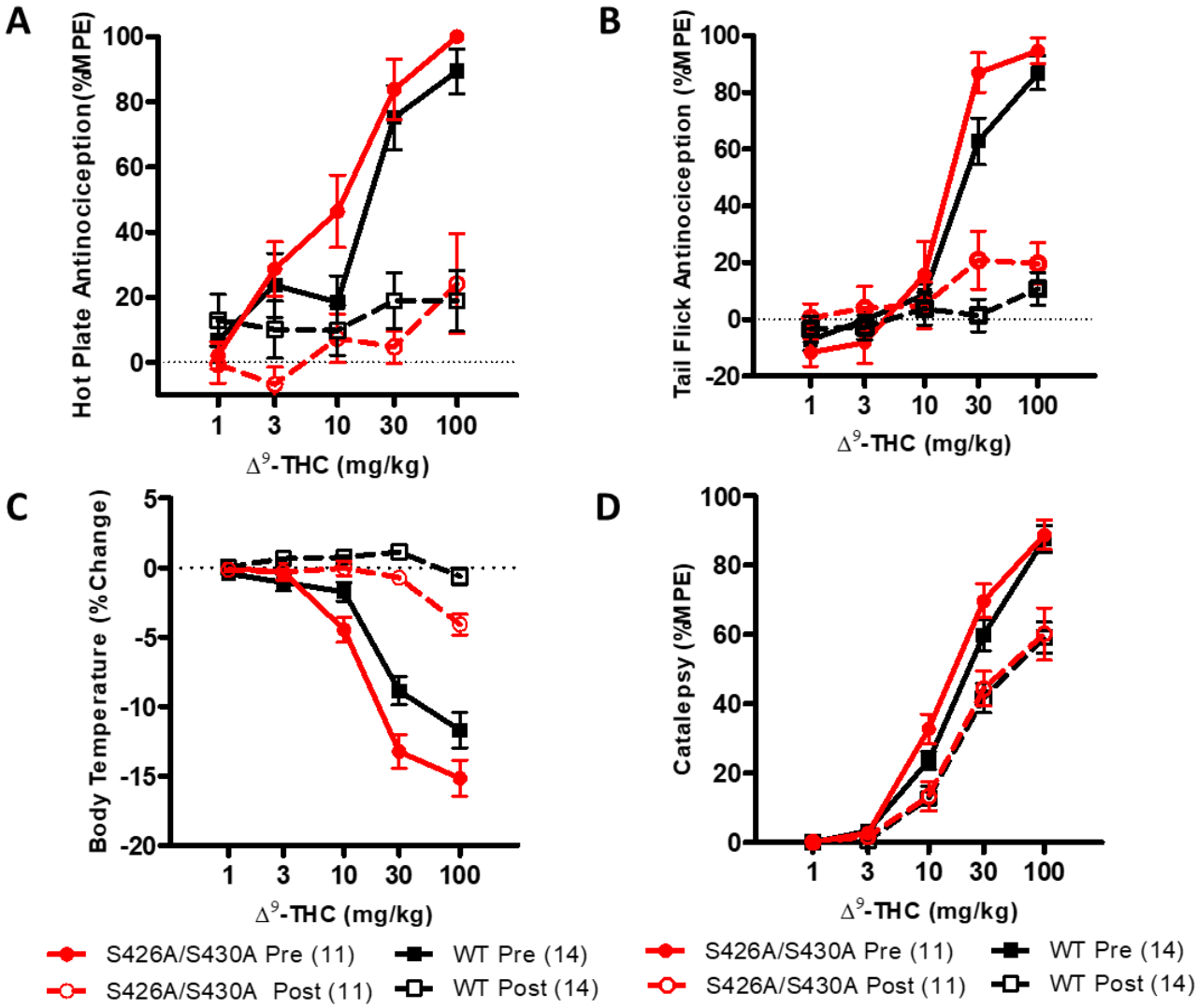

Figure 3.1. Wild-type and S426A/S430A mutant mice demonstrate significant tolerance to the antinociceptive and hypothermic effects of Δ^9-THC following chronic treatment. Wild-type (WT) and S426A/S430A mutant mice were tested for antinociceptive, hypothermic, and cataleptic responses across a cumulative dose range of 1, 3, 10, 30, and 100 mg/kg Δ^9-THC as drug naïve (Pre; filled symbols and solid lines) or following six days of once-daily treatment with either 30 mg/kg Δ^9-THC (Post; open symbols and dashed lines). The mean represents percent maximal possible effect (%MPE) and error bars indicate the standard error of the mean (SEM). The number of mice in each group are indicated in parenthesis. Lines represent the distance between two points.

(p=0.3512, suggesting that S426A/S430A mutant mice are not more sensitive than wild-type mice to the acute antinociceptive effects of Δ^9-THC in the hot plate test. S426A/S430A mice demonstrated greater response to the antinociceptive effects of acutely administered Δ^9-THC,

indicated by a leftward shift in the dose response curve and a significantly lower calculated ED_{50} than the calculated ED_{50} value for wild type mice ($F_{1,121}$=5.471, p=0.0210; Table 3.1).

Both wild-type and S426A/S430A mutant mice developed significant tolerance to the antinociceptive effects of Δ^9-THC in the hot plate test after six consecutive days of drug administration (30 mg/kg) (Figure 3.1 A). Wild-type mice ($F_{1,26}$=13.00, p=0.0014) and S426A/S430A mutant mice ($F_{1,20}$=49.84, p<0.0001) demonstrated markedly reduced responses to the antinociceptive effects of Δ^9-THC following six days of administration. Tolerance to the antinociceptive effects of Δ^9-THC in the hot plate test was observed for mice of both genotypes, as a significant dose x time interaction effect was observed for both wild-type ($F_{4,104}$=10.97, p<0.0001) and S426A/S430A mutant mice ($F_{4,80}$=21.54, p<0.0001). Post hoc analyses revealed that these interaction effects were driven by differences in antinociceptive responses at 30 and 100 mg/kg in wild-type mice (30, p<0.001; 100, p<0.001) and at 3 (p<0.05), 10 (p<0.01), 30 (p<0.001), and 100 mg/kg (p<0.001) in S426A/S430A mutant mice. Both wild-type ($F_{1,134}$=57.95, p<0.0001) and S426A/S430A mutant mice ($F_{1,108}$=113.2, p<0.0001) demonstrated a significant increase in calculated ED_{50} values after six days of once-daily treatment with 30 mg/kg Δ^9-THC (Table 3.1), suggesting that tolerance develops to the antinociceptive effects of Δ^9-THC in both genotypes. Unlike the trend observed in drug-naïve mice, calculated ED_{50} values for tolerant wild-type and S426A/S430A mutant mice were not significantly different (p=0.1468).

3.2.1.2 Tail flick antinociceptive tolerance

Tolerance to Δ^9-THC-induced antinociception was also assessed in wild-type and S426A/S430A mutant mice using the tail flick assay. Acute Δ^9-THC treatment (1, 3, 10, 30, 100 mg/kg) dose-dependently increased Δ^9-THC-induced antinociceptive responses in the tail-

flick test ($F_{4,92}$=114.19, p<0.0001; Figure 3.1 B). Wild-type and S426A/S430A mutant mice did not differ in their acute responses to Δ^9-THC, as neither a main effect of genotype (wild type vs S426A/S430A mutant mice) (p=0.2573) nor a genotype x dose interaction effect (p=0.0888) were observed in acute Δ^9-THC dose-responses. Likewise, the calculated ED_{50} values for Δ^9-THC in wild-type and S426A/S430A mutant mice were not significantly different (p=0.0871).

	Δ^9-THC Tail Flick	
	WT	S426A/S430A
Pre drug	29.71 (14.83-59.52)	19.02 (1157-31.25)
Post drug	106.5 (0.03120-363374)	296.5 (140.4-626.3)
F statistic	0.07168 (p=0.7893)	56.88 (p<0.0001)

Table 3.2. Calculated ED50 values (mg/kg) from tail flick tests following treatment with Δ^9-THC. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-14 mice were tested for each group. Data were analyzed using F tests.

Similar to the results obtained from the hot plate test, all mice developed significant tolerance to the antinociceptive effects of Δ^9-THC following six consecutive days of treatment. Wild-type ($F_{1,26}$=38.23, p<0.0001) and S426A/S430A mutant mice ($F_{1,20}$=11.82, p=0.0026) demonstrated significantly attenuated responses to the antinociceptive effects of Δ^9-THC after daily administration in the tail flick test (Figure 3.1 B). Wild-type ($F_{4,104}$=30.43, p<0.0001) and S426A/S430A mutant mice ($F_{14,80}$=22.87, p<0.0001) showed significant tolerance following chronic administration of Δ^9-THC as mice of both genotypes demonstrated a significant interaction effect (dose x time) in antinociceptive responses in post drug (chronically treated) mice. Post hoc analyses revealed that this interaction was due to differences in responses to 30 (wild-type, p<0.001; S426A/S430A, p<0.001) and 100 mg/kg (wild-type, p<0.001; S426A/S430A, p<0.001) cumulative doses of Δ^9-THC. Consistent with this finding, calculated ED_{50} values for S426A/S430A mutant mice were significantly increased following six days of daily administration of 30 mg/kg Δ^9-THC ($F_{1,108}$=56.88, p<0.0001; Table 3.2). In contrast, calculated ED_{50} values for wild-type mice following Δ^9-THC treatment were not significantly different from those calculated in drug-naïve mice (p=0.7893). This unexpected result is likely driven by the substantial error in the

calculation of the post-drug ED_{50} values based on a sigmoidal curve from a dose-response curve in fully tolerant wild-type mice that is almost linear. Therefore, this observation may not represent a reliable estimation of tolerance to the antinociceptive effects of Δ^9-THC in wild-type mice. Overall, both wild-type and S426A/S430A mutant mice demonstrated significant tolerance to the antinociceptive effects of Δ^9-THC in the tail flick test following six days of chronic administration of drug.

3.2.1.3 Hypothermic tolerance

Tolerance to the hypothermic effects of Δ^9-THC was also assessed following acute administration of Δ^9-THC and again after six days of daily administration of 30 mg/kg Δ^9-THC. Dose-dependent hypothermia developed in wild-type and S426A/S430A mutant mice following acute Δ^9-THC administration (1, 3, 10, 30, 100 mg/kg) ($F_{4,92}$=139.42, p<0.0001; Figure 3.1 C). A main effect of genotype on hypothermic responses to acute Δ^9-THC administration was observed ($F_{1,23}$=139.42, p=0.0379), as S426A/S430A mutant mice demonstrated greater decreases in body temperature after acute administration of Δ^9-THC. Wild-type and S426A/S430A mutant mice differed significantly in their pre-chronic dosing dose responses ($F_{4,92}$=4.99, p=0.0011), which was driven by differences in the hypothermic responses to 30 and 100 mg/kg Δ^9-THC in wild-type mice (30 and 100 mg/kg, p<0.001) and 10, 30, and 100 mg/kg Δ^9-THC in S426A/S430A mutant mice (10, 30, and 100 mg/kg, p<0.001). However, while calculated ED_{50} values for wild-type mice were larger than those for S426A/S430A mutant mice, the calculated ED_{50} values were not significantly different (p=0.0869).

	Δ^9-THC Hypothermia	
	WT	S426A/S430A
Pre drug	21.34 (15.21-29.95)	14.24 (10.59-19.15
Post drug	Not Converged	34.68 (very wide)
F statistic	--------	8.215 (p=0.0050)

Table 3.3. Calculated ED50 values (mg/kg) from body temperature measurements following treatment with Δ^9-THC. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-14 mice were tested for each group. Data were analyzed using F tests.

Robust tolerance to the hypothermic effects of Δ^9-THC was observed following six days of administration of 30 mg/kg Δ^9-THC in both wild-type ($F_{1,26}$=38.23, p<0.0001) and S426A/S430A mutant mice ($F_{1,20}$=68.07, p<0.0001). A genotype x time interaction was also observed for mice of both genotypes (wild-type, $F_{4,104}$=30.43, p<0.0001; S426A/S430A $F_{4,80}$=43.41, p<0.0001), indicating that both wild-type and S426A/S430A mutant mice develop significant tolerance to the hypothermic effects of Δ^9-THC following six days of chronic administration of drug. S426A/S430A mutant mice demonstrated a significant shift in calculated ED_{50} values after six consecutive days of 30 mg/kg Δ^9-THC ($F_{1,102}$=8.215, p=0.0050; Table 3.3). However, since wild-type mice did not demonstrate any sensitivity to the hypothermic effects of Δ^9-THC following six consecutive days of treatment, a post drug ED_{50} could not be calculated for wild-type mice. Taken together, these results suggest that robust tolerance develops to the hypothermic effects of Δ^9-THC in both wild-type and S426A/S430A mutant mice, although this tolerance is slightly reduced in S426A/S430A mutant mice compared to wild-type mice.

3.2.1.4 Cataleptic tolerance

In order to determine how tolerance develops to the cataleptic effects of Δ^9-THC following chronic treatment, wild-type and S426A/S430A mutant mice were assessed for cataleptic responses to Δ^9-THC following both acute administration of a cumulative dose-response and in a second cumulative dose response after six days of chronic treatment with 30 mg/kg Δ^9-THC. Acute treatment with Δ^9-THC produced dose-dependent catalepsy in wild-type and S426A/S430A mutant mice ($F_{4,92}$=342.10, p<0.0001; Figure 3.1 D). Mice of both genotypes demonstrated similar cataleptic responses following acute treatment with Δ^9-THC,

	Δ^9-THC Catalepsy	
	WT	S426A/S430A
Pre drug	23.10 (20.36-26.21)	17.39 (14.94-20.75)
Post drug	57.60 (46.81-70.86)	53.22 (40.02-70.77)
F statistic	63.45 (p<0.0001)	55.78 (p<0.0001)

Table 3.4. Calculated ED50 values (mg/kg) from catalepsy tests following treatment with Δ^9-THC. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-14 mice were tested for each group. Data were analyzed using F tests.

as no significant effects of genotype (p=0.1378), or interaction (genotype x dose) (p=0.1378), or a difference in calculated ED_{50} values (p=0.0820; Table 3.4) were observed.

Wild-type ($F_{1,26}$=27.75, p<0.0001) and S426A/S430A mutant mice ($F_{1,20}$=18.45, p=0.0004; Figure 3.1 D) demonstrated altered cataleptic responses to Δ^9-THC following chronic treatment. Daily administration of Δ^9-THC caused tolerance to the cataleptic effects of Δ^9-THC, as indicated by a significant rightward shift in dose responses and an interaction effect (dose x time) in both wild-type ($F_{4,104}$=8.01, p<0.0001) and S426A/S430A mutant mice ($F_{4,80}$=7.14, p<0.0001). Post-hoc analyses indicated that this interaction was driven by shifts in cataleptic responses to 10 (S426A/S430A, p<0.01), 30 (wild-type and S426A/S430A, p<0.001), and 100 mg/kg (wild-type and S426A/S430A, p<0.001) of Δ^9-THC. Interestingly, while the calculated ED_{50} values for cataleptic responses to Δ^9-THC in both wild-type ($F_{1,134}$=63.45, p<0.0001) and S426A/S430A mutant mice ($F_{1,108}$=55.78, p<0.0001) were significantly different from the ED_{50} values calculated in drug naïve mice (Table 3.4), the post-drug ED_{50} values for both genotypes were not significantly different form each other (p=0.0820), suggesting that tolerance to the cataleptic effects of Δ^9-THC develops at the same rate in both wild-type and S426A/S430A mice.

3.2.2 Discussion

Consistent with previous findings from our lab (Morgan et al., 2014), both wild-type and S426A/S430A mutant mice demonstrated significant tolerance to the antinociceptive, hypothermic, and cataleptic effects of Δ^9-THC after chronic administration of 30 mg/kg Δ^9-THC. The dose responses were measured on the seventh day following six days of consecutive administration of Δ^9-THC, and all mice tested demonstrated nearly complete antinociceptive tolerance in the hot plate and tail flick assays and hypothermic tolerance. When considered in the context of results from daily tolerance studies previously performed in the lab (Morgan et al., 2014), where wild-type mice developed tolerance to the antinociceptive and hypothermic effects

of Δ^9-THC after five consecutive days of administration of Δ^9-THC, this finding is not unanticipated.

The robust tolerance to Δ^9-THC observed in both wild-type and S426A/S430A mice after six days of drug treatment limits the assessment of the relative contribution of GRK/β-arrestin-mediated desensitization of CB_1 on tolerance to Δ^9-THC. Ideally, assessment of tolerance (post-drug dose response) would be timed to assess tolerance prior to the onset of complete tolerance in all animals. Assessing dose-response after two or three days of drug administration (rather than seven, as reported here) would be more likely to produce results which allow assessment of genotype effects between wild-type and S426A/S430A mice, based on previous results (Morgan et al., 2014). In addition, assessment of tolerance at higher doses of Δ^9-THC (300 mg/kg) in tolerant (post-drug) mice could also be used to better assess the drug response and produce a dose-response curve from which reliable calculation of ED_{50} values would be possible.

	Wild-type	**S426A/S430A**
Hot Plate		
Tolerance (ΔED_{50})	↑	↑
Tail Flick		
Tolerance (ΔED_{50})	↔	↑
Hypothermia		
Tolerance	↑	↑
Catalepsy		
Tolerance (ΔED_{50})	↑	↑

Table 3.5. Tolerance to the effects of Δ^9-THC develops following six days of chronic administration. A summary table is presented for all data indicating how Δ^9-THC responses changed in wild-type and S426A/S430A mutant mice following chronic Δ^9-THC administration compared to acute drug responses. Responses are shown as either change in ED_{50} (hot plate, tail flick, catalepsy) or change in total hypothermia. Arrows indicate the direction of response.

Overall, we observed that tolerance to the antinociceptive and hypothermic effects of Δ^9-THC was slightly reduced in S426A/S430A mutant mice following six consecutive days of administration, confirming the results observed in previous studies in the lab. However, many of the genotype effects were obscured by the complete tolerance which was observed in mice of both genotypes, which complicates the determination of effects of the S426A/S430A mutation on Δ^9-THC tolerance.

3.3 Tolerance to CP55,940

3.3.1 Shifts in cumulative dose responses to CP55,940

3.3.1.1 Hot plate antinociceptive tolerance

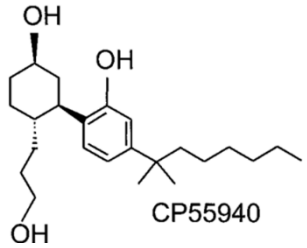

Figure 3.2. Structure of CP55,940. Adapted from (Howlett, 2002).

In order to determine whether wild-type and S426A/S430A mutant mice differ in their antinociceptive responses to CP55,940, changes in CP55,940-induced nociception were assessed using the hot plate and tail flick tests. Dose-dependent antinociception was induced following acute administration of CP55,940 (0.01, 0.03, 0.1, 0.3, and 1 mg/kg) ($F_{4,76}$=47.62, p<0.0001; Figure 3.2 A). Wild-type and S426A/S430A mutant mice did not differ in their responses to acute CP55,940 cumulative dose response administration, as neither a main effect of genotype (p=0.9830) nor a genotype x dose interaction (p=0.1409) were observed following acute administration of CP55,940. Calculated ED_{50} values for hot plate responses from acute administration of CP55,940 also did not differ between wild-type and S426A/S430A mutant mice (p=0.4113, Table 3.6).

	CP55,940 Hot Plate	
	WT	S426A/S430A
Pre drug	0.2347 (0.1608-0.3425)	0.1907 (0.1192-0.3053)
Post drug	0.893 (0.3458-1.004)	0.4220 (0.2755-0.6462)
F statistic	8.792 (p=0.0037)	6.356 (p=0.0133)

Table 3.6. Calculated ED50 values (mg/kg) from hot plate tests following treatment with CP55,940. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11 mice were tested for each group. Data were analyzed using F tests.

Neither wild-type nor S426A/S430A mice demonstrated tolerance (indicated by a significant right-ward shift in dose response curves) following six consecutive days of administration of 0.3 mg/kg CP55,940 (Figure 3.1 A). Post-drug dose hot plate dose response curves did not differ significantly from pre-drug dose response curves for either wild-type (p=0.0765) or S426A/S430A mutant mice (p=0.1714). However, in contrast to the results from multifactorial analyses of dose-response curves, significant increases in calculated ED_{50} values were observed in mice of both genotypes following prolonged CP55,940 treatment (wild-type,

$F_{1,106}$=8.792, *p*=0.0037; S426A/S430A, $F_{1,96}$=6.356, *p*=0.0133; Table 3.6). Together, these results suggest that while tolerance develops to the antinociceptive effects of CP55,940 in the hot plate test following chronic administration, the tolerance which develops is minimal.

3.3.1.2 Tail flick antinociceptive tolerance

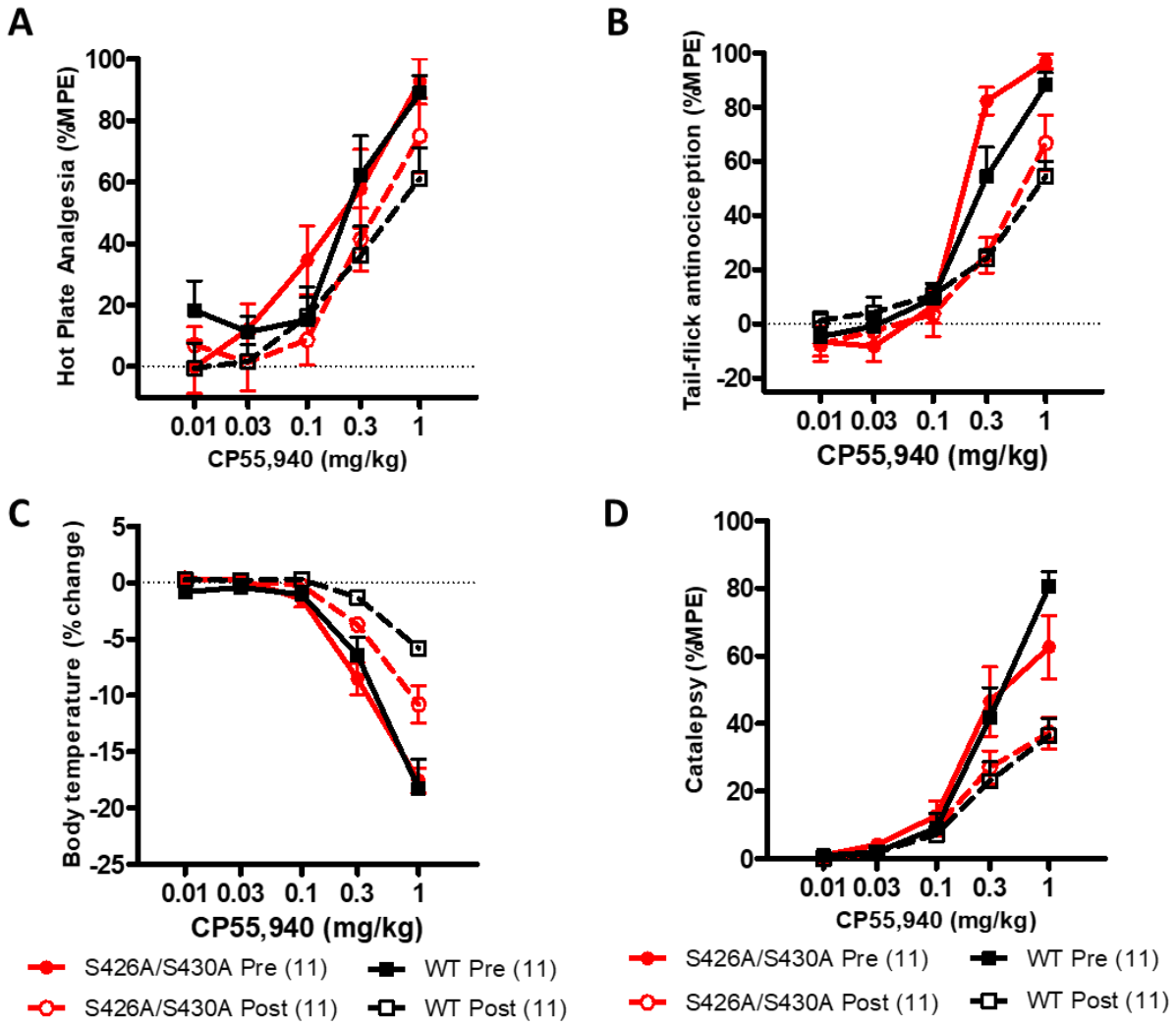

Figure 3.2. Wild-type and S426A/S430A mutant mice demonstrate significant tolerance to the antinociceptive and hypothermic effects of CP55,940 following chronic treatment. Wild-type and S426A/S430A mutant mice were tested for antinociceptive, hypothermic, and cataleptic responses across a cumulative dose range of 0.01, 0.03, 0.1, 0.3, and 1 mg/kg CP55,940 as drug naïve (Pre; filled symbols and solid lines) or following six days of once-daily treatment with 0.3 mg/kg CP55,940,940 (Post; open symbols and dashed lines). The mean represents percent maximal possible effect (%MPE) and error bars indicate the standard error of the mean (SEM). The number of mice in each group are indicated in parenthesis.

Acute treatment with CP55,940 dose-dependently increased antinociceptive responses in the tail flick assay ($F_{4,76}$=144.94, p<0.0001; Figure 3.2 B). While wild type and S426A/S430A mutant mice did not differ in their acute responses to CP55,940 by genotype overall (p=0.3223), a significant dose x genotype interaction was observed ($F_{4,80}$=3.50, p=0.0110). Post hoc analyses showed that S426A/S430A mice showed a greater antinociceptive response to a cumulative dose of 0.3 mg/kg of CP55,940 versus wild-type mice (p<0.01). Likewise, no significant genotype differences were observed in the ED_{50} values calculated from the pre-drug dose response curves (p=0.1024, Table 3.7). Overall, S426A/S430A mutant mice and wild type mice produced similar responses to acute CP55,940 administration.

	CP55,940 Tail-flick	
	WT	S426A/S430A
Pre drug	0.2860 (0.2259-0.3622)	0.2005 (0.2118-0.5517)
Post drug	0.8539 (0.6348-1.149)	0.6471 (0.4472-0.9363)
F statistic	33.06 (p<0.0001)	26.59 (p<0.0001)

Table 3.7. Calculated ED50 values (mg/kg) from tail-flick tests following treatment with CP55,940. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11 mice were tested for each group. Data were analyzed using F tests.

Following six once-daily injections of 0.3 mg/kg of CP55,940, a second cumulative CP55,940 dose response curve was performed in order to evaluate the development of tolerance to the antinociceptive responses of this agonist using tail-flick assay. While wild-type mice demonstrated only a trend toward altered antinociceptive responses to CP55,940 after six days of drug administration (p=0.0617), there was a main effect of time for S426A/S430A mutant mice ($F_{1,18}$=8.25, p=0.0094). Significant tolerance developed to the antinociceptive effects of CP55,940 in mice of both genotypes in the tail-flick assay, indicated by significant dose x time interactions for both wild-type ($F_{4,80}$=9.14, p<0.0001) and mutant ($F_{4,72}$=10.11, p<0.0001) mice (Figure 3.2 B). Post hoc analyses showed that both wild-type and S426A/S430A mice showed a significant reduction in antinociceptive response to cumulative doses of 0.3 (wild-type, p<0.01; S426A/S430A, p<0.001) and 1.0 mg/kg CP55,940 (wild-type, p<0.01; S426A/S430A, p<0.01), indicating that tolerance was due to differences at these higher doses. Both wild-type and

S426A/S430A mice demonstrated a significant increase in the calculated ED_{50} values following six consecutive days of WIN55,212-2 treatment (Table 3.7), suggesting that all mice demonstrated tolerance to CP55,940. However, the magnitude of shift between the pre- versus post ED_{50} values was greater in wild-type versus S426A/S430A mutant mice, suggesting that tolerance to the antinociceptive effects of CP55,940 is reduced in desensitization-resistant mice. Together, these results indicate that tolerance to the antinociceptive effects of CP55,940 is reduced in S426A/S430A mutant mice in the tail-flick test.

3.3.1.3 Hypothermic tolerance to CP55,940

Acute treatment with CP55,940 (0.01, 0.03, 0.1, 0.3 and 1 mg/kg) produced dose-dependent reduction in body temperature in all mice tested ($F_{4,76}$=138.58, p<0.0001; Figure 3.2 C). Wild type and S426A/S430A mutant mice did not differ in their acute hypothermic responses to CP55,940, as neither a genotype (p=0.6803) nor a dose x genotype interaction (p=0.3179) were observed. Following six once-daily injections of 0.3 mg/kg CP55,940, both wild-type ($F_{1,20}$=23.60, p<0.0001) and S426A/S430A ($F_{1,18}$=7.22, p=0.0151) mice demonstrated significant alterations in hypothermic responses to CP55,940. Significant tolerance (dose x time interaction) was observed in both wild type and ($F_{4,80}$=24.00, p<0.0001) and S426A/S430A mutant ($F_{4,72}$=8.39, p<0.0001) mice. Post hoc analyses revealed that these interaction effects were driven by differences in the hypothermic responses to 0.3 mg/kg (wild-type, p<0.001; S426A/S430A, p<0.01) and 1 mg/kg (wild-type, p<0.001; S426A/S430A, p<0.001) CP55,940. The calculated ED_{50} values for the hypothermic effects of CP55,940 in wild-type mice significantly differed between drug naïve and drug treated mice (p=0.0412) but did not

	CP55,940 Hypothermia	
	WT	S426A/S430A
Pre drug	0.2631 (0.03875-1.438)	0.3394 (0.2078-0.5544)
Post drug	1.901 (0.1310-27.59)	0.4415 (0.1143-1.706)
F statistic	4.521 (p=0.0417)	0.1735 (p=0.6780)

Table 3.8. Calculated ED50 values (mg/kg) from body temperature measurements following treatment with CP55,940. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11 mice were tested for each group. Data were analyzed using F tests.

differ in S426A/S430A mutants (p=0.6780) (Table 3.8). Taken together, these results suggest that tolerance to the hypothermic effects of CP55,940 is delayed in S426A/S430A mutant mice but not in wild type mice.

3.3.1.4 Cataleptic tolerance to CP55,940

To determine the how tolerance develops to the cataleptic effects of CP55,940, wild-type and S426A/S430A mutant mice were assessed for cumulative dose responses in drug naïve mice and in mice treated with 0.3 mg/kg CP55,940 for six days. Acute treatment with CP55,940 (0.01, 0.03, 0.1, 0.3 and 1 mg/kg) produced dose-dependent catalepsy in all mice ($F_{4,76}$=81.39, p<0.0001; Figure 3.2 D). Wild-type and S426A/S430A mutant mice demonstrated similar cataleptic responses to acute administration of CP55,940, as neither a main effect of genotype (p=0.7860) nor a genotype x dose interaction effect (p=0.1184) were observed.

	CP55,940 Catalepsy	
	WT	S426A/S430A
Pre drug	0.3848 (0.3140-0.4715)	0.4824 (0.3258-0.7143)
Post drug	1.878 (1.099-3.211)	1.785 (1.089-2.927)
F statistic	66.72 (p<0.0001)	17.08 (p<0.0001)

Table 3.9. Calculated ED50 values (mg/kg) from catalepsy tests following treatment with CP55,940. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11 mice were tested for each group. Data were analyzed using F tests.

Following six days of once-daily administration of 0.3 mg/kg CP55,940, wild-type ($F_{1,20}$=17.35, p=0.0005) but not S426A/S430A (p=0.0551) mice demonstrated significantly altered responses to the cataleptic effects of CP55,940, although S426A/S430A demonstrated a trend toward altered responses. Despite this, significant tolerance was observed in both wild-type ($F_{4,80}$=10.87, p<0.0001) and S426A/S430A mutant ($F_{4,72}$=3.55, p=0.0106) mice, indicated by a significant dose x time interaction, which was driven by differences in cataleptic responses to 0.3 and 1 mg/kg CP55,940 in wild-type mice (0.3, p<0.05; 1, p<0.01) and at 1 mg/kg in desensitization-resistant mice (p<0.01). The post-drug calculated ED_{50} values for the cataleptic effects of CP55,940 in significantly differed from calculated ED_{50} values in drug naïve mice in both wild-type ($F_{1,106}$=66.72, p<0.0001) and

S426A/S430A mutants ($F_{1,96}$=17.08, p<0.0001; Table 3.9). Overall, wild-type and S426A/S430A mutant mice did not differ in development of tolerance to the cataleptic effects of CP55,940 after six days of consecutive administration, in contrast to the results observed for the other effects of CP55,940 measured (antinociception and hypothermia).

3.3.2 Tolerance to daily administration of CP55,940

To assess how the S426A/S430A mutation alters the rate of tolerance development to CP55,940, wild-type and S426A/S430A mutant mice were tested for antinociceptive, hypothermic, and cataleptic responses to 0.3 mg/kg CP55,940 daily for 15 days. Tolerance to

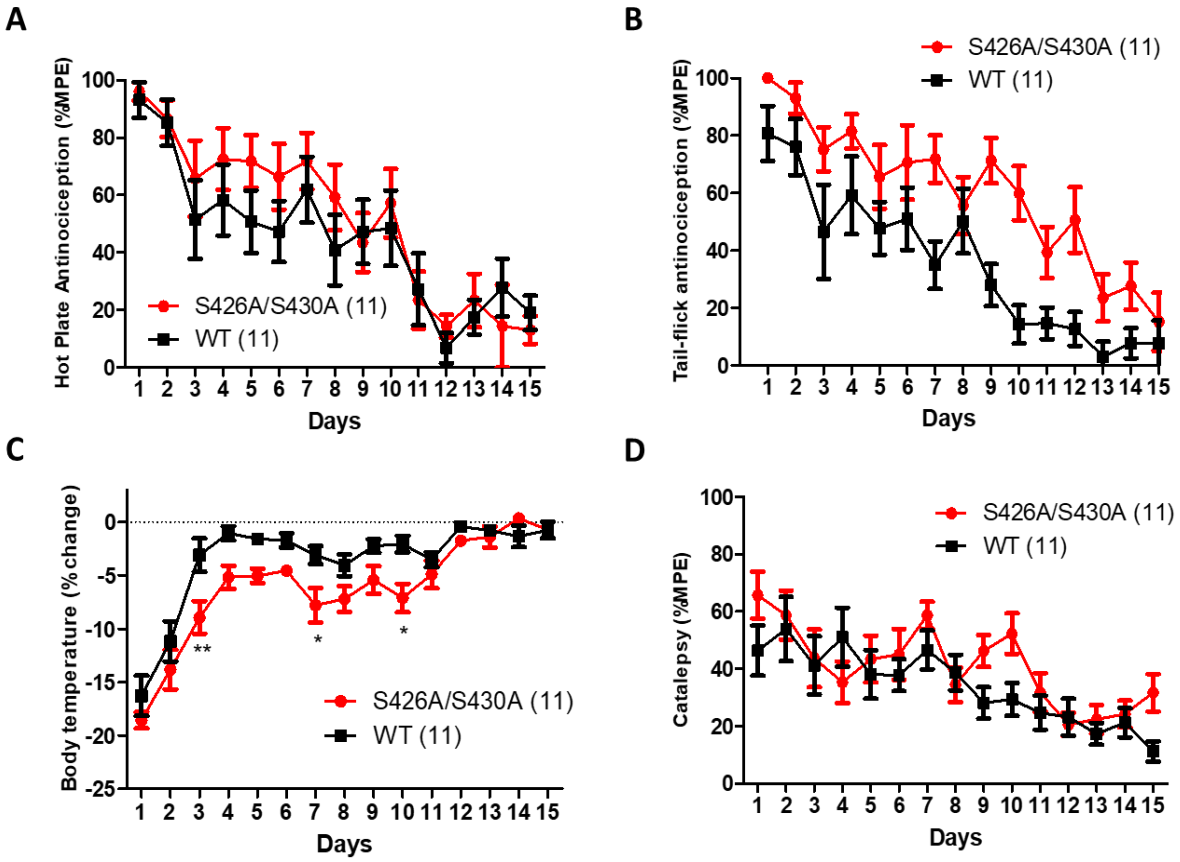

Figure 3.3. Antinociceptive tolerance to CP55,940 is not altered in S426A/S430A mice. Wild-type and S426A/S430A mice were assessed for the development of tolerance to the antinociceptive (A, hot plate; B, tail flick), hypothermic (C), and cataleptic (D) effects of once-daily 0.3 mg/kg CP55,940 (A, C) in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Means represent %MPE (A, B, D) and %ΔBT (C) and error bars represent the standard error of the mean (SEM). The number of mice in each group are shown in parentheses. *p<0.05, **p<0.01.

the antinociceptive effects of CP55,940 developed in all mice, as antinociceptive responses in both the hot plate ($F_{14,280}$=15.96, p<0.0001; Figure 3.3 A) and tail-flick ($F_{14,280}$=15.93, p<0.0001 Figure 3.3 B) were altered as an effect of number of administrations of CP55,940. However, the effect of the S426A/S430A mutation produced slightly different effects on the development of tolerance to the antinociceptive effects of CP55,940 in these assays. In the hot plate test, prolonged administration of 0.3 mg/kg CP55,940 did not produce either a main effect of genotype (p=0.3883) or a significant time x genotype interaction (p=0.8344), suggesting that the S426A/S430A mutation does not affect the development of tolerance in a supraspinal acute thermal nociceptive pain assay. A main effect of genotype ($F_{1,20}$=23.07, p=0.0001) was observed in the development of tolerance to CP55,940 in the tail-flick test, suggesting that S426A/S430A mutant mice are more sensitive to the effects of CP55,940 in the tail-flick test, however there was only a trend towards a significant time x genotype interaction (p=0.0517), suggesting that antinociceptive tolerance to CP55,940 in the tail-flick assay is almost but not quite significantly delayed by the S426A/S430A mutation.

Tolerance to the hypothermic effects of CP55,940 developed following 15 days of chronic administration of CP55,940 ($F_{14,280}$=40.46, p<0.0001; Figure 3.3 C). S426A/S430A mutant mice were more sensitive to the hypothermic effects of CP55,940 than wild-type mice ($F_{1,20}$=16.18, p=0.0007). In addition, tolerance to the hypothermic effects of CP55,940 was faster to develop in S426A/S430A mice than in wild-type mice, as significant time x genotype interaction was observed ($F_{14,280}$=1.98, p=0.0191). Post hoc analyses revealed that S426A/S430A mutant mice were more sensitive to the hypothermic effect of 0.3 mg/kg CP55,940 on days 3 (p<0.01), 7 (p<0.05), and 10 (p<0.05) of treatment than their wild-type littermates and were slower to develop tolerance to these effects. However, while tolerance to the cataleptic effects of CP55,940 developed following to daily treatment with 0.3 mg/kg CP55,940 ($F_{14,280}$=6.06, p<0.0001, Figure 3.3 D), an effect of the S426A/S430A mutation was not observed, as wild-

type and S426A/S430A mutant mice did not differ in their responses to CP55,940 ($F_{1,19}$=6.47, *p*=0.0198), and a genotype x time interaction was not observed (*p*=0.3759). Taken together, these results suggest that only tolerance to the hypothermic and not the antinociceptive or cataleptic effects of CP55,940 is altered in S426A/S430A mutant mice.

3.3.3 Formalin test

3.3.3.1 Acute phase (Phase I) of the formalin test

Tolerance to the antinociceptive effects of 0.3 mg/kg CP55,940 was also assessed in wild type and S426A/S430A mutant mice using the formalin test (Figure 3.4). All mice (wild type and S426A/S430A mutant) treated once-daily with 0.3 mg/kg CP55,940 developed significant, if incomplete tolerance ($F_{3,27}$=37.61, *p*<0.0001) in the acute phase of the formalin test (Phase I; Figure 3.4 A and C). However, the S426A/S430A mutation did not alter tolerance to CP55,940 in the acute phase of the formalin test, as there was neither a main effect of genotype (*p*=0.0584) nor a time x genotype interaction (*p*=0.8610). Therefore, while tolerance did develop to CP55,940 in the acute phase (Phase I) of the formalin test, the rate of tolerance development did not vary as a function of the S426A/S430A mutation.

3.3.3.2 Inflammatory phase (Phase II) of the formalin test

Tolerance to the antinociceptive effects of once-daily treatment with CP55,940 also occurred in the inflammatory phase of the formalin test in wild type and S426A/S430A mutant mice ($F_{3,27}$=32.63, *p*<0.0001; Phase II, Figure 3.4 B and D). S426A/S430A mutant mice were more sensitive to the effects of CP55,940 in the inflammatory phase of the formalin test ($F_{1,27}$=4.39, *p*=0.0457), however the mutation did not alter the rate of tolerance development in S426A/S430A mutant mice, as a genotype x time interaction effect was not observed in the inflammatory phase ($F_{3,27}$=0.99, *p*=0.4102) of the formalin test. Overall, these data suggest that while S426A/S430A mice are more sensitive to the effects of CP55,940, the S426A/S430A

mutation does not alter tolerance to the antinociceptive effects of CP55,940 in a model of inflammatory pain compared to wild-type mice.

3.3.4 Tolerance to CP55,940 in mice with cisplatin-evoked chronic neuropathic pain

Tolerance to the antiallodynic effects of 0.3 mg/kg CP55,940 in mice with cisplatin-induced neuropathic pain was assessed in wild-type and S426A/S430A mutant mice (Figure 3.5). Mice demonstrated significant neuropathic pain following four weeks of cisplatin treatment

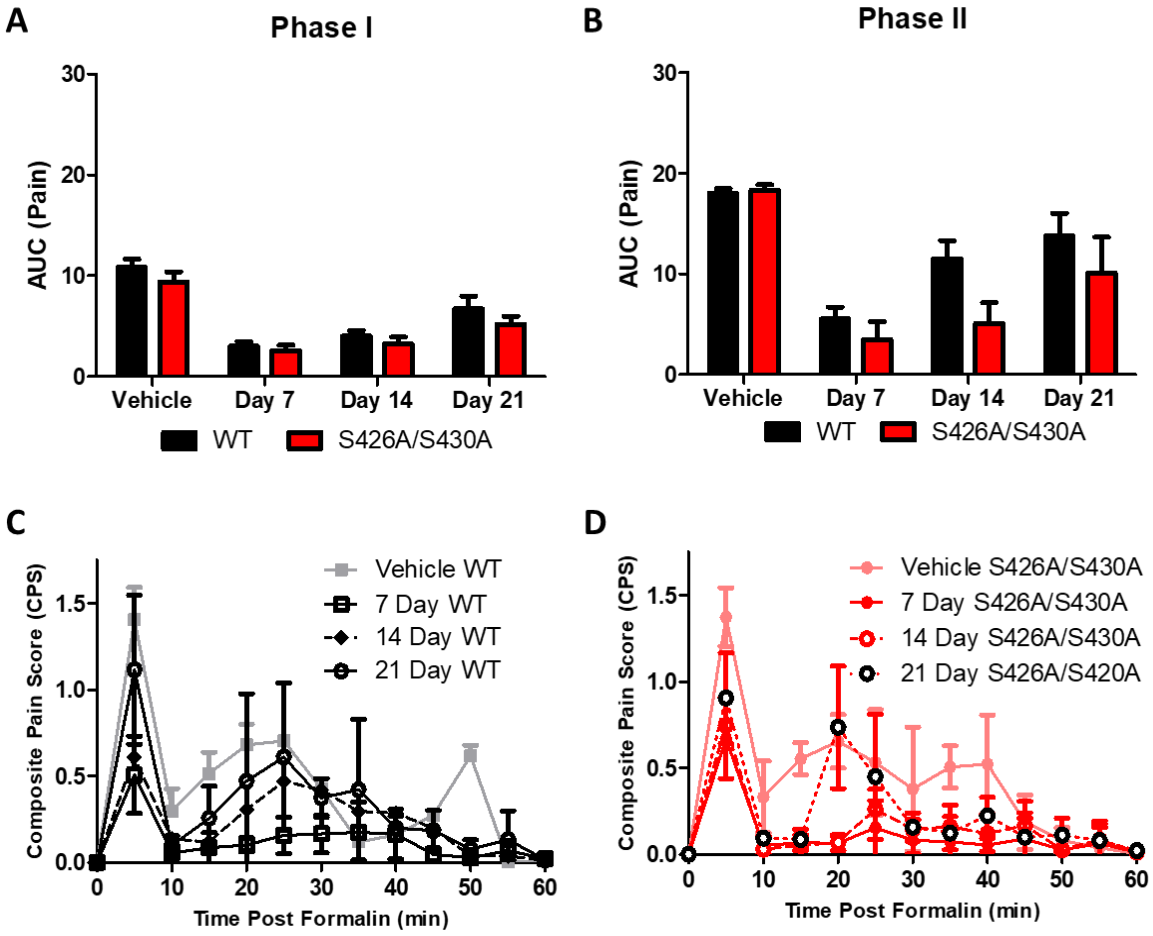

Figure 3.4. The S426A/S430A mutation does not delay the rate of antinociceptive tolerance development to in a model of acute inflammatory pain. Wild-type (black bars; n=4-5/day) and S426A/S430A mutants (red bars; n=4-6/day) were assessed for tolerance to the antinociceptive effects of or 0.3 mg/kg CP55,940 in the acute (Phase I; A, C) and inflammatory (Phase II; B, D) phases of the formalin test. Means represent the AUC of the composite pain score (CPS) (A, B) or the CPS (C, D) and error bars represent the standard error of the mean (SEM). Data were analyzed with two-way ANOVA and Bonferroni multiple comparisons test.

(t(2)=9.918, *p*=0.0100) compared to the pre-cisplatin baseline allodynic responses. Likewise, acute treatment with 0.3 mg/kg CP55,940 reversed neuropathic pain (t(2)=15.46, *p*=0.0042). Tolerance to the antiallodynic effects of CP55,940 ($F_{9,225}$=16.57, *p*<0.0001) developed across 19 consecutive days of treatment with 0.3 mg/kg CP55,940. There was neither a main effect of genotype (*p*=0.1637) nor a genotype x time interaction ($F_{9,225}$=1.07, *p*=0.3878), suggesting that tolerance to the antiallodynic effects of CP55,940 is not altered in S426A/S430A mutant mice.

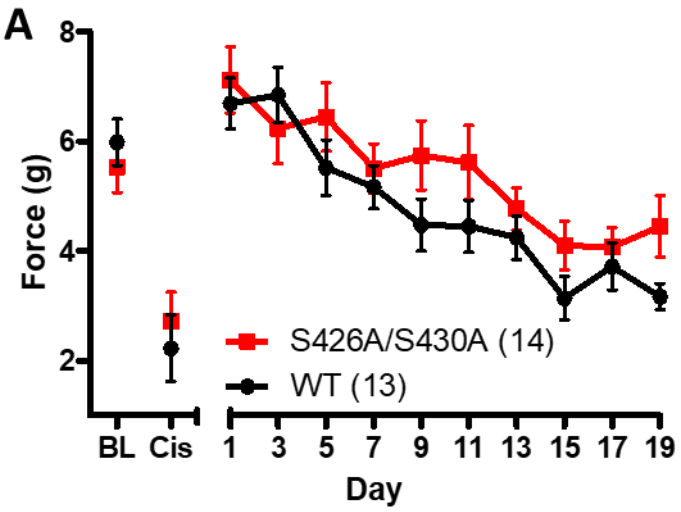

Figure 3.5. The S426A/S430A mutation does not delay tolerance to the antiallodynic effects of CP55,940 in a model of chronic neuropathic pain. The von Frey test was used to assess the antiallodynic effects of once-daily treatment with either in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Baseline (BL) and cisplatin-induced neuropathic (Cis) allodynic responses and CP55,940 antiallodynic responses (Days 1-19) are shown. The mean represents paw withdrawal thresholds (grams of force) and error represents standard error of the mean (SEM) with the number of mice in each group in parentheses. Data were analyzed with two-way RM ANOVA.

3.3.5 Discussion

Overall, we did not observe significant differences in the development of tolerance to CP55,940 between wild-type and S426A/S430A mutant mice. This suggests that GRK/β-arrestin 2 mediated desensitization of CB_1 is not a primary mechanism for the development of tolerance to CP55,940. We did observe increased sensitivity to the effects of CP55,940 in S426A/S430A mice, which is in contrast to previous studies in β-arrestin2 knockout mice that did not observe a difference in acute CP55,940 responses despite disruption of the same pathway (Breivogel et al., 2008). This difference could be explained by changes in compensatory signaling mechanisms in β-arrestin2 knockout mice which lead to a lack of

Hot Plate		S426A/S430A vs Wild type
Tolerance (ΔED_{50})		↓
Daily Tolerance		↔
Tail Flick		
Tolerance (ΔED_{50})		↓
Daily Tolerance		↓ (trend)
Hypothermia		
Tolerance		↓
Daily Tolerance		↓
Catalepsy		
Tolerance (ΔED_{50})		↔
Daily Tolerance		↔
Formalin (AUC)		S426A/S430A vs Wild type
Saline	Phase I	↔
	Phase II	↔
CP55,940	Phase I	↔
	Phase II	↑ (trend)
Neuropathic Pain		S426A/S430A vs Wild type
Tolerance		↔

Table 3.10. Tolerance to the effects of CP55,940 is not increased in S426A/S430A mutant mice. A summary table is presented for all data indicating how CP55,940 responses changed in wild-type and S426A/S430A mutant mice following chronic CP55,940 administration. Responses are shown as either change in ED_{50} (hot plate, tail flick, catalepsy) or change in total hypothermia for dose-responses and as total shift in responses for daily tolerance measurements. Responses are shown as change in total pain behaviors for formalin and allodynic responses for cisplatin-induced neuropathy. Arrows indicate the direction of response, with ↔ indicating no difference when comparing S426A/S430A and wild type mice. Trends are noted in parenthesis ($0.05<p<0.1$).

alteration in CP55,940 responses that are not found in S426A/S430A mutant mice, as β-arrestin2 is still present.

An interesting finding is the prolonged time to tolerance development to CP55,940. Wild-type mice treated with chronic CP55,940 develop tolerance over a period of time which is substantially longer than tolerance to either Δ^9-THC (Morgan et al., 2014) or WIN55,212-2 (Section 3.4). This may be an indication that the mechanisms which are responsible for the development of tolerance to CP55,940 are very different from those involved in tolerance to Δ^9-THC and WIN55,212-2. The exact nature of these mechanisms and how they relate specifically to CP55,940 tolerance will be addressed in Chapter 4.

3.4 Tolerance to WIN55,212-2

3.4.1 Shifts in WIN55,212-2 dose response curves

3.4.1.1 Hot plate antinociception

In order to determine whether wild-type and S426A/S430A mutant mice differ in their antinociceptive responses to WIN55,212-2, changes in WIN55,212-2-induced antinociception were assessed via the hot plate assay. Acute administration of WIN55,212-2 produced dose-dependent antinociception (0.3, 1, 3, 10, and 30 mg/kg) ($F_{4,84}$=117.96, p<0.0001; Figure 3.6 A).

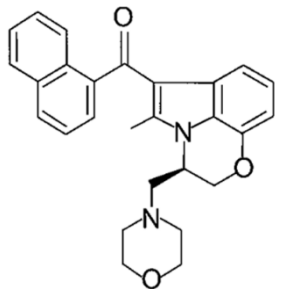

Figure 3.3. Structure of WIN55,212-2. Adapted from (Howlett, 2002).

Antinociceptive responses following acute administration of WIN55,212-2 did not differ between wild-type and S426A/S430A mutant mice (p=0.2039), and a genotype x dose interaction was not observed (p=0.2871). However, calculated ED_{50} values for hot plate responses from acute administration of WIN55,212-2 differed between wild-type and S426A/S430A mutant mice, where the calculated ED_{50} values were greater for S426A/S430A mice ($F_{1,107}$=4.670, p=0.0328; Table 3.11).

Both wild-type and S426A/S430A mice demonstrated tolerance following six consecutive days of administration of 10 mg/kg WIN55,212-2 in the hot plate test (Figure 3.6 A). Post-drug dose hot plate dose response curves differed significantly from pre-drug dose response curves for both wild-type ($F_{1,22}$=8.94, p=0.0068) and S426A/S430A mutant mice ($F_{1,20}$=18.12, p=0.0004), indicating the development of tolerance. Significant increases in calculated ED_{50} values were observed in mice of both genotypes following prolonged WIN55,212-2 treatment (wild-type, $F_{1,111}$=4.670, p=0.0328; S426A/S430A, $F_{1,106}$=45.15, p<0.0001; Table 3.11), although the magnitude of the shift was greater in S426A/S430A mutant mice. Taken

	WIN55,212-2 Hot Plate	
	WT	S426A/S430A
Pre drug	4.847 (3.271-7.184)	3.301 (1.619-6.372)
Post drug	12.36 (1.324-115.5)	9.394 (very wide)
F statistic	3.340 (p=0.0703)	45.15 (p<0.0001)

Table 3.11. Calculated ED50 values (mg/kg) from hot plate tests following treatment with WIN55,212-2. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-12 mice were tested for each group. Data were analyzed using F tests.

together, these results suggest that while tolerance to WIN55,212-2 develops in both wild-type and S426A/S430A mutant mice in the hot plate test, that tolerance is significantly greater in wild-type than in S426A/S430A mutant mice.

3.4.1.2 Tail flick antinociception

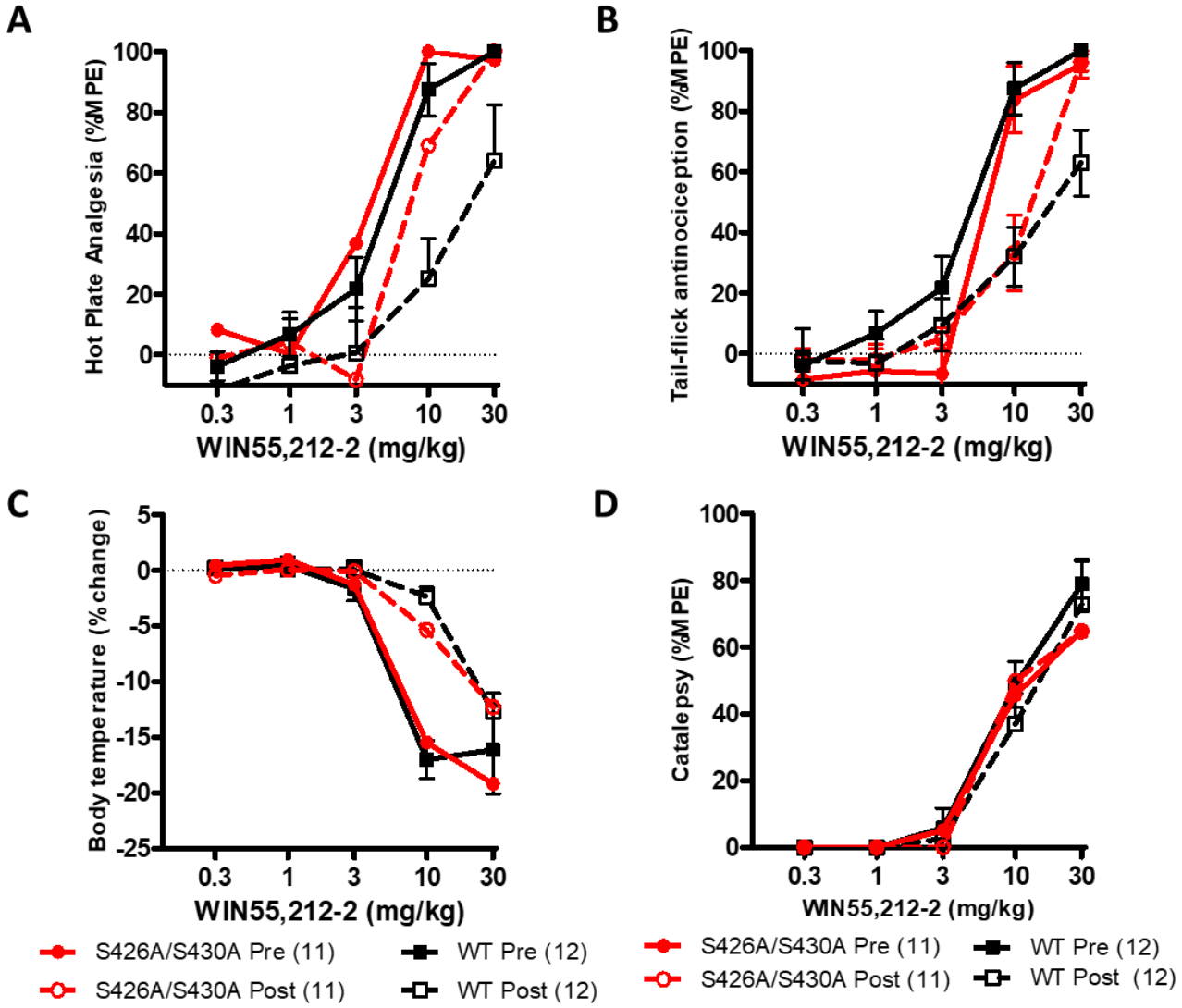

Figure 3.6. Wild-type and S426A/S430A mutant mice demonstrate significant tolerance to the antinociceptive and hypothermic effects (but not cataleptic) of WIN55,212-2 following chronic treatment. Wild-type (WT) and S426A/S430A mutant mice were tested for antinociceptive, hypothermic, and cataleptic responses across a cumulative dose range of 0.3, 1, 3, 10, and 30 mg/kg WIN55,212-2 as drug naïve (Pre; filled symbols and solid lines) or following six days of once-daily treatment with 30 mg/kg WIN55,212-2 (Post; open symbols and dashed lines). The mean represents percent maximal possible effect (%MPE) and error bars indicate the standard error of the mean (SEM). The number of mice in each group are indicated in parenthesis.

The tail flick test was used to determine whether wild-type and S426A/S430A mutant mice differ in the development of tolerance to the antinociceptive effects of WIN55,212-2 across a range of cumulative doses of WIN55,212-2 (0.3, 1, 3, 10 and 30 mg/kg) following both acute WIN55,212-2 administration and after six days of administration of 10 mg/kg WIN55,212-2. Acute treatment with WIN55,212-2 produced dose-dependent antinociception in the tail flick assay ($F_{4,36}$=36.34, p<0.0001; Figure 3.6 B). However, wild-type and S426A/S430A mutant mice did not demonstrate differences in acute antinociceptive responses to WIN55,212-2 in the tail-flick test, as there were no genotype differences between wild-type and S426A/S430A mutant mice observed across the dose response (p=0.2134) or in the ED_{50} values that were calculated from the dose response curves (p=0.4806; Table 3.12). A dose x genotype interaction was also not observed (p=0.7945) between wild type and S426A/S430A mutant mice in acute WIN55,212-2 responses.

Following six days of once-daily administration of 10 mg/kg WIN55,212-2, wild-type mice developed tolerance as indicated by a rightward shift in the cumulative dose-response curve for WIN55,212-2 ($F_{1,22}$=6.39, p=0.0192; Figure 3.6 B). In contrast, S426A/S430A mice did not show a rightward shift in their pre- versus post dose-response curve (p=0.3996). However, a significant dose x time interaction effect was observed in both wild-type ($F_{4,88}$=9.00, p<0.0001) and S426A/S430A ($F_{4,80}$=7.60, p<0.0001) mice, suggesting that tolerance developed in both wild type and S426A/S430A mutant mice as an effect of the dose of WIN55,212-2 administered. Post hoc analyses revealed that this interaction effect was driven entirely by differences in the responses to 10 mg/kg WIN55,212-2 in wild-type (p<0.001) and S426A/S430A mutant mice (p<0.001).

	WIN55,212-2 Tail-flick	
	WT	S426A/S430A
Pre drug	7.668 (4.647-12.65)	8.624 (very wide)
Post drug	15.90 (12.93-19.55)	12.28 (10.01-15.06)
F statistic	22.87 (p<0.0001)	4.892 (p=0.0291)

Table 3.12. Calculated ED_{50} values (mg/kg) from tail flick tests following treatment with WIN55,212-2. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-12 mice were tested for each group. Data were analyzed using F tests.

Both wild-type and S426A/S430A mice demonstrated a significant increase in the calculated ED_{50} values following six consecutive days of WIN55,212-2 treatment (Table 3.12), suggesting that all mice demonstrated some degree of tolerance. However, the magnitude of shift in the pre- versus post ED_{50} values was greater in wild-type versus S426A/S430A mutant mice, suggesting that tolerance is reduced in these desensitization-resistant mice. These results suggest that after six days of treatment with 10 mg/kg WIN55,212-2, tolerance is slower to develop in S426A/S430A mutant mice than in wild-type mice.

3.4.1.3 Hypothermia

Shifts in dose responses for the hypothermic effects of WIN55,212-2 were also assessed in wild-type and S426A/S430A mutant mice. Treatment with WIN55,212-2 (0, 0.3, 1, 3, 10 and 30 mg/kg) dose-dependently induced hypothermia ($F_{4,36}$=49.69, p<0.0001; Figure 3.6 D). However, wild-type and S426A/S430A mutant mice did not differ in their acute responses to WIN55,212-2, as neither a main effect of genotype (p=0.9561) nor a dose x genotype interaction (p=0.7762) was observed in acute hypothermic responses to WIN55,212-2. Following six days of once-daily injections of 10 mg/kg WIN55,212-2, tolerance to the hypothermic effects of WIN55,212-2 developed in both wild-type ($F_{1,22}$=24.61, p<0.0001) and S426A/S430A mutant ($F_{1,20}$=11.35, p=0.0031) mice (Figure 3.6 D), indicated by significant rightward shifts in the dose response curves for both groups of mice. Dose x time interaction effects were observed for both wild-type ($F_{4,88}$=17.72, p<0.0001) and S426A/S430A ($F_{4,36}$=10.19, p<0.0001) mice. Post hoc analyses revealed that these interaction effects were driven by differences in the hypothermic

	WIN55,212-2 Hypothermia	
	WT	S426A/S430A
Pre drug	5.878 (4.148-8.330)	6.396 (5.041-8.115)
Post drug	20.01 (very wide)	10.09 (very wide)
F statistic	9.859 (p=0.0022)	2.675 (p=0.1050)

Table 3.13. Calculated ED_{50} values (mg/kg) from body temperature measurements following treatment with WIN55,212-2. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-12 mice were tested for each group. Data were analyzed using F tests.

responses to 10 mg/kg (wild-type, $p<0.001$; S426A/S430A, $p<0.001$) and 30 mg/kg (wild-type, $p<0.001$; S426A/S430A, $p<0.001$) doses of WIN55,212-2 for mice of both genotypes. Analysis of calculated ED_{50} values for the hypothermic effects of WIN55,212-2 found that while the calculated ED_{50} values for both drug-treated wild-type and S426A/S430A mice were higher than the ED_{50} values calculated for the drug naïve controls, the shift in ED_{50} was only significant for wild-type ($F_{1,116}=9.859$, $p=0.0022$) and not for S426A/S430A mice ($p=0.1050$; Table 3.13). Overall, these results suggest that tolerance to the hypothermic effects of WIN55,212 develops in both wild type and S426A/S430A mutant mice after six days of treatment, but the tolerance that develops is slightly greater in wild type mice than in S426A/S430A mutant mice.

3.4.1.4 Catalepsy

To determine the effects of chronic treatment with WIN55,212-2 on tolerance to its cataleptic effects, wild-type and S426A/S430A mutant mice were assessed for cataleptic responses in cumulative dose responses following acute administration of WIN55,212-2 and after six days of chronic administration of 10 mg/kg WIN55,212-2. Acute treatment with WIN55,212-2 (0.3, 1, 3, 10, and 30 mg/kg) produced dose-dependent catalepsy ($F_{4,36}=81.39$, $p<0.0001$; Figure 3.6 D). Wild type and S426A/S430A mutant mice did not differ in their acute responses to WIN55,212-2, as neither a main effect of genotype ($p=0.3027$) nor a genotype x dose interaction effect ($p=0.7663$) were observed.

	WIN55,212-2 Catalepsy	
	WT	S426A/S430A
Pre drug	5.588 (3.601-8.6700	5.718 (1.643-16.32)
Post drug	9.612 (7.408-12.47)	9.653 (very wide)
F statistic	2.035 (p=0.1566)	0.1214 (p=0.7282)

Table 3.14. Calculated ED_{50} values (mg/kg) from catalepsy tests following treatment with WIN55,212-2. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 11-12 mice were tested for each group. Data were analyzed using F tests.

Following six days of once-daily administration of 10 mg/kg WIN55,212-2, tolerance to the cataleptic effects of WIN55,212-2 did not develop in either wild type ($p=0.2287$) or S426A/S430A ($p=0.1985$) mice. Dose x time interaction effects were also not observed in either

wild type (p=0.4032) and S426A/S430A mutant mice (p=0.1135). In addition, the post-drug calculated ED_{50} values for the cataleptic effects of WIN55,212-2 did not significantly differ from calculated ED_{50} values in drug naïve mice in either wild-type (p=0.1566) or S426A/S430A mutants (p=0.7272; Table 3.14). These results suggest that tolerance did not develop to the cataleptic effects of WIN55,212-2 after six days of treatment.

3.4.2 Daily tolerance to the effects of WIN55,212-2

To assess changes in WIN55,212-2 response during chronic administration, mice were tested for antinociceptive responses to once-daily 10 mg/kg WIN55,212-2 injections for 20 days. Tolerance to the antinociceptive effects of WIN developed in all mice after 20 days of treatment with 10 mg/kg WIN55,212-2 in the hot plate ($F_{19,627}$=13.65, p<0.0001; Figure 3.7 A) and tail-flick tests ($F_{19,627}$=10.03, p<0.0001; Figure 3.7 B). S426A/S430A mutant mice were more sensitive to the effects of WIN55,212-2 in both antinociceptive assays (hot plate, $F_{1,33}$=6.41, p=0.0163; tail-flick, $F_{1,33}$=95.89, p<0.0001). The S426A/S430A mutation altered the rate of tolerance development to the antinociceptive effects of WIN55,212-2, leading to attenuated development of tolerance in S426A/S430A mutant mice compared to wild type mice (time x genotype interaction). In the hot plate test, a significant time x genotype interaction ($F_{19,627}$=1.96, p=0.0087), although post-hoc analyses failed to reveal any differences. In the tail-flick test, a significant time x genotype interaction effect ($F_{19,627}$=1.73, p=0.0275) was also observed, and post hoc analyses revealed that S426A/S430A mutant mice were slower to develop tolerance to the antinociceptive effects of 10 mg/kg WIN55,212-2 in the tail flick test compared to their wild-type littermates after 3-11 (3, 7, 8, p<0.01; 4, 5, 6, 9, 10, 11, p<0.001) and 13-17 (13, p<0.01; 14-17, p<0.001) days of WIN55,212-2 treatment. The half-time for complete tolerance to 10 mg/kg WIN55,212-2 in S426A/S430A mice in the tail-flick test was significantly longer (16.62 d, 95%CI 4.5954-27.83 d) compared to wild-type controls (1.746 d, 95%CI 1.187-3.301 d).

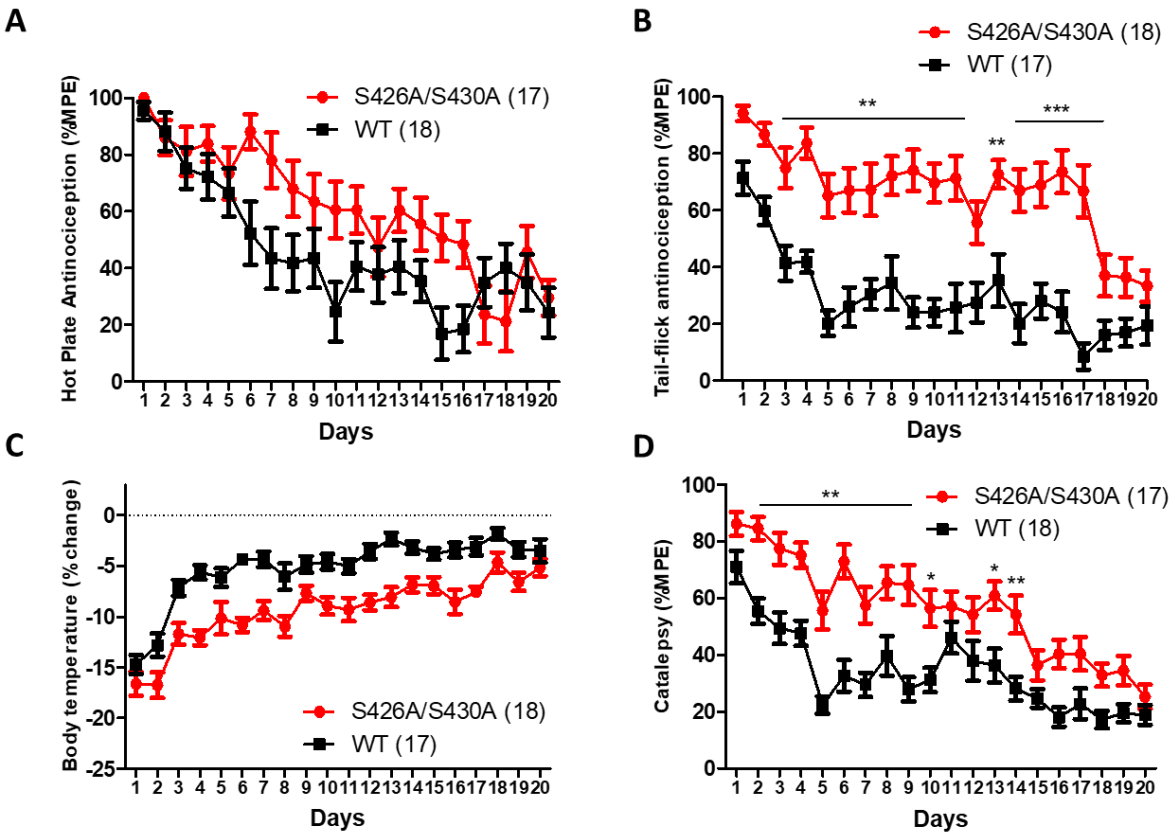

Figure 3.7. Antinociceptive tolerance to WIN55,212-2 is significantly delayed in S426A/S430A mice. Wild-type and S426A/S430A mice were assessed for the development of tolerance to the antinociceptive (A, hot plate; B, tail flick), hypothermic (C), and cataleptic (D) effects of once-daily 10 mg/kg WIN55,212-2 (A, C) in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Means represent %MPE (A, B, D) and %ΔBT (C) and error bars represent the standard error of the mean (SEM). The number of mice in each group are shown in parentheses. ***p*<0.01, ****p*<0.001.

Tolerance to the hypothermic effects of WIN55,212-2 also developed in wild-type and S426A/S430A mutant mice following 20 days of drug treatment ($F_{19,627}$=27.60, *p*<0.0001; Figure 3.7 C). S426A/S430A mutant mice were also more sensitive to the hypothermic effects of WIN55,212-2 ($F_{1,33}$=58.23, *p*<0.0001), however the S426A/S430A mutation did not alter the rate of tolerance development to WIN55,212-2 compared to wild type mice as a time x genotype interaction was not observed (*p*=0.2267). Thus, while S426A/S430A mutant mice, overall, were more sensitive to the hypothermic effects of WIN55,212-2 compared to their wild-type littermates, there was no difference in tolerance to the hypothermic effects of this agonist. In

addition, tolerance to the cataleptic effects of WIN55,212-2 was observed in all mice ($F_{19,627}$=19.34, p<0.0001, Figure 3.7 D). S426A/S430A mutant mice were more sensitive to the cataleptic effects of WIN55,212-2 ($F_{1,33}$=64.79, p<0.0001). Unlike tolerance to the hypothermic effects of WIN55,212-2, the S426A/S430A mutation altered tolerance to the cataleptic effects of WIN55,212-2, where S426A/S430A mutant mice were slower to develop cataleptic tolerance, indicated by a significant genotype x time interaction ($F_{19,627}$=1.65, p=0.0412). Post hoc analyses revealed that this interaction was driven by differences on days 2-10 and 13-14 (p<0.05 days 8, 10, and 13; p<0.01 days 2, 3, 4, 7, and 14; p<0.001 day 5, 6, and 9). Taken together, these results suggest that the S426A/S430A mutation leads to significant delays in tolerance to the antinociceptive and cataleptic effects of WIN55,212-2 but not its hypothermic effects.

3.4.3 Antinociceptive effects of WIN55,212-2 in the formalin test

3.4.3.1 Acute phase (Phase I) of the formalin test

Tolerance to the antinociceptive effects of WIN55,212-2 on acute and inflammatory pain was assessed in wild-type and S426A/S430A mutant mice using the formalin test. Mice pre-treated with once-daily injections of 3 mg/kg WIN55,212-2 developed tolerance to the antinociceptive effects of WIN55,212 in the acute phase (Phase I; $F_{4,31}$=52.25, p<0.0001) of the formalin test (Figure 3.8 A and C). However, the S426A/S430A mutation did not alter either sensitivity or tolerance to the effects of WIN55,212-2 in the acute phase of the formalin test, as neither a main effect of genotype (p=0.8740) nor a genotype x time interaction (p=0.8716) was observed.

3.4.3.2 Inflammatory phase (Phase II) of the formalin test

Tolerance to the antinociceptive effects of once-daily WIN55,212-2 was also assessed in the inflammatory phase (Phase II) of the formalin test (Figure 3.8 B and D). In the inflammatory phase of the formalin test, there was a main effect of the duration of once-daily WIN55,212-2

injections ($F_{4,30}$=70.66, p<0.0001). The S426A/S430A mutation produced a significant effect on tolerance to WIN55,212-2 in the inflammatory phase of the formalin test. S426A/S430A mutant mice were more sensitive to the antinociceptive effect of WIN55,212-2 ($F_{1,31}$=18.46, p=0.0002), and demonstrated a significant delay in the development of tolerance comparted to wild type mice ($F_{4,31}$=4.20, p=0.0081). Post hoc analyses revealed that tolerance to the effects of WIN 55,212-2 differed between wild-type and S426A/S430A mutant mice following 14 (p<0.01) and 21 (p<0.001) days of treatment, where significant tolerance developed in wild-type mice while S426A/S430A remained fully responsive to the effects of WIN55,212-2. These data suggest

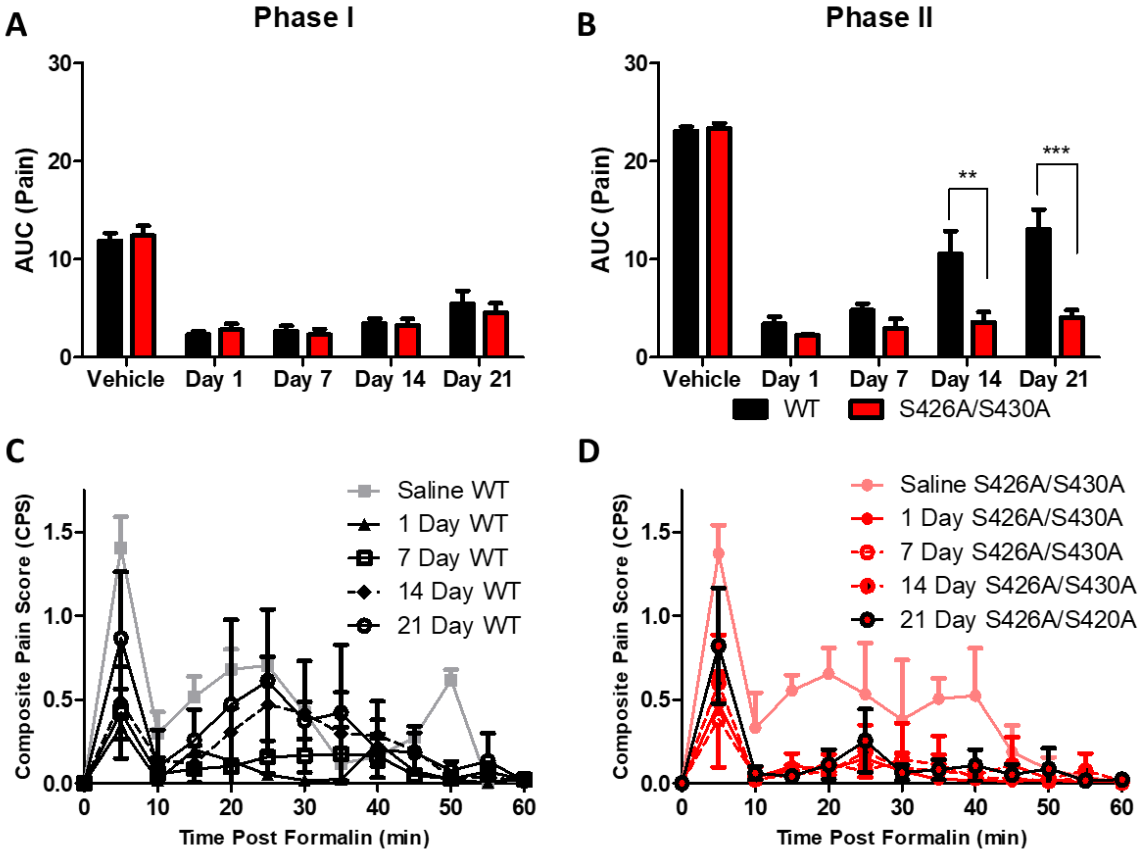

Figure 3.8. The S426A/S430A mutation delays the rate of antinociceptive tolerance development to WIN55,212-2 in a model of acute inflammatory pain. Wild-type (black bars; n=4-5/day) and S426A/S430A mutants (red bars; n=4-6/day) were assessed for tolerance to the antinociceptive effects of 3 mg/kg WIN55,212-2 in the acute (Phase I; A, C) and inflammatory (Phase II; B, D) phases of the formalin test. Means represent the AUC of the composite pain score (CPS) (A, B) or the CPS (C, D) and error bars represent the standard error of the mean (SEM). Data were analyzed with two-way ANOVA and Bonferroni multiple comparisons test.

that the S426A/S430A mutation selectively alters the rate at which tolerance develops to the antinociceptive effects of WIN55,212-2 but only in the inflammatory phase of the formalin test.

3.4.4. Tolerance to WIN55,212-2 in cisplatin-evoked chronic neuropathic pain

Following cisplatin treatment, wild-type and S426A/S430A mutant mice displayed mechanical allodynia in the von Frey test. Paw pressure thresholds required to elicit an allodynic paw withdrawal response in the von Frey test were reduced after cisplatin treatment compared to baseline allodynic responses (p=0.0230) (Figure 3.9). Cisplatin-induced neuropathic pain was reversed by treatment with 3 mg/kg WIN55,212-2 (p=0.0451). During 25 days of once daily treatment with 3 mg/kg WIN55,212-2, mice developed tolerance to the antiallodynic effects of WIN55,212-2 ($F_{12,252}$=14.97, p<0.0001). Further analysis indicated a significant genotype x time interaction effect for the antiallodynic effects of WIN55,212-2 treatment, indicating that tolerance is significantly delayed in S426A/S430A mutant mice ($F_{12,252}$=4.60, p<0.0001).

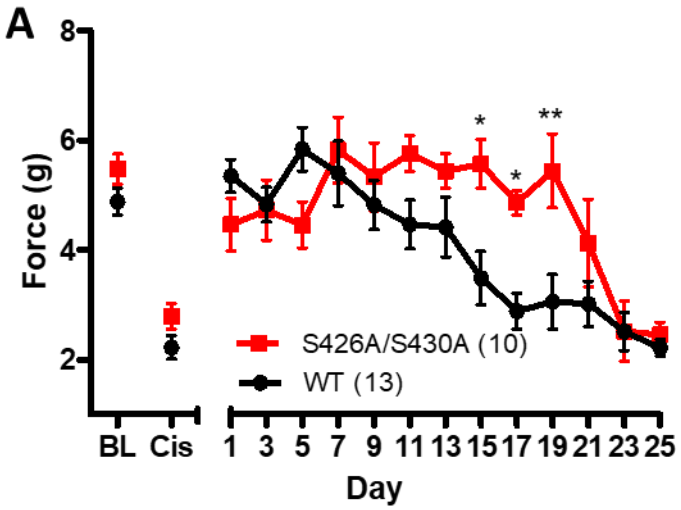

Figure 3.9. The S426A/S430A mutation delays tolerance to the antiallodynic effects of WIN55,212-2 in a model of chronic neuropathic pain. The von Frey test was used to assess the antiallodynic effects of once-daily treatment with either in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Baseline (BL) and cisplatin-induced neuropathic (Cis) allodynic responses and antiallodynic responses to 3 mg/kg WIN55,212-2 (Days 1-25) are shown. The mean represents paw withdrawal thresholds (grams of force) and error represents standard error of the mean (SEM) with the number of mice in each group in parentheses. Data were analyzed with two-way RM ANOVA and Bonferroni multiple comparisons test. *p<0.05, **p<0.01

However, in contrast to previous studies of WIN55,212-2 tolerance in acute pain models, S426A/S430A mutant mice were not more sensitive to the antiallodynic effects of WIN55,212-2 (p=0.1225). These results suggest that while the S426A/S430A mutation disrupts the

development of tolerance to the antiallodynic effects of WIN55,212-2, tolerance to WIN55,212-2 may develop via a slightly different mechanism during chronic pain conditions versus acute pain conditions.

3.4.4 Discussion

Desensitization-resistant S426A/S430A mutant mice demonstrate attenuated tolerance to the effects of WIN55,212-2 in acute nociceptive, inflammatory, and chronic cisplatin-induced neuropathic pain. Unlike mice chronically administered Δ^9-THC or CP55,940, S426A/S430A mutant mice also demonstrated a significant delay in the development of tolerance to the cataleptic effects of WIN55,212-2, suggesting potential tissue-specific interaction of WIN55,212-2 and the S426A/S430A CB_1 receptor.

We report that tolerance to WIN55,212-2 was profoundly delayed but not entirely blocked in desensitization-resistant S426/S430A mutant mice. One advantage of this study is the long duration of chronic drug administration. Most studies of tolerance look for the effects of an intervention on tolerance but do not continue long-term drug administration to induce tolerance in all subjects (Martini et al., 2010; Wallace et al., 2009); the current study provides a rare look at the development of delayed tolerance. However, the mechanisms which might contribute to the eventual tolerance development to WIN55,212-2 in S426A/S430A mice remains unknown. Several studies have previously reported potential mechanisms responsible for tolerance to WIN55,212-2 (Martini et al., 2010; Wallace et al., 2009). Mice lacking GPCR-associated sorting protein 1 (GASP-1), a protein involved in CB_1 trafficking and degradation, exhibit delayed tolerance to the antinociceptive but not the hypothermic effects of WIN55,212-2 (Martini et al., 2010). GASP-1 does not appear to interact with CB_1 in the regions of the C-terminal tail responsible for internalization (residues 418-438) (Hsieh et al., 1999; Jin et al., 1999; Tappe-Theodor et al., 2007), but it is unknown if GASP-1 interacts with CB_1 in the separate arrestin-binding regions responsible for CB_1 desensitization. Additionally, mice with genetic disruption of

the epsilon isoform of protein kinase C (PKCε) demonstrate accelerated tolerance to WIN55,212-2 (Wallace et al., 2009). Similar to our findings, an agonist bias was observed in PKCε knockout mice as tolerance to CP55,940 was not affected by deletion of PKCε. Either one of these proteins could be responsible for the delayed tolerance to WIN55,212-2 which eventually developed in the desensitization-resistant mice. A better understanding of how these two proteins interact with the arrestin-binding region mutated in the S426A/S430A mice would provide valuable insight into how other mechanisms might contribute to tolerance to WIN55,212-2.

Hot Plate		S426A/S430A vs Wild type
Tolerance (ΔED_{50})		↓
Daily Tolerance		↓
Tail Flick		
Tolerance (ΔED_{50})		↓
Daily Tolerance		↓
Hypothermia		
Tolerance		↔
Daily Tolerance		↔
Catalepsy		
Tolerance (ΔED_{50})		No tolerance
Daily Tolerance		↓
Formalin (AUC)		S426A/S430A vs Wild type
Saline	Phase I	↔
	Phase II	↔
WIN55,212	Phase I	↔
	Phase II	↑
Neuropathic Pain		S426A/S430A vs Wild type
Tolerance		↑

Table 3.15. Tolerance to the effects of WIN55,212-2 is delayed in S426A/S430A mutant mice. A summary table is presented for all data indicating how WIN55,212-2 responses changed in wild-type and S426A/S430A mutant mice following chronic WIN55,212-2 administration. Responses are shown as either change in ED_{50} (hot plate, tail flick, catalepsy) or change in total hypothermia for dose-responses and as total shift in responses for daily tolerance measurements. Responses are shown as change in total pain behaviors for formalin and allodynic responses for cisplatin-induced neuropathy. Arrows indicate the direction of response, with ↔ indicating no difference when comparing S426A/S430A and wild type mice. Trends are noted in parenthesis ($0.05<p<0.1$).

Another interesting aspect of the delayed tolerance to WIN55,212-2 in S426A/S430A mutant mice is the very rapid pace at which tolerance eventually develops. This may be due to changes in internalized CB_1 receptor recycling and degradation. Previous studies have demonstrated that internalized CB_1 co-localizes with signaling proteins, including β-arrestin, in endosomes (Delgado-Peraza et al., 2016; Rozenfeld and Devi, 2008). CB_1 receptors remain associated with G proteins in endosomes

and with use of highly lipophilic and membrane permeable cannabinoid agonists

internalized CB_1 receptors form a “functionally active intracellular receptor pool” (Rozenfeld and Devi, 2008). Other studies have found that β-arrestin1 does not couple to CB_1 after activation of wild-type receptors with WIN55,212-2 (Flores-Otero et al., 2014; Gyombolai et al., 2013); however, the S426A/S430A mutant CB_1 receptor colocalizes with β-arrestin1 after WIN55,212-2 activation, demonstrating a significant change in coupling. The rapid development of delayed WIN55,212-2 tolerance in S426A/S430A mice could develop as a result of changes in receptor recycling, where β-arrestin1-bound or active CB_1 receptors in endosomes are rapidly trafficked for degradation instead of recycling, leading to the rapid development of tolerance.

Chapter 4

Role of c-Jun N-terminal Kinase (JNK) in tolerance to synthetic cannabinoid agonists.

Adapted from: c-Jun N terminal kinase signaling pathways mediate cannabinoid tolerance in an agonist specific manner. Henderson-Redmond AN*; **Nealon CM***; Davis BJ*; Yuill MB*; Blanton H; Sepulveda DE; Haskins CP; Marcus DJ; Mackie K; Guindon J; Morgan DJ. Submitted to Neuropharmacology January 2018.

*These authors contributed equally to this work

4.1 Rationale

Desensitization-resistant mice are slower to develop tolerance to the antinociceptive effects of Δ^9-THC, however tolerance develops in both wild-type and S426A/S430A mice in the tail flick test after four days of administration (Morgan et al., 2014). We have previously observed that inhibition of JNK activation by pretreatment with the small molecule JNK inhibitor SP600125 (3 mg/kg, SP6) eliminates the tolerance observed in S426A/S430A mutant mice following repeated administrations of Δ^9-THC, causing sustained drug response after seven consecutive days of drug administration (Unpublished results). While tolerance to Δ^9-THC was attenuated in both wild-type and S426A/S430A mutant mice, the delay in development of tolerance was much longer in S426A/S430A mutant mice than in wild-type mice. This finding suggested that JNK is responsible for mediating development of tolerance to the antinociceptive effects of Δ^9-THC. In particular, these results suggest that tolerance to the antinociceptive effects Δ^9-THC is mediated by two pathways, GRK/β-arrestin2-mediated CB_1 desensitization and JNK activation, which coordinate to cause tolerance development in acute pain.

In the context of these findings, we aimed to identify whether JNK activation plays a role in the development of antinociceptive tolerance to other cannabinoid agonists, specifically CP55,940 and WIN55,212-2. We have shown that tolerance develops to both agonists following repeated

administration, even in desensitization-resistant S426A/S430A mutant mice (Chapter 3). The goal of this study was to identify potential agonist biases for JNK inhibition in tolerance to the antinociceptive effects of cannabinoid agonists.

4.2 Role of JNK activation in tolerance to WIN55,212-2

4.2.1 Antinociceptive tolerance in the tail flick test

These next experiments determined whether JNK inhibition could alter cannabinoid tolerance for two structurally distinct, synthetic, high potency, high efficacy cannabinoid agonists, WIN55,212-2 and CP55,940. In this first experiment, we determined whether pretreatment with 3 mg/kg SP600125 could alter tolerance to the antinociceptive effects of once-daily injections of 10 mg/kg WIN55,212-2 in wild-type and/or S426A/S430A mice. We found that tolerance developed to the antinociceptive effects of WIN55,212-2 in both wild-type ($F_{19,285}$=6.55, p<0.001) and S426A/S430A ($F_{19,304}$=6.59, p<0.001) mice. However, a main effect of SP600125 pretreatment on WIN55,212-2 tolerance was not observed in either wild-type (p=0.113) or S426A/S430A mutant mice (p=0.687). A treatment x day interaction was also not observed in either wild-type (p=0.884) or S426A/S430A mutant mice (p=0.510; Figure 4.1 A, B). These results indicate that inhibition of JNK activation does not alter tolerance to WIN55,212-2, suggesting that JNK activation is not involved in tolerance to the antinociceptive effects of WIN55,212-2.

4.2.2 Hypothermic tolerance

Likewise, we found significant main effects of both day/number of WIN55,212-2 injections in both wild-type ($F_{19,285}$=16.08, p<0.0001) and S426A/S430A ($F_{19,304}$=14.72, p<0.0001) mice, indicating that tolerance develops to the hypothermic effects of WIN55,212-2. We failed to see an effect of SP600125 pretreatment in wild-type mice (p=0.209) but did observe a significant effect of SP6 pretreatment in S426A/S430A mutant mice (p=0.010) compared to vehicle-treated

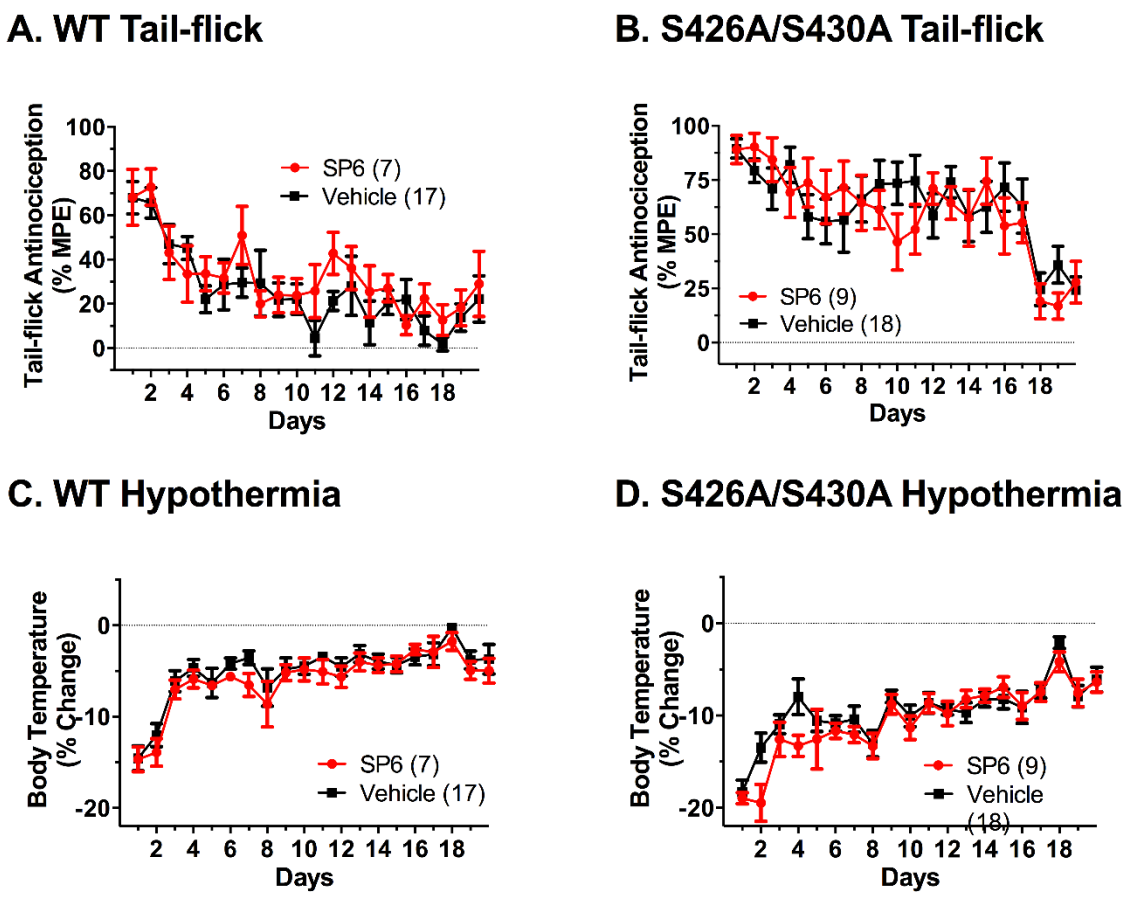

Figure 4.1. Tolerance to the antinociceptive and hypothermic effects of WIN 55,212-2 is not altered by SP600125. Wild-type and S426A/S430A mutant mice were pretreated with vehicle (black line with squares) or 3 mg/kg SP600125 (SP6; red line with circles) one hour prior to administration of 10 mg/kg WIN55,212-2 for 20 consecutive days. Tail-flick antinociception (%MPE, A, B) and hypothermia (%ΔBT, C, D) were assessed in both wild-type (A, C) and S426A/S430A mutant (B, D) mice one hour after WIN55,212-2 treatment. Error bars represent the SEM and data analysis was performed using two-way ANOVAs. Sample sizes for each group are in parentheses.

controls. However, we did not observe a significant treatment x day interaction in either wild-type (p=0.998) or S426A/S430A mutant mice (p=0.501; Figure 4.1 C, D). Taken together, these results indicate that, unlike with Δ^9-THC, SP600125 pretreatment fails to alter tolerance to the antinociceptive or hypothermic effects of once-daily administration of 10 mg/kg of WIN55,212-2 in either wild-type or S426A/S430A mutant mice.

4.3 Role of JNK inactivation in tolerance to CP55,940

4.3.1 Antinociceptive tolerance in the tail flick test

When determining whether pretreatment with 3 mg/kg SP600125 could alter tolerance development to once-daily administration of 0.3 mg/kg of CP55,940, we found tolerance developed to the antinociceptive effects of CP55,940 ($F_{9,207}$=5.96, p<0.001) and observed main effects of SP610025 pretreatment on CP55,940 antinociceptive responses ($F_{1,23}$=4.81, p=0.039). A significant treatment x day interaction was not observed in wild-type mice (p=0.760), suggesting that although pretreatment of wild-type mice with 3 mg/kg of SP600125 decreased antinociceptive responses to 0.3 mg/kg CP55,940, it did not alter the rate at which

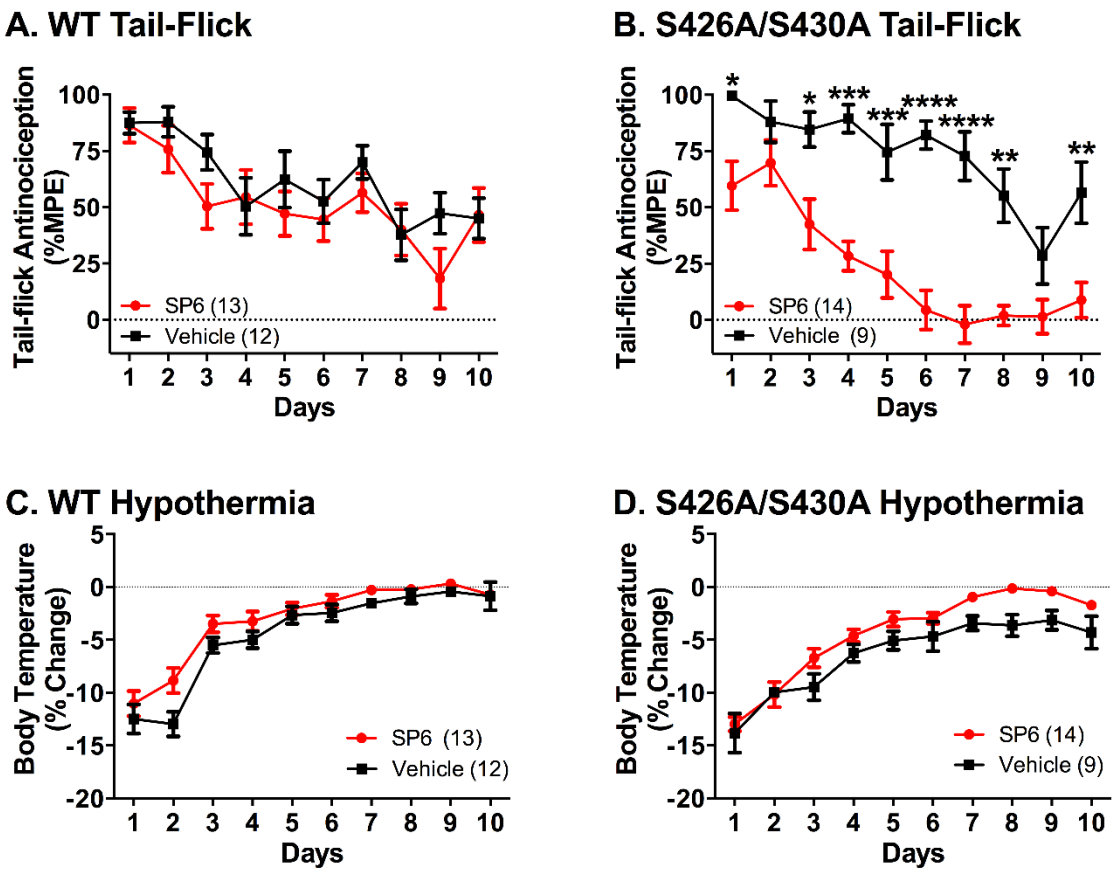

Figure 4.2. Tolerance to the hypothermic and antinociceptive effects of 0.3 mg/kg (-)-CP55,940 is accelerated following pretreatment with 3 mg/kg of SP600125. Wild-type and S426A/S430A mutant mice were pretreated with vehicle (black line with squares) or 3 mg/kg SP600125 (red line with circles) one hour prior to administration of 0.3 mg/kg CP55,940 for 10 consecutive days. Tail-flick antinociception (%MPE, A, B) and hypothermia (%ΔBT, C, D) were assessed in both wild-type (A, C) and S426A/S430A mutant (B, D) mice one hour after CP55,940 treatment. Error bars represent the SEM and data analysis was performed using two-way ANOVAs with Bonferroni post-hoc tests. Sample sizes for each group are in parentheses. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

tolerance to CP55,940 developed (Figure 4.2 A). However, S426A/S430A mutant mice showed a main effect of SP600125 pretreatment ($F_{1,21}$=79.82, p<0.0001), an effect of day ($F_{9,189}$=10.82, p<0.001), and a treatment x day interaction ($F_{9,189}$=2.09, p=0.033). Post hoc analyses revealed that pretreatment of S426A/S430A mutant mice with SP600125 not only decreased their sensitivity to 0.3 mg/kg CP55,940 (noted by the difference in response on day 1), but also accelerated the rate at which antinociceptive tolerance to CP55,940 developed in mutant mice compared to those pretreated with vehicle (Figure 4.2 B).

4.3.2 Hypothermic tolerance

In contrast, we failed to find an effect of SP600125 pretreatment (p=0.053) on tolerance to the hypothermic effects of 0.3 mg/kg of CP55,940 in wild-type mice following once-daily administration (Figure 4.2 C). Interestingly, we did find main effects of both SP600125 pretreatment ($F_{1,21}$=7.94, p=0.010) and day/number of CP55,940 injections ($F_{9,189}$=54.69, p<0.001) on tolerance to the hypothermic effects of CP55,940 in S426A/S430A mutant mice suggesting that S426A/S430A mutant mice are less sensitive to the effects of JNK pretreatment on CP55,940 responses. However, we did not observe a significant treatment x day interaction (p=0.432) on the development of tolerance to these effects. Thus, while pretreatment with SP600125 results in mutant mice being, overall, less sensitive to the hypothermic effects of 0.3 mg/kg CP55,940, it does not alter the rate at which hypothermic tolerance develops to 0.3 mg/kg CP55,940 compared to mice pretreated with vehicle (Figure 4.2 D).

4.4 Discussion

We report that tolerance to WIN55,212-2 was not affected by pretreatment with JNK inhibitor SP600125 in either wild-type or desensitization-resistant S426A/S430A mutant mice. This is not unanticipated, as studies in related systems did not observe an effect of JNK inhibition on acute tolerance to fentanyl, an opioid agonist of similar potency to WIN55,212-2 (Melief et al., 2010). WIN55,212-2 and fentanyl are both synthetic, high-efficacy agonists and tolerance to

both drugs is blocked by disruption of GRK-mediated desensitization of their respective receptors (CB_1, see Chapter 3; MOR, (Melief et al., 2010)). This provides further evidence that cannabinoid and opioid systems induce signaling via similar pathways, as we have observed an agonist bias that is similar to one observed in the opioid system.

We also report a significant effect of JNK inhibition on tolerance to CP55,940. Interestingly, inhibition of JNK activation with SP600125 accelerated antinociceptive tolerance to CP55,940, which is not a response we have previously observed in other tolerance interventions. However, tolerance to CP55,940 was only altered in S426A/S430A mutant mice and not in wild-type mice. This is an opposite effect to what we observed in mice chronically treated with 30 mg/kg Δ^9-THC, where tolerance to Δ^9-THC was significantly attenuated in S426A/S430A mutant mice pretreated with SP600125. While this could be caused by differences in agonist efficacy- Δ^9-THC is a partial CB_1 agonist, while CP55,940 is a full agonist for CB_1- this is not likely, as JNK inhibition did not affect tolerance to WIN55,212-2 in S426A/S430A mutant mice. However, the significant structural variation between WIN55,212-2 and Δ^9-THC and CP55,940 could undermine this interpretation. CP55,940 and Δ^9-THC are of a similar chemotype and may be activating similar pathways, while WIN55,212-2, which is of a very different chemotype (aminoalkylindole) and may activate entirely different signaling mechanisms, may not be a reliable reference for agonist efficacy in this situation. Assessing the effects of JNK inhibition on tolerance to a different full CB_1 agonist that is of a similar chemotype like HU-210 would assist in identification of the role of agonist efficacy in JNK activation.

Responses to CP55,940 and Δ^9-THC were only attenuated in S426A/S430A mutant mice, suggesting that disruption of CB_1 phosphorylation and subsequent binding of β-arrestin2 is essential for full activation of the effects of JNK signaling. It may be that β-arrestin2 association with CB_1 blocks the access of JNK to phosphorylate CB_1, which is removed in S426A/S430A mutant mice. Previous studies have found that the S426A/S430A receptor demonstrates a

significant bias toward β-arrestin1-mediated signaling over G protein-mediated signaling (Delgado-Peraza et al., 2016). β-arrestin1 may be a potential target to identify the role of JNK in cannabinoid tolerance, either through direct association with CB_1 in sites phosphorylated by JNK or as an essential component of scaffolding that facilitates signal transduction of a different pathway.

Chronic administration of Δ^9-THC and CP55,940 produces agonist-specific effects on antinociceptive tolerance in β-arrestin1 knockout mice (Breivogel and Vaghela, 2015). β-arrestin1 knockout does not alter acute sensitivity or antinociceptive tolerance to Δ^9-THC from wild-type mice. However, β-arrestin1 knockout mice are less sensitive to the effects of CP55,940 and show a trend toward faster development of antinociceptive tolerance to CP55,940 in β-arrestin1 knockout mice. Considered in this context, it is possible that the accelerated tolerance to CP55,940 which we observed in SP600125-treated S426A/S430A mutant mice may indicate that JNK activation leads to β-arrestin1 activation, which in turn is involved in preventing the development of tolerance to CP55,940. Another potential role for β-arrestin involvement in JNK-mediated tolerance development to CP55,940 is through internalization of the receptor. Previous studies have observed that S426A/S430A mutant CB_1 remains coupled to β-arrestin1 after endocytosis with a strong interaction that can be identified by co-immunoprecipitation (Delgado-Peraza et al., 2016). It is possible that JNK signaling is responsible for activation of key components of the endocytic machinery, including β-arrestin2 (Flores-Otero et al., 2014), and that inhibition of JNK activation allows CB_1 signaling via either G proteins of β-arrestin1 to become desensitized without internalization and recycling, which would lead to accelerated tolerance development.

It is difficult to make a concrete determination about agonist biases in the role of JNK signaling in cannabinoid tolerance, particularly because we have only assessed the effects of JNK inhibition on tolerance to CP55,940 in acute nociceptive pain. We have not yet assessed the

role of JNK inhibition on tolerance to CP55,940 in S426A/S430A mutant mice in pathologically relevant pain models. Future studies focused on identifying the subtle regulation and interaction of JNK and CP55,940-bound CB_1 will need to include other pain models to confirm that the acceleration of tolerance observed in these studies is not an artifact of assessing tolerance solely via the tail-flick test.

Collectively, these results identify a novel, non-classical signaling mechanism with an agonist-specific role in mediating tolerance to synthetic, high-efficacy cannabinoid agonists. We found that JNK inhibition does not significantly alter tolerance to WIN55,212-2 or CP55,940 in wild-type mice, nor does SP600125 pretreatment alter antinociceptive tolerance to WIN55,212-2 in S426A/S430A mutant mice. However, tolerance to CP55,940 is accelerated in S426A/S430A mutant mice, suggesting that there may be agonist biases in the effects of JNK activation on development of tolerance to high-efficacy synthetic cannabinoid agonists.

Chapter 5

General Discussion and Model

5.1 Summary

The antinociceptive effects of cannabinoid agonists have been well described (Morgan et al., 2014; Sim-Selley and Martin, 2002). However, the molecular mechanisms underlying the development of tolerance to cannabinoids are still being elucidated. Previous work performed *in vitro* suggested that desensitization of the cannabinoid receptor 1 (CB_1) occurs as a result of phosphorylation of two serine residues (426 and 430) by GRK3 and subsequent association with β-arrestin2 (T. Daigle et al., 2008; Jin et al., 1999). Our lab has previously shown that mice expressing a mutant desensitization-resistant form of CB_1 (S426A/S430A) develop tolerance to Δ^9-tetrahydrocannabinol (Δ^9-THC) more slowly than wild-type littermates (Morgan et al., 2014). There is growing evidence to suggest that some cannabinoid agonists, including WIN55,212-2, induce biased signaling mediated through β-arrestin pathways (Flores-Otero et al., 2014). Furthermore, evidence in cell culture models suggests the S426A/S430A mutant form of CB_1 exhibits biased signaling through β-arrestin1 versus G-protein-mediated signaling mechanisms (Delgado-Peraza et al., 2016). In addition, studies in related GPCR systems have indicated that JNK activation may be a potential non-classical mechanism for the development of tolerance to cannabinoid agonists. While the underlying mechanisms of desensitization have been extensively studied in transfected cells expressing CB_1, these studies do not provide specific insight into how these mechanisms contribute to the development of tolerance in a living animal. Thus, the current study provides an analysis of the roles of GRK/β-arrestin-mediated desensitization of CB_1 and CB_1-mediated JNK activation in the development of tolerance to CP55,940, and WIN55,212-2, two structurally diverse synthetic cannabinoid agonist chemotypes that exhibit known signaling bias.

Tolerance develops following prolonged administration of drug, where a consistent treatment with a compound leads to reduced efficacy over time (Martin et al., 2004; Taylor and Fleming, 2001). In this study, we used two models of acute nociceptive pain as well as a model of chronic neuropathic pain to assess tolerance to WIN55,212-2 and CP55,950 in desensitization deficient S426A/S430A mutant mice. We found that tolerance for WIN55,212-2 was attenuated in S426A/S430A mutant mice consistent with previous studies examining tolerance to Δ^9-THC (Morgan et al., 2014). S426A/S430A mice demonstrated a longer half-time to tolerance for both WIN55,212-2 and a smaller magnitude of ED_{50} shift of drug responses in the tail-flick test. Tolerance was also slower to develop to the antinociceptive effects of WIN55,212-2 in desensitization-resistant mice in the formalin test. Our results agree with reports of delayed tolerance for cannabinoids in β-arrestin2 knock-out mice (Nguyen et al., 2012), showing a similar effect on tolerance development with a different disruption of the classical desensitization mechanism. Collectively, these studies suggest that interference with β-arrestin2-mediated signaling, either through removal of the protein or its binding sites on CB_1, plays a role in the development of tolerance to many cannabinoid agonists.

We also observed that JNK signaling plays a role in cannabinoid tolerance in an agonist-specific manner. Disruption of JNK activation with a JNK inhibitor (SP600125) had no effect on tolerance to WIN55,212-2. Interestingly, inhibition of JNK activation produced an acceleration of tolerance to CP55,940 in desensitization-resistant S426A/S430A mutant mice, suggesting that JNK signaling plays a role in preventing tolerance to the antinociceptive effects of CP55,940. Previous studies of opioid agonists (a related GPCR system to the cannabinoid system) have found that tolerance to morphine is delayed following JNK inhibition with SP600125 (Melief et al., 2010), but accelerated tolerance after SP600125 treatment has not been reported for opioid agonists. Our lab has demonstrated that JNK inhibition in S426A/S430A mutant mice delays tolerance to Δ^9-THC, suggesting that there may be significant ligand biases in the activation and

role of JNK signaling in the development of tolerance to different cannabinoid agonists, particularly between Δ^9-THC and CP55,940.

5.2 Tolerance to WIN55,212-2 develops via GRK/β-arrestin mediated desensitization of CB_1

The effects of blocking GRK/β-arrestin2-mediated desensitization of CB_1 on tolerance were partially agonist-specific. Mutation of putative GRK phosphorylation sites in CB_1 delayed tolerance to WIN55,212-2 but not to CP55,940. Desensitization-resistant mice administered WIN55,212-2 demonstrated significantly slower development of tolerance to its antinociceptive effects in both the tail-flick and formalin tests. The modest effect of the S426A/S430A mutation on tolerance to CP55,940 was similar to what we previously observed for Δ^9-THC in these mutant mice (Morgan et al., 2014), suggesting that GRK/β-arrestin-mediated desensitization of CB_1 only partially mediates tolerance to both CP55,940 and Δ^9-THC and that other mechanisms contribute to tolerance for both agonists. Previous *in vitro* studies demonstrated that CP55,940 and Δ^9-THC binding induced greater association of β-arrestin2 with CB_1 compared to WIN55,212-2 (Laprairie et al., 2014), which might suggest that interfering with β-arrestin2-mediated desensitization would have a greater effect on tolerance to CP55,940 and Δ^9-THC. Instead, we see the opposite effect on tolerance for these agonists *in vivo*, highlighting the necessity of investigating agonist biases in whole-animal models. Further studies are needed to better elucidate the other pathways that may contribute to tolerance to these agonists.

	WIN55,212-2	CP55,940
Tail-flick (Acute Thermal)	**Genotype Effect**	No genotype effect
Formalin (Acute Inflammatory)	**Genotype Effect**	No genotype effect
Von Frey/Cisplatin (Chronic Neuropathy)	**Genotype Effect**	No genotype effect

Table 5.1 Summary of the effects of the S426A/S430A mutation on tolerance to the effects of WIN55,212-2 and CP55,940 on pain. Results from the primary antinociceptive and antiallodynic assays utilized in these studies are represented alongside the type of pain determined in each assay and the role of the S426A/S430A mutation (genotype effect) on tolerance in each assay.

Interestingly, the results we observed for tolerance to these agonists in acute pain assays did not fully manifest in the assay for chronic chemotherapy-evoked neuropathic pain. We found that disruption of GRK/β-arrestin-mediated CB_1 desensitization produced only a more modest effect on tolerance to WIN55,212-2 in mice experiencing chronic cisplatin-induced neuropathy, while tolerance to CP55,940 was not delayed at all in this model. It may be that the effects of the S426A/S430A mutation are masked in chronic pain conditions where CB_2 signaling pathways play a more important role in modulating antinociception. CB_2 expression is upregulated in chronic pain states following peripheral nerve injury but not in inflammatory pain models (Wotherspoon et al., 2005; Zhang et al., 2003). Both WIN55,212-2 and CP55,940 are mixed dual agonists for CB_1 and CB_2 (Howlett, 2002) and multiple studies demonstrate a lack of CB_2-mediated cannabinoid tolerance (Yuill et al., 2017). Therefore, it is possible that upregulation of CB_2 in cisplatin-treated mice leads to a sufficient level of CB_2-mediated nociceptive signaling to mask the effects of the S426A/S430A mutation on cannabinoid tolerance. Previous work has demonstrated that WIN55,212-2 elicits biased signaling at the CB_2 receptor compared to CP55,940 (Atwood et al., 2012). CP55,940 suppressed paclitaxel-induced pain responses via CB_2 in CB_1-knockout mice without inducing the development of tolerance (Deng et al., 2015). While the current studies have focused on tolerance to WIN55,212-2 and CP55,940 signaling via the CB_1 receptor, these findings suggest that the patterns of tolerance which we observed using the cisplatin-induced model of chronic neuropathic pain may be due to changes in CB_2 expression which have masked the effects of interfering with GRK/β-arrestin-mediated desensitization. Future studies should investigate this possibility by using a CB_1-selective agonist such as ACEA (Hillard et al., 1999) or by co-administering these mixed agonists in the presence of a CB_2 antagonist to isolate the specific contribution of the S426A/S430A mutation to tolerance for these agonists in this chronic pain model. Nonetheless, our findings suggest that tolerance to WIN55,212-2 is mediated almost

entirely by GRK/β-arrestin-mediated desensitization, while this pathway plays only a minor role in tolerance to CP55,940 and Δ^9-THC (Morgan et al., 2014).

Previous studies have found that WIN55,212-2 and CP55,940 show distinct biases in G protein activation via CB_1 (Georgieva et al., 2008). WIN55,212-2- bound CB_1 was found to be more effective at both recruiting and activating G_{il} proteins to the receptor than CB_1 bound to CP55,940. That study did not address whether the observed ligand bias extended to other G protein types, but biases in WIN55,212-2 activation of other G proteins have been reported (Flores-Otero et al., 2014; Glass and Northup, 1999; Mukhopadhyay, 2005; Prather et al., 2000). The S426A/S430A mutation disrupts desensitization of G protein-mediated signaling via CB_1, which has interesting implications in the context of potential for cannabinoid agonist biases in G protein activation on our own findings in behavioral assessments of tolerance. We did not observe a significant effect of the S426A/S430A mutation on tolerance to CP55,940, but if G protein-mediated signaling induced by CP55,940 is reduced this could provide an explanation for why disruption of GRK/β-arrestin2-mediated desensitization did not affect tolerance to CP55,940.

Tolerance to WIN55,212-2 was significantly and profoundly delayed but not entirely blocked in S426A/S430A mutant mice. Other studies have identified potential mechanisms which contribute to tolerance to WIN55,212-2, including PKCε, GASP-1, and CRIP1a (See section 3.4.4, (Blume et al., 2016; Martini et al., 2010; Tappe-Theodor et al., 2007; Wallace et al., 2009)). The central theme among these studies, our own included, is that disruption or removal of proteins that either phosphorylate or associate with CB_1 leads to a delay in antinociceptive tolerance to WIN55,212-2. The present study observes attenuated WIN55,212-2 tolerance by reducing the binding of a potential source of steric hindrance to G protein coupling with CB_1 (β-arrestin2). Steric hindrance is not highly specific, which opens the possibility for other proteins to interfere with G protein coupling in a similar manner. These other mechanisms may also lead

to G protein desensitization via non-classical pathways; removal of β-arrestin2 binding site from the 418-439 region (Jin et al., 1999) of the intracellular tail of CB_1 could delay activation of these responses, which may contribute to the eventual tolerance observed in S426A/S430A mutant mice treated with WIN55,212-2.

5.3 JNK signaling may have agonist-specific effects on cannabinoid tolerance

Our study is the first to address the role of JNK signaling in agonist-specific cannabinoid tolerance in a model of acute nociceptive pain. Similar to our previous findings in the opioid system (Marcus et al., 2015; Yuill et al., 2017), we found that the role of JNK signaling in cannabinoid tolerance was agonist-specific. Tolerance to WIN55,212-2 was not affected by SP600125 pretreatment while inhibition of JNK signaling drastically accelerated tolerance to CP55,940 in S426A/S430A mutant mice in both acute and inflammatory pain. The mechanism underlying the different effects of SP600125 in S426A/S430A mutant mice is unknown. However, previous work has shown that the S426A/S430A mutant form of CB_1 differentially couples to β-arrestin1 instead of β-arrestin2, and such a switch in arrestin coupling could impact the dynamics of JNK modulation of cannabinoid signaling for certain agonists (Delgado-Peraza et al., 2016). Of particular note is that we observed an opposite effect of JNK inhibition on CP55,940 tolerance from previous studies our lab has performed investigating antinociceptive tolerance to Δ^9-THC. In these studies, antinociceptive tolerance to Δ^9-THC was modestly delayed following pretreatment with SP600125 in wild-type mice, but antinociceptive tolerance to Δ^9-THC was abolished in S426A/S430A mutant mice. Δ^9-THC and CP55,940 are cannabinoid agonists of a similar chemotype, so this stark difference in response to SP600125 pretreatment was surprising. However, CP55,940 is a full agonist of CB_1 while Δ^9-THC is only a partial agonist. Differential

Agonist	JNK Inhibition
Δ^9-THC	Yes (↓ tolerance)
CP55,940	Yes (↑ tolerance)
WIN55,212-2	No effect

Table 5.2 Summary of the effects of JNK inhibition on tolerance in S426A/S430A mutant mice. Results from tail-flick antinociceptive assays and antiallodynic assays are represented. Δ^9-THC results are currently in submission but are not part of the work performed as part of this dissertation.

activity levels of CB_1 may explain why the effects of JNK activation vary between the two agonists, although further investigation of the role of JNK signaling in tolerance to another structurally similar cannabinoid (e.g. HU-210, full CB_1 agonist) would be necessary to either confirm this observation or eliminate agonist efficacy as a potential factor.

JNK inhibition produced significant effects on tolerance to Δ^9-THC and CP55,940, but only in S426A/S430A mutant mice. This finding has interesting implications for the role of JNK activation in cannabinoid tolerance, suggesting that both mechanisms coordinate to mediate antinociceptive tolerance to Δ^9-THC and CP55,940 but not WIN55,212-2. It is possible that β-arrestin2 binding to CB_1 limits the effects of JNK activation in signaling which leads to tolerance development, and interference with β-arrestin2 and CB_1 binding allows the full effects of JNK signaling on CB_1 activity to proceed. However, even without identification of a direct JNK/β-arrestin2 interaction, out findings implicate a novel non-classical mechanism for cannabinoid tolerance, specifically to Δ^9-THC and CP55,940.

We investigated JNK signaling using a small molecule JNK inhibitor, SP600125. SP600125 is highly specific inhibitor for JNK, but is not selective among its three isoforms, JNK1, JNK2, and JNK3. This lack of selectivity limits the use of SP600125 as a tool to investigate JNK signaling. From our reported results, we cannot identify which JNK isoforms are responsible for the effects of JNK inhibition on tolerance to CP55,940. Indeed, activity of different JNK isoforms may help to explain the opposite effects we observed on tolerance to Δ^9-THC and CP55,940, where the difference could be due to activity of different JNK isoforms that are differentially activated by various cannabinoid agonists. Continuing studies in S426A/S430A and JNK 1, 2, or 3 knockout double-mutant mice would help to elucidate how these pathways interact to modulate cannabinoid tolerance. In addition, we could also be observing off-target effect of SP600125 inhibition of either ERK1/2- or p38-mediated signaling, although this potential interaction could also be eliminated

by reevaluating the current results using JNK1, 2, or 3 knockout mice instead of a pharmacological inhibitor.

5.4 Synthetic cannabinoid agonists demonstrate biased signaling in tolerance development

Cannabinoid agonists demonstrate significant biases in tolerance development (Breivogel and Vaghela, 2015; Sim-Selley and Martin, 2002). However, confirming agonist biases requires tight control of experimental conditions to ensure that effects of other receptors or effector proteins are not attributed to functionally selective agonist biases (Kenakin, 2007). This is doubly important for analyses of behavioral effects of different agonists, where behavioral responses are particularly sensitive to variations in animal species, genetic background, or environment (Belzung and Griebel, 2001; Bolles, 1970; Peirson et al., 2017; Sorge et al., 2014; Witte et al., 2010). A strength of the current study is that tolerance to multiple cannabinoid agonists was assessed across multiple assays of both acute and chronic pain, which identifies a role for CB_1 desensitization on tolerance development in treatment of multiple types of acute and chronic pain. Moreover, the results we observed are consistent across different pain models, suggesting that the agonist biases we have observed are driven solely by the agonists and not by subtle differences between different tissues responsible for processing painful stimuli.

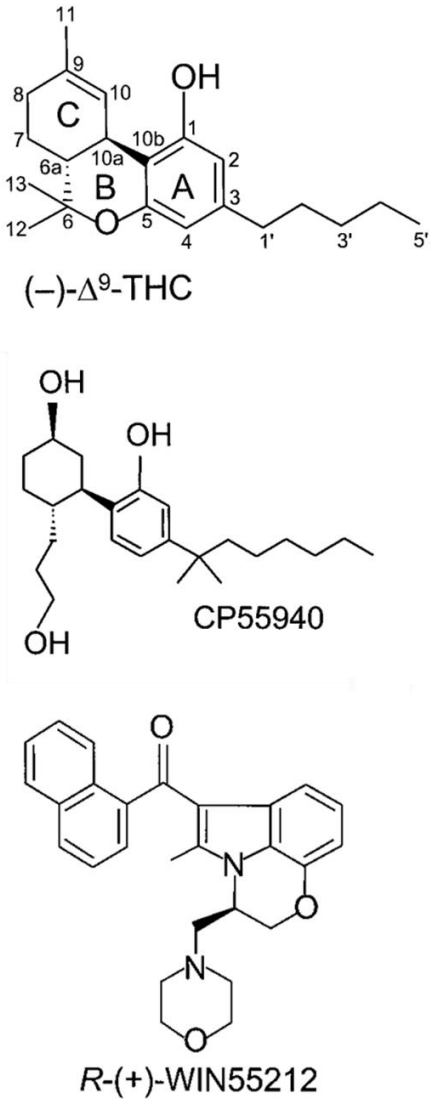

Figure 5.1. Chemical structures of Δ^9-THC, CP55,940, and WIN55,212-2. Adapted from (Howlett, 2002).

Assessment of equally efficacious cannabinoid agonists supports identification of agonist biases in the current study.

The significant differences in tolerance attenuation observed between S426A/S430A mutant mice chronically treated with Δ^9-THC (Morgan et al., 2014) and those treated with WIN55,212-2 could be the result of differences in efficacy between cannabinoid agonists. However, a significant effect on tolerance development to CP55,940, another synthetic, high-efficacy cannabinoid agonist, was not observed, suggesting that the differences on tolerance observed between the cannabinoid agonists tested is agonist-specific and not a result of relative agonist efficacy. In addition, WIN55,212-2 and CP55,940 are both synthetic cannabinoid agonists, suggesting that the observed variations in tolerance development are not due to origin (plant-derived vs. synthetic).

The clear agonist biases we observed may be a result of structural differences in agonist binding to CB_1. Binding to WIN55,212-2 or CP55,940 has been demonstrated to produce agonist-specific ligand-receptor conformations in CB_1 (Georgieva et al., 2008). Specifically, the conformational changes in CB_1 after binding WIN55,212-2 or CP55,940 produced equivalent, but opposing, shifts in spectral analyses of agonist-bound CB_1. WIN55,212-2 is of significantly different structure from the other two cannabinoid agonists investigated in this study (Figure 1.1). This structural variation may underlie the biased agonism we observed in development of tolerance, leading to regulation of tolerance to WIN55,212-2 by different signaling mechanisms than either CP55,940 or Δ^9-THC.

Biased agonism in JNK signaling in the development of tolerance has been previously reported. In a study of acute opioid tolerance in mice, pretreatment with SP600125 dose-dependently rescued acute morphine tolerance but did not alter acute tolerance to fentanyl (Melief et al., 2010). Our own results show a similar effect, where tolerance to Δ^9-THC – like morphine, a plant-derived, moderate efficacy agonist – is delayed by SP600125 pretreatment while tolerance to WIN55,212-2 – like fentanyl, a synthetic, high-efficacy agonist with a substantially different structure – is not altered by JNK inhibition. Interestingly, acute tolerance to fentanyl was

attenuated in GRK3 knockout mice (Melief et al., 2010), showing a similar effect on interference with MOR desensitization as we observed with WIN55,212-2 treatment and GRK/β-arrestin2-mediated CB_1 desensitization in S426A/S430A mutant mice. These similar results in two different GPCR systems throw into sharp relief the necessity of investigating agonist biases in pain responses to different classes of analgesics. We have demonstrated that biased agonism observed in the opioid system has a cognate in the cannabinoid system, which has substantial implications for the applicability of determinations of agonist biases outside of their individual receptor systems. Biased signaling mechanisms are not often considered outside of their respective neuronal pathways and may represent global GPCR biases which could benefit from further investigation in other neuronal systems.

Overall, we have observed that three different cannabinoid agonists induce signaling and the development of tolerance through three different pathways, where tolerance to WIN55,212-2 is mediated by GRK/β-arrestin2 desensitization of G protein signaling through CB_1, tolerance to CP55,940 is prevented by JNK signaling, and tolerance to THC is mediated by both classical CB_1 desensitization and JNK signaling.

5.5 Concluding remarks and model

The following model is proposed to explain the functions of GRK/β-arrestin2-mediated CB_1 desensitization and JNK signaling in cannabinoid tolerance. Binding of WIN55,212-2 and CP55,940 induces different agonist-receptor conformations which significantly alter the association of different scaffolding and effector molecules with CB_1. Binding to WIN55,212-2 induces a receptor conformation that is most likely biased toward G protein activation and signaling and does not facilitate interaction of JNK and CB_1. Subsequent desensitization of G protein signaling leads to the development of tolerance to the antinociceptive and antiallodynic effects of WIN55,212-2. However, binding to CP55,940 may induce CB_1 into a conformation which does not facilitate the development of tolerance through β-arrestin2-mediated signaling,

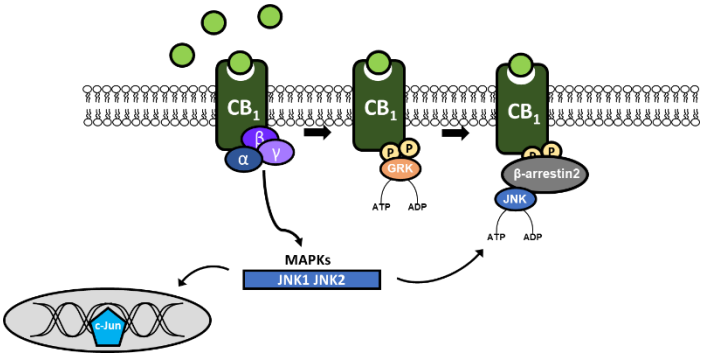

Figure 5.2. Proposed model for the development of antinociceptive tolerance to cannabinoids. Prolonged activation of CB_1 by WIN55,212-2 and Δ^9-Δ^9-THC leads to phosphorylation of the receptor by GRK and subsequent association of β-arrestin2 with CB_1. This blocks G protein coupling with CB_1 and leads to desensitization of the receptor and tolerance which is attenuated by the S426A/S430A mutation. Activation of CB_1 by Δ^9-THC and CP55,940 leads to activation of JNK, which contributes to the development of tolerance. Disruption of this activation leads to alteration of the rate of tolerance development to Δ^9-THC and CP55,940.

which renders any disruption of GRK and β-arrestin2-mediated desensitization ineffective on the development of tolerance. However, this conformation may facilitate phosphorylation of CB_1 by JNK which results in sustained CP55,940 signaling through CB_1 and prevents the development of antinociceptive tolerance.

The current study raises many further questions to be explored. We report significant agonist biases among synthetic, high-efficacy cannabinoid ligands in tolerance development via GRK/β-arrestin2-mediated CB_1 desensitization. The role of this mechanism of tolerance development has not been investigated in endocannabinoid signaling. Significant differences in β-arrestin1/2 activation and recruitment between WIN55,212-2 and 2-AG have been reported in cell culture models (Delgado-Peraza et al., 2016; Flores-Otero et al., 2014), which suggests that classical CB_1 desensitization may vary even among endocannabinoid agonists. We observed increased sensitivity to the antinociceptive and hypothermic effects of anandamide following acute administration in S426A/S430A mutant mice (Morgan et al., 2014), but we did not investigate tolerance development. Future investigations of the effects of GRK/β-arrestin2-mediated CB_1 desensitization on either chronic administration of endogenous cannabinoid agonists (2-AG and anandamide) or disruption of endocannabinoid degradation in the synapse [via inhibitors of FAAH (PF-3845 (Booker et al., 2012) or URB-597 (Russo et al., 2007)) or MAGL (JZL-184 (Long et al., 2009))] in S426A/S430A mutant mice could provide

essential insights in to how CB_1 desensitization regulated endocannabinoid signaling and could assist in identifying endocannabinoid ligand biases.

We have also previously identified a role for GRK/β-arrestin2-mediated desensitization in cannabinoid dependence, where S426A/S430A mutant mice demonstrated significant increases in antagonist-precipitated withdrawal behaviors following chronic Δ^9-THC administration (Morgan et al., 2014). Whether there are potential agonist biases in cannabinoid dependence has not been investigated and given the long delays we observed in development of antinociceptive tolerance we observed in S426A/S430A mutant mice treated with WIN55,212-2, the potential for increased dependence is particularly important. Further studies of the role of GRK/β-arrestin2-mediated CB_1 desensitization on cannabinoid dependence, particularly to WIN55,212-2, would provide a broader understanding of the implications of the prolonged drug sensitivity we observed for non-pain cannabinoid behavioral effects.

While we have established JNK signaling as a novel mechanism for the development of tolerance, we have not investigated whether agonist biases exist in cannabinoid tolerance in pathological pain models. In particular, we have not investigated the role of JNK inhibition on the development of tolerance to CP55,940 in desensitization-resistant S426A/S430A mutant mice in either acute inflammatory (formalin test) or chronic cisplatin-induced neuropathic pain models. The results we have observed in the tail-flick test are encouraging and suggest that a clear agonist bias exists for JNK activation in cannabinoid tolerance, but we cannot confirm any potential agonist biases without further investigation in different pain models. Accelerated tolerance to CP55,940 in SP600125-pretreated S426A/S430A mutant mice in either the formalin test or in mice experiencing chronic cisplatin-induced neuropathic pain would confirm this finding and suggest very complex regulation of JNK activation exists between CB_1 receptors bound to Δ^9-THC, CP55,940, and WIN55,212-2.

In summary, the findings from our study show that tolerance to WIN55,212-2 and CP55,940 develops through different mechanisms, where tolerance to WIN55,212-2 is primarily mediated by GRK/β-arrestin-mediated desensitization of CB_1. Desensitization-resistant S426A/S430A mice demonstrated agonist-specific delayed tolerance to WIN55,212-2 in models of both acute and chronic pain. The same mice demonstrated accelerated tolerance to CP55,940 when pretreated with SP600125, an inhibitor of activation of JNK signaling. The evidence supports previous *in vitro* studies of cannabinoid agonist biases, particularly in arrestin-mediated signaling, and suggests a critical agonist-specific role of both CB_1 desensitization and JNK signaling in the development of antinociceptive tolerance.

	GRK and β-arrestin Signaling			JNK Signaling	
Agonist	*Nociceptive Pain*	*Inflammatory Pain*	*Neuropathic Pain*	*Nociceptive Pain*	*Inflammatory Pain*
Δ^9-THC	Genotype Effect *	Genotype Effect °	In Process	**Yes ° (↓ tolerance)**	In Process
CP55,940	No effect	No effect	No effect	**Yes (↑ tolerance)**	In Process
WIN55,212-2	**Genotype Effect**	**Genotype Effect**	**Genotype Effect**	No effect	-----

Table 5.3 Summary of the effects of disruption of GRK and β-arrestin-mediated CB_1 desensitization and JNK signaling on tolerance to Δ^9-THC, CP55,940, and WIN55,212-2. Results from the primary antinociceptive and antiallodynic assays utilized in these studies are represented alongside the type of pain determined in each assay and the role of the S426A/S430A mutation (genotype effect) on tolerance in each assay. * Results reported in Morgan et al., 2014. ° Results are reported in Henderson-Redmond et al. (in submission) and are not part of the scope of this dissertation but are included for completion.

Appendix

Alterations in nociception and morphine antinociception in mice fed a high-fat diet.

Adapted from: Nealon, CM; Patel, C; Worley, B; Henderson-Redmond, AN; Morgan, DJ; Czyzyk TA. Alterations in Nociception and Morphine Antinociception in Mice Fed a High-fat Diet. *Brain Research Bulletin,* In Press, 2017. http://dx.doi.org/10.1016/j.brainresbull.2017.06.019

Abstract

Currently, more than 78.6 million adults in the United States are obese. A majority of the patient population receiving treatment for pain symptoms is derived from this subpopulation. Environmental factors, including the increased availability of food high in fat and sugar, contribute to the continued rise in the rates of obesity. The focus of this study was to investigate whether long-term exposure to a high-fat, energy-dense diet enhances baseline thermal and inflammatory nociception while reducing sensitivity to morphine-induced antinociception. Antinociceptive and hypothermic responses to morphine were determined in male and female C57BL/6N mice fed either a “western-style” diet high in fat and sucrose (HED) or a standard low-fat chow diet for 15 weeks. Antinociception was assessed using both the hot plate and tail flick tests of acute thermal pain and the formalin test of inflammatory pain. Acute administration of morphine dose-dependently increased antinociception in the hot plate and tail flick assays for mice of both sexes fed either chow or HED. However, female mice displayed lower antinociceptive response to morphine, regardless of diet, when compared to males in the tail-flick test. Hypothermic responses to acute morphine were also assessed in mice fed chow or HED. Male and female mice fed chow, and female mice fed HED displayed similar hypothermic responses to morphine. However, males fed HED did not exhibit normal morphine-induced hypothermia. Tolerance to the antinociceptive and hypothermic effects of morphine was assessed after ten days of repeated daily administration (10 mg/kg morphine). Male mice fed chow or HED developed tolerance to morphine in the hot plate test. However, females fed HED

did not. In the tail flick assay, under the treatment conditions of this study, only mice fed HED developed tolerance to morphine. All groups showed tolerance to morphine-induced hypothermia. In the formalin test, we found that both male and female mice fed HED had reduced sensitivity to the antinociceptive effects of morphine (6 mg/kg). Collectively, these data suggest that sensitivity and tolerance to the antinociceptive effects of morphine may be dependent on diet and sex in the hot plate and tail flick tests for thermal pain, and that the acute antinociceptive effects of morphine in the formalin inflammatory pain model may also be dependent on these two factors. In addition, diet and sex can influence morphine-induced hypothermia. Exposure to a HED may lead to changes in neuronal signaling pathways that alter nociceptive responses to noxious stimuli in a sex-specific manner. Thus, dietary modifications might be a useful way to impact pain therapy.

A1. Introduction

Obesity is one of the major epidemics challenging global health and wellness. Currently, 35% of Americans are obese (defined as a body mass index (BMI) ≥ 30 kg^2/m (Centers for Disease Control and Prevention, 2011; World Health Organization, 2016) and there are more overweight Americans than those of healthy weight (Finkelstein et al., 2012). The prevalence of obesity and co-morbid conditions is expected to increase in the coming decades (Ogden et al., 2014).

Clinical studies have demonstrated that obesity is associated with persistent pain complaints (Higgins et al., 2014). Obese patients report 20% greater total pain than normal weight patients (Stone and Broderick, 2012). Obesity can be a predictor for the onset and progression of chronic pain. The incidence of chronic, non-cancer pain rises with increasing BMI, leading to a four times greater likelihood of morbidly obese patients reporting chronic pain compared to non-obese patients (Hitt et al., 2007). The comorbidity of obesity and pain can be a significant barrier for weight loss, further exacerbating the pain. Obesity leads to a persistent, pro-inflammatory state which might be expected to increase pain, yet a study by Ray, et al (2011) showed that the

association of waist circumference and the increased risk for chronic pain in elderly obese subjects was not dependent on markers of insulin resistance or inflammation. In addition, it has been reported that obese subjects have a decreased ability to consistently score painful stimuli of similar intensity (McKendall and Haier, 1983; Pradalier et al., 1981) which is significant for the treatment of post-surgical pain as the number of bariatric and other surgeries are increasing in obese patients (Nguyen et al., 2016). Despite these studies, there remains a large gap of knowledge in the relationship of pain and obesity, and whether this relationship is distinct in males and females.

There have been very few clinical studies that have directly measured nociception in obesity. While the existing studies suggest that nociception is altered in obese patients, there is no clear consensus on how obesity affects overall sensitivity to pain. Evidence indicates that sensitivity to nociceptive stimuli is reduced in obesity (Price et al., 2013; Torensma et al., 2017). However, this sensitivity might vary between the type of noxious stimuli and the location to which it is applied. For example, Price et al. (2013) demonstrated that obese subjects were able to better tolerate thermal (cold and hot) stimuli applied to the abdominal area compared to non-obese controls. However, no differences in sensitivity were found when the stimuli were applied on the forehead or hand suggesting that the reduced pain sensitivity in obese subjects is dependent upon the increases in subcutaneous fat. In contrast, other studies have reported an increase in nociception in obese subjects (McKendall and Haier, 1983; Pradalier et al., 1981). None of these studies investigated acute inflammatory pain. Therefore, in addition to BMI, the type and location of the stimuli are also important when interpreting clinical studies of obesity and pain.

Pre-clinical studies of pain sensitivity induced by obesity have produced mixed results, with some suggesting obesity increases pain hypersensitivity (Pradalier et al., 1981; Sugimoto et al., 2008), others suggesting obesity reduces pain hypersensitivity (Frye et al., 1993; Kanarek et al., 2001, 1997; Ramzan et al., 1993), and still others indicating obesity has no effect on pain

hypersensitivity (Iannitti et al., 2012; Rossi et al., 2013). The wide range of results suggests that further investigation is necessary to evaluate the relationship between obesity and pain. The diet-induced obese (DIO) model is one of the most commonly used models of obesity in rodents yet no systematic studies have been done in this model to parse out the effects of obesity per se and effects of diet on nociception.

Prescription opioids are among the most commonly used pharmacotherapies to treat pain. Studies suggest that obese patients consume greater amounts of opioid analgesics at a higher rate than normal weight patients (D'Arcy, 2015) and are more likely to be prescribed opioids for pain therapy (Liabaud et al., 2013). Studies of obese patients report rates of narcotic analgesic usage as high as 21% (Raebel et al., 2004). Despite these observations, the efficacy and dosage of opioids required for effective analgesia has not been systematically explored in the obese population. Moreover, how an individual's diet can affect sensitivity to opioids has also not been studied. Increasing availability of highly palatable foods, which are high in fat and sugar and fuel the rise in obesity, and opioid analgesics, which have a high abuse liability and cause rapid tolerance, present a growing concern. As the availability of these expand, it is critical to understand how obesity could alter baseline nociception and overall sensitivity to opioids. Furthermore, a better understanding how diet influences both nociceptive pain and morphine antinociception raises the possibility that dietary interventions might be sufficient to reduce pain and enhance sensitivity to prescription opioids.

The overall objective of this study was to understand how long-term exposure to a nutritionally complete western-style, high fat, energy-dense diet (HED) in mice affects baseline nociception in acute thermal and inflammatory pain. In addition, we sought to understand how exposure to this diet in mice can alter the antinociceptive responses to acute and chronic morphine treatment. We investigated the hypothesis that mice fed a HED are less sensitive to and develop greater tolerance to the antinociceptive effects of morphine. Collectively, these studies

demonstrate that mice fed HED have reduced sensitivity to morphine in both thermal and inflammatory pain models. Furthermore, these studies demonstrated that the widely accepted preclinical diet-induced obese model can be successfully used to better understand how metabolic disease impacts pain.

A2. Materials and Methods

2.1 Subjects

This study was performed using 46 experimentally naïve adult male (n=20) and female (n=26) C57BL/6N mice (Envigo, Indianapolis, IN) obtained from an in-house breeding colony. Upon weaning, mice were group housed and maintained on a standard 12:12h light/dark cycle (lights on at 07:00, lights off at 19:00) with *ad libitum* access to water and a standard chow diet (Teklad 2018, 18% calories from fat, 3.1 kcal/g, Harlan Laboratories, Madison, WI). At 11-15 weeks of age, mice were divided into two groups that were fed either standard chow or a high-fat, energy dense diet (HED) (Teklad 95217, 39.8% calories from fat, ~30% calories from sucrose, 4.3 kcal/g, Harlan Laboratories, Madison, WI). Mice were maintained on chow (n=23) or HED (n=23) for fifteen weeks prior to testing and drug administration. All animal care procedures were carried out in accordance with the Guidelines of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 2011) and with approval from the Pennsylvania State College of Medicine Institutional Animal Care and Use Committee (IACUC).

2.2 Drugs

Morphine sulfate was obtained from the National Institute on Drug Abuse (NIDA) Drug Supply (Bethesda, MD). Morphine was dissolved in 0.9% saline and administered via subcutaneous (s.c.) injection in a single volume of 5 ml/kg body weight. Aqueous formalin solution (2.5% v/v) was prepared by diluting formaldehyde (Fisher Scientific, Pittsburgh, PA) with distilled water.

2.3 Whole body composition

Whole body composition was measured using a minispec LF90 Body Composition Analyzer (Bruker Optics, Billerica, MA), which measures fat mass, lean tissue mass, and free body fluid mass, in live subjects using time-domain nuclear magnetic resonance (TD-NMR) spectroscopy. Body composition was assessed in drug naïve mice before the initial administration of morphine.

2.4 Morphine dose-response and tolerance

Male and female mice fed chow or HED were assessed for antinociceptive and hypothermic responses to 0 (saline), 1, 3, 10, and 30 mg/kg morphine. Baseline nociception and morphine-induced antinociception were measured using a Columbus Instruments hot plate analgesia meter (Columbus, OH) and a TF-1 tail flick analgesia meter (Columbus, OH), with heat sources set to 55°C (hot plate) or to an intensity level of 5 (tail flick) as previously described (Henderson-Redmond et al., 2015; Marcus et al., 2015). To avoid tissue damage, cutoff times of 30 s and 10 s were used for the hot plate and tail flick tests, respectively. Nociception was measured immediately prior to drug injection, and at 30 min following treatment with saline or each morphine dose and the percent maximal possible effect (%MPE) of each dose was calculated, where %MPE = [post-test latency - pre-test latency) / (cut off time - pre-test latency)] * 100. Hypothermia was measured by determining body temperature with a mouse rectal thermometer (Physitemp Instruments, Clifton, NJ). Temperature values were converted to percent change in body temperature (%ΔBT), where %ΔBT = [(post-injection temp - pre-injection temp) / (pre-injection temp)] * 100.

Following assessment of the dose-response in drug naïve mice, tolerance to morphine was assessed by performing a second dose-response on the eleventh day after ten consecutive days of once-daily administration of 10 mg/kg morphine (s.c.).

2.5 Formalin testing

Male and female mice fed either chow or HED were assessed for differences in inflammatory pain response using the formalin test, which produces a characteristic transient, biphasic pattern of pain behavior (Rosland et al., 1990; Tjølsen et al., 1992). After a two-week washout period from the morphine dose-response tests described above, mice were administered either 0.9% saline (vehicle) or 6 mg/kg morphine (s.c.) 30 min prior to testing.

Mice were acclimated in a Plexiglass chamber (5”x5”x5”) on a transparent, elevated platform for 15 min prior to injection. Before and during testing, mice were observed using a mirror placed at a 45° angle underneath the platform to allow constant observation of the paws of the mouse. Mice were also recorded using a high-definition digital camera (Logitech, Newark, CA) placed underneath the transparent platform. After the acclimation period, 10 µl of 2.5% formalin was injected into the plantar surface of a single hind paw (0.5 ml syringe, 28 ½ gauge needle, Becton Dickson, Franklin Lakes, NJ). Immediately following formalin administration, mice were returned to the observation chamber.

Pain behaviors were measured for 60 min and quantified within 12 five-minute bins during the full observation period. Behaviors were quantified as the amount of time each mouse spent engaged in one of three behaviors: the injected paw bears little weight; the injected paw is held elevated above the surface of the platform; or, the injected paw is licked, shaken, or bitten. Quantified behaviors were recorded and weighted using the composite pain score-weighted scores technique, which resulted in composite pain scores (CPS) between 0 (indicating no pain behavior during the five-minute time bin) and 2 (indicating continuous, active pain behavior for the entire five-minute bin) (Henderson-Redmond et al., 2015; Marcus et al., 2015; Watson et al., 1997). The area under the curve (AUC) was calculated for the acute phase (0-15 min; Phase I) and inflammatory phase (15-60 min; Phase II).

2.6 Data analysis and statistics

All mice were age-matched littermate controls that were block randomized into chow or HED groups based on initial body weight. Pain behaviors were expressed as mean ± standard error of the mean (SEM) for all treatment groups. Body composition data were analyzed with unpaired t-tests. Morphine dose-response data were first analyzed using two-way analyses of variance with repeated measures (RM ANOVA) to assess the effects of drug (i.e. dose of morphine) as the within-subjects factor, and diet or sex (i.e., chow vs. HED, male vs. female) as the between subjects factor. Morphine tolerance data were analyzed using RM ANOVA to assess the effects of the drug (i.e., dose of morphine) as the within-subjects factor, and time point (i.e., pre vs. post ten days morphine treatment) as the between subjects factor. Post-hoc analyses were performed using Bonferroni's multiple comparisons test. ED_{50} values were calculated by generating dose response curves for each group using a four-parameter, non-linear regression analysis allowing for variable slope and taking into account that all data were normalized in the calculation of %MPE. ED_{50} values were compared across diet (chow vs. HED) or treatment length (i.e. pre vs. post ten days morphine treatment) using extra sum-of-squares F tests. Formalin experiments were analyzed using a two-way ANOVA with drug (i.e., saline or morphine) and diet or sex (i.e., chow vs. HED, male vs. female) as factors, and followed by comparisons with multiple t-tests using the Holm-Sidak method. Significance was set at $p<0.05$. All data were analyzed using Prism 6 statistical software (Graphpad, La Jolla, California, USA).

A3. Results

3.1 Body weight and composition of C57BL/6N mice fed chow or a high-energy diet

Fifteen-weeks of exposure to HED increased body weight by 5.9% in males and 19.2% in females compared to chow-fed, littermate controls (Table 1). These differences were significant in female but not in male mice. However, TD-NMR analysis of body composition demonstrated that all mice fed HED had significant increases in percent body fat (Table 1). While more robust

changes in body weight might have been expected, mice were already 11-15 weeks old when HED feeding commenced. The fat composition of the diet and the age when exposure to HED is initiated can significantly impact both body weight and fat-mass gain, with older mice showing partial resistance to weight and fat-mass gain compared to mice given HED upon weaning (Wang and Liao, 2012; Winzell and Ahrén, 2004). In summary, the HED-fed mice used in these studies were diet-induced obese (DIO) in a manner that is consistent with their advanced age and exposure to the HED.

	Males			**Females**		
	Chow	*HED*	*P*	*Chow*	*HED*	*P*
Lean, %	61.5 ± 0.7	62.0 ± 0.6	(*ns*)	67.50 ± 0.7	59.78 ± 3.1*	0.0310
Fat, %	14.4 ± 0.2	22.4 ±0.7****	<0.0001	11.18 ± 0.5	15.15 ± 1.5*	0.0261
BW, g	40.4 ± 1.1	42.8 ± 1.7	(*ns*)	26.1 ± 0.5	31.1 ± 2.1*	0.0383

Table A1. Body weight and body composition analysis. Values are mean ± SEM. Shown are body weights, and percent lean and fat mass values in C57BL/6N mice fed chow or a high-fat, energy dense diet (HED). Body composition was analyzed by TD-NMR after 15 weeks of diet exposure and just prior to starting morphine injections. Data were analyzed with unpaired t-tests (n= 10 males; n= 8 females per diet). BW, body weight in grams (g).

3.2 Sensitivity and tolerance to the antinociceptive effects of morphine using the hot plate test

Male and female mice fed chow or HED were tested for nociceptive pain responses using the hotplate assay. Acute treatment with morphine produced dose-dependent antinociception in the hot plate test (Figure 1, solid lines). A dose-dependent increase in antinociception was observed following acute morphine administration (0, 1, 3, 10, and 30 mg/kg; s.c.) in both male mice fed chow ($F_{3,54}$=56.66, p<0.0001) or a high-fat, energy dense diet (HED, $F_{3,54}$=64.37, p<0.0001) (Figure 1A and B, solid lines). Female mice fed either chow ($F_{3.42}$=27.54, p<0.0001) or HED ($F_{3,42}$=62.90, p<0.0001) (Figure 1 C and D, solid lines) also displayed significant main effects of dose. The magnitude of the antinociceptive response to morphine in the hotplate test did not differ in males compared to females fed either chow ($F_{1,16}$=0.01, p=0.9215) or HED ($F_{1,16}$=0.6997, p=0.4152).

After ten consecutive days of 10 mg/kg morphine (s.c.), male mice fed chow ($F_{1,18}$=6.309, *p*=0.0218) or HED ($F_{1,18}$=11.73, *p*=0.0030) (Figure 1A and B, solid vs. dotted lines) and female mice fed chow ($F_{1,14}$=12.33, *p*=0.0035) (Figure 1C, solid vs. dotted lines) developed tolerance as evidenced by a right-ward shift in the morphine dose-response data compared to the dose response data observed for naïve mice. However, unlike males, there was no significant effect of time (i.e., pre vs. post) in female mice fed HED ($F_{1,14}$=3.444, *p*=0.0846) (Figure 1D, solid vs. dotted lines) suggesting that there is reduced tolerance to the antinociceptive effects of morphine in the hot plate test in female mice. The development of morphine tolerance in the hot plate test is further supported by an analysis of calculated morphine ED_{50} values (Table 2). Male mice fed chow or HED demonstrated a significant increase in ED_{50} values following ten days of morphine treatment. In contrast to the results from the multifactorial analysis of antinociceptive responses in Figure 1D, a significant increase was observed in morphine ED_{50} values in female mice suggesting that tolerance also develops in female mice fed HED. Collectively, these data suggest that the development of tolerance to the antinociceptive effects of morphine in the hot plate test is dependent on diet and sex.

	Males		**Females**	
Hot Plate	*Chow*	*HED*	*Chow*	*HED*
Pre	8.99	6.54	9.91	9.75
Post	22.63#	22.71#	27.43#	21.23#
Tail Flick	*Chow*	*HED*	*Chow*	*HED*
Pre	2.30	2.32	8.80	3.57*
Post	3.67	3.85#	27.07#	54.01#

Table A2. Calculated ED50 values (mg/kg) from hot plate and tail flick tests. ED_{50} values were calculated from dose response curves generated by non-linear regression analysis. Values shown are mean ± SEM, and 8-10 mice were tested for each group. Data were analyzed using F tests. Statistical significance (p<0.05) is indicated with asterisks (*) for comparisons of chow versus high-fat, energy dense diet (HED) and number signs (#) for comparisons of acute exposure (pre) versus after 10 days of once daily treatment with 10 mg/kg morphine (post). v

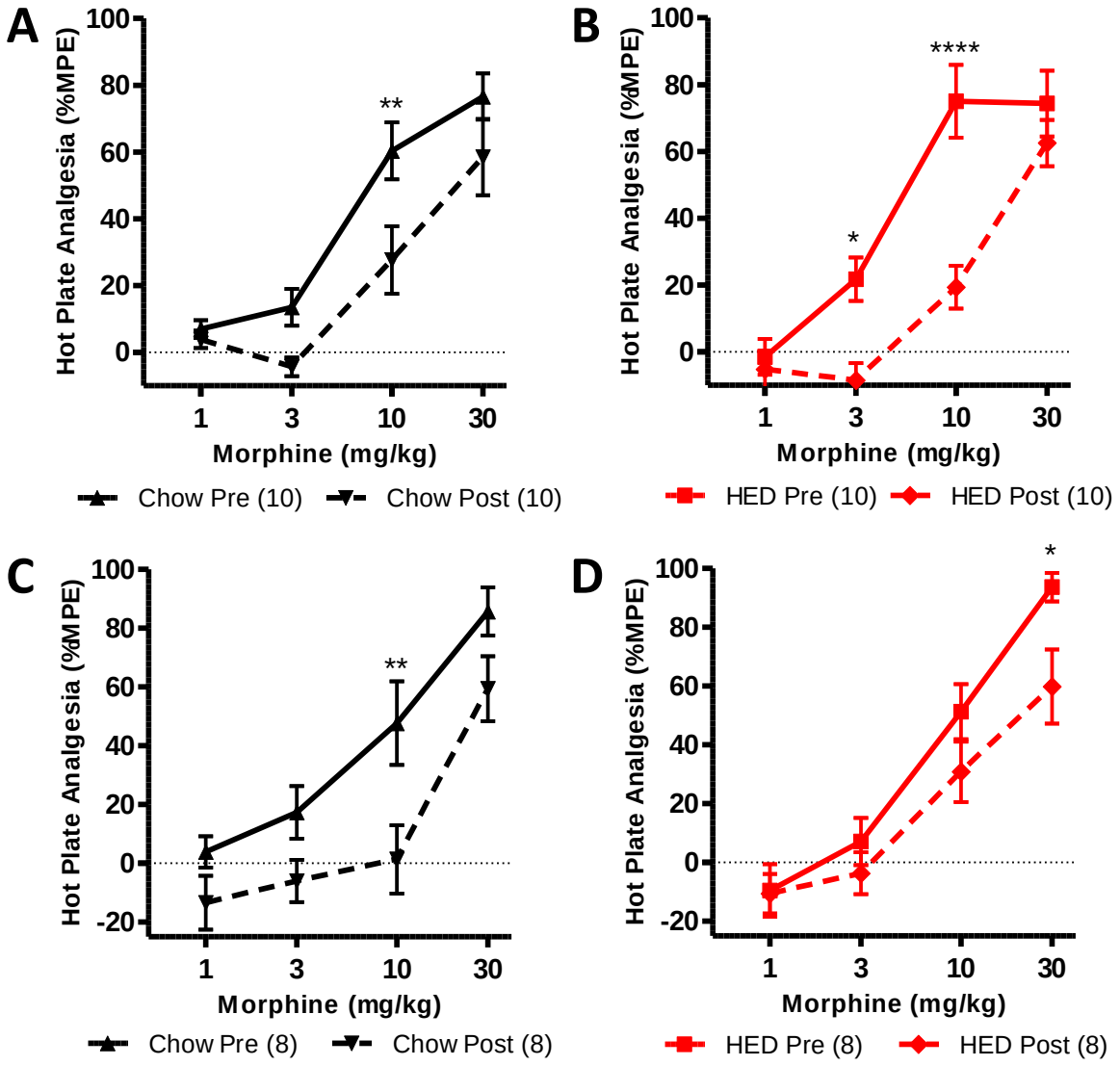

Figure A1. Sex and diet-specific differences in sensitivity to the antinociceptive effects of morphine in the hot plate test. Male (A, B) and female (C, D) mice fed chow (A, C) or high energy diet (HED) (B, D) for 15 weeks were assessed for the antinociceptive effects of 1, 3, 10, and 30 mg/kg morphine via the hot plate test. Mice were injected (s.c.) with the indicated dose of morphine and antinociception was measured 30 minutes later. The mean levels of antinociception (as percent maximal possible effect, %MPE) are shown for each of the doses tested in drug-naïve mice (Pre, solid lines), and the same mice treated with 10 mg/kg morphine once daily for ten days following the initial dose-response and again assessed for morphine antinociception (Post, dotted lines). Values shown are mean ± SEM, and 8-10 mice were tested for each group. Data were analyzed with two-way RM ANOVA and Bonferroni multiple comparisons test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

response data observed for naïve mice. However, unlike males, there was no significant effect of time (i.e., pre vs. post) in female mice fed HED ($F_{1,14}$=3.444, p=0.0846) (Figure 1D, solid vs. dotted lines) suggesting that there is reduced tolerance to the antinociceptive effects of morphine in the hot plate test in female mice. The development of morphine tolerance in the hot plate test is further supported by an analysis of calculated morphine ED_{50} values (Table 2). Male mice fed chow or HED demonstrated a significant increase in ED_{50} values following ten days of morphine treatment. In contrast to the results from the multifactorial analysis of antinociceptive responses in Figure 1D, a significant increase was observed in morphine ED_{50} values in female mice suggesting that tolerance also develops in female mice fed HED. Collectively, these data suggest that the development of tolerance to the antinociceptive effects of morphine in the hot plate test is dependent on diet and sex.

3.3 Sensitivity and tolerance to the antinociceptive effects of morphine using the tail flick assay

Mice were also assessed for morphine-induced antinociception using the tail flick assay. Acute morphine treatment (0, 1, 3, 10, and 30 mg/kg; s.c.) dose-dependently increased tail withdrawal latencies in the tail flick test (Figure 2, solid lines). Male mice fed chow ($F_{3,54}$=61.52, p<0.0001) or HED ($F_{3,54}$=56.67, p<0.0001) demonstrated dose-dependent morphine-induced antinociception in the tail flick test (Figure 2A and B, solid lines). Morphine also increased antinociception in female mice fed either chow ($F_{3,42}$=20.81, p<0.0001) or HED ($F_{3,42}$=21.78, p<0.0001) (Figure 2C and D, solid lines). The magnitude of these responses between males and females differed, with female mice on both chow ($F_{1,16}$=15.23, p=0.0013) and HED ($F_{1,16}$=7.966, p=0.0123) less sensitive to the antinociceptive effects of morphine as indicated by reduced %MPE values at doses of 3, 10 and 30 mg/kg. This is further supported by the generally higher calculated morphine ED_{50} values in female mice across all diets (Table 2).

In contrast to studies assessing tolerance to the antinociceptive effects of morphine in the hot plate test described previously, no significant effect of prolonged morphine administration (i.e. pre vs. post) was observed in the tail flick test in either male ($F_{1,18}$=1.786, *p*=0.1980) or female ($F_{1,14}$=3.879, *p*=0.0690) mice fed chow (Figure 2A and C, solid vs. dotted lines) following ten consecutive days of administration of 10 mg/kg morphine (s.c.). Consistent with this finding, morphine ED_{50} values were not changed by repeated daily morphine injection in chow fed male mice (Table 2); however, morphine ED_{50} values were significantly increased in chow-fed females. In contrast, robust tolerance to the antinociceptive effects of morphine was observed in male ($F_{1.18}$=6.690, *p*=0.0186) (Figure 2B, solid vs. dotted lines) and female ($F_{1,14}$=25.91, *p*=0.0002) (Figure 2D, solid vs. dotted lines) mice fed HED. Furthermore, significant increases in ED_{50} values for morphine were observed in male and female mice fed HED (Table 2).

Further analysis of dose-response data after ten days of repeated morphine injections demonstrated that there was a significant interaction between diet and dose in both male ($F_{3,54}$=9.646, *p*<0.0001) and female ($F_{3,42}$=2.940, *p*=0.0440) mice fed HED, but not in chow-fed mice (Male $F_{3,54}$=2.244, *p*=0.0936; Female $F_{3,42}$=3.879, *p*=0.1572). Thus, as in the hot plate test, acute morphine-induced antinociception and tolerance in the tail flick test is dependent upon both diet and sex.

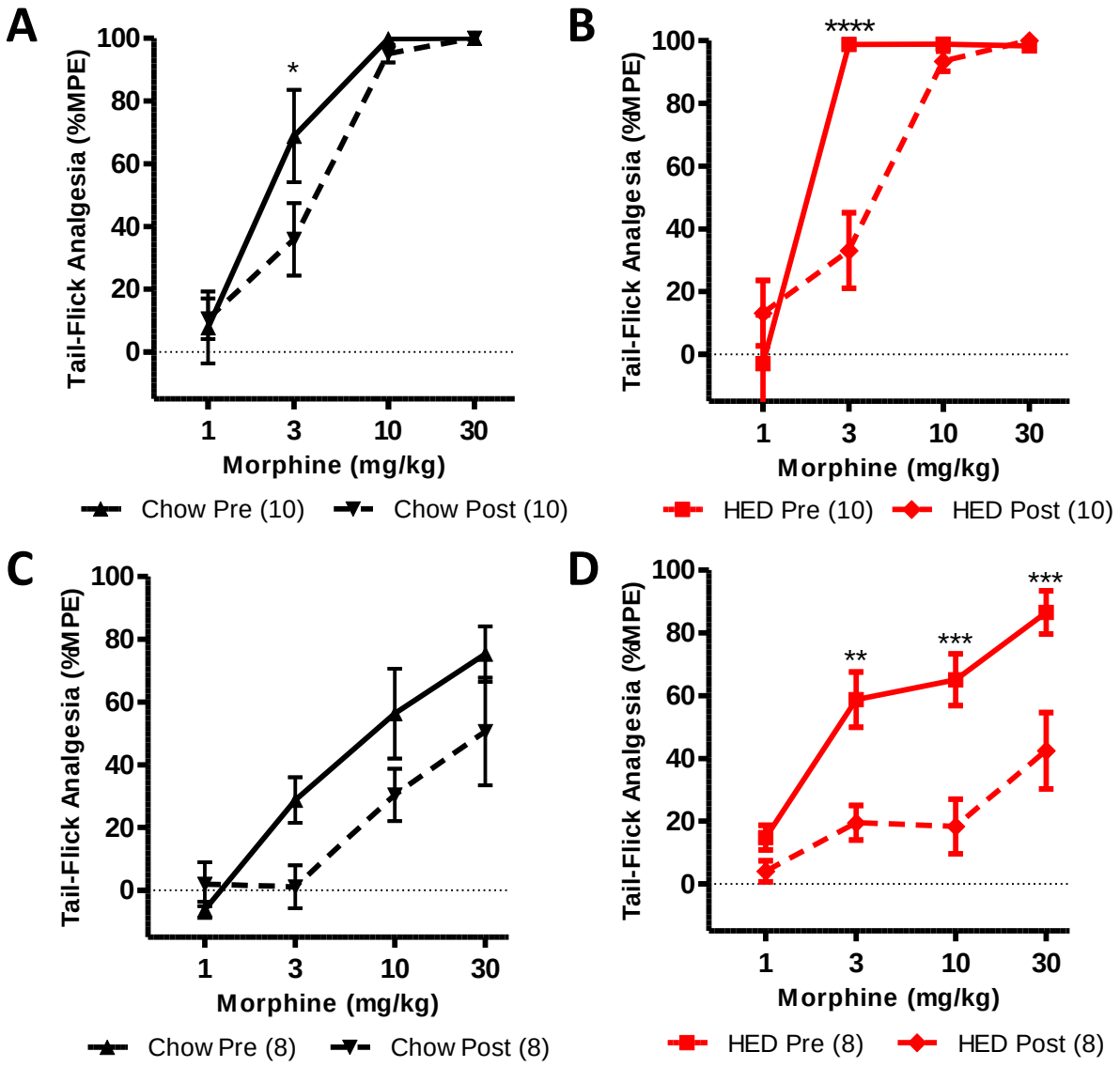

Figure A2. Sex and diet-specific differences in sensitivity in morphine-induced tail flick antinociception. The tail flick test was used to assess the antinociceptive effects of 1, 3, 10, and 30 mg/kg morphine (s.c.) in male (A, B) and female (C, D) mice fed chow (A, C) or high energy diet (HED) (B, D) for 15 weeks. Mice were injected (s.c.) with the indicated dose of morphine and antinociception was measured 30 minutes later. The mean levels of antinociception (as percent maximal possible effect, %MPE) are shown for each of the doses tested in drug-naïve mice (Pre, solid lines), and the same mice treated with 10 mg/kg morphine once daily for ten days following the initial dose-response and again assessed for morphine antinociception (Post, dotted lines). Values shown are mean ± SEM, and 8-10 mice were tested for each group. Data were analyzed with two-way RM ANOVA and Bonferroni multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 Effect of diet on tolerance to the hypothermic effects of morphine

In order to determine whether mice fed a HED differ in their hypothermic responses to morphine, changes in body temperature were measured in male and female mice fed HED and compared to chow-fed controls. Dose-dependent hypothermia was induced following acute morphine dosing (0, 1, 3, 10, and 30 mg/kg; s.c.) in male mice fed chow ($F_{3,54}$=25.84, p<0.0001), but not in male mice fed HED ($F_{3,54}$=1.108, p=0.3540) (Figure 3A and B, solid lines). Morphine treatment dose-dependently increased hypothermia in female mice fed either chow ($F_{3,42}$=37.87, p<0.0001) or HED ($F_{3,42}$=23.44, p<0.0001) (Figure 3C and D, solid lines). Daily injection of morphine (ten days, 10 mg/kg s.c.) caused a significant rightward shift in the dose response data after morphine treatment in male mice fed chow ($F_{3,54}$=7.865, p=0.0117) (Figure 3A, dotted lines). This rightward shift (tolerance) for the hypothermic effects of morphine was also observed in female mice fed chow ($F_{3,42}$=74.50, p<0.0001) or HED ($F_{3,42}$=47.00, p<0.0001) (Figure 3C and D, dotted lines). A dose x diet interaction was observed in males fed chow ($F_{3,54}$=42.81, p<0.0001), females fed chow ($F_{3,42}$=29.30, p<0.0001) and females fed HED ($F_{3,42}$=28.97, p<0.0001). Male mice fed HED did not develop tolerance to the hypothermic effects of morphine ($F_{3,54}$=0.9799, p=0.3353) and an interaction between diet and dose was not observed in these mice ($F_{3,54}$=0.7264, p=0.5407). In summary, sensitivity and tolerance to the hypothermic effects of morphine are dependent on both diet and sex, with females more sensitive to morphine-induced hypothermia as indicated by significant reductions in body temperature after 3mg/kg. These results are consistent with previous studies in mice (Kest et al., 2000).

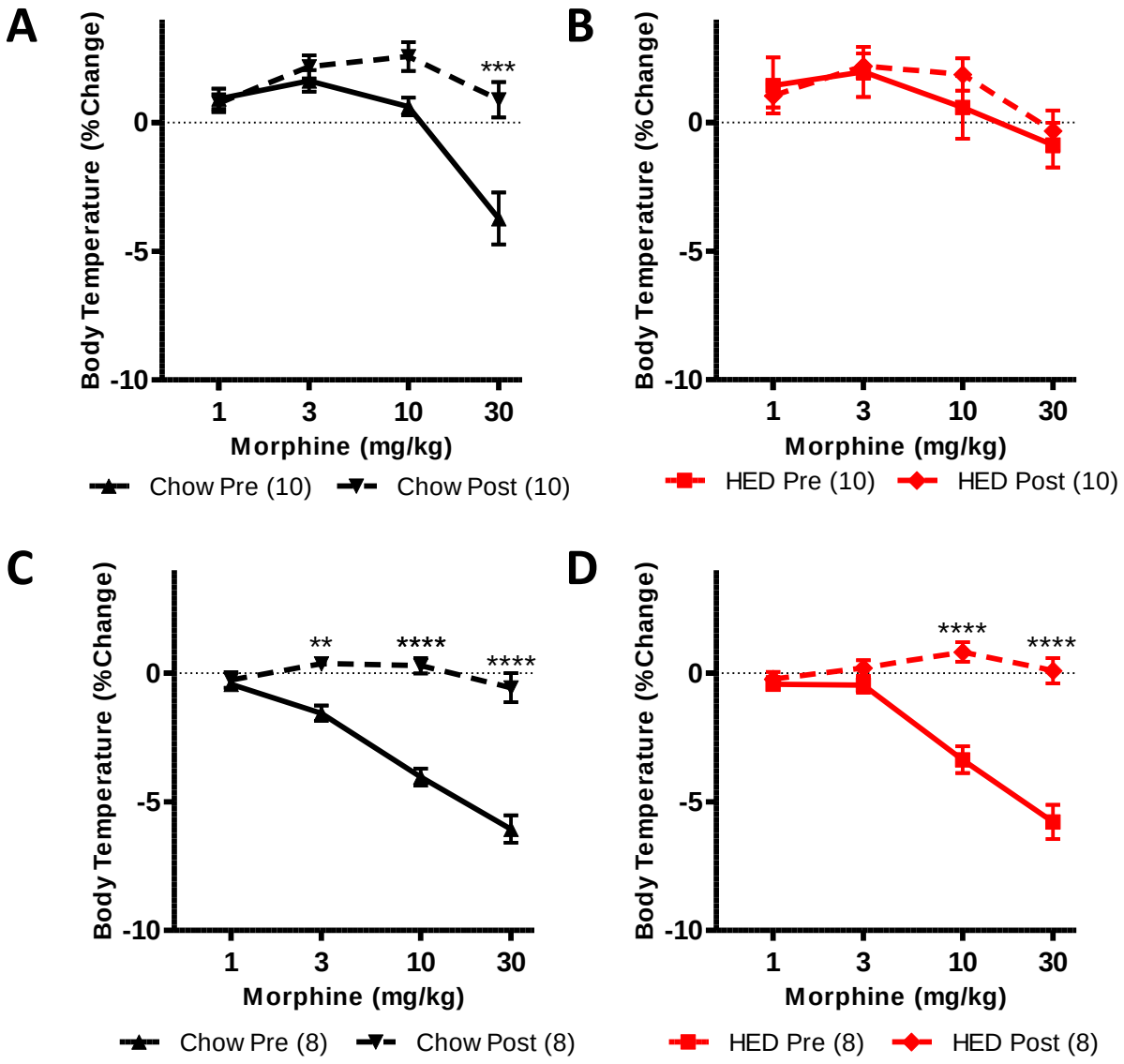

Figure A3. Sex and diet-specific differences in the hypothermic response to morphine**.** Male (A, B) and female (C, D) mice fed chow (A, C) or high energy diet (HED) (B, D) for 15 weeks were assessed for the hypothermic effects of 1, 3, 10, and 30 mg/kg morphine (s.c.) via the hot plate test. The mean degree of hypothermia (percent change in body temperature; %ΔBT) is shown for each of the doses tested in in drug-naïve mice (Pre, solid lines), and the same mice treated with 10 mg/kg morphine once daily for ten days following the initial dose-response and again assessed for morphine antinociception (Post, dotted lines). Values shown are mean ± SEM, and 8-10 mice were tested for each group. Data were analyzed with two-way RM ANOVA and Bonferroni multiple comparisons test. **p*<0.05, ***p*<0.01, ***p<0.001, ****p<0.0001.

3.5 Baseline nociception and sensitivity to the antinociceptive effects of morphine in the formalin test

An injection of dilute formalin (2.5%) into a single hind paw of mice produces a biphasic pain response corresponding to acute pain in the first 15 minutes (Phase I), followed by inflammatory pain in the subsequent 45 minutes (Phase II). In order to assess the effects of exposure to HED on inflammatory pain responses, formalin nociception was assessed in male and female mice fed chow or HED. Treatment with saline revealed that there were no differences in acute pain responses during Phase I between mice fed chow or HED (Figure 4A and B, solid bars).

Treatment with morphine (6 mg/kg, s.c.) produced significant antinociception in male mice fed either chow or HED. Male mice fed chow or HED displayed morphine-induced antinociception in both the acute phase ($F_{1,16}$=22.86, p=0.0002) (Figure 4A, hatched bars) and the inflammatory phase ($F_{1,16}$=32.00, p<0.0.0001) (Figure 4C, hatched bars). No significant interaction was found between diet and drug in either phase of the formalin test (Phase I $F_{1,16}$=0.3834, p=0.5445; Phase II $F_{1,16}$=0.05813, p=0.4576). However, post-hoc analysis revealed that there was a significant difference in morphine-induced antinociception between chow and HED fed male mice in Phase I (p=0.0304) but not in Phase II (p=0.1064).

Treatment with morphine (6 mg/kg, s.c.) also produced significant antinociception in female mice fed either chow or HED. Female mice fed either chow or HED demonstrated antinociception relative to saline treatment in both the acute ($F_{1,25}$=35.14, p<0.0.0001) (Figure 4B, hatched bars) and inflammatory phase ($F_{1,25}$=31.59, p<0.0001) of the formalin test (Figure 4D, hatched bars). Female mice fed chow did not exhibit significantly altered morphine antinociception compared to female mice fed HED in the acute phase (p=0.1342). However, a small but significant decrease in morphine-induced antinociception between chow and HED fed mice was observed in the inflammatory phase (p=0.0329) during posthoc analysis. Similar to males, no significant interaction between diet and drug was observed among female mice

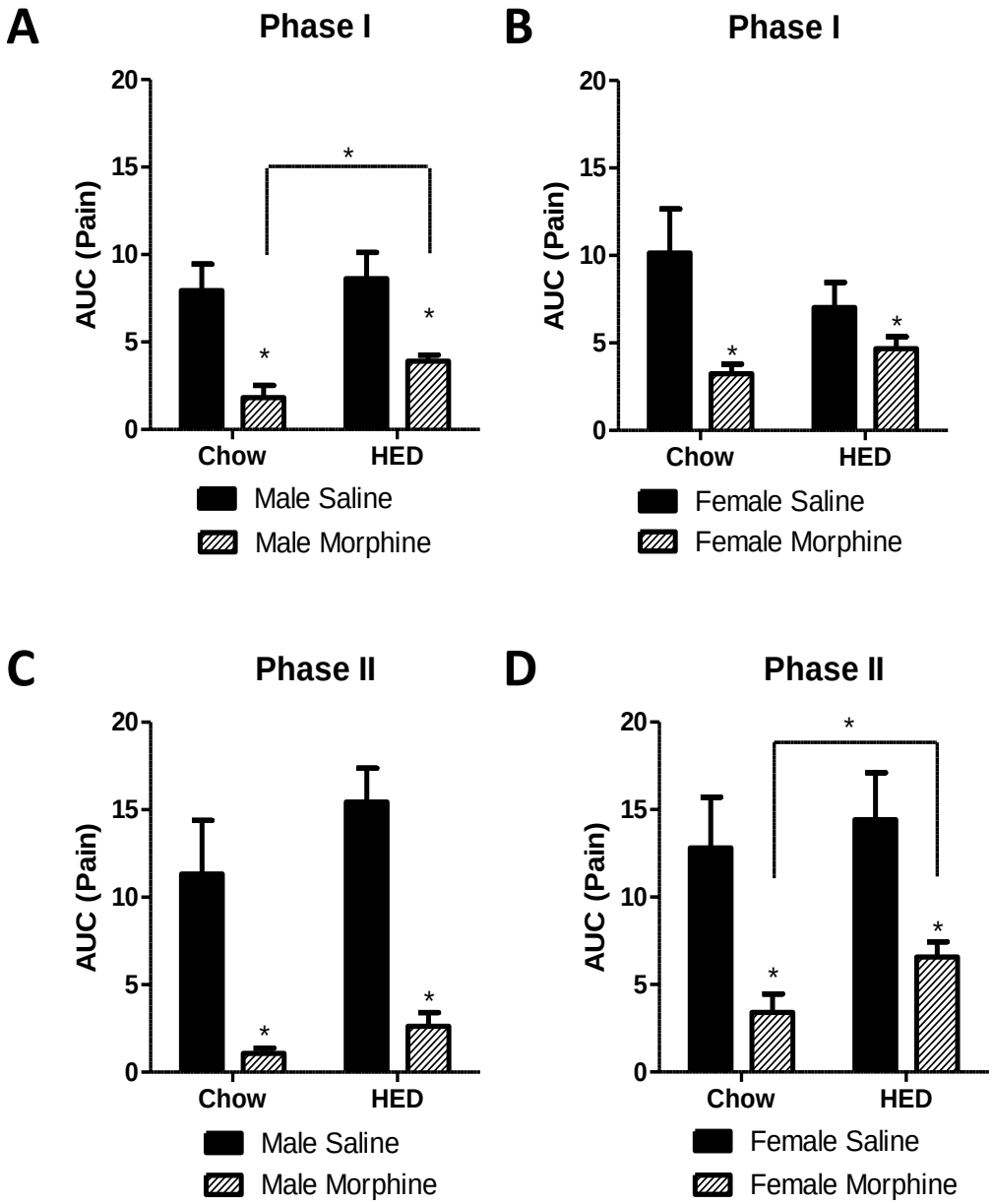

Figure A4. Morphine response in the formalin test model of inflammatory pain. The formalin testing paradigm was used to assess basal pain responses after saline injection (solid bars) and the antinociceptive effects of 6 mg/kg morphine (hatched bars) in male (A, C) and female (B, D) mice fed chow or a high energy diet (HED). Pain behaviors are shown as the mean area under the curve (AUC) of the composite pain score collapsed across Phase I (first fifteen minutes of testing, top panels) and Phase II (minutes 15-60, bottom panels). Groups represent mice that were treated with either saline [Male chow (n=5); Male HED (n=6); Female chow (n=5); Female HED (n=5)] or morphine [Male chow (n=6); Male HED (n=5); Female chow (n=9); Female HED (n=8)] prior to injection with 2.5% formalin. Means are shown as a function of each group; errors bars represent the standard error of the mean (SEM). Data were analyzed with two-way ANOVA and Bonferroni multiple comparisons test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

(Phase I $F_{1,17}$=0.02211, p=0.8855; Phase II $F_{1,17}$=4.432, p=0.0504). Further comparisons between males and females revealed that there were no differences in baseline (i.e. saline-treatment) pain scores between males and females fed chow or HED in either the acute or inflammatory phases. However, morphine was less effective in female mice in the inflammatory phase as evidenced by increased pain scores in females and a significant effect of sex ($F_{1,25}$=9.709, p=0.0046) in Phase II compared to males. In addition, a significant effect of diet ($F_{1,25}$=5.424, p=0.0282) was found supporting that mice fed HED had higher pain scores overall. These data suggest that the antinociceptive effects of morphine in the formalin test may be reduced by long-term exposure to HED in a sex-specific manner.

A4. Discussion

The present study sought to investigate how a 'western-style', high energy diet (HED) alters acute morphine response or tolerance and whether these effects are specific to sex or type of pain. Male and female C57BL/6N mice fed either regular chow or HED were assessed for differences in sensitivity and tolerance to the antinociceptive effects of morphine on pain from an external noxious stimulus in two models of acute thermal nociceptive pain: the tail flick test, which measures a primarily spinally-mediated nociceptive reflexive pain response; and the hotplate test, which measures a pain response that is mediated through both spinal and supraspinal mechanisms. The formalin test was also used to assess the antinociceptive effects of morphine in an inflammatory pain model. Our findings suggest that a HED alters both efficacy of and tolerance to morphine in mice and that these effects are sex-specific across different types of pain. Furthermore, we showed that the commonly used diet-induced obese mouse model can be successfully utilized for common nociceptive measurements and might be a more clinically relevant model given the increase in obesity among the general population.

Many studies of diet-induced changes in nociception utilize non-nutritive palatable sweet solutions to induce changes in basal antinociception, which is not the most physiologically

relevant feeding model. However, the current study utilized a nutritionally complete diet that more closely resembles high-energy (western-style) consumption in obese patients. Obese patients report greater pain and require increased doses of postoperative morphine than individuals of normal weight (Liabaud et al., 2013). To assess the effects of diet on morphine-induced antinociception, male and female mice fed either regular chow or a HED were tested for nociceptive responses in the hot plate and tail flick acute pain assays. In our study, morphine produced greater acute antinociceptive effects, as mice fed an HED demonstrated longer latencies in both the hot plate and tail flick tests than chow-fed counterparts. We observed an effect of diet in both assays, where male and female mice fed a HED demonstrated greater thermal hyperalgesia and reduced antinociceptive effects of morphine. These results are consistent with findings demonstrating increased nociception in the tail flick test following acute administration in rats fed hydrogenated vegetable fat (Kanarek et al., 1997, 1991). Male and female Long-Evans rats chronically fed sucrose solution also demonstrate greater tail flick (Kanarek et al., 2001; Roane and Martin, 1990) and hot plate (Kanarek and Homoleski, 2000) latencies.

We also assessed the effect of diet on nociception in a pathological pain model, the formalin test. We found the effect of diet was reversed in the formalin test of inflammatory pain. HED-fed mice of both sexes also trended toward elevated basal pain responses in the acute and inflammatory phases of the formalin test. Increased basal inflammatory pain was not sex-specific. We also observed an effect of sex on response to morphine in inflammatory pain. Female mice fed either lean chow or a HED demonstrated greater pain responses following morphine administration than male mice in the formalin test. Chow- and HED-fed mice demonstrated intriguing patterns of morphine-induced antinociception in the formalin test. Male HED-fed mice were significantly less sensitive to the antinociceptive effects of morphine than chow-fed mice in the acute phase of the formalin test, but not in the inflammatory phase. This

suggests male mice fed a HED are less sensitive to the antinociceptive effects of morphine on acute pain in the formalin test, which is in contrast to the increased efficacy of morphine following acute administration that we observed in HED-fed mice in the hot plate and tail flick nociceptive tests. This may be due to differences in processing and motor output between the thermal pain assays (hot plate, tail flick) and the formalin assay. Interestingly, female mice demonstrated an inverse relationship between diet and morphine-induced antinociception. Female mice fed HED showed greater pain behaviors than chow-fed mice only in the inflammatory phase of the formalin test, suggesting that HED-fed female mice are less sensitive to the antinociceptive effects of morphine on inflammatory hyperalgesia. Because basal pain responses did not differ between male and female mice, the difference in morphine response is not due to an underlying difference in baseline formalin pain. Although we observed reduced efficacy of morphine in our inflammatory pain assay, a study by Iannitti and colleagues (2012) using male lean (*fa/-*) and obese (*fa/fa*) Zucker rats did not observe any differences in morphine sensitivity in an inflammatory (carrageenan) pain model. In their model, obesity was driven by genetic changes as opposed to difference in diet between lean and obese animals. Conversely, our study sought to elucidate the effects of diet, not genetics, on morphine sensitivity. Therefore, our results suggest that a HED alone may be sufficient to alter morphine sensitivity independent of changes in body weight. Indeed, a study of chronic pain sensitivity in insulin treated- and untreated- obese Zucker rats (Sugimoto et al., 2008) found that increased tail flick antinociception could be observed before any significant changes in body weight.

Obesity is considered to be a low-grade inflammatory disorder (Greenberg and Obin, 2006; Hotamisligil, 2006), characterized by increases in circulating pro-inflammatory adipokines including leptin (Kershaw and Flier, 2004) and lipopolysaccharide (LPS) (Cani et al., 2007). Inflammatory changes are particularly prevalent in models of diet-induced obesity, including enhanced toll-like receptor 4 (TLR4) sensitivity (Tramullas et al., 2016) and changes in intestinal

microbiota (Ding et al., 2010; McAllan et al., 2014). We observed increased basal hyperalgesia in mice fed HED that was independent of sex that may be driven by a diet-induced increase in pro-inflammatory signaling. The immunosuppressive role of the mu opioid receptor (MOR) in innate and adaptive immune response has been well established (Ninković and Roy, 2013). The reduced morphine efficacy that we observed in HED-fed mice may be a result of an elevated inflammatory state. Diminished anti-inflammatory signaling via the MOR may also reduce peripheral pain signaling through the same receptor. Morphine treatment and HED consumption seem to induce opposing immunomodulatory adaptations in mice, which may explain the decreased efficacy of morphine on inflammatory pain. Mice fed a high-fat diet show changes in peripheral immune signaling, including increased circulating pro-inflammatory cytokines (Tramullas et al., 2016) and enhanced helper T cell (Th1) differentiation (Hong et al., 2013); however, treatment with morphine leads to Th2 differentiation in mice (Roy et al., 2005). A HED and morphine treatments induce opposing alterations in adaptive immunity, so it is possible that the pro-inflammatory state induced by feeding with a HED inhibits the normal response to morphine in the periphery. These underlying differences in immunity may affect the sensation from an immune-driven noxious stimulus, which is the basis for the formalin test. Peripheral immune changes may not affect centrally- and spinally-mediated antinociceptive responses equally, which may explain our opposing findings between our acute nociceptive pain assays (hot plate, tail flick) and our inflammatory pain assay (formalin).

The hypothermic effects of morphine were more pronounced in female mice than male mice. Our results agree with previous studies in mice (Kest et al., 2000), particularly regarding acute response to morphine hypothermia in drug naïve mice. Kest and colleagues reported a sex-specific effect of hypothermic tolerance to morphine which we did not observe, where male mice developed significant hypothermic tolerance to morphine while female mice did not, but our study included ten days of chronic administration of morphine while their previous study only

included four. Our study utilized a longer period of chronic morphine administration that may have eliminated differences in tolerance to the hypothermic effects of morphine between males and females by driving all of the mice in the study to tolerance. We did not observe an effect of diet on morphine tolerance in female mice, but a significant effect of diet was observed for hypothermic tolerance to morphine in male mice. Male mice fed a HED did not develop hypothermia following the initial exposure to morphine, unlike chow-fed counterparts. HED-fed male mice also did not show any tolerance to the hypothermic effects of morphine following chronic administration, while chow-fed mice developed significant hypothermic tolerance to morphine. Hypothalamic signaling is a primary mediator of body temperature that may be dysregulated in male mice fed a HED. The lack of hypothermia in male HED-fed mice presents an interesting anomaly, particularly because female mice fed a HED did not differ from chow-fed counterparts in hypothermia. When mice are housed at thermal neutral temperatures hypothermia does not develop in morphine-treated mice. Morphine lowers the hypothermic set point, but the set point may already be altered in mice fed a HED. However, alterations in hypothalamic signaling occur as a result of leptin insensitivity (Pinto et al., 2004; Sun et al., 2016; Yu et al., 2016). Extra-hypothalamic regulation of energy homeostasis, particularly through alterations in hormonal signaling, which is more effective than the subtle changes caused by diet may account for the discrepancy in hypothermia between both sexes in mice fed a HED.

Tolerance to the antinociceptive effects of opioid analgesics develops when a specific dose of opioid becomes ineffective for pain relief during chronic use, requiring greater doses to achieve the same effect. We examined whether tolerance to the antinociceptive effects of morphine was altered as an effect of diet or of gender and found tolerance to morphine developed in at least one thermal pain assay in all groups of mice. Both male and female mice fed either chow or a HED developed tolerance to the antinociceptive effects of morphine in the hot plate test.

Using the tail flick test, tolerance was observed only in HED-fed mice. These two thermal pain assays measure nociception that is processed in different ways (hot plate spinal and supraspinal, tail flick primarily spinally-mediated), which may explain the differences in morphine tolerance we observed in each test. Mice of both sexes fed a HED developed more robust tolerance to morphine than chow-fed counterparts in both antinociceptive assays, suggesting that a HED increases morphine tolerance. Our findings are not consistent with previous studies which reported that long-term sucrose feeding does not alter antinociceptive tolerance to morphine in the tail flick test (Schoenbaum et al., 1989) or the findings of D'Anci (1999), who reported reduced tolerance to the antinociceptive effects of morphine in the tail flick assay in male Long-Evans rats following chronic intake of palatable fluids. Our study used higher doses of morphine for both chronic administration and dose-responses than reported by either Schoenbaum or D'Anci, which will lead to greater development of tolerance in morphine treated animals.

The mechanism for enhanced morphine tolerance in mice fed a HED is unclear but may involve changes in the Mu-opioid receptor (MOR) that are induced by the diet and/or obesity. Morphine is a potent MOR agonist. In humans, MOR binding is reduced in brain regions involved in reward in obese woman and this reduction is reversed following weight loss after bariatric surgery (Karlsson et al., 2015a, 2015b). Chronic sucrose feeding increases binding affinity for opioid receptors throughout the brain (Marks-Kaufman et al., 1989). MOR changes may therefore explain the elevated acute response and overall enhanced tolerance to morphine that we observed in HED-fed mice. MORs in the periaqueductal grey (PAG) have been shown to mediate the effects of long-term sucrose feeding on pain (Kanarek et al., 2001). However, the potential for changes in morphine pharmacokinetics caused by metabolic alterations in mice fed HED also cannot be eliminated at this point. Future studies of the influence of HED on morphine sensitivity should look at changes in MOR expression and binding in brain regions such as the

PAG which are part of known nociceptive pathways and should also investigate the plasma morphine levels in HED-fed mice after morphine injection to determine the contributions of diet-induced metabolic changes on morphine-induced antinociception.

While we are the first to report sex-specific effects of a nutritionally complete, high-fat and -sugar diet on pain, our results corroborate extensive related clinical and pre-clinical studies demonstrating sex-specific differences in pain and morphine response. Female mice display greater baseline pain behaviors and require more morphine for effective anti-nociception than male mice (Bartley and Fillingim, 2013; Niesters et al., 2010). We observed an effect of sex on morphine efficacy in the tail flick assay that was independent of diet, as reported previously (Craft et al., 1999). Male mice displayed longer tail-flick latencies than female mice on both types of diet. Interestingly, this was not consistent between our two thermal acute pain assays, as male responses in the hot plate test were not significantly different from those of female mice. After acute morphine treatment, all mice had similar maximal pain responses (80-85% MPE) in the hot plate test, while in the tail flick test only male mice demonstrated maximal drug responses (100% MPE) independent of diet. Morphine was less effective in female mice in the tail flick test; females did not show maximal drug responses, even in drug naïve mice. Female mice were more sensitive to the hypothermic effects of morphine than male mice, suggesting that the sex effects of morphine may be tissue/response-specific. A HED also produced effects that were sex-specific. In contrast to male mice, where consuming a HED had a similar effect on antinociceptive tolerance to morphine in both hot plate and tail flick assays, female mice demonstrated varying sensitivity to the effects of a HED. Tolerance to the antinociceptive effects of morphine in the hot plate test developed only in female mice fed regular chow; however, female mice fed a HED developed substantial tolerance to morphine in the tail flick test while chow-fed female mice did not.

The sex-specific morphine tolerance we observed may be a result of changes in MOR signaling outside of primary pain-processing regions of the brain, particularly the periaqueductal gray (PAG). Modulation of MOR signaling in the PAG was not found to be responsible for the sex-specific development of morphine tolerance (Bernal et al., 2007; Kanarek et al., 2001), suggesting that the alterations we observed in morphine tolerance occur as a result of modulation of MOR function in either the periphery or other pain-processing regions of the brain or ganglia. Estrous cycle was not monitored in our female subjects. The contribution of the estrous cycle to pain behaviors of female mice has not been clearly defined. Female mice in proestrous exhibit increased pain sensitivity and decreased tail flick latencies during fluctuation of levels of estrogen and progesterone (Frye et al., 1992), although results from the same lab indicated that estrous cycle does not have an effect on tail flick latencies (Frye et al., 1993). Any hormonal effect on tail flick latency was eliminated following chronic sucrose feeding (Frye et al., 1993, 1992). We did not observe any differences in morphine response in drug naïve female mice fed either chow or a HED. If estrous cycle-dependent effects on pain sensitivity are reduced by a consuming a palatable diet and we see no difference in initial pain sensitivity, it is likely that estrous cycle did not alter morphine responses in female mice.

Weight loss required of obese patients to return to a normal BMI is not insignificant. Major weight loss is a prolonged process, and a return to normal BMI may not occur in sufficient time to provide improved pain management. Indeed, for obese patients suffering from chronic pain and disability, the necessary weight loss may not be feasible. We have demonstrated altered morphine-induced antinociception in mice fed HED without any significant change in body weight in males (Table A1) which is consistent with weight-independent effects observed in other studies (Kanarek et al., 1997, 1991). These findings may help position dietary intervention as an ideal adjuvant to traditional pain management. In comparison to major weight loss, dietary changes are easier and would provide more immediate effect on clinical pain

management. Patients experiencing chronic pain could see improvements in pain and quality of life and a reduction in opioid use.

While we have demonstrated that an increase in dietary fat and sugar leads to altered morphine sensitivity, the clinical implications of our findings are limited. The primary concern for patients is not how to reduce opioid efficacy, but rather how to preserve or rescue morphine sensitivity in obese patients. Future studies need to focus on the relationship between reduction of dietary fat and recovery of morphine sensitivity. Chronic sucrose-fed rats develop morphine sensitivity similar to basal levels when returned to chow (D'Anci et al., 1996), but the effect of replacing a highly palatable, nutritionally complete diet with chow on morphine sensitivity has not been investigated. Determining whether the physiological changes caused by consumption of a HED can be reversed will be critical to understanding the clinical implications of a dietary intervention for pain management.

Overall, our results support that a nutritionally complete, high fat and sucrose diet leads to significant alterations in nociception in inflammatory pain, acute morphine-induced antinociception, and the development of morphine tolerance. Future studies should address the relationship between dietary changes and morphine-induced antinociception, specifically the full extent of the inverse relationship between dietary fat content and morphine antinociception. In addition, determining whether the physiological changes caused by consumption of an HED can be reversed will be critical to understanding the clinical implications of a dietary intervention for pain management. This could help physicians provide more complete pain management to and provide an avenue to reduce opioid use in obese patients.

		Males	Females
Hot Plate		*HED vs. Chow*	*HED vs. Chow*
Acute (ED_{50})		↓ (trend)	↔
Tolerance (ΔED_{50})		↑	↓
Tail Flick		*HED vs. Chow*	*HED vs. Chow*
Acute (ED_{50})		↔	↓
Tolerance (ΔED_{50})		↑	↑
Hypothermia		*HED vs. Chow*	*HED vs. Chow*
Acute		↓	↔
Tolerance		↔	↔
Formalin (AUC)		*HED vs. Chow*	*HED vs. Chow*
Saline	Phase I	↔	↔
	Phase II	↔	↔
Morphine	Phase I	↑	↑ (trend)
	Phase II	↑ (trend)	↑

Table A3. Morphine efficacy is reduced and tolerance is increased in mice fed an HED. A summary table is presented for all data indicating how morphine-induced antinociceptive responses changed in mice fed HED compared to chow-fed same-sex controls. Responses are shown as either change in ED_{50} (hot plate, tail flick), change in total hypothermia, or change in total pain behaviors (formalin). Data for both male and female mice are shown. Arrows indicate the direction of response, with ↔ indicating no difference when comparing mice fed HED to chow. Trends are noted in parentheses ($0.05<p<0.1$).

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VITA
Caitlin Marie Nealon

EDUCATION

2012 - 2018	PhD in Biomedical Sciences, Pennsylvania State University College of Medicine, Hershey, PA
2010 - 2011	MS in Biotechnology, Georgetown University, Washington, DC
2006 - 2010	BA in Chemistry, Sweet Briar College, Sweet Briar, VA

SELECTED PUBLICATIONS

Nealon, CM; Hale, DE; Henderson-Redmond, AN; Morgan, DJ. Tolerance to WIN55,212-2 but not CP55,940 is significantly delayed in desensitization-resistant S426A/S430A mice. Submitted to *Neuropharmacology* Feb 2018.

Henderson-Redmond, AN*; **Nealon, CM***; Davis, BJ*; Yuill, MB*; Muller, J; Haskins, CP; Marcus, DJ; Czyzyk, TA; Mackie, K; Guindon, J; Morgan, DJ. c-Jun N-terminal Kinase Signaling Pathways Mediate Cannabinoid Tolerance in and Agonist-Specific Manner. Submitted to *Neuropharmacology* Jan 2018.

Nealon, CM; Patel, C; Worley, B; Henderson-Redmond, AN; Morgan, DJ; Czyzyk TA. Alterations in Nociception and Morphine Antinociception in Mice Fed a High-fat Diet. *Brain Research Bulletin,* In Press, 2017. http://dx.doi.org/10.1016/j.brainresbull.2017.06.019

SELECTED PRESENTATIONS

Nealon, CM; Hale, DE; LeFleur, RL; Morgan, DJ. Agonist-specific mechanisms of cannabinoid tolerance in desensitization-resistant mice. Talk presented at the Annual International Cannabinoid Research Society Symposium on the Cannabinoids, Montreal, QC, Canada, June 23, 2017.

Nealon, CM; Zee, ML; Kline AM; DeTurk N; Morgan DJ. Investigating Agonist-Specific Mechanisms of Cannabinoid Tolerance. Invited talk presented at the Pharmacology of Drugs of Abuse Conference at Northeastern University, Boston MA, June 6, 2016.

Nealon, CM; Zee, ML; Morgan DJ. Where the wild mice are: Ligand biases in cannabinoid tolerance. Travel fellow talk presented to students of Gill Center for Biomedical Sciences at Indiana University, Bloomington, IN, October 1, 2015.

HONORS AND AWARDS

2015, 2016, 2017	Gill Symposium Conference Travel Award
2017	Penn State College of Medicine Class of 1971 and 1974 Award
2016, 2017	Penn State College of Medicine Graduate Alumni Endowed Scholarship
2017	Cayman Chemical Student Travel Award
2017	Penn State University Life Sciences Symposium, Fourth Place Poster
2017	ASPET/Experimental Biology Travel Award
2016	Chemistry and Pharmacology of Drugs of Abuse Conference Travel Award
2016	AAAS/Science Program for Excellence in Science

SERVICE AND OUTREACH

2017-2018	Vice President, Outreach Director, Graduate Women in Science, Kappa Rho Chapter
2017	Mentor, Summer Undergraduate Research Program
2016, 2017	Judge, Capital Area Science and Engineering Fair
2015-2016	Co-Chair, Career Day Committee
2015	Keynote speaker, Pennsylvania Society for Biomedical Research Annual Awards Dinner
2014-2015	Chair, Fundraising Committee, Graduate Student Association
2013-2015	Academic Integrity Officer, Graduate Student Association