2018

Complexities of Chronic Opioid Exposure

Maciej Gonek
Virginia Commonwealth University

Complexities of Chronic Opioid Exposure

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Maciej Gonek

Bachelor of Science, University of North Carolina at Chapel Hill 2013

Director: Dr. William L. Dewey
Professor and Chair, Pharmacology & Toxicology

Virginia Commonwealth University
Richmond, Virginia
May, 2018

Acknowledgments

Trying to thank everyone that I would like to thank while keeping this concise would be impossible. Nevertheless, I am eternally gratefully to my mentor Dr. Bill Dewey. He was able to inspire me during my first meeting with him and has ever since. Since he offered me to join his lab, he has guided my growth as a scholarly thinking scientist. He has constantly shown me either directly or indirectly how to handle any challenge that life throws at me with grace and humility. Thank you Dr. Dewey for continually picking me up when I was down and then continually picking on me every other chance you had. So much of our success is also due to the front office, Pat, Sheryol, Sallie, and Laura. Thank you to all of you. To my committee members: Dr. Hamid Akbarali, Dr. Steve Negus, Dr. Carmen Sato-Bigbee, Dr. John Bigbee, as well as the late Dr. Lou DeFelice. It was a pleasure to learn from you all in classes, committee meetings, and in person. Thank you all for the valuable input into this project and my progress as a scientist. Thank you also to each of you for letting me use your labs for resources as well as your minds to gather some snippets of wisdom. Thank you to our collaborator in the UK, Dr. Graeme Henderson, who has been working with us on the reversal of tolerance project. Every time I've interacted with him has led to me learning something new. To all the members of the Pharm/Tox department: Thank you so much for all your teachings. As Dr. Dewey frequently states "It takes all kinds." Our department here really exemplifies how a collaborative environment really benefits us all. In particular, I would like to thank Dr. Bettinger, Dr. Eddy Ishac, Dr. Patrick Beardsley, Dr. Laura Sim-Selley Dr. Gerard Moeller, and Dr. Aron Lichtman for helping me when I needed guidance. Thank you as well to Dr. Travis Grim for being a good friend as well as a semi-mentor before I officially joined a lab. Thank you to all of the Dewey and Akbarali lab members during this time: Aravind, Joy, Sukhada, Bethany, Dwight, Ken, Minho, Essie, Ryan, Karan, Kumiko, Jacy, Krista, and Dave. You all have been fantastic coworkers and also good friends. Thank you for all your scientific input as well as the laughs. Thank you to all the friends I've made here in Richmond, especially Dave Brohawn with whom I've had more laughs per minute spent than with anyone else.

Special thanks to those who directly helped me with these studies. Thank you to Dr. Jason Paris and Dr. Kurt Hauser especially for collaborating with us in order to help answer the questions we posed in Chapter 3 of this dissertation. Your ways of tacking scientific questions had me on my toes and I appreciated every minute of it. Thank you so much for your help, teaching, and guidance. I would also like to thank Dr. Virginia McLane for her part in performing the cytokine assay and allowing me to shadow her. Thank you to Dr. Ryan Mischel and future doctor Karan Muchala for their help in performing the DRG electrophysiology in Chapter 4.

Thank you to my family here and in Poland for supporting me. Last but certainly not least, thank you so much to my parents. Twoje miłość dla siebie i dla mnie inspiruje mnie każdego dnia

Table of Contents

List of Tables

List of Figures

Chapter 2.

Chapter 3.

List of Abbreviations

AIDS	Acquired immune deficiency syndrome
AP	Action potential
BBB	Blood-brain-barrier
BCA	Bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
CBX	Carbenxolone
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CPP	Conditioned Place Preference
CCR5	C-C chemokine receptor type 5
CDC	Centers for Disease Control
CNS	Central nervous system
CSF	Cerebral spinal fluid
Cx43	Connexin 43
DZ	Diazepam
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin)
DOR	δ-opioid receptor
DRG	Dorsal root ganglia
ED_{50}	Effective Dose (half-maximal)

FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric acid
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
HIV	Human Immunodeficiency virus
IDU	Injection drug use
i.p.	Intraperitoneal injection
ISTD	Internal Standard
JNK	c-Jun N-terminal kinase
KOR	κ-opioid receptor
MOR	μ-opioid receptor
MP	Morphine Pelleted
MPE	Maximum possible effect
mRNA	messenger ribonucleic acid
NMDA	N-Methyl-D-aspartate
PAG	Periaqueductal gray
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
p.o	orally “Per os”
PP	Placebo Pelleted
QD	one a day
SAL	Saline
s.c.	Subcutaneous injection

Tat	Trans-Activator of Transcription
Veh	Vehicle
WT	Wild type

Abstract

COMPLEXITIES OF CHRONIC OPIOID EXPOSURE

By Maciej Gonek, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2018

Mentor: Dr. William L. Dewey, Chairman Pharmacology & Toxicology

Studies on repeated exposure to opioids have been carried out for decades yet the mechanisms for certain phenomena such as tolerance are still not fully understood. Furthermore, different medications, such as frequently prescribed benzodiazepines, or different disease states, such as HIV, have their own effects and interactions with chronic opioid exposure that are not fully understood. The overall objective of this dissertation was to investigate the complexities of chronic opioid exposure and how different disease states and medications may modulate the effects of chronic opioids. Our findings demonstrate that the administration of diazepam, at doses that are not antinociceptive or have any motor effects, reverse both antinociceptive and locomotor tolerance to orally active opioids. These doses of diazepam did not potentiate the acute effects of these prescription opioids. We also found that HIV-1 Tat expression significantly attenuated the antinociceptive potency of acute morphine in non-tolerant mice while not significantly altering the antinociceptive tolerance to morphine. Consistent with this, Tat attenuated withdrawal symptoms among morphine-tolerant mice. Pretreatment with maraviroc, a CCR5 antagonist blocked the effects of Tat, reinstating morphine potency in non-tolerant mice and restoring withdrawal

symptomology in morphine-tolerant mice. Protein array analyses revealed only minor changes to cytokine profiles whether morphine was administered acutely or repeatedly; however, 24 h post repeated morphine administration, the expression of several cytokines was greatly increased. Tat further elevated levels of several cytokines and maraviroc pretreatment attenuated these effects. With the understanding that gap junctions may be involved in both HIV-Tat effects on opioid antinociception as well as tolerance, we investigated the role of gap junctions in opioid antinociceptive tolerance. We observed that carbenoxolone, a gap junction inhibitor, administered systemically attenuated the development of opioid antinociceptive tolerance. Furthermore, we observed a small percentage of carbenoxolone in brain tissue compared to the amount found in blood, suggesting a peripheral site of action. Finally, we show preliminary evidence that in vivo administration of carbenoxolone is able to attenuate tolerance to morphine in DRG neurons.

CHAPTER 1

INTRODUCTION

I. A History of Opioids

An ancient culture originating in southern Mesopotamia, modern day Iraq, the Sumerians were the first in recorded history to cultivate what they called “hul gil”, or plant of joy around 3400 B.C. (Brownstein, 1993). The opium poppy (papaver somniferum) is species of plant from which the dried latex opium can be extracted. Contained within that latex reside many alkaloid compounds called opiates.

Although many used opium for religious ceremonies, medicinal purposes, as well as for abuse, further understanding of opium’s effects occurred in 1805 after Friedrich Sertürner, a German pharmacist, isolated the first active alkaloid in opium. He named it “morphium” after Morpheus, the Greek god of dreams (Brownstein, 1993). Known today as morphine, it is the most abundant opiate found within opium followed by codeine. When consumed orally, morphine was found to be unreliable in its desired effects due to poor oral bioavailability. Following the invention of the hypodermic needle and syringe, morphine quickly became a useful analgesic for use in surgical procedures and postoperative pain. It was quickly discovered, however, that morphine still shared opium’s additive properties as well as its undesirable side effects. Chemists took it upon themselves to search for safer opioid compounds; opioids that are efficacious but are non-addicting.

This led to a new generation of semi-synthetic opioid compounds that were orally bioactive with similar properties to morphine, alas including addictive properties. In 1917, oxycodone was synthesized from thebaine, another alkaloid found in opium. The synthesis of hydrocodone from codeine was followed shortly in 1920. Both of these compounds can be considered opiates since they are structurally similar to naturally occurring opium compounds. Opiates fall under the

general class of "opioid" compounds which include a broad spectrum of drug molecules that have morphine-like agonist activity, which can be structurally similar or dissimilar from traditional opiates and may be either naturally occurring or synthesized (Kreek et al., 2005).

Both oxycodone and hydrocodone were synthesized in the early part of the 20th century. Yet, they would not be approved as pain reliving medications in the United States until more than 20 years after their discovery. Many in the United States remembered the promises of heroin (an acetylated form of morphine) being advertised as a non-addictive morphine substitute. In 1963, California's attorney general estimated that oxycodone abuse made up one-quarter of all drug addiction in the state. Top executives at the company who marketed the drug disputed this claim and stated that the analgesic possessed "relatively little or no addicting liability" (Meier, 2003). This pattern continued to repeat with Purdue Pharma L.P. who began marketing oxycodone in the mid-1990s as a safer and appropriate compound for the relief of acute and chronic pain. Around this time, there was growing concern regarding the undermanagement of pain. Therefore, in his 1995 Presidential Address to the American Pain Society, Dr. James Campbell presented the idea of pain being the "fifth vital sign" (Campbell, 1996). Even though it cannot be measured objectively like other vital signs (e.g. blood pressure, heart rate, etc.), the idea rapidly spread and was adopted by the Veterans' Health Administration (Booss et al., 2000). Although the Joint Commission on Accreditation of Healthcare Organizations maintains that they never stated that pain needed to be treated like a vital sign, they did suggest making it a patient rights issue and emphasized the quantitative aspects of pain such as placing it on a 10-point scale (Phillips and Chapman, 2000). These guidelines aided the perception that pain was undertreated and provoked the overprescription of opioid painkillers.

The amount of prescription opioids sold to pharmacies, hospitals, and doctors' offices nearly quadrupled from 1999 to 2010 (Paulozzi et al., 2011). Yet there had not been an overall change in the amount of pain that Americans reported (Daubresse et al., 2014). With this fuel, the U.S. opioid epidemic exploded with the number of overdose deaths involving opioids (including prescription opioids and heroin) being 5 times higher in 2016 than in 1999. As of 2016, an average of 115 Americans die every day from an opioid overdose (Rudd et al., 2016). Not all opioid users started with opioid prescription analgesics. However, a 2014 study found 75 percent of heroin users in treatment started with prescription opioids (Cicero et al., 2014). Furthermore, in 2015 the CDC observed people who are addicted to prescription opioids are 40 times more likely to be addicted to heroin. However, limited research has been done with these drugs when compared to the opioid standard, morphine, and the illicit heroin. It is common to assume that these opioids all act through a similar mechanism. However, these compounds have been shown to have different pharmacokinetic properties, varying affinities for the μ opioid receptor, and have different off-target effects (Nielsen et al., 2007). They are different molecules and it is disadvantageous and maybe dangerous to assume they all act through the same mechanism. Therefore, it is important to properly investigate how commonly prescribed opioids compare in their effects as opposed to morphine.

II. Opioid Receptors & Opioid Pharmacology

Comprehending the mechanisms of how opioids induce their powerful effects has been a goal for many pharmacologists. The idea being that understanding these mechanisms may one day lead to a compound that lacks side effects. After the discovery of many opioid compounds, the structure–activity relationships from the vast number of opioid derivatives led to the concept of the opioid receptors (Portoghese, 1965). Among the early neurotransmitter receptors found were

opioid receptors which was quickly followed by the proposal and eventual discovery of endogenous opioid peptide ligands. These peptide compounds are organized into one of three families: the enkephalins, endorphins, and dynorphins. These peptides arise from much larger precursor proteins called proenkephalin, proopiomelanocortin, prodynorphin, respectively. Followed by the discovery of different classes of ligands, multiple opioid receptor types were immediately suggested. The existence of specific opioid receptors in the central nervous system was proposed after studies in the chronic spinal dog model (Martin et al., 1976). The existence of μ, δ, and κ opioid receptors was ultimately confirmed and characterized using selective radioactive ligands, cross tolerance studies, and the cloning of each receptor subtype (Chang et al., 1979; Chang and Cuatrecasas, 1979; Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993; Porreca et al., 1982; Schulz et al., 1980; Thompson et al., 1993)

Opioids play a role in the modulation of pain, affective behavior, locomotor activity, learning and memory, neuroendocrine function, autonomic function, and immune function. Primarily used as analgesics, opioids are also used clinically as antitussives and antidiarrheals. Noxious effects include nausea, constipation, and respiratory depression, the cause of many opioid overdose deaths. Opioids tend to have euphoric, rewarding properties that can cause people to abuse them and over time become addicts.

Commonly used opioid drugs exert nearly all of their clinically relevant actions through stimulation of the μ-opioid receptor (MOR). Activation of MORs by an agonist such as morphine can cause analgesia, euphoria, sedation, nausea, respiratory depression, miosis, and constipation. The multiple effects throughout the body may be explained by MORs being highly concentrated in certain peripheral and central areas of the nervous system. In terms of antinociception, opioid receptors are expressed in pain-modulating descending pathways, which include the

periaqueductal gray (PAG) and the rostroventral medulla (RVM). They are also expressed in limbic, midbrain, and cortical structures (Al-Hasani and Bruchas, 2011) These multiple sites of opioid action have been deduced from the effects of local agonist and antagonist infusion.

The most common understanding of the mechanism of acute opioid administration is the inhibitory effects on pain transmission. Opioid receptors, including MORs, are members of the G protein-coupled receptor (GPCR) superfamily characterized by the presence of 7 transmembrane regions. MORs are primarily considered to be part of Gi/o class of GPCRs, characterized by the ability of the receptor to activate Gi alpha subunit, inhibiting adenylate cyclase activity thereby lowering cAMP levels. The inhibition of adenylyl cyclase and the further inhibition of ion channels are commonly accepted as the mechanism by which opioids block pain transmission. When activated, MORs produce hyperpolarization of neurons (primarily through the activation of potassium ion channels yielding an efflux of potassium ions) and the inhibition of calcium ion channels, decreasing the transmission of neurotransmitters or nociceptive information (Fornasari, 2012; Williams et al., 2013).

III. Tolerance(s)

Repeated exposure to opioids is now more common than ever before. This observation is seen in the clinic with enough opioid analgesics sold in 2010 to medicate every American adult with a typical dose every 4 hours for 1 month (Paulozzi et al., 2011). This is also seen in the rising abuse of opioids with 2 million Americans having a substance use disorder involving prescription opioids and 591,000 using heroin (Center for Behavioral Health, 2016). Chronic exposure to opioids exhibit different issues than short term, acute exposure. One common issue that arises after repeated opioid exposure is tolerance.

Tolerance is generally defined as a reduction or loss of responsiveness to an agonist after continued exposure. Tolerance has been often studied in either the whole animal where the cellular and molecular mechanisms are difficult to determine or in isolated tissues which may make translational conclusions difficult. Typically, tolerance is measured as a rightward shift in the dose-response curve that may also be associated with a reduction in the maximum response. Tolerance after chronic exposure to opioids is commonly seen both in vivo and in vitro. Interestingly, tolerance occurs to some, but not all opioid induced effects. Antinociception, respiratory depression, and euphoria are all opioid effects susceptible to the development of tolerance. For reasons still not fully understood, little to no tolerance is seen with opioid-induced constipation or miosis (excessive pupillary constriction). It has been also shown that tolerance develops to these different effects at different rates. Opioid tolerance development is fastest for the analgesic effects, followed by respiratory depressant actions, and least for peripheral effects such as the slowing of gastrointestinal motility (Dumas and Pollack, 2008; Hayhurst and Durieux, 2016; Hill et al., 2016). Therefore, it is important to note that using the term tolerance to describe these generalities may be somewhat of a red herring. In other words, using the general term tolerance to describe all of these observed effects may be misleading because of the multiplicity of opioid tolerance expression. A more accurate term may be the plural form, as opioid *tolerances*. Many mechanisms have been discovered to underlie opioid tolerances. Despite intensive studies, neither the mechanisms themselves nor the connections between them are fully understood.

Most studies have concentrated on the receptor level, with opioid receptors belonging to the GPCR family. Consequently, opioid receptors are generally susceptible to a series of adaption processes that occur in response to persistent GPCR activation. These adaption processes include desensitization, internalization, and downregulation. Considered to be fairly rapid (seconds to

minutes), desensitization is usually defined as the uncoupling of the receptor from its G-protein and therefore a decrease in effector response. Mechanisms involved in desensitization may include the activation of one or many kinase pathways which can lead to phosphorylation or binding to the receptor. Intracellular kinases indicated in the phosphorylation of MORs include G protein receptor kinases (GRKs), protein kinase C (PKC), protein kinase A (PKA), c-Jun N-terminal kinases (JNKs), and many more (Williams et al., 2013). These kinases each contribute to their own downstream signaling pathways. Downregulation refers to a reduced number of functional receptors present in cells which occurs as a result of increased degradation and/or reduced biosynthesis of receptors (Tsao and Zastrow, 2000; Williams et al., 2013).

Tolerance may occur differently depending on what opioid compound the MOR is exposed to. Different agonists may initiate different receptor conformations and different downstream signaling pathways. High efficacy agonists, such as the peptide [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) have been observed to produce tolerance through internalization and downregulation of MOR through GRKs and β-arrestin (Stafford et al., 2001). However, partial agonists such as morphine do not lead to internalization, as tolerance seems to be driven more by desensitization, which may be driven primarily by PKC-mediated phosphorylation, although PKA has been implicated as well (Bailey et al., 2009, 2006; Dalton, 2005; Hull et al., 2010).

The issue of tolerance continues to be more complex due to differences observed in cell and tissue types. For example, morphine fails to induce MOR internalization in spinal cord in vivo but it efficiently induces endocytosis in dendrites of nucleus accumbens neurons (Haberstock-debic et al., 2005, 2003; Trafton and Basbaum, 2004; Williams et al., 2013). It is argued that this may be due to different isoforms of select kinases involved in opioid tolerance present in different cell types. However, recent evidence shows different subtypes of MORs may also play an

important role in opioid-mediated effects and may play a role in tolerance, specifically cross-tolerance (Pasternak, 2010; 2018).

Preclinical and clinical studies have shown that tolerance to opioid-mediated effects are susceptible to reversal by a number of agents. In the clinic, anesthesiologists reported having to use less opioid medications for patients that were previously administered ketamine (Trujillo and Akil, 1991). Recent publications that investigated the issue of polydrug abuse observed reversal of opioid antinociceptive and respiratory depressive tolerance after the administration of ethanol or diazepam (Gonek et al., 2017; Hill et al., 2016; Hull et al., 2013; Jacob et al., 2017). The list of compounds that can reverse or attenuate opioid tolerances could be expanded to include different kinase inhibitors, antibiotics, gap junction inhibitors, astrocyte inhibitors, and even the administration of stem cells (Bailey et al., 2006; Dalton, 2005; Kang et al., 2017; Li et al., 2017, 2015; Shen et al., 2014; Smith et al., 1999). The sheer variety of agents that are able to attenuate opioid tolerances highlight that opioid tolerance may not be explained by one cellular mechanism. The underlying mechanisms of these and other reversal of opioid tolerances are poorly understood. However, some key players emerge. Ketamine has many targets but it is primarily known as an antagonist at NMDA receptors, which have been indicated as potential mechanism by which opioid tolerance is reversed (Trujillo and Akil, 1991). Diazepam-induced reversal of morphine antinociceptive tolerance was blocked by the $GABA_A$ antagonist bicuculline, suggesting that $GABA_A$ receptors may play a role. However, ethanol-induced reversal of morphine antinociceptive tolerance was not fully blocked by bicuculline as it required the administration of both bicuculline and phaclofen, a $GABA_B$ antagonist (Hull et al., 2013). In a study investigating ethanol reversal of morphine tolerance to respiratory depressive effects, an interaction involving

PKC was proposed (Hill et al., 2016). Interestingly, activation of NMDA receptors are involved with the activation of specific isoforms of PKC (Abraham, 2008; Lau and Zukin, 2007).

Many of these substances interact with inflammatory processes which have been observed to interact with opioid tolerance. Increasing evidence suggests that opioids can also produce neuroinflammatory responses in both central and peripheral nervous systems. Chronic morphine treatment has been associated with increased astrocytic activity in several parts of the CNS and increased the expression of proinflammatory cytokines TNFα, IL1-β and IL-6 (Shen et al., 2011; Song and Zhao, 2001). Inhibiting these cytokines either by inhibiting glial cells release of them or by directly preventing their interaction with receptors attenuates the development of opioid tolerance (Shen et al., 2011). Chemokines, small cytokines secreted by cells that have the ability to induce directed chemotaxis in nearby responsive cells, are also modulated by morphine exposure within the central nervous system. The blockade of chemokine receptor CX3CR1 attenuated the development of antinociceptive tolerance and allodynia to chronic morphine (Johnston et al., 2004). Activation of C-C Chemokine receptor type 5 (CCR5) can lead to cross-desensitization of MOR by phosphorylation by intracellular kinases (Szabo et al., 2003, 2002; Zhang et al., 2004).

IV. Scope of Dissertation and Aims

The overall objective of this dissertation was to investigate the complexities of chronic opioid exposure and how different disease states and medications may modulate the effects of chronic opioids. We decided to primarily focus on opioid tolerances because this phenomena plays an important role in the clinic (patients) and society (addicts) as a whole. The studies described here lead to new understandings of what occurs on a behavioral and cellular level during repeated exposure to opioids.

Our first series of experiments characterized the effect of diazepam, a commonly prescribed and abused anti-anxiety medication, to reverse the tolerances to prescription opioids oxycodone and hydrocodone. In the next series of experiments, we examined the intertwined health epidemic of HIV and investigated the role of the CCR5 receptors in proinflammatory HIV-1 Tat's effects on morphine tolerance, dependence, and reward. Lastly, we investigated how the blockade of gap junctions influence the development of tolerances to morphine on a behavioral and cellular level.

CHAPTER 2

REVERSAL OF OXYCODONE AND HYDROCODONE TOLERANCE BY DIAZEPAM

This chapter has been published in Brain Research (Maciej Gonek, Hamid I. Akbarali, Graeme Henderson. William L. Dewey (2017) "Reversal Of Oxycodone And Hydrocodone Tolerance By Diazepam")

Summary

The Centers for Disease Control has declared opioid abuse to be an epidemic. Overdose deaths are largely assumed to be the result of excessive opioid consumption. In many of these cases, however, opioid abusers are often polydrug abusers. Benzodiazepines are one of the most commonly co-abused substances and pose a significant risk to opioid users. In 2016, the FDA required boxed warnings – the FDA's strongest warning – for prescription opioid analgesics and benzodiazepines about the serious risks associated with using these medications at the same time. The point of our studies was to evaluate the interactions between these two classes of drugs. We investigated whether diazepam adds to the depressant effects of opioids or does it alter the levels of tolerance to opioids. In the present study, we have found that the antinociceptive tolerance that developed to repeated administration of oxycodone was reversed by an acute dose of diazepam. Antinociceptive tolerance to hydrocodone was also reversed by acute injection of diazepam; however, a four-fold higher dose of diazepam was required when compared to reversal of oxycodone-induced tolerance. These doses of diazepam did not potentiate the acute antinociceptive effect of either opioid. The same dose of diazepam that reversed oxycodone antinociceptive tolerance also reversed oxycodone locomotor tolerance while having no potentiating effects. These studies show that diazepam does not potentiate the acute effect of prescription opioids but reverses the tolerance developed after chronic administration of the drugs.

1. Introduction

Reducing opioid overdose deaths is an important public health and drug policy goal. Much of the current opioid epidemic can be attributed to the rise in use of prescription opioid pain analgesics such as oxycodone and hydrocodone. These drugs have become widely prescribed, with enough opioid analgesics sold in 2010 to medicate every American adult with a typical dose every 4 hours for 1 month. In the United States, at least half of all opioid overdose deaths involve a prescription opioid (Paulozzi et al., 2011). The dangers of accidental opioid overdose are mainly due to respiratory depressive effects. Chronic use of opioids results in the development of tolerance (a decrease in pharmacologic response following repeated or prolonged drug administration) to the analgesic, euphoric, and respiratory depressive effects. This leads to addicts and patients taking higher doses in order to obtain the euphoric high or the analgesic effects, respectively. However, it has been shown that tolerance to different effects of opioids do not occur at the same rate or to the same extent (Hill et al., 2016). It has been suggested that, in man, tolerance to euphoria develops to a greater extent than to respiratory depression (White and Irvine, 1999).

Opioid overdose deaths are largely assumed to result from excessive opioid administration alone. However, opioid abusers are often polydrug users, consuming benzodiazepines, ethanol, cocaine and/or gabapentoids along with opioid drugs. Benzodiazepines and ethanol have been found to pose a significant risk to chronic opioid users, particularly in those taking methadone (National Treatment Agency for Substance Misuse [NTA], 2007). The CDC has reported that benzodiazepines were involved in 31% of opioid related drug poisoning deaths in recent years (Chen et al., 2014). Benzodiazepines, ethanol, and opioids are all considered central nervous system depressants and their effects may be additive or synergistic. Recently, our lab has published that low doses of ethanol and diazepam, which have no observable effect of their own, significantly

and dose-dependently reduced the antinociceptive tolerance produced by morphine while not affecting the acute responses (Hull et al., 2013). Low doses of ethanol reversed morphine tolerance at the level of single brain neurons (Llorente et al., 2013) and in a rodent model of respiratory depression (Hill et al., 2016).

A major limitation of the previously described studies is that they have not investigated oxycodone and hydrocodone, two commonly prescribed opioid analgesics. Limited research has been done with these drugs when compared to the opioid standard, morphine, and the illicit compound heroin. Although all are considered opioids, these compounds may differently interact through the μ opioid receptor (MOR). They have been shown to have different pharmacokinetic properties, varying affinities for the μ opioid receptor, potentially interact with other opioid receptors and have different off-target effects (Nielsen et al., 2007). Therefore, it is important to investigate how commonly prescribed opioids compare in their effects to morphine.

The goals of this study were to determine if benzodiazepines potentiate the acute antinociceptive effects of commonly prescribed opioids as well as to determine if they act to reduce tolerance to these opioids. We characterized the development of antinociceptive tolerance to oxycodone and hydrocodone in mice and investigated whether diazepam could reverse tolerance as it does morphine-induced tolerance. Antinociception, as measured by the rodent warm water tail-immersion assay, was chosen for these studies because it has been shown to be a good predictor of antinociception and tolerance for a wide range of compounds in humans. Furthermore, our group has used this assay to extensively investigate the mechanisms of opioid tolerance (Hull et al., 2013, 2010; Smith et al., 2007).

2. Experimental Procedure

2.1. Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g were housed five to a cage in animal care quarters and maintained at 22 +/- 2°C on a 12-hour light-dark cycle. Food and water were available ad libitum. The mice were brought to the test room (22 +/- 2°C, 12-hour light-dark cycle), marked for identification, and allowed 18 hours to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the International Association for the Study of Pain (IASP).

2.2. Drugs and Chemicals

Oxycodone HCl and hydrocodone bitartrate were obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD) and were each dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL). Diazepam was obtained from Sigma-Aldrich Corporation (St Louis, MO, USA) and was dissolved in 45% hydroxypropyl beta-cyclodextrin (HPBCD).

2.3. Antinociceptive Testing

Antinociception was assessed using the 56°C warm water tail immersion test performed according to Coderre and Rollman (1983). Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 seconds were used. The test latency after opioid treatment was assessed at the peak time point of 20 minutes with a 10-second maximum cut-off time imposed to prevent tissue damage. Antinociception was quantified

according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (%MPE), which was calculated as: %MPE = [(test latency – control latency) / (10 – control latency)]* 100. Percent MPE was calculated for each mouse.

2.4. Tolerance Studies

A 7 hour antinociceptive tolerance model for oxycodone and hydrocodone was developed. Mice were injected subcutaneously (s.c.) once every hour for 7 hours (total of 7 injections) with an acute ED80 dose of the opioid (1.25 mg/kg for oxycodone and 6 mg/kg for hydrocodone, as previously determined). An hour after the final dose, mice were administered diazepam or vehicle by intraperitoneal injection and 30 minutes later were challenged with subcutaneous doses of oxycodone or hydrocodone to construct dose-response curves for calculation of ED50 values.. The warm water tail immersion test was performed 20 minutes after the injection of the challenge dose of opioid.

For maximum tolerance to oral administration of oxycodone, the route of administration used by humans, a 4-day antinociceptive tolerance model was developed. Mice were orally gavaged with 256 mg/kg oxycodone twice a day (9 AM & 5 PM) on day 1, 2, and 3. On the fourth day, mice were gavaged only in the morning. A full 24 hours after their last pretreatment dose, baseline latencies were recorded and immediately afterwards diazepam or vehicle was administered by intraperitoneal injection. 30 minutes later, mice were challenged with oral doses of oxycodone to construct dose-response curves for calculation of ED50 values. The warm water tail immersion test began 20 minutes after the administration of the challenge dose.

2.5. Locomotor Activity

Spontaneous motor activity was assessed using activity chambers (Med Associates, St. Albans, VT). Each individual activity chamber has closeable doors and a ventilation system. The interior of the chamber consists of a 27 × 27 cm Plexiglas enclosure that is wired with photo-beam cells connected to a computer console that counts the activity of the animal contained within the enclosure. For locomotor tolerance, a 4-day antinociceptive tolerance model was developed where mice were orally gavaged with 64 mg/kg oxycodone twice a day (9 AM & 5 PM) on day 1, 2, 3, and 4. On the fifth day, mice were habituated to the chamber for 30 minutes before any drug administration. Afterwards, mice were administered an intraperitoneal injection of vehicle or 0.5 mg/kg diazepam and placed in home cage. 30 minutes later, mice were administered 64 mg/kg oxycodone by oral gavage. 10 minutes later, mice were placed in separate activity chambers. Ambulatory counts for spontaneous activity were obtained over a 40-minute time period.

2.6. Statistical Analysis

Opioid dose-response curves were generated for calculation of ED_{50} values using least-squares linear regression analysis followed by calculation of 95% confidence limits (95% CL) by the method of Bliss (Bliss, 1967). Significance was determined by non-overlapping 95% confidence limits. Locomotor data was represented as mean ± standard error of the mean. Locomotor data was analyzed using two-way analysis of variance (ANOVA) with factors for time, treatment, and their interactions with locomotor activity followed by Tukey's post hoc analyses to determine statistical significance (Prism 6). Analyses were considered significant when $p < 0.05$.

3. Results

3.1. Effects of Acute Diazepam in Drug-Naïve Mice

Diazepam was administered at doses of 0.5, 1, and 2 mg/kg i.p. and mice were monitored over a 3-hour period for behavioral changes and assessed in the warm-water tail immersion test at 30-minute intervals over 2 hours. No antinociceptive effects or behavioral changes, including locomotor activity, were observed at any of these doses.

3.2. Tolerance Development to Oxycodone

Baseline latencies were taken prior to the beginning of the hourly subcutaneous injections. Mice were randomly assigned to either a chronic saline or chronic opioid schedule whereby seven hourly injections of isotonic saline or an ED80 dose of oxycodone were given s.c. After seven injections, mice were injected with a challenge dose of oxycodone (0.25, 0.5, 1, 2 or 4 mg/kg) at the 8-hour time point (Figure 1). Dose-response curves for oxycodone after chronic injections of saline generated similar ED50 values to those in acute dose response experiments (1.19 mg/kg (1.00-1.41, 95% CL)). The ED50 was significantly shifted to the right 1.6-fold, indicating tolerance was observed, in the animals chronically injected with oxycodone prior to receiving the challenge injections (1.84 mg/kg (1.58 - 2.14, 95% CL)). The sample size of each group was 20 animals [Acute Oxycodone, n =20; Chronic Oxycodone, n = 20].

3.3. Reversal of Oxycodone Antinociceptive Tolerance with Diazepam in Tail Immersion Assay

Baseline latencies were obtained in the tail immersion test in the morning before any injections. Following the development of tolerance (single day tolerance model), diazepam (0.5 mg/kg i.p.) was administered. Thirty minutes later, the mice were challenged with doses of

oxycodone s.c. for construction of dose-response curves for calculation of the ED_{50} values (Figure 1; Table 1). Diazepam fully reversed the oxycodone-induced tolerance. The sample size of each group was between 10–20 animals [Acute Oxycodone, n =20; Chronic Oxycodone, n= 20; Chronic Oxycodone + 0.5 mg/kg DZ, n= 12]. The same dose of diazepam did not potentiate the antinociception produced by acute doses of oxycodone in naive mice (Figure 2) [Acute Oxycodone, n =20; Acute Oxycodone + 0.5 mg/kg DZ, n= 11].

Table 1: Diazepam Reversal of Oxycodone Tolerance	
Treatment	Oxycodone ED_{50} (mg/kg (95% C.L.))
Acute Oxycodone + Vehicle	1.19 (1.00-1.41)
Acute Oxycodone + Diazepam (0.5 mg/kg)	1.25 (1.04-1.50)
Chronic Oxycodone + Vehicle	1.84 (1.58-2.14)*
Chronic Oxycodone + Diazepam (0.5 mg/kg)	1.12 (0.88-1.43)

***** Significantly different than Acute Oxycodone + Vehicle group based on non-overlapping 95%.

3.4. Tolerance Development to Hydrocodone

Baseline latencies were taken prior to the beginning of hourly subcutaneous injections. Mice were assigned randomly to either a chronic saline or chronic opioid schedule whereby seven hourly injections of isotonic saline or an ED_{80} dose of hydrocodone were given s.c. After seven injections, all mice were injected with final challenge doses of hydrocodone (1, 2, 4, 8, 16 or 32 mg/kg) at the 8-hour time point (Figure 3). Dose response curves of hydrocodone after chronic injections of saline generated similar ED_{50} values to those in acute dose response experiments (5.51 mg/kg (4.97-6.12, 95% CL)). The ED_{50} was significantly shifted 2.4-fold to the right in the animals chronically injected with hydrocodone prior to receiving the challenge injections (13.18 mg/kg

(11.00-15.80, 95% CL)). The sample size of each group was between 6–12 animals [Acute Hydrocodone, n =6; Chronic Hydrocodone, n= 12]

3.5. Reversal of Hydrocodone Antinociceptive Tolerance with Diazepam in Tail Immersion Assay

Baseline latencies were obtained in the tail immersion test in the morning before any injections. Following the development of tolerance (single day tolerance model), diazepam was administered i.p. Thirty minutes later, the mice were challenged with doses of hydrocodone s.c. for construction of dose-response curves for calculation of the ED_{50} values (Table 2). In contrast with oxycodone, 0.5 mg/kg diazepam did not fully reverse antinociceptive tolerance to hydrocodone (Figure 3). 2 mg/kg diazepam fully reversed hydrocodone tolerance and actually significantly potentiated the antinociceptive effect of hydrocodone after chronic administration (Figure 4). However, 2 mg/kg diazepam did not potentiate the antinociception produced by an acutely administered dose of hydrocodone (Figure 5). The sample size of each group was between 6–12 animals [Figure 3: Acute Hydrocodone, n =6; Chronic Hydrocodone, n= 12; Chronic Hydrocodone + 0.5 mg/kg DZ n = 8; Figure 4: Acute Hydrocodone, n =12; Chronic Hydrocodone, n= 12; Chronic Hydrocodone + 2 mg/kg DZ n = 6; Figure 5: Acute Hydrocodone, n =12; Acute Hydrocodone + 2 mg/kg DZ n = 6].

Table 2: Diazepam Reversal of Hydrocodone Tolerance	
Treatment	Hydrocodone ED_{50} (mg/kg (95% C.L.))
Acute Hydrocodone + Vehicle	5.51 (4.97-6.12)
Acute Hydrocodone + Diazepam (2 mg/kg)	4.48 (3.22-6.25)
Chronic Hydrocodone + Vehicle	13.18 (11.00-15.80)*
Chronic Hydrocodone + Diazepam (0.5 mg/kg)	9.92 (7.74-12.70)*
Chronic Hydrocodone + Diazepam (2 mg/kg)	3.77 (2.83-5.04)

***** Significantly different than Acute Oxycodone + Vehicle group based on non-overlapping 95%.

3.6. Reversal of Oral Oxycodone Antinociceptive Tolerance with Diazepam in Tail Immersion Assay

Following the development of maximum tolerance (4-day oral tolerance model), diazepam was administered i.p. Thirty minutes later, the mice were challenged with doses of oxycodone p.o. for construction of dose-response curves for calculation of the ED50 values. The ED_{50} of the acute oral oxycodone was 8.33 (5.77-12.03, 95% CL). There was marked tolerance to oral oxycodone following the 4-day oral tolerance model. The observation that 0.5 mg/kg diazepam was able to fully reverse the antinociceptive tolerance to oral oxycodone (Figure 6) was supported by the overlapping ED_{50}: 13.24 mg/kg (4.00 - 43.93, 95% CL). Administering 0.5 mg/kg diazepam to animals given acute oral oxycodone generated the ED_{50} value of 7.27 mg/kg (5.01 - 10.54, 95% CL), indicating that it did not potentiate the antinociception produced by acute doses of oxycodone (Figure 7). The sample size of each group was between 6-7 animals [Figure 6: Acute Oral Oxycodone, n =6; Chronic Oral Oxycodone, n= 7; Chronic Oral Oxycodone + 0.5 mg/kg DZ n = 6; Figure 7: Acute Oral Oxycodone, n =6; Acute Oral Oxycodone + 2 mg/kg DZ n = 6].

3.7. The Lack of the Development of Antinociceptive Tolerance to Oral Hydrocodone in Tail Immersion Assay

We attempted a model to produce tolerance to oral hydrocodone in the tail immersion assay. A dose response curve of acute oral hydrocodone was established and reliably repeated. However, we did not succeed in developing tolerance to oral gavages of hydrocodone (data not shown). Methods to establish such a model included a 4-day antinociceptive tolerance model using a twice a day dosing schedule of 32, 64, 128, or 256 mg/kg.

3.8. Development of Locomotor Tolerance to Oral Oxycodone and Subsequent Reversal with Diazepam

Tolerance was examined by assessing changes of locomotor activity after acute and repeated oxycodone gavages. A main effect was noted for treatment group [$F(3,15) = 5.49$, $p < 0.001$] , time [$F(7,105) = 10.89$, $p < 0.0001$], and treatment × time interaction [$F(21,105) = 4.29$, $p < 0.001$]. Post hoc tests demonstrated acutely administered 64 mg/kg oral oxycodone produced significant increased ambulatory counts starting from 20 minutes (with the exception at the 25-minute time point) until the 40-minute endpoint (Figure 8). Using a modified 4-day oral tolerance model, tolerance to oxycodone's stimulatory effects was observed. After the administration of 0.5 mg/kg diazepam to mice that were repeatedly treated with oral oxycodone, 64 mg/kg oral oxycodone produced significant increased ambulatory counts starting from the 35-minute time point until the 40-minute endpoint. In determining the effects of acute diazepam by itself or on acute oxycodone's stimulatory effects, a two-way ANOVA revealed a main effect for treatment group [$F(3,16) = 9.37$, $p < 0.001$] , time [$F(7,112) = 34.62$, $p < 0.0001$], and treatment × time interaction [$F(21,112) = 10.42$, $p < 0.001$]. Post hoc tests revealed that 0.5 mg/kg diazepam did

not potentiate acute oxycodone's stimulatory effects in the locomotor assay. This dose of diazepam did not show any stimulatory effects on its own (Figure 9). The sample size of each group was five animals with the exception of the chronic oxycodone group with four animals.

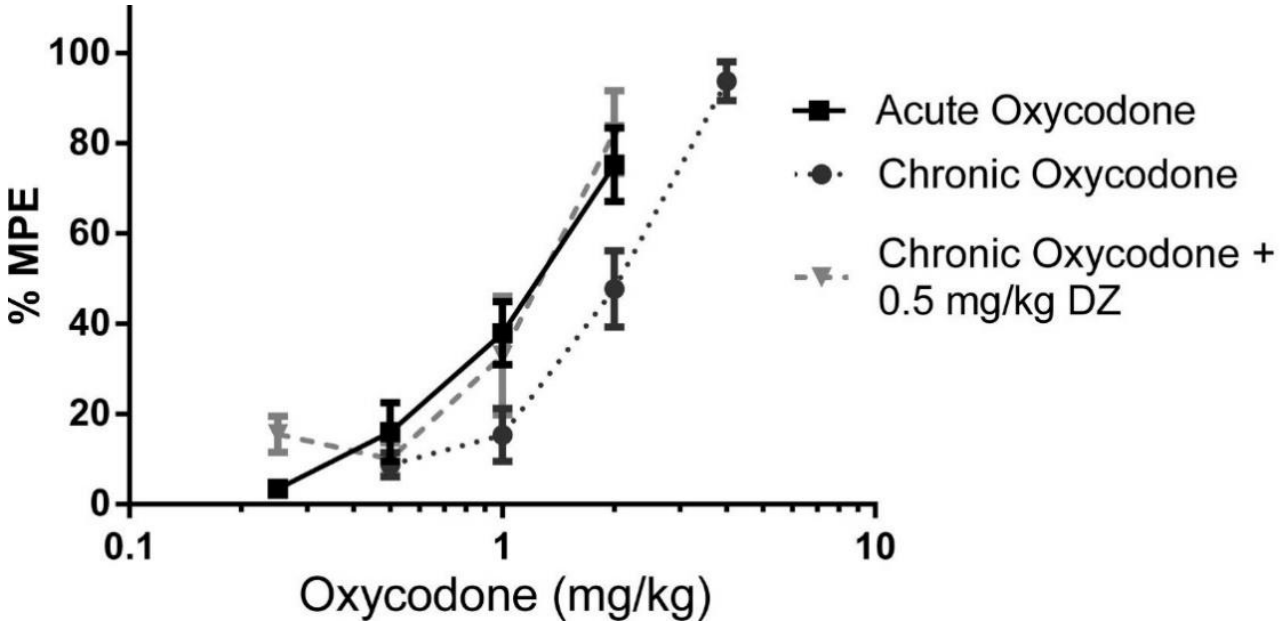


Fig. 1. Tolerance to oxycodone, developed using a single-day injection paradigm, was significantly reversed by 0.5 mg/kg diazepam (n = 10–20). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve, repeatedly treated with oxycodone, or repeatedly treated with oxycodone and diazepam pretreatment 30 min before testing. Various doses of the oxycodone were used for construction of dose-response curves for calculation of ED_{50} values.

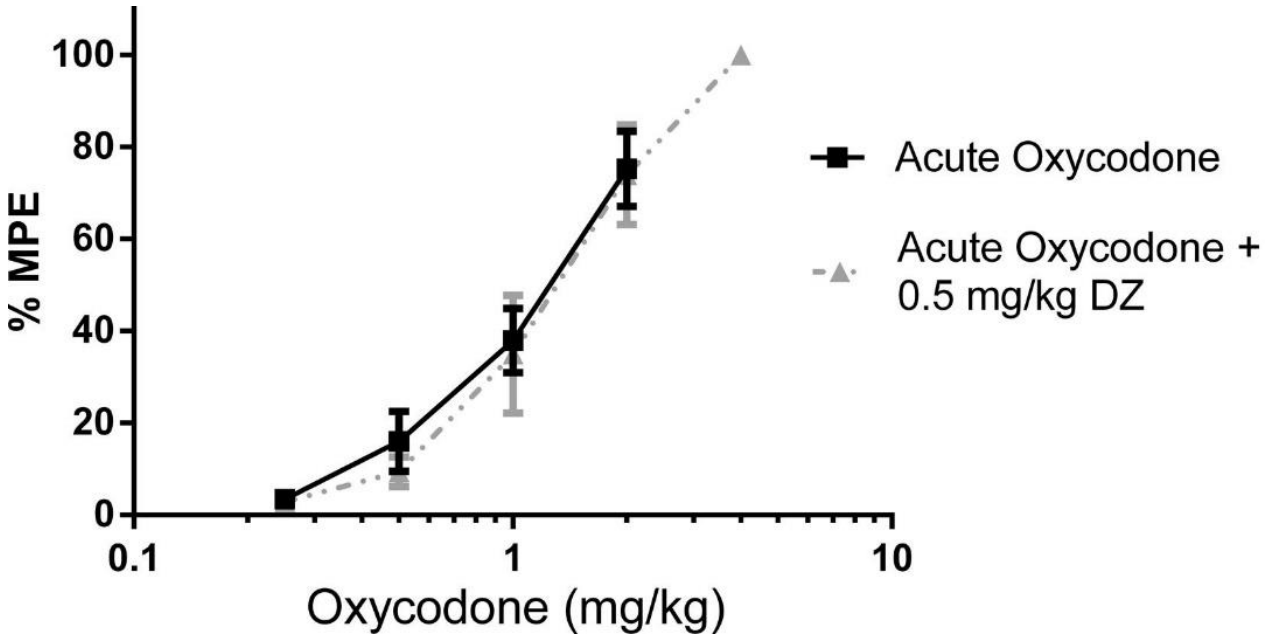


Fig. 2. 0.5 mg/kg Diazepam did not potentiate the antinociception produced by oxycodone in the tail immersion test. (n = 10–20). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve or pretreated with diazepam 30 min before testing. Various doses of the oxycodone were used for construction of dose response curves for calculation of ED_{50} values.

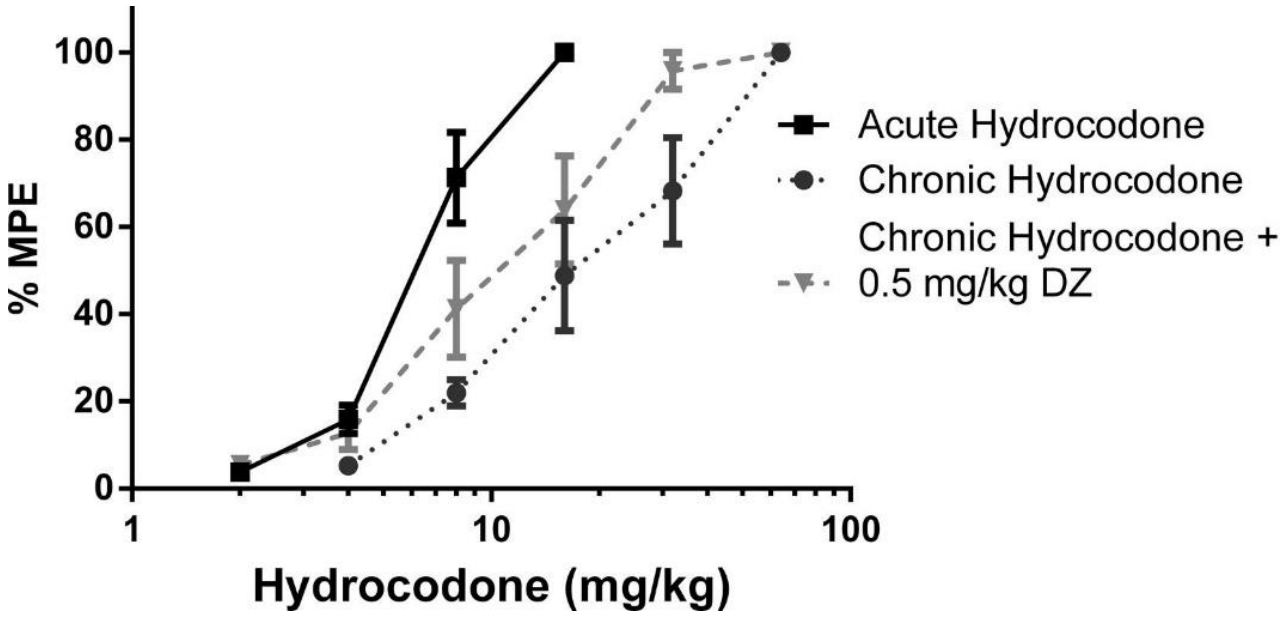


Fig. 3. 0.5 mg/kg Diazepam did not fully reverse the antinociception produced by hydrocodone in the tail immersion test (n = 6–12). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve, repeatedly treated with hydrocodone, or repeatedly treated with hydrocodone and pretreated with diazepam 30 min before testing. Various doses of the hydrocodone were used for construction of dose-response curves for calculation of ED_{50} values.

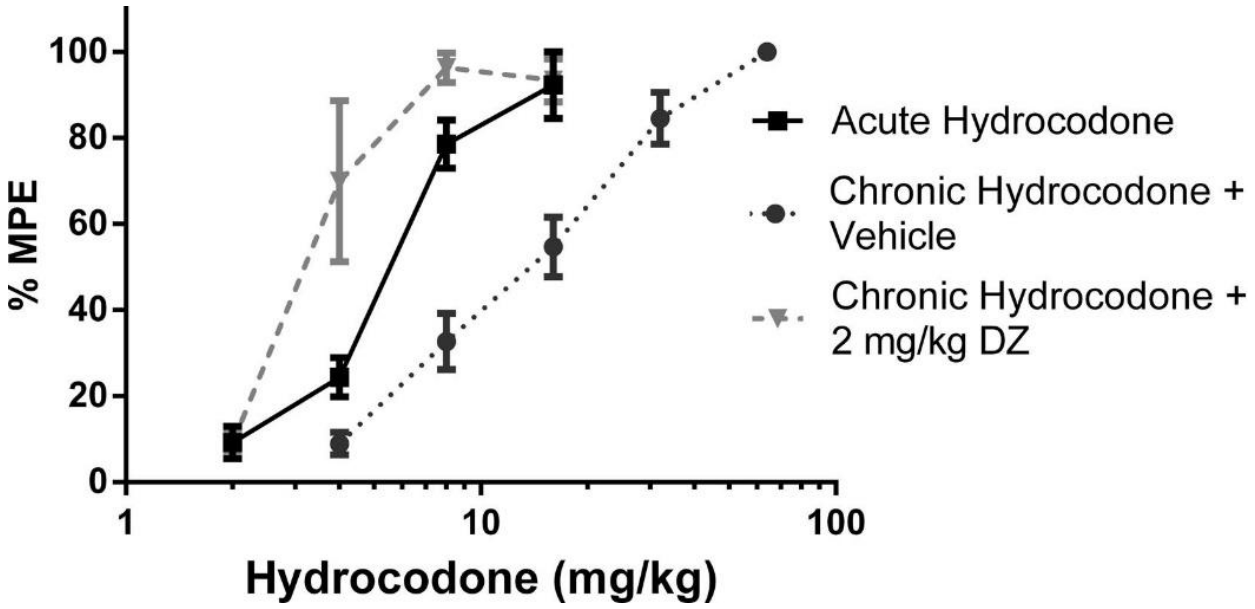


Fig. 4. 2 mg/kg Diazepam fully reversed the antinociception produced by hydrocodone in the tail immersion test (n = 6–12). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve, repeatedly treated with hydrocodone or repeatedly treated with hydrocodone and pretreated with diazepam 30 min before testing. Various doses of the hydrocodone were used for construction of dose-response curves for calculation of ED_{50} values.

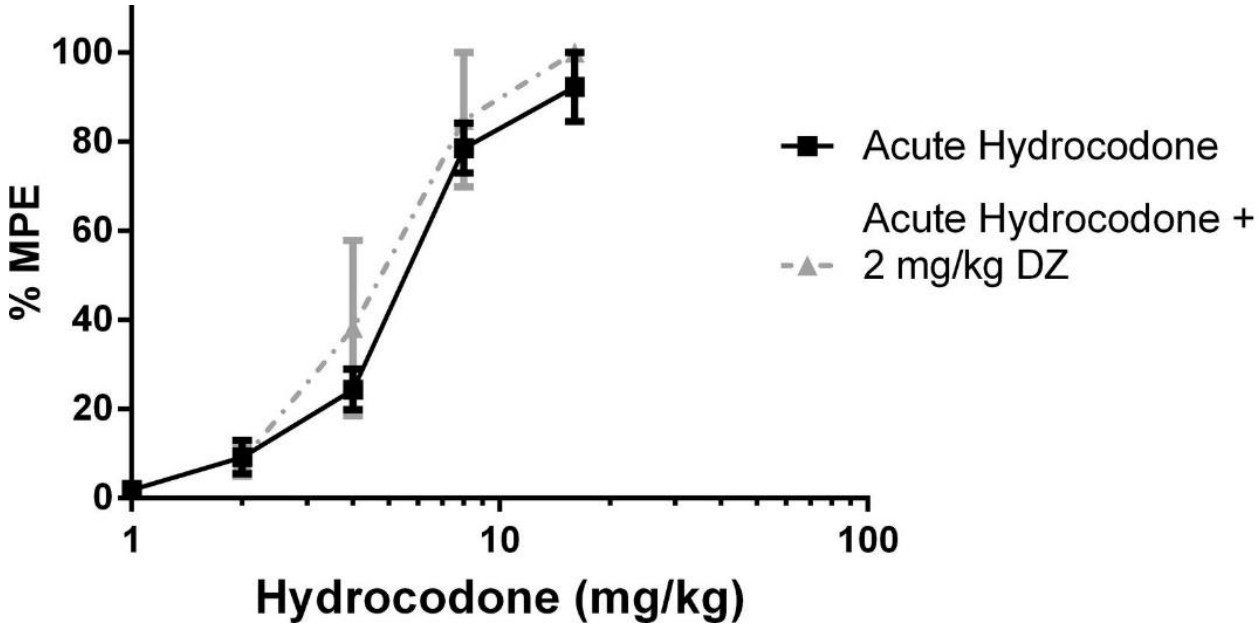


Fig. 5. 2 mg/kg Diazepam did not potentiate the antinociception produced by hydrocodone in the tail immersion test (n = 6–12). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve or pretreated with diazepam 30 min before testing. Various doses of the hydrocodone were used for construction of dose-response curves for calculation of ED_{50} values.

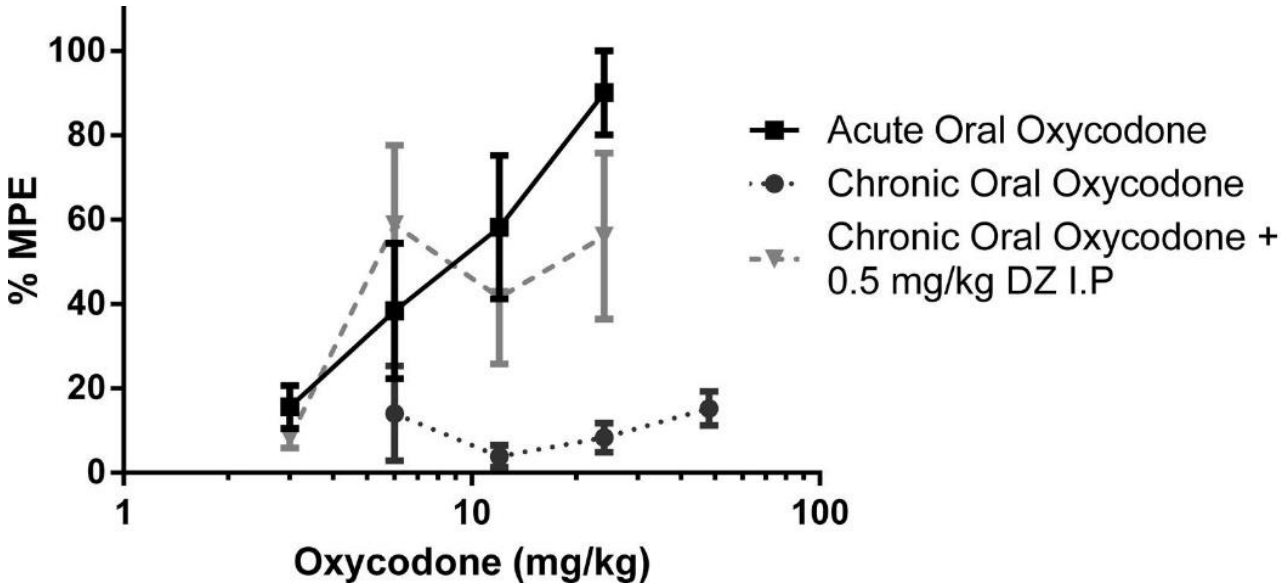


Fig. 6. 0.5 mg/kg Diazepam fully reversed the antinociception produced by multiple-day paradigm of oral oxycodone in the tail immersion test. (n = 6–7). Latency to tail withdrawal (% MPE ± SEM) among mice that were drug naïve, repeatedly gavaged with oxycodone over 4 days, or repeatedly gavaged with oxycodone over 4 days and pretreated with diazepam 30 min before testing. Various doses of the oxycodone were used for construction of dose-response curves for calculation of ED_{50} values.

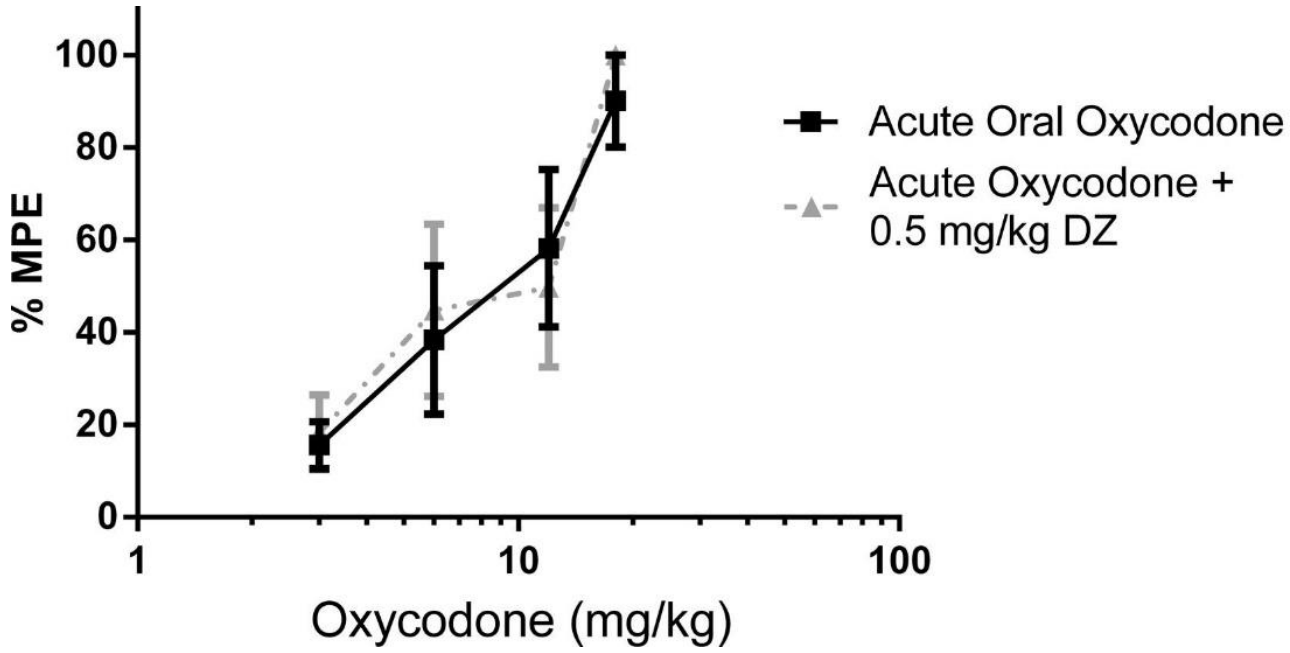


Fig. 7. 0.5 mg/kg Diazepam did not potentiate the antinociception produced by oxycodone (p.o.) in the tail immersion test (n = 6). Latency to tail withdrawal (%MPE ± SEM) among mice that were drug naïve or pretreated with diazepam 30 min before testing. Various doses of the oxycodone were used for construction of dose response curves for calculation of ED_{50} values.

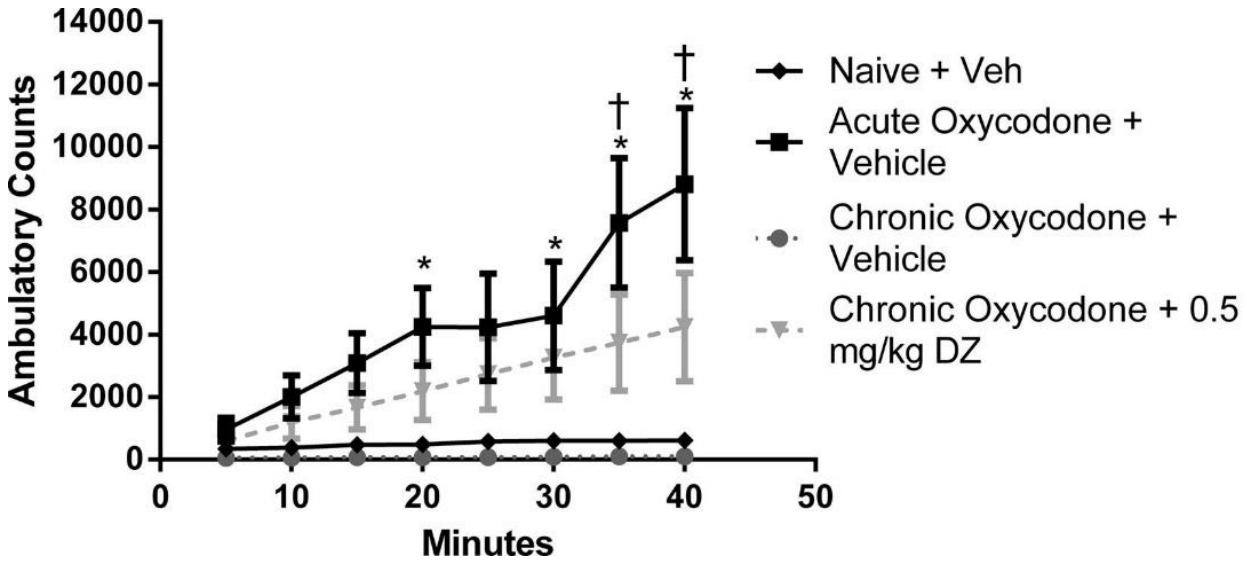


Fig. 8. Locomotor stimulation in response to vehicle or oxycodone (64 mg/kg p.o.) was assessed (n = 5). * Indicates a significant effect of acute oxycodone increasing ambulatory counts as compared to mice naïve to the opioid (treated with vehicle). Indicates a significant effect of chronic oxycodone + 0.5 mg/kg diazepam increasing ambulatory counts as compared to mice naïve to the opioid ($p < 0.05$)

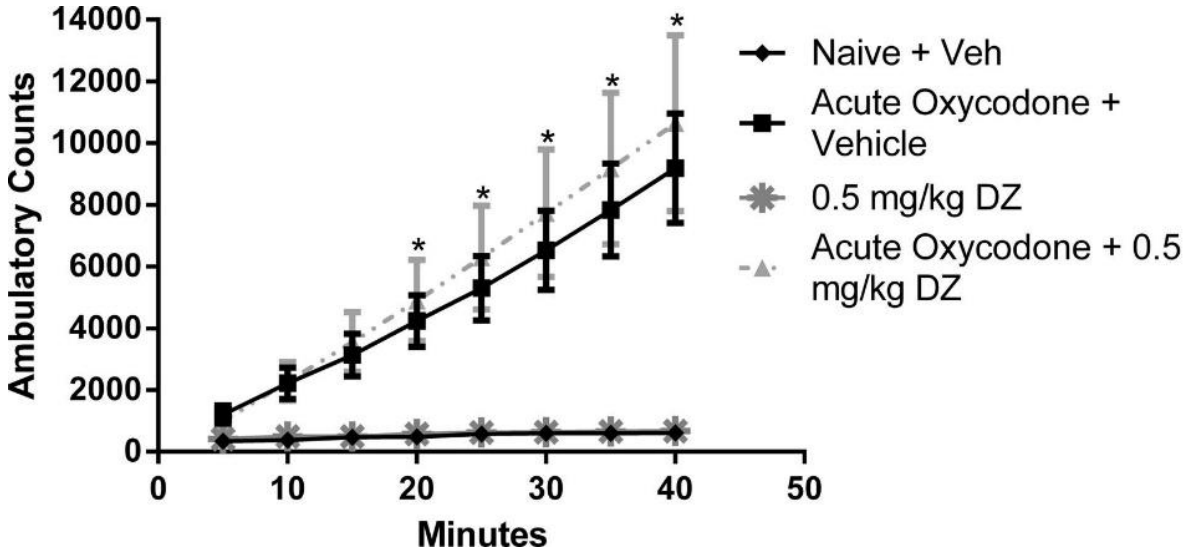


Fig. 9. Locomotor stimulation in response to vehicle, diazepam (0.5 mg/kg i.p.) or oxycodone (64 mg/kg p.o.) was assessed (n = 5). * Indicates a significant effect of both acute oxycodone and acute oxycodone + 0.5 mg/kg diazepam increasing ambulatory counts as compared to mice naïve to the opioid ($p < 0.05$). 0.5 mg/kg diazepam does not potentiate acute oxycodone's stimulatory effects. 0.5 mg/kg diazepam does not produce a difference in stimulatory counts when compared to naïve controls.

4. Discussion

Oxycodone and hydrocodone remain among the most commonly prescribed drugs for relief of acute and chronic pain. However, tolerance limits the long-term utility of these opioid agonists, leading to escalating doses of opioids to achieve the same analgesic effect while increasing the risks for abuse liability and death from respiratory depression. Additionally, people who become tolerant and then addicted to opioids usually are consuming other substances as well. Therefore, it is imperative to investigate the effects of these other substances on opioid tolerance. In this study, we investigated the effects of diazepam, a widely coabused substance, on oxycodone and hydrocodone antinociceptive tolerance and oxycodone locomotor stimulating tolerance in mice.

We found that diazepam reversed the antinociceptive tolerance that developed after repeated injections of subcutaneous oxycodone. The observation that an acute dose of diazepam, that was inactive alone, reversed tolerance to the antinociceptive effects of oxycodone is in agreement with our previous studies in which we demonstrated that diazepam reversed tolerance to the antinociceptive effects of morphine (Hull et al., 2013). We also demonstrated that the same dose of diazepam that reversed oxycodone tolerance did not significantly enhance the antinociceptive effect of acute oxycodone.

Patients are often administered oxycodone in tablets or solutions intended for oral use. Therefore, in a separate series of experiments, we investigated the ability of diazepam to reverse maximum tolerance of oral oxycodone. In these studies, to achieve maximum tolerance, we administered 256 mg/kg oxycodone orally twice a day for four days. Testing on the fifth day, this protocol produced significant tolerance illustrated by the lack of effective doses as high as 64 mg/kg. The observation that 0.5 mg/kg diazepam was able to reverse this maximum tolerance further supports the phenomenon of oxycodone tolerance reversal by benzodiazepines.

Furthermore, we found that diazepam reversed the antinociceptive tolerance that developed after repeated subcutaneous injections of hydrocodone. However, the reversal of tolerance to hydrocodone required a larger dose of diazepam than needed to reverse oxycodone or morphine tolerance. It is clear that diazepam did not potentiate the acute antinociceptive effect of hydrocodone. Further investigation is needed into why diazepam reversed oxycodone and morphine antinociceptive tolerance at lower doses than it reversed hydrocodone tolerance. It is possible that this difference occurred because hydrocodone produced a greater amount of tolerance than oxycodone. However, this is unlikely because the greatest tolerance was achieved using a multiple day oral oxycodone model and this tolerance was reversed by 0.5 mg/kg diazepam. Hydrocodone is also commonly administered to patients in tablets or solutions intended for oral use. We were not able to achieve tolerance when hydrocodone was given orally. This can be due to the fact that hydrocodone has been found to be less potent in certain measures of opioid effects (Zacny and Gutierrez, 2009). However, it is uncertain as to why we observed a greater amount of tolerance to repeated subcutaneous injections of hydrocodone compared to oxycodone yet no significant tolerance to hydrocodone given repeatedly orally. Further studies are needed to examine whether there is an important pharmacokinetic difference between oral dosing of oxycodone and hydrocodone.

The interaction between benzodiazepines and opioids on antinociception has been reported in the literature but with disagreement. Doses of diazepam, up to 5 mg/kg, were inactive in our warm water tail-withdrawal assays. Several studies reported antinociception with diazepam (Jiménez-Velázquez et al., 2010, 2008; Sierralta and Miranda, 1992). This difference may lie in the different noxious stimuli used (chemical versus thermal) and route of administration (intracerebroventricular injection versus intraperitoneal). In our studies, there was no potentiation

of oxycodone or hydrocodone induced antinociception with pretreatment doses of diazepam up to 0.5 mg/kg and 2 mg/kg, respectively. Whereas in other studies, intrathecal administration of benzodiazepines were found to enhance opioid antinociception (Bergman et al., 1988; Rattan et al., 1991). In agreement with our studies, others found neither an antinociceptive effect of benzodiazepines nor a potentiation effect of benzodiazepines on opioid induced antinociception (Mantegazza et al., 1982; Rodgers and Randall, 1987; Rosland et al., 1990). To our knowledge, benzodiazepines' interaction with oxycodone or hydrocodone has not been previously investigated.

In light of these results on antinociceptive tolerance, we tested the hypothesis that diazepam will have a similar effect in another paradigm of tolerance. We observed that oxycodone increased stimulatory activity in the locomotor activity assay. Marked tolerance was observed after repeated exposure to oxycodone over four days. Diazepam significantly reversed this marked tolerance. To rule out potentiation, we tested whether diazepam simply had an additive effect when combined with oxycodone. Diazepam did not potentiate acute oxycodone's stimulatory effects nor did it have any stimulatory effects on its own. At this dose of diazepam, we did not detect either depressing or stimulatory effects. Diazepam is classified as a CNS depressant and has been shown to decrease locomotor activity (Spyraki and Papadopoulou, 1980). In certain cases (such as prolonged social isolation), low doses of diazepam have been shown either to be inactive or have a stimulatory effect, resulting in a bimodal dose response curve (Pinna et al., 2006). The similar reversal of tolerance seen in both antinociception and in locomotor activity suggest that diazepam acts on a mechanism of tolerance that is shared by both opioid-induced effects.

The precise mechanisms and neuronal circuitry involved remain to be elucidated. This tolerance-reversal effect could be responsible, in part, for the high incidence of polydrug use

among opioid abusers. It has been suggested that opioid abusers occasionally combine the use of benzodiazepines with opioids to achieve a greater high (Jones et al., 2012). Whether this is simply an additive or synergistic effect is not clear and in a previous publication, we have suggested that compounds that act on GABA receptors increase the rewarding effects of opioid abuse by reducing tolerance (Hull et al., 2013). It has been theorized that both benzodiazepines and opioids produce a hyperpolarization of GABA interneurons which causes a reduction in the release of GABA which results in the disinhibition of dopaminergic neurons and an increase in extracellular dopamine in areas such as the striatum (Tan et al., 2011). The phenomenon of opioid-induced locomotion is due to mu opioid receptor-mediated increases in striatal dopamine release (Johnson and Glick, 1993; Kalivas and Duffy, 1987; Piepponen et al., 1999). GABAergic interneurons also play an important role in opioid antinociception (Lau et al., 2014). This suggests a potential site of action that should be further examined.

Our observations suggest this phenomenon is not due to the additive or synergistic effects of these CNS depressants but instead results from a reversal of tolerance. The result may be the same but the mechanism should be considered as new understandings of the tolerance mechanism will lead to new drug therapies such as tolerance-resistant analgesics. Similar reversal of opioid tolerance has been observed in models of respiratory depression and at the single cell level (Hill et al., 2016; Llorente et al., 2013). The reversant (reversing agent) in their studies was ethanol, which along with benzodiazepines, interacts with $GABA_A$ receptors.

The neurochemical mechanism of this reversal is likely due to diazepam's action on $GABA_A$ receptors. Our laboratory demonstrated that the diazepam reversal of morphine tolerance was fully inhibited by bicuculline, a $GABA_A$ antagonist, but not by phaclofen, a $GABA_B$ antagonist. This was in contrast to ethanol as neither inhibitor fully reversed ethanol's reversal or

morphine tolerance until they were administered in combination (Hull et al., 2013). Future studies should include determining whether the reversal of oxycodone and hydrocodone tolerance is due to diazepam's effects on $GABA_A$ receptors, if any subtypes are significant for the effect, and the necessary intracellular pathways for this phenomenon.

5. Conclusion

Collectively, our findings demonstrate that the administration of diazepam, at doses that are not antinociceptive or have any motor effects, reverses both antinociceptive and locomotor tolerance to orally active opioids. These doses of diazepam did not potentiate the acute effects of these prescription opioids. The findings reported here suggest that individuals who are taking oral opioids for chronic pain relief and an anxiety agent such as diazepam need be cognizant of the risk of reversal of tolerance to opioids that could lead to unintentional overdose deaths.

CHAPTER 3

CCR5 MEDIATES HIV-1 TAT-INDUCED NEUROINFLAMMATION AND INFLUENCES MORPHINE TOLERANCE, DEPENDENCE, AND REWARD

This chapter has been published in Brain, Behavior, and Immunity (Maciej Gonek, Virginia D. McLane, David L. Stevens, Kumiko Lippold, Hamid I. Akbarali, Pamela E. Knapp, William L. Dewey, Kurt F. Hauser, Jason J. Paris (2017) "CCR5 Mediates Hiv-1 Tat-Induced Neuroinflammation And Influences Morphine Tolerance, Dependence, And Reward")

Summary

The HIV-1 regulatory protein, trans-activator of transcription (Tat), interacts with opioids to potentiate neuroinflammation and neurodegeneration within the CNS. These effects may involve the C-C chemokine receptor type 5 (CCR5); however, the behavioral contribution of CCR5 on Tat/opioid interactions is not known. Using a transgenic murine model that expresses HIV-1 Tat protein in a GFAP-regulated, doxycycline-inducible manner, we assessed morphine tolerance, dependence, and reward. To assess the influence of CCR5 on these effects, mice were pretreated with oral vehicle or the CCR5 antagonist, maraviroc, prior to morphine administration. We found that HIV-1 Tat expression significantly attenuated the antinociceptive potency of acute morphine (2 – 64 mg/kg, i.p.) in non-tolerant mice. Consistent with this, Tat attenuated withdrawal symptoms among morphine-tolerant mice. Pretreatment with maraviroc blocked the effects of Tat, reinstating morphine potency in non-tolerant mice and restoring withdrawal symptomology in morphine-tolerant mice. Twenty-four hours following morphine administration, HIV-1 Tat significantly potentiated (~3.5-fold) morphine-conditioned place preference and maraviroc further potentiated these effects (~5.7-fold). Maraviroc exerted no measurable behavioral effects on its own. Protein

array analyses revealed only minor changes to cytokine profiles when morphine was administered acutely or repeatedly; however, 24 h post morphine administration, the expression of several cytokines was greatly increased, including endogenous CCR5 chemokine ligands (CCL3, CCL4, and CCL5), as well as CCL2. Tat further elevated levels of several cytokines and maraviroc pretreatment attenuated these effects. These data demonstrate that CCR5 mediates key aspects of HIV-1 Tat-induced alterations in the antinociceptive potency and rewarding properties of opioids.

1. Introduction

There is a dynamic relationship between opioid use, human immunodeficiency virus-1 (HIV-1) acquisition, and disease progression. Worldwide, injection drug use (IDU) accounts for ~30% of new HIV-1 infections outside of sub-Saharan Africa (WHO, 2016). Within the United States, over 3,500 new infections involved IDU in 2015 (CDC 2016), a year in which overall drug overdose deaths rose another 11%, the majority (63%) of which involved opioids (Rudd et al., 2016). The convergence of the HIV and opioid epidemics is particularly concerning given evidence that opioid usage increases the progression of HIV-1 to acquired immune deficiency syndrome (AIDS) and promotes neurocognitive impairment in humans and non-human primates (Bell et al., 2006, 2002; Bokhari et al., 2011; Chuang et al., 2005; Donahoe et al., 1993; Kumar et al., 2006; R et al., 2004; Rivera et al., 2013). Moreover, HIV-infected individuals are at risk for the development of neuropathic pain (Malvar et al., 2015) for which prescription opioids remain a common treatment (Kremer et al., 2016; Zilliox, 2017). Pharmacological treatment for opioid abuse includes substitution therapies (e.g. methadone, buprenorphine, buprenorphine/naloxone; Moatti et al., 1998; Roux et al., 2008; Sambamoorthi et al., 2000; Woody et al., 2014), which may exert neurotoxic interactions with HIV-1 proteins (Fitting et al., 2014b).

As such, the mechanisms and physiological consequences of HIV/opioid interactions need to be understood in order to improve outcomes for HIV seropositive patients that are pharmacologically managed for pain and/or addiction.

The biological mechanisms that underlie HIV-1 and opioid interactions in the central nervous system (CNS) likely involve the HIV-1 regulatory protein, trans-activator of transcription (Tat). Tat is critical for efficient HIV replication; however, Tat is soluble and can be secreted from infected cells to exert direct and indirect neurotoxicity in vitro (reviewed in King et al., 2006; Nath et al., 2002). Tat promotes neuroinflammation via NF-κB signaling (El-Hage et al., 2008b; Herbein et al., 2010), upregulation of proinflammatory cytokines [particularly the endogenous β-chemokine ligands for the C-C "motif" chemokine receptor type 5 (CCR5): C-C chemokine ligand 3 (CCL3, also known as "macrophage inflammatory protein-1α" or MIP-1α), CCL4 (also known as "macrophage inflammatory protein-1β" or MIP-1β), and CCL5 (also known as "regulated on activation normal T-cell expressed and secreted" or RANTES); El-Hage et al., 2005; Hahn et al., 2010]. And subsequent recruitment of neuroimmune cells promoting neuroinflammation In vitro, morphine exacerbates Tat effects to activate microglia (Bokhari et al., 2009; Gupta et al., 2010; Sorrell and Hauser, 2014), increase cytokine production (Bokhari et al., 2009; El-Hage et al., 2005; Fitting et al., 2014b.; Turchan-Cholewo et al., 2009), drive oxidative stress (Dalvi et al., 2016; Fitting et al., 2014a,b; Malik et al., 2011; Turchan-Cholewo et al., 2009), increase intracellular calcium (El-Hage et al., 2005; Fitting et al., 2014a,b), and promote neurotoxicity (Fitting et al., 2014a,b.; Gurwell et al., 2001; Malik et al., 2011). Morphine and Tat interactions may depend on μ opioid receptors (MORs) given that neurotoxic synergy is observed in co-cultures when mixed glia express MORs, but not when they are derived from MOR−/− mice (Zou et al., 2011). These data support the notion that glial MORs are critical for the indirect neurotoxic effects of Tat.

The proinflammatory effects of HIV-1 Tat at CCR5 may directly influence opioid sensitivity. In studies of opioid-mediated antinociception in rats, activation of CCR5 or CXCR4 can rapidly (within 30 min) desensitize μ- or δ-opioid-receptors (Chen et al., 2007). Blocking actions at CCR5 in proinflammatory states may attenuate heterologous desensitization of MORs and increase therapeutic efficacy. In support, intrathecal administration of the CCR5 antagonist, maraviroc, attenuated chronic constriction injury induced microgliosis, astrogliosis, upregulation of CCR5 protein, and mRNA expression of CCR5-ligands (CCL3, CCL4, and CCL5) in the spinal cord and dorsal root ganglion concurrent with reduced neuropathic pain (Kwiatkowski et al., 2016). Moreover, CCR5 and MORs may form functionally active heteromers. A bivalent ligand derived from a MOR agonist (oxymorphone) and a CCR5 antagonist (TAK-220) had ~2000× greater antinociceptive potency than morphine in mice experiencing LPS-mediated inflammation (Akgün et al., 2015). Another bivalent ligand comprised of an opioid receptor antagonist (naltrexone) and maraviroc reduced the infectivity of human astrocytes when cultured with R5-tropic HIV (Arnatt et al., 2016; El-Hage et al., 2013; Yuan et al., 2013). These data suggest a dynamic relationship between MOR and CCR5 activation that may contribute to HIV pathology; however, the functional effects are poorly understood. As such, we investigated morphine tolerance, dependence, and reward in a transgenic murine model that conditionally-expresses the proinflammatory HIV-1 regulatory protein, Tat_{1-86}. Using a transgenic mouse approach, conditional Tat expression has been demonstrated to reduce the antinociceptive potency of morphine (Fitting et al., 2016, 2012), while potentiating psychostimulant reward in acute drug withdrawal (24 h post drug administration; (Paris et al., 2014a,b). We hypothesized that HIV-1 Tat expression would attenuate morphine antinociceptive potency and that the CCR5 antagonist,

maraviroc, would reverse these effects. Further, we hypothesized that 24 h post morphine, Tat- and cytokine-mediated effects would be potentiated.

2. Materials and Methods

The use of mice in these studies was pre-approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and the experiments were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

2.1. Subjects and housing

Adult male mice expressed (or did not express) an HIV-1_{IIIB} tat_{1-86} transgene (N = 245) as previously described (Bruce-Keller et al., 2008; Fitting et al., 2013; Hauser et al., 2009) and were generated in the vivarium at Virginia Commonwealth University (MCV campus). Briefly, HIV-1 Tat_{1-86} is conditionally expressed in a CNS-targeted manner via a GFAP-driven, Tet-on promoter (activated by consumption of doxycycline-containing chow) in Tat(+) mice. Tat(-) control littermates express the Tet-on transcription factor without the tat_{1-86} transgene. While Tat can induce astrogliosis in transgenic mice (El-Hage et al., 2008b; Hahn et al., 2015; Paris et al., 2015), this has not been observed to impair Tat production (Fitting et al., 2012; Paris et al., 2014b). Additional control experiments were carried out in adult, male, C57BL/6J mice (N = 30). All mice (~70 days of age) were housed 4 - 5 / cage and were maintained in a temperature- and humidity controlled room on a 12:12 h light / dark cycle (lights off at 18:00 h) with ad libitum access to food and water.

2.2. Chemicals

To induce HIV-1 Tat_{1-86} expression, Tat(+) transgenic mice [and Tat(-) controls] were placed on doxycycline chow (Dox Diet #2018, 6 g/kg; Harlan Laboratories, Madison, WI, USA) for 28 days, unless otherwise specified. Some mice received subcutaneous implants of placebo or morphine pellets (75 mg; National Institute on Drug Abuse, Rockville, MD, USA), the latter of which induces tolerance and dependence in the present strain of mice (Fitting et al., 2016). To precipitate morphine withdrawal, mice were administered (-)naloxone (1 mg/kg, s.c.; Sigma-Aldrich, St. Louis, MO; (Fitting et al., 2016). To investigate morphine reward, mice were administered morphine sulfate (#M8777; Sigma-Aldrich) at a concentration of 10 mg/kg, i.p. (0.1 ml per 10 g body weight) which has been demonstrated to produce morphine-conditioned place preference (CPP; Zhu et al., 2015). To investigate the contribution of CCR5 to these effects, some mice were administered the CCR5-selective antagonist, maraviroc (62 mg/kg, p.o.; #376348-65-1; BOC Sciences, Shirley, NY, USA) which was dissolved in 100% DMSO, then diluted to 5% DMSO in vegetable oil. Using inter-species allometric scaling (by a factor of 12.3; (Freireich et al., 1966; Reagan-Shaw et al., 2008) others have determined maraviroc (62 mg/kg, p.o.) dosing for mice from clinical formulations (Neff et al., 2010).

2.3. Surgical manipulation

Mice received subcutaneous implants of placebo or morphine (75 mg) pellets under isoflurane (2.5 %) anesthesia as previously reported (Fitting et al., 2016; Ross et al., 2008). Following surgery, mice were monitored to ensure weight gain, muscle tone, proper neurological response, and general health (Crawley and Paylor, 1997). No mice failed to recover.

2.4. Behavioral assays

Prior to all behavioral testing, mice were acclimated to the testing room for 24 h. For assessments of morphine tolerance and morphine dependence, mice received subcutaneous implants of placebo or morphine pellets. Five days later, mice were assessed for morphine tolerance in a warm-water tail-withdrawal assay. Following tail withdrawal testing, mice underwent naloxone-precipitated withdrawal and were assessed for morphine dependence. For assessments of morphine reward, Tat-transgenic mice were assessed in a CPP paradigm with psychomotor sensitization assessed on conditioning days. Some mice were additionally assessed on a rotarod to rule out potential locomotor confounds.

2.4.1. Warm-water tail-withdrawal test

A warm-water tail-withdrawal test was conducted with a water-bath maintained at 56 ± 0.1°C as previously described (Coderre and Rollman, 1983; Fitting et al., 2016). Briefly, mice were gently wrapped in a cloth and the distal one-third of the tail was immersed in a water bath. The mice rapidly removed their tail from the bath at the first sign of discomfort and the tail-withdrawal latency was recorded. Tail-withdrawal latency was then assessed using a cumulative dosing procedure. Mice were injected with a starting dose of morphine and were tested for antinociception 20 min later. Mice that did not reach a 10 sec cut-off threshold received an additional cumulative dose of morphine and were retested. This process was repeated until the animals reached the cut-off value of 10 sec. Baseline latency ranged from 2 to 4 sec. The 10 sec maximum cutoff latency was used to prevent tissue damage. Antinociception was quantified as the

percentage of maximal possible effect (%MPE): %MPE = [(Test latency − Baseline latency) / (10 – Baseline latency)] × 100 (Harris and Pierson, 1964).

2.4.2. Antagonist-precipitated withdrawal

Mice were administered an injection of the opioid receptor antagonist, naloxone (1 mg/kg, s.c.), in order to precipitate withdrawal. The primary symptom assessed was jumping from an elevated platform (32 cm high, 17 cm diameter). The proportion of mice that jumped from their individual platforms was recorded over a 10 min trial. The proportion of jumping mice is considered an index of withdrawal (Fitting et al., 2016). This test was followed by an evaluation of additional signs of withdrawal. Mice were placed in a rectangular, clear, plastic observation box (16 × 16 × 30 cm) and observed for 5 min. The concomitant number of jumps, forepaw tremors, and wet-dog shakes was recorded. The frequency of jumps, tremors, and shakes are considered additional indices of withdrawal (Fitting et al., 2016).

2.4.3. Conditioned Place Preference

Behavior in the CPP test was recorded and digitally-encoded by an ANY-maze behavioral tracking system (Stoelting Co., Wood Dale, IL, USA). Morphine-CPP and locomotor sensitization were assessed simultaneously (Zhu et al., 2015). CPP was conducted as modified from previous methods (Paris et al., 2014). The apparatus (#64101; Stoelting Co.) consisted of two black conditioning chambers (18 × 20 × 35 cm), each visually-distinguished by the presence of white circles or horizontal stripes on the chamber walls, as well as ~30 lux difference in ambient lighting. Conditioning chambers were connected by a start box/transition chamber (10 × 20 cm). A biased conditioning design was utilized (Semenova et al., 1995). On day 1, mice were allowed to freely

explore the apparatus for 10 min in order to establish an initial chamber-preference (there was no significant side preference observed across experimental groups). On days 2 – 5 mice underwent one cycle of morphine conditioning per day (receiving an i.p. saline injection paired with confinement to the preferred chamber for 30 min, followed 4 h later by an i.p. morphine, 10 mg/kg, injection paired with confinement to the less preferred chamber for 30 min). Twenty-four hours after the last morphine-conditioning cycle, mice were allowed to freely explore the apparatus in order to assess their final chamber preference. The amount of time that mice spent in the chambers or the start/transition box, as well as the distance traveled, and the frequency of rearing was recorded on each day. CPP was quantified as a difference score: CPP d-score = (time spent in the morphine-paired chamber) – (time spent in the saline-paired chamber) (Paris et al., 2014a).

2.4.4. Rotarod

Locomotor coordination was assessed on an accelerated rotarod as previously described (Paris et al., 2013). Briefly, mice were trained to balance on an immobile rotarod (3 cm in diameter and suspended 44.5 cm high; Columbus Instruments, Columbus, OH, USA) for 30 sec. Mice were then trained to navigate the task across two 30 sec fixed speed trials (10 rpm) and two 180 sec fixed speed trials (10 rpm). Lastly, mice were tested on two accelerated speed trials (180 sec max. latency at 0 - 20 rpm). The mean latency to fall from the rotarod and the maximum RPM achieved across the two accelerated trials were utilized as indices for locomotor performance. Decreased latencies to fall and lower maximal RPM on the accelerated test indicate an impaired motor phenotype.

2.5. Cytokine assay

The head of the caudate nucleus has been identified as a central reservoir for maximal HIV viral load in humans (reviewed in Nath, 2015) and we have found the striatum to host a cell

population that may be selectively vulnerable to HIV-1 Tat (Schier et al., 2017). As such, cytokine analyses were conducted on caudate/putamen (dorsal striatum) in the present mouse model. Mice underwent cervical dislocation and bilateral dorsal striata were immediately dissected, flash-frozen in liquid nitrogen, and stored at - 80°C until assay. At the time of assay, tissues were homogenized in IP lysis buffer (#87787; Pierce Biotechnology, Rockford, IL, USA) with a protease/phosphatase inhibitor cocktail (#04693159001; Roche, Mannheim, Germany). Protein concentrations were determined via bicinchoninic acid (BCA) assay per kit manufacturer instructions (#23224; Pierce Biotechnology).

Cytokines were assessed using a Bio-Plex Pro™ Mouse Cytokine 23-plex assay kit (#M60009RDPD; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed on a Bio-Plex 200 system. Samples were diluted to a concentration of 500 µg/mL. Unknown samples and standards were incubated with fluorescent, antibody-tagged microspheres and detected via streptavidin-phycoerythrin-labeled detection antibodies. Cytokine concentrations were calculated from respective standard curves via Bio-Plex Manager 4.0 software. All samples were analyzed in duplicate. Limits of detection ranged from 2 to 20 pg/mL. Mean intra- and inter-assay coefficients of variance were 7.2 % and 5.3 %, respectively.

2.6. Statistical analyses

Median effective doses (ED50; reported with 95 % confidence intervals) were determined via non-linear regression (sigmoidal curvilinear modeling with variable slope) using a least-squares fit for each treatment group (bottom and top values constrained to 0 and 100, respectively). Dependent measures for additional behavioral analyses were assessed via ANOVA (to assess dependence) or repeated measures ANOVA (to assess psychostimulation or CPP over multiple trials) with drug condition (placebo or morphine), inhibitor condition (vehicle or maraviroc), and

genotype [Tat(-) or Tat(+)] as factors. Cytokine arrays were assessed via separate three-way ANOVAs. Mice treated acutely with doxycycline (48 h) were analyzed with morphine condition (acute saline or acute morphine), inhibitor condition (vehicle or maraviroc), and genotype [Tat(-) or Tat(+)] as factors. Mice treated chronically with doxycycline for 28 days were analyzed with morphine condition (morphine-naïve, repeated morphine, or 24 h post morphine), inhibitor condition (vehicle or maraviroc), and genotype [Tat(-) or Tat(+)] as factors. Fisher's Protected Least Significant Difference post-hoc tests determined group differences following main effects. Interactions were delineated via simple main effects and main effect contrasts with alpha controlled for multiple comparisons. Analyses were considered significant when $p < 0.05$.

3. Results

3.1. HIV-1 Tat decreased morphine potency in non-tolerant mice; tolerance or pharmacological antagonism of CCR5 attenuated Tat effects

As negative control measures before proceeding to tests involving Tat-transgenic mice, morphine dosing, non-specific interactions with maraviroc, and warm-water tail withdrawal test conditions were confirmed in C57BL/6J mice. In a 52°C water bath, morphine administered at 5 mg/kg (Fig. 1A) or 10 mg/kg (Fig. 1B) produced antinociception that was present for at least 2 h and peaked at 60 min, commensurate with observations in other animal models (Altun et al., 2015; Williams et al., 2008). In a 56°C water bath, cumulative morphine-dosing produced antinociception commensurate with what we have previously observed in C57BL/6J mice (Fitting et al., 2016; Fig. 1C). Maraviroc did not significantly influence the acute time-course (Fig. 1A-B) or the antinociceptive effects of morphine in C57BL/6J mice [vehicle/morphine ED_{50} = 2.0 (95% CI: 0.9 – 4.3), maraviroc/morphine ED50 = 3.0 (95% CI: 2.2 – 4.1)] (Fig. 1C).

Tat(-) and Tat(+) mice were placed on doxycycline for 28 days to induce Tat expression (or not). On day 28, mice were implanted with a subcutaneous placebo pellet or morphine pellet to induce morphine tolerance. From days 28 – 32, mice were administered vehicle or maraviroc (p.o., QD). On day 33, mice received their last dose of vehicle or maraviroc and were tested 30 min later.

Tat induction, morphine tolerance, and maraviroc pretreatment significantly influenced the antinociceptive response to acute morphine (Fig. 2A-B). Among nontolerant, placebo-pelleted mice, Tat exposure produced a modest but significant shift to the right in the antinociceptive potency of acutely-administered morphine [$F(1,48) = 7.97$, $p < 0.05$] [Fig. 2A; Tat(-)ED_{50} = 4.5 (95% CI: 3.6 - 5.5), Tat(+)ED_{50} = 6.8 (95% CI: 5.6 - 8.0)]. This effect was obviated by maraviroc pretreatment [Fig. 2B; Tat(-)ED50 = 6.0 (95% CI: 4.9 - 7.1), Tat(+)ED_{50} = 5.8 (95% CI: 4.9 - 6.7)]. Morphine tolerance significantly shifted the ED_{50} for morphine-induced antinociception to the right in all treatment groups [Fig. 2A-B; ED_{50} range = 17.8 - 53.8]. Notably, baseline tail-withdrawal latencies were ~0.4 sec greater among morphine-pelleted (2.7 ± 0.1 sec) vs. placebo pelleted mice (2.3 ± 0.1 sec) [$F(1,44) = 10.91$, $p < 0.05$]. Neither Tat induction nor maraviroc pretreatment influenced baseline tail-withdrawal latencies.

3.2. Among tolerant mice, Tat-exposure decreased primary withdrawal behavior; pharmacological antagonism of CCR5 attenuated Tat effects

Following testing for antinociception, mice were administered the opioid receptor antagonist, naloxone, and precipitated withdrawal behaviors were assessed. Tat induction, morphine tolerance, and maraviroc pretreatment significantly interacted to influence the primary measure of withdrawal, the proportion of mice that jumped from an elevated platform [$F(1,44) =$

6.77, $p < 0.05$] (Fig. 3A). Unlike other withdrawal-related behaviors assessed, elevated platform jumping was only observed among previously morphine-tolerant mice (a significant difference from their placebo-pelleted counterparts [$F(1,44) = 122.27$, $p < 0.05$]; Fig. 3A). Among morphine-tolerant mice, withdrawal precipitated jumping was significantly attenuated by Tat exposure [Tat(+) mice significantly differed from Tat(-) controls ($p = 0.03$)] or maraviroc pretreatment [maraviroc treated, Tat(-) mice significantly differed from Tat(-) controls ($p = 0.005$)] (Fig. 3A). However, combined Tat exposure and maraviroc pretreatment restored withdrawal precipitated jumping [maraviroc-treated Tat(+) mice significantly differed from maraviroc treated Tat(-) mice ($p = 0.004$) or vehicle-treated Tat(+) mice ($p = 0.03$)] (Fig. 3A).

When observed for additional, simultaneous withdrawal-precipitated behaviors, the frequency of spontaneous jumping was significantly greater [$F(1,44) = 10.83$, $p < 0.05$] (Fig. 3B) and paw tremors were significantly reduced [$F(1,44) = 10.79$, $p < 0.05$] (Fig. 3C) among morphine-tolerant mice compared to placebo-pelleted controls, irrespective of Tat or maraviroc exposure. There was a main effect for wet-dog shakes to be significantly greater among Tat(+) mice compared to Tat(-) mice, irrespective of morphine or maraviroc exposure [$F(1,44) = 3.97$, $p = 0.05$] (Fig. 3D).

3.3. HIV-1 Tat exposure and CCR5 antagonism attenuated the psychomotor response to morphine

Both Tat exposure and maraviroc pretreatment interacted with repeated morphine injections to influence locomotor behavior (Fig. 4AB). Among vehicle-pretreated mice, morphine administration significantly increased the distance traveled in the CPP apparatus [$F(7,147) = 16.66$, $p < 0.05$] compared to saline administration, irrespective of Tat exposure ($p < 0.0001$ per day; Fig. 4A). However, among maraviroc-pretreated mice, Tat-exposure and morphine administration significantly interacted [$F(7,147) = 2.46$, $p < 0.05$] such that Tat(-) mice receiving morphine demonstrated significantly greater distances traveled on days 1 ($p = 0.03$) and 4 ($p = 0.003$), compared to Tat(+) mice receiving morphine (Fig. 4B). Tat(-) mice also displayed enhanced locomotion to a significantly greater degree following morphine administration than following saline administration ($p < 0.0001$ per day); whereas, locomotion among Tat(+) mice did not differ between morphine or saline administration (Fig. 4B). Notably, Tat exposure can impair locomotion on its own; however, these effects are not usually observed in the present model until Tat has been induced for over one month (Hahn et al., 2012). To rule out nonspecific effects of Tat on locomotion, some Tat(-) and Tat(+) mice from the present experiment were assessed on an accelerated rotarod after testing. No significant differences in locomotor capacity were observed following fixed speed trials (10 rpm) lasting 30 s [Tat(-): 9 ± 2, Tat(+): 12 ± 2] or 180 s [Tat(-): 56 ± 16, Tat(+): 81 ± 20], or following accelerated speed trials (0 – 20 rpm) lasting 180 s [Tat(-): 132 ± 10, Tat(+): 128 ± 14].

3.4. CCR5 antagonism potentiated Tat-related morphine reward

To assess the contribution of HIV-1 Tat on the rewarding properties of morphine, mice were assessed for morphine-CPP. Tat(-) and Tat(+) mice were exposed to two morphine conditioning cycles (saline followed by morphine) and assessed for chamber preference 24 h after the last morphine administration (Fig. 5A). Following two conditioning cycles, morphine significantly increased the time that mice spent in the previously non-preferred chamber [$F(1,24) = 26.84$, $p < 0.05$] (Fig. 5A'). However, significant differences between Tat(-)and Tat(+) mice were not observed (Fig. 5A').

Another group of mice underwent four morphine-conditioning cycles and were assessed for final chamber preference 24 h later (Fig. 5B). Under these conditions, Tat genotype significantly interacted with the final preference trial [$F(1,22) = 5.43$, $p < 0.05$] (Fig. 5B'). Tat(-) and Tat(+) mice spent a commensurate amount of time in the least preferred chamber prior to conditioning; but, Tat(+) mice spent a significantly greater amount of time in the morphine-paired chamber following four cycles of morphine conditioning compared to Tat(-) controls ($p = 0.04$; Fig. 5B').

To assess the influence of CCR5 on Tat-potentiated CPP, Tat(-) and Tat(+) mice were pretreated with vehicle or maraviroc 30 min prior to the start of conditioning over four cycles (Fig. 5C). As previously observed, mice significantly preferred the morphine paired chamber 24 h following conditioning, irrespective of treatment [$F(1,42) = 100.80$, $p < 0.05$] (Fig. 5C'). There was significant interaction between Tat-genotype and maraviroc condition [$F(1,42) = 4.13$, $p < 0.05$], such that Tat(+) mice spent a significantly greater amount of time in the morphine-paired chamber when pretreated with maraviroc than did any other group ($p = 0.0006 – 0.02$; Fig. 5C').

3.5. Prior morphine exposure and HIV-1 Tat increase striatal cytokine content and CCR5 antagonism attenuates Tat effects

Cytokine protein expression was assessed in the dorsal striatum (caudate/putamen) of Tat (-) and Tat(+) mice. In order to parse the acute- vs. chronic influences of Tat, maraviroc, and morphine on the immune effectors examined, tissues from two separate groups of mice were assessed. The first group was acutely exposed to Tat (48 h of doxycycline to induce Tat), maraviroc (a single administration of vehicle or maraviroc, 62 mg/kg, p.o., 30 min prior to i.p. injection), and morphine (a single administration of saline or morphine, 10 mg/kg, i.p.). The second group was chronically exposed to Tat (28 d of doxycycline to induce Tat), repeated maraviroc (vehicle or maraviroc, 62 mg/kg, p.o., QD for 4 d, 30 min prior to i.p. injection), and repeated morphine (saline or morphine, 10 mg/kg, i.p., QD for 4 d). Tissues were collected either
1 h after the last treatment, or 24 h after the last treatment (the latter timeframe being commensurate with that which produced Tat-potentiated morphine-CPP).

3.5.1. Chemotactic Cytokines

Among mice that were acutely-exposed to Tat and drug manipulations (Fig. 6, left-hand panels, 48 h doxycycline exposure), acute Tat induction and acute maraviroc pretreatment significantly interacted to increase striatal CCL3, compared to vehicle treated, Tat(-) controls [$F(1,31) = 4.69$, $p < 0.05$] (Fig. 6B, left panel, white bars). There was an additional interaction for acute morphine to significantly increase CCL5, but only among Tat(+) mice [$F(1,31) = 4.59$, $p < 0.05$] (Fig. 6G, left panel, cyan bars). Main effects were observed for acute Tat exposure to significantly increase CCL4 [$F(1,31) = 15.56$, $p< 0.05$] (Fig. 6C, left panel, white and cyan bars) and CCL11 [$F(1,31) = 5.56$, $p < 0.05$] (Fig. 6H, left panel, white and cyan bars), irrespective of

maraviroc or morphine administration. Lastly, there was a main effect for morphine to significantly elevate CXCL1 [$F(1,31) = 10.55$, $p < 0.05$], irrespective of Tat or maraviroc exposure (Fig. 6D, left panel, cyan bars). Acute treatments did not otherwise influence the chemokines examined.

Among mice exposed to chronic Tat and repeated morphine, no significant differences were observed until morphine was withheld for 24 h (Fig. 6, right-hand panels, 28 d doxycycline exposure). Three-way interactions were revealed 24 h post morphine treatment for CCL2 [$F(2,45) = 3.64$, $p < 0.05$] (Fig. 6A, right panel, gray bars), CCL3 [$F(2,45) = 6.14$, $p < 0.05$] (Fig. 6B, right panel, gray bars), CCL4 [$F(2,45) = 4.55$, $p < 0.05$] (Fig. 6C, right panel, gray bars), CXCL1 [$F(2,45) = 4.21$, $p < 0.05$] (Fig. 6D, right panel, gray bars), G-CSF [$F(2,45) = 5.42$, $p < 0.05$] (Fig. 6E, right panel, gray bars), and GMCSF [$F(2,45) = 6.11$, $p < 0.05$] (Fig. 6F, right panel, gray bars). In each case, chronic Tat significantly potentiated the increase of chemokines and maraviroc pretreatment significantly ameliorated this potentiation (see outlined chemokines in Fig. 6). Additionally, a main effect was observed for CCL5 to be increased 24 h post morphine administration, irrespective of Tat or maraviroc exposure [$F(2,45) = 115.48$, $p < 0.05$] (Fig. 6G, right panel, gray bars). Intriguingly, repeated morphine significantly reduced CCL11 [$F(2,45) = 3.25$, $p < 0.05$], an effect that was prevented by maraviroc pretreatment (Fig. 6H, right panel, magenta bars). Once morphine was withheld for 24 h, CCL11 declined in all groups (Fig. 6H, right panel, gray bars).

3.5.2. Pro-inflammatory cytokines

Acute HIV-1 Tat and maraviroc/morphine exposure (Fig. 7, left-hand panels, 48 h doxycycline exposure) influenced pro-inflammatory cytokine levels. A significant interaction was observed for acute morphine to modestly elevate IL-1α, but only in maraviroc-pretreated mice

[F(1,31) = 6.11, $p < 0.05$] (Fig. 7G, left panel, cyan bars). Acute Tat also significantly increased IL-3 (which was attenuated by maraviroc) [F(1,31) = 3.82, p = 0.05] (Fig. 7A, left panel, white bars) and IL-9 (which was attenuated by morphine and reinstated by maraviroc) [F(1,31) = 3.96, p = 0.05] (Fig. 7C, left panel, white and cyan bars). Lastly, there was a main effect for acute Tat exposure to cause a modest, significant increase in IL-1α [F(1,31) = 7.03, $p < 0.05$] (Fig. 7G, left panel, white and cyan bars) and a main effect for acute maraviroc to increase IL-6 [F(1,31) = 4.07, $p < 0.05$] (Fig. 7B, left panel, white bars). No additional effects of acute treatments were observed on pro-inflammatory cytokines.

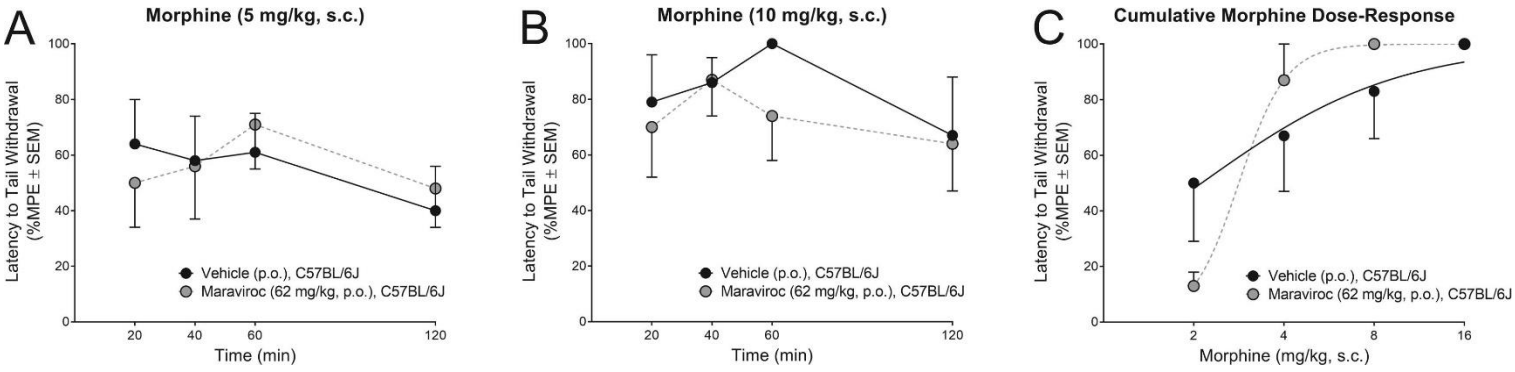
In mice exposed to chronic Tat and repeated maraviroc/morphine treatment, significant elevations were observed in all pro-inflammatory cytokines examined (Fig. 7, right-hand panels, 28 d doxycycline exposure). Three-way interactions were revealed 24h post morphine treatment for IL-3 [F(2,45) = 6.73, $p < 0.05$] (Fig. 7A, right panel, gray bars), IL-6 [F(2,45) = 3.14, p = 0.05] (Fig. 7B, right panel, gray bars), IL-9 [F(2,45) = 5.18, $p < 0.05$] (Fig. 7C, right panel, gray bars), IL-12p40 [F(2,45) = 4.02, $p < 0.05$] (Fig. 7D, right panel, gray bars), IL-12p70 [F(2,45) = 5.43, $p < 0.05$] (Fig. 7E, right panel, gray bars), and IL-17A [F(2,45) = 5.65, $p < 0.05$] (Fig. 7F, right panel, gray bars). In each case, chronic Tat significantly potentiated the increase of proinflammatory cytokines and maraviroc pretreatment significantly ameliorated this potentiation (see outlined cytokines in Fig. 7). A 3-way interaction was also revealed for IFN-γ to be significantly increased among mice that were exposed to chronic Tat and repeated maraviroc/morphine [F(2,45) = 3.23, $p < 0.05$] (Fig, 7J, right panel, magenta bars). These effects were significantly exacerbated 24 h post morphine; however, this potentiation was not significantly attenuated by maraviroc-pretreatment (Fig, 7J, right panel, gray bars). Interestingly, repeated morphine also significantly increased IL-9 content in maraviroc-pretreated mice [F(2,45) = 5.63,

$p < 0.05$] (Fig. 7C, right panel, magenta bars). Irrespective of Tat and maraviroc exposure, main effects were observed for cytokines to be increased 24 h post morphine for IL-1α [$F(2,45) = 80.55$, $p < 0.05$] (Fig. 7G, right panel, gray bars), IL-1β [$F(2,45) = 288.09$, $p < 0.05$] (Fig. 7H, right panel, gray bars), IL-2 [$F(2,45) = 475.92$, $p < 0.05$] (Fig. 7I, right panel, gray bars), and TNF-α [$F(2,45) = 42.36$, $p < 0.05$] (Fig. 7K, right panel, gray bars).

3.5.3. Anti-inflammatory cytokines

Among the anti-inflammatory cytokines assessed, only IL-10 demonstrated a significant response to acute manipulations (Fig. 8, left-hand panels, 48 h doxycycline exposure), [$F(1,31) = 5.56$, $p < 0.05$]. Acute Tat exposure significantly increased IL-10, and this effect was attenuated by maraviroc unless co-administered with acute morphine (Fig. 8A, left panel, white and cyan bars).

When assessed for chronic Tat exposure and repeated maraviroc/morphine administration (Fig. 8, right-hand panels, 28 d doxycycline exposure), IL-10 was the only anti-inflammatory cytokine that demonstrated Tat-mediated potentiation 24 h post morphine that was reversible by pretreatment with maraviroc [$F(2,45) = 6.46$, $p < 0.05$] (Fig. 8A, right panel, gray bars). Other anti-inflammatory cytokines demonstrated significant main effects for enhanced protein expression 24 h post morphine, but not in a Tat- or maraviroc-sensitive manner: IL-4 [$F(1,45) = 499.77$, $p < 0.05$] (Fig. 8B, right panel, gray bars), IL-5 [$F(2,45) = 3.45$, $p < 0.05$]] (Fig. 8C, right panel, gray bars), and IL-13 [$F(2,45) = 313.62$, $p < 0.05$]] (Fig. 8D, right panel, gray bars). Additionally, a main effect for 28 d Tat exposure to significantly increase IL-4 was observed [$F(1,45) = 9.13$, $p < 0.05$], independent of maraviroc or morphine administration (Fig. 8B, right panel, yellow/magenta/gray bars).



<u>Figure 1</u>: Maraviroc did not significantly influence the antinociceptive response to morphine in C57BL/6J mice. Latency to tail withdrawal (% MPE ± SEM) among C57BL/6J mice (n = 5 / group) that were administered vehicle (p.o.; black circles) or maraviroc (62 mg/kg, p.o.; blue circles) prior to (A) morphine (5 mg/kg, s.c.), (B) morphine (10 mg/kg, s.c.), or (C) a cumulative morphine dosing regimen (2 – 16 mg/kg, s.c.).

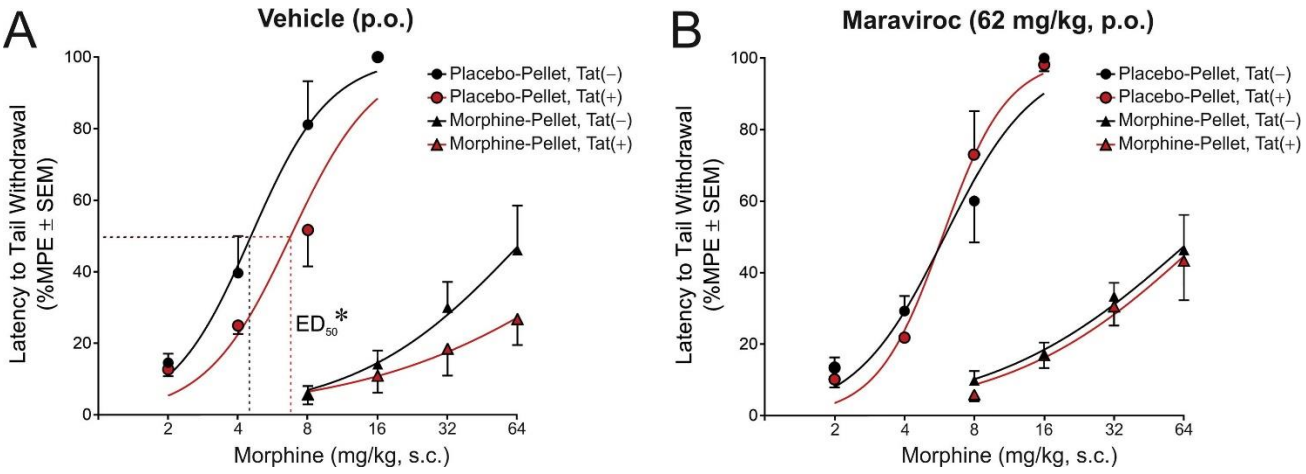


Figure 2: HIV-1 Tat significantly shifted the ED50 for morphine to the right in nontolerant mice and maraviroc pretreatment obviated this effect. Latency to tail withdrawal (% MPE ± SEM) among Tat(-) or Tat(+) mice (n = 6 - 7 / group) that were morphine-naïve (placebo-pelleted; circles) or morphine-tolerant (morphine-pelleted; triangles). Mice [Tat(-) in black, Tat(+) in red] were administered (A) daily vehicle (p.o.) or (B) daily maraviroc (62 mg/kg, p.o.) following pelleting and were assessed for tail withdrawal reflex in response to a cumulative morphine regimen (2 - 64 mg/kg, s.c.). * indicates a significant shift in ED50, $p < 0.05$.

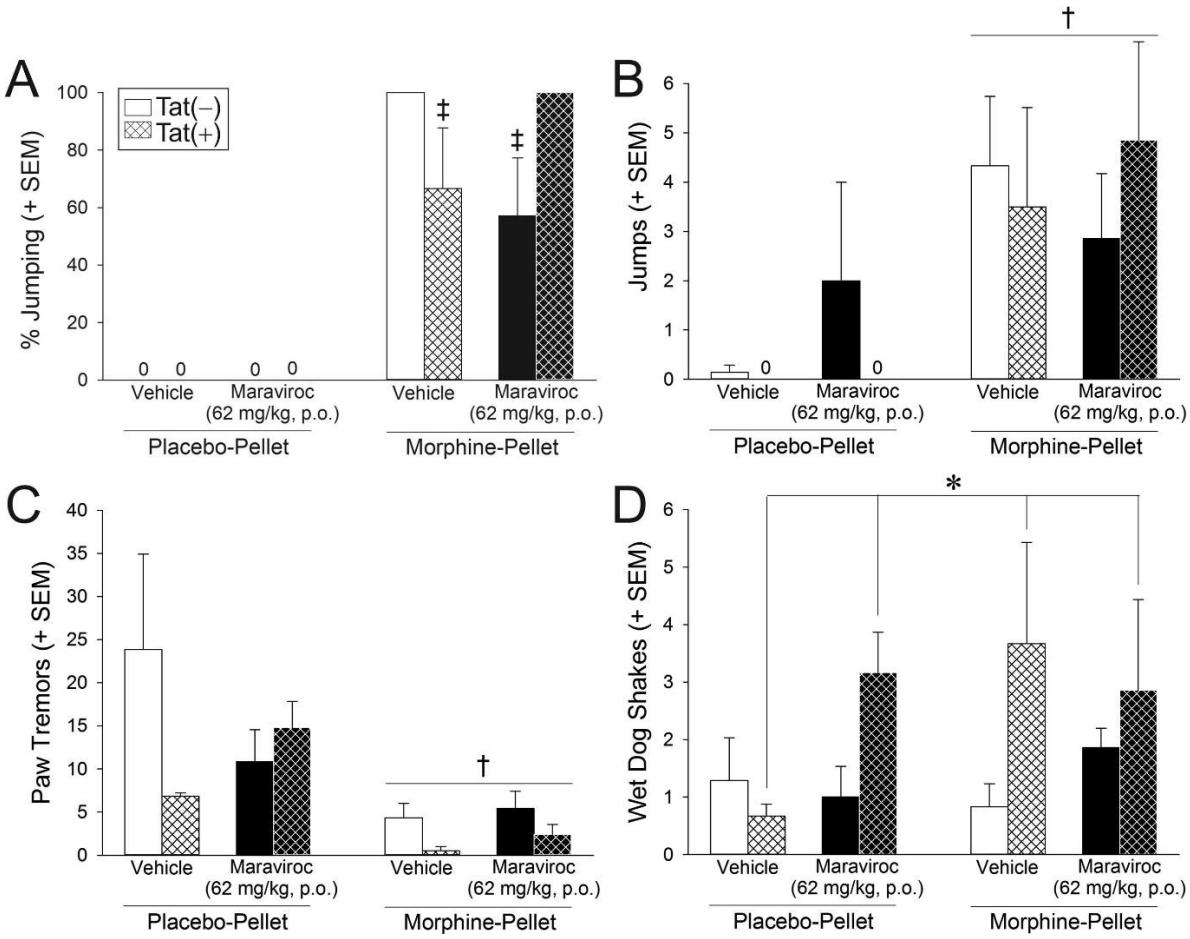


Figure 3: HIV-1 Tat significantly attenuated withdrawal-precipitated jumping and maraviroc pretreatment reversed this effect; Tat and morphine tolerance influenced additional withdrawal symptomology. Measures of naloxone-precipitated withdrawal among Tat(-) or Tat(+) mice [n = 6 - 7 / group; Tat(-) in open bars, Tat(+) in hatched bars] that were morphine-naïve (placebo-pelleted; left bars in each panel) or morphine-tolerant (morphine-pelleted; right bars in each panel), administered daily vehicle (p.o.; white bars) or maraviroc (62 mg/kg, p.o.; black bars), and assessed for warm water tail withdrawal. (A) The proportion of mice jumping from an elevated platform over 10 min, as well as the concomitant frequency of (B) spontaneous jumping, (C) paw tremor, and (D) wet dog shakes over 5 min in an observation box, were recorded. * main effect of Tat-exposure [greater wet-dog shakes among Tat(+) mice vs. Tat(-

) mice]. † main effect of prior morphine tolerance (greater frequency of spontaneous jumps and fewer simultaneous paw tremors among morphine-tolerant vs. non-tolerant mice). ‡ interaction for indicated groups to differ from morphine-tolerant Tat(-) mice or morphine-tolerant, maraviroc treated Tat(+) mice, $p < 0.05$.

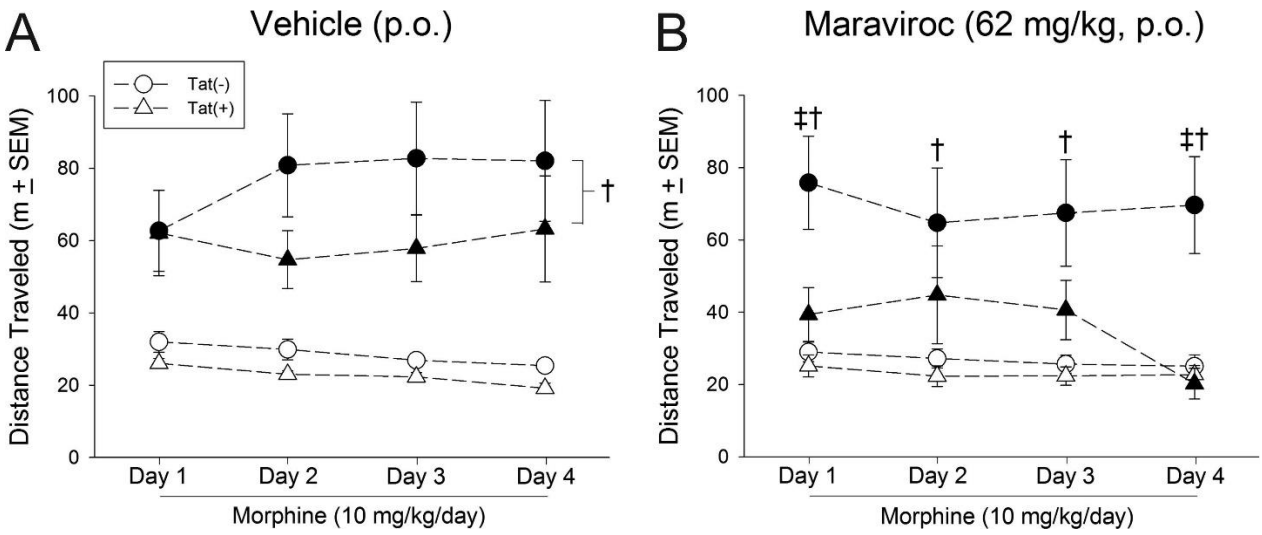


Figure 4: **HIV-1 Tat and maraviroc significantly attenuate morphine-mediated psychomotor behavior.** Locomotor behavior in response to saline (i.p. for 4 days; open circles) morphine (10 mg/kg, i.p. for 4 days; closed circles) was assessed during conditioning cycles among Tat(-) or Tat(+) mice that were pretreated with (A) vehicle (p.o.) or (B) maraviroc (62 mg/kg, p.o.; n = 11 - 12 / group). † main effect of morphine (greater locomotion following morphine vs. saline treatment). ‡ interaction for indicated Tat(-) groups differ from their respective Tat(+) counterparts, $p < 0.05$.

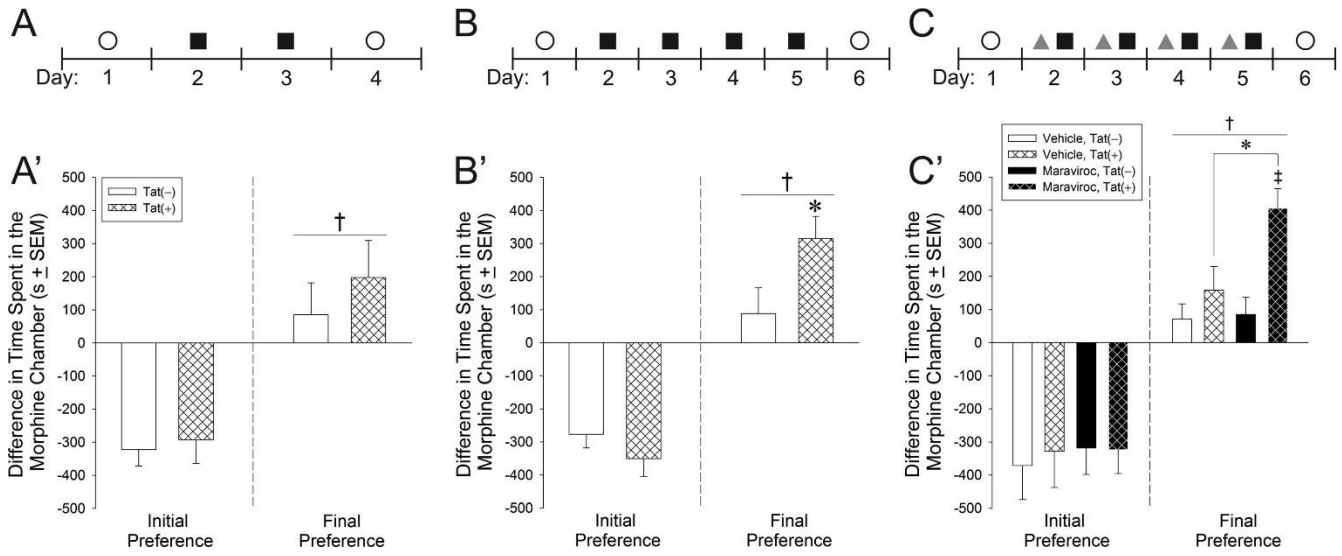


Figure 5: HIV-1 Tat potentiates morphine-conditioned place preference 24 h post morphine and maraviroc pretreatment exacerbates this effect. Tat(-) or Tat(+) mice (n = 11 - 14 / group) were assessed for their chamber preference (open circles) in a biased conditioned place preference (CPP) test consisting of either (A) two cycles of saline (i.p.)/morphine (10 mg/kg, i.p.) conditioning (closed squares), (B) four cycles of saline/morphine conditioning (closed squares), or (C) four cycles of vehicle (p.o.) or maraviroc pretreatment (gray triangles) followed by saline/morphine conditioning (closed squares). Initial preferences for the morphine-paired chamber (left of dashed line) are depicted followed by final CPP (right of dashed line) after (A') two conditioning cycles, (B') four conditioning cycles, or (C') vehicle/maraviroc pretreatment prior to four conditioning cycles. Tat(-) mice are depicted in open bars, Tat(+) mice are depicted in hatched bars, vehicle (p.o.)-pretreated mice are depicted with white bars, and maraviroc (62 mg/kg, p.o.)-pretreated mice are depicted with black bars. † main effect for the final chamber preference to differ from the initial preference. * interaction for Tat(-) mice to differ from Tat(+) mice. ‡ interaction for maraviroc-pretreated Tat(+) mice to differ from all other groups, $p < 0.05$.

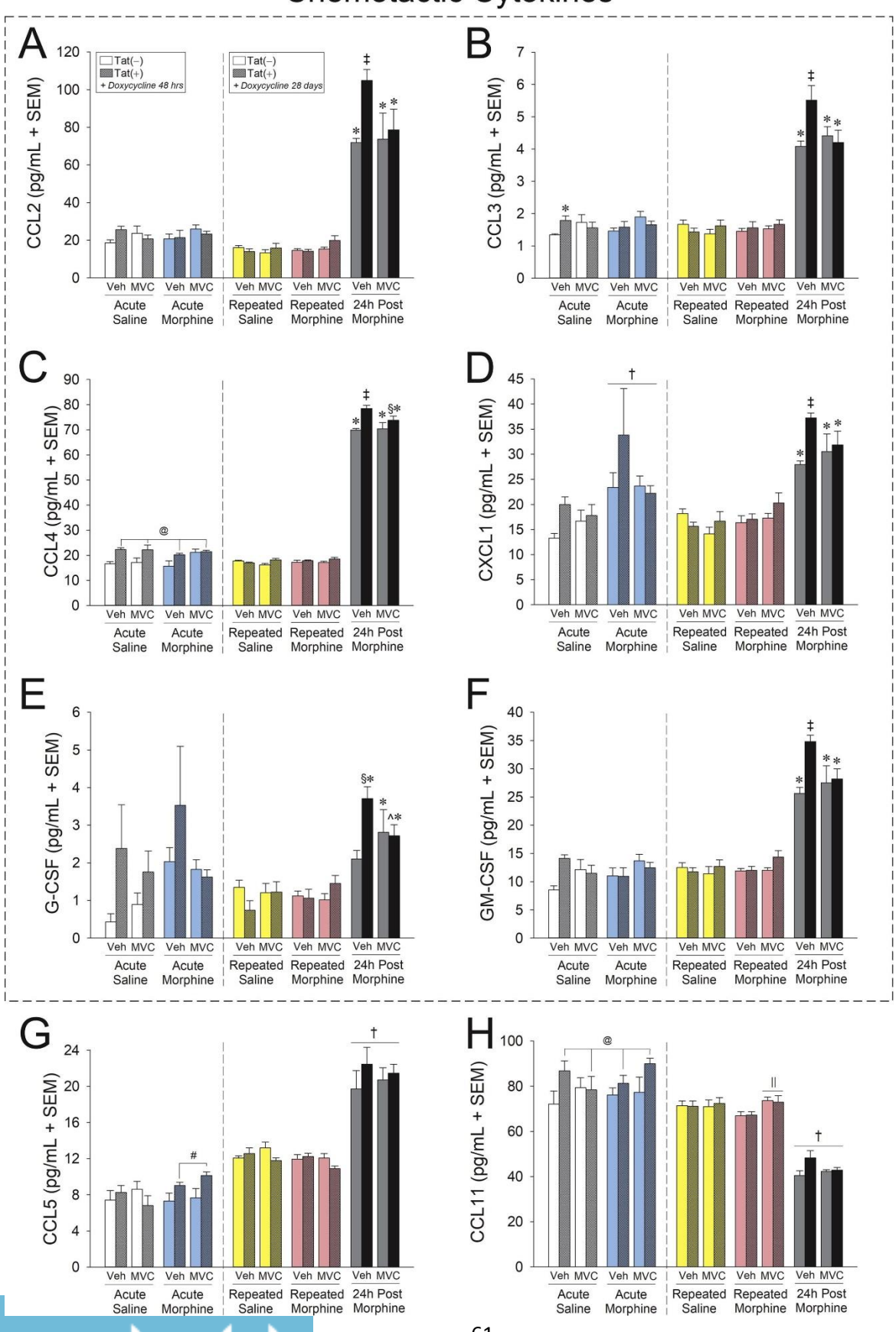


Figure 6: Chemokine expression in caudate/putamen is largely upregulated 24 h following repeated morphine exposure; HIV-1 Tat potentiated this effect on some analytes (outlined) which was ameliorated by maraviroc pretreatment. Chemotactic cytokine protein expression (pg/mL ± SEM) in dorsal striatum (caudate/putamen) of vehicle- (p.o.) or maraviroc- (62 mg/kg, p.o.) exposed mice [n = 4 – 5 / group; Tat(-) in open bars, Tat(+) in stippled bars] that had Tat expressed for 48 h or 28 days (via doxycycline). Mice were administered saline (i.p.; white bars) or morphine (10 mg/kg, i.p., QD) acutely (cyan bars), repeatedly (4 consecutive days; repeated saline in yellow bars and repeated morphine in magenta bars), or were 24 h post repeated morphine treatment (gray bars). Cytokines within the dashed box demonstrated Tat-potentiation that was ameliorated by maraviroc. @ main effect of Tat [Tat(+) mice have greater expression than Tat(-) mice, irrespective of morphine or maraviroc condition]. † main effect of morphine (indicated morphine group has greater expression than the respective saline group, irrespective of Tat or maraviroc condition). || maraviroc-morphine interaction (indicated maraviroc-treated group differs from vehicle-treated control within the same morphine condition). * 3-way interaction for indicated groups to differ from vehicle-treated, saline administered, Tat(-) controls. ‡ 3-way interaction for indicated group to differ from all other groups. § 3-way interaction for indicated groups to differ from respective vehicle treated, Tat(-) control within the 24 h post morphine group. ^ 3-way interaction for indicated maraviroc-treated Tat(+) group be reduced compared to respective vehicle treated Tat(+) group, $p < 0.05$.

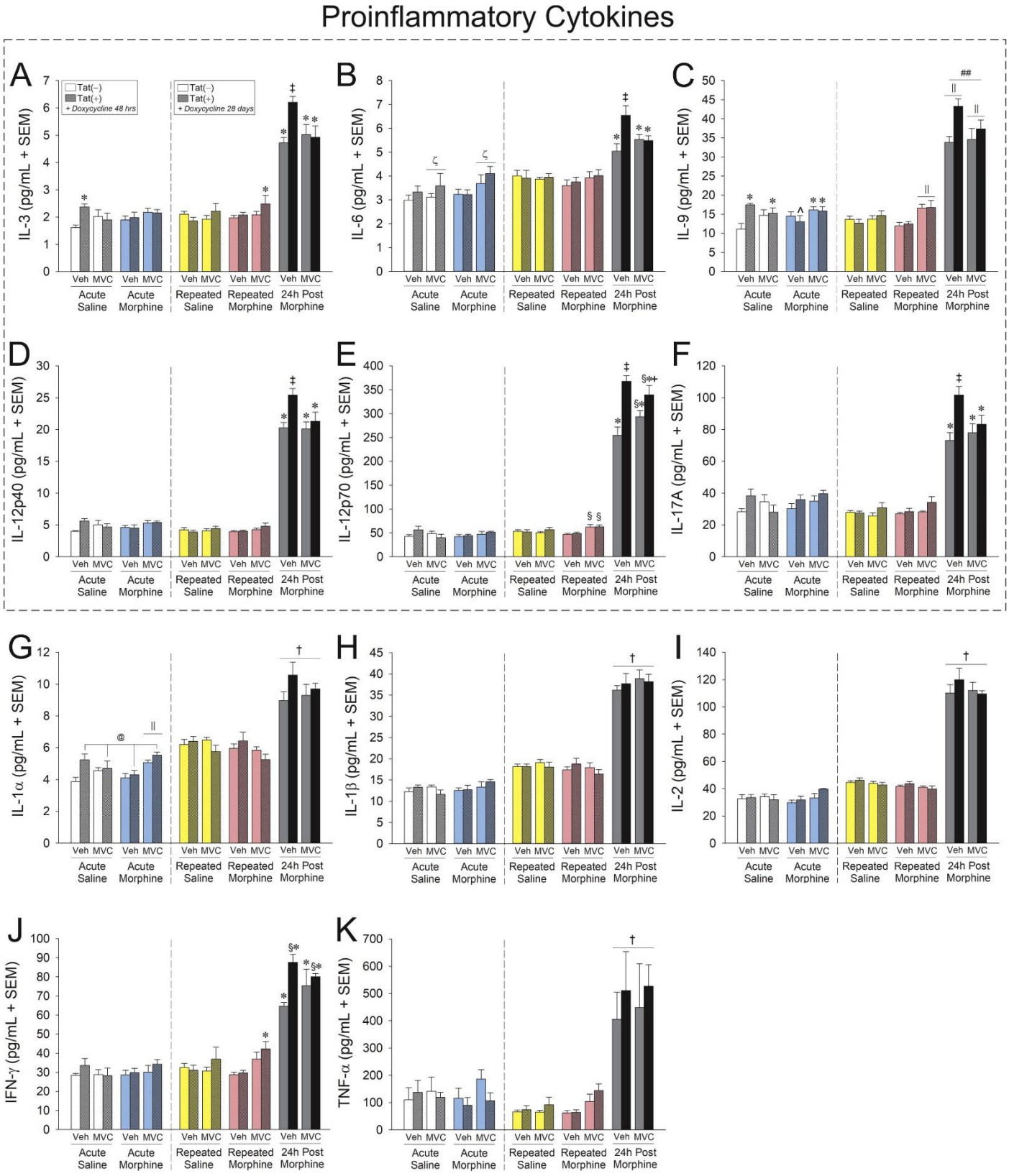


Figure 7: Proinflammatory cytokine expression in caudate/putamen is partly upregulated 24 h following repeated morphine exposure; HIV-1 Tat potentiated this effect on some analytes (outlined) which could be ameliorated by maraviroc pretreatment. Proinflammatory cytokine protein expression (pg/mL ± SEM) in dorsal striatum (caudate/putamen) of vehicle- (p.o.) or maraviroc- (62 mg/kg, p.o.) exposed mice [n = 4 – 5 / group; Tat(-) in open bars, Tat(+) in stippled bars] that had Tat expressed for 48 h or 28 days (via doxycycline). Mice were administered saline (i.p.; white bars) or morphine (10 mg/kg, i.p., QD) acutely (cyan bars), repeatedly (4 consecutive days; repeated saline in yellow bars and repeated morphine in magenta bars), or were 24 h post repeated morphine treatment (gray bars). Cytokines within the dashed box demonstrated Tat-potentiation that was ameliorated by maraviroc. @ main effect of Tat [Tat(+) mice have greater expression than Tat(-) mice, irrespective of morphine or maraviroc condition]. † main effect of morphine (indicated morphine group has greater expression than the respective saline group, irrespective of Tat or maraviroc condition). ζ main effect of maraviroc (maraviroc-treated groups have significantly greater expression that vehicle-treated groups, irrespective of Tat or morphine condition). ## Tat-morphine interaction wherein Tat(-) and Tat(+) mice in the indicated morphine group are greater than those in other morphine groups and differ from each other (irrespective of maraviroc condition). || maraviroc-morphine interaction wherein maraviroc-pretreatment differs from vehicle-pretreatment within the indicated morphine groups (irrespective of Tat condition). * 3-way interaction for indicated groups to differ from respective vehicle-treated, saline administered, Tat(-) controls. ‡ 3-way interaction for indicated group to differ from all other groups. § 3-way interaction for indicated groups to differ from respective vehicle treated, Tat(-) control within the 24 h post morphine group. ^ 3-way interaction for indicated group to differ from respective vehicle-treated, acute-saline-administered, Tat(+) mice. + 3-way interaction for

indicated group to be greater than their maraviroc treated, Tat(-) counterparts within the 24 h post morphine group, $p < 0.05$.

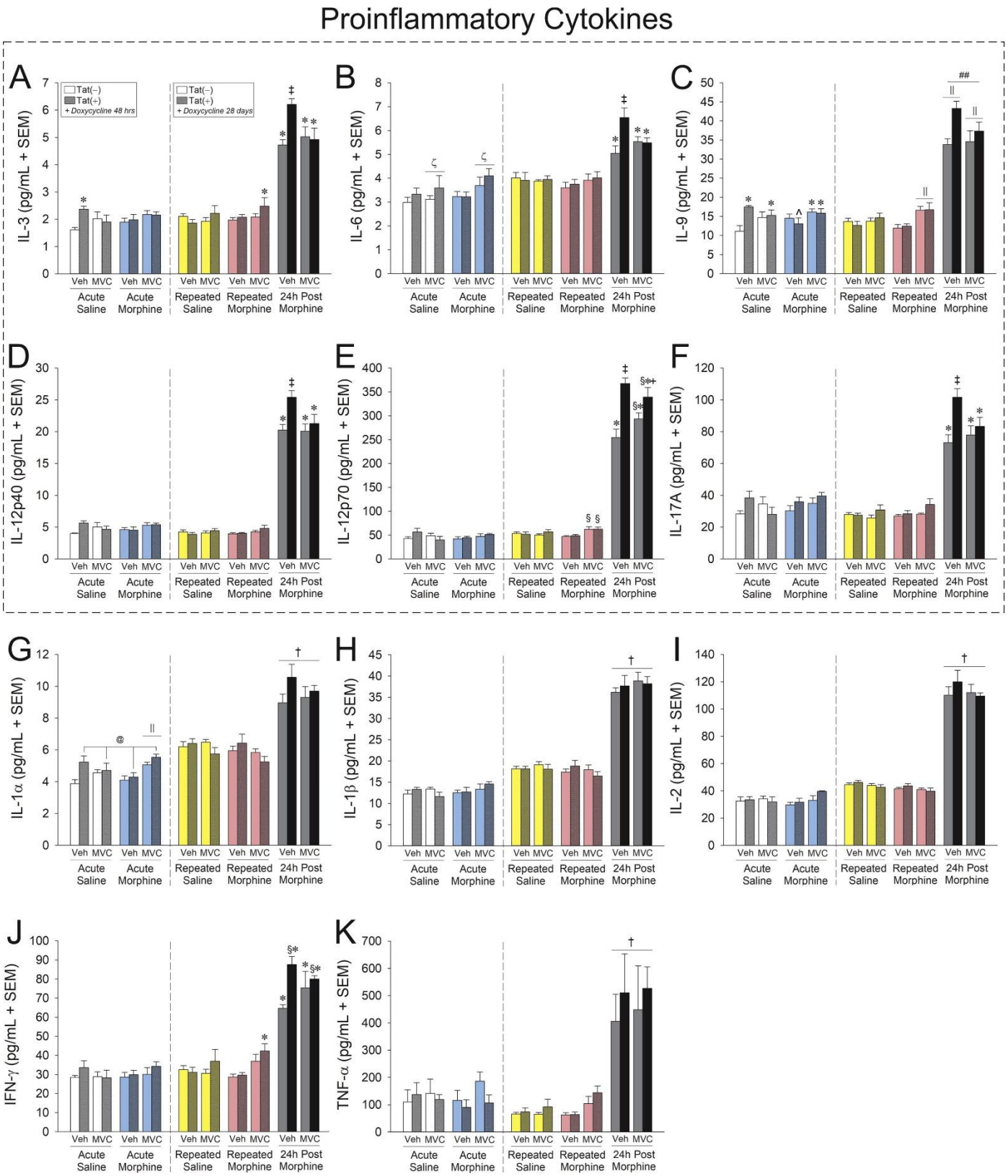


Figure 8: Anti-inflammatory cytokine expression in caudate/putamen is upregulated 24 h following repeated morphine exposure; HIV-1 Tat potentiated this effect on IL-10 and maraviroc pretreatment ameliorated this effect. Anti-inflammatory cytokine protein expression (pg/mL ± SEM) in dorsal striatum (caudate/putamen) of vehicle- (p.o.) or maraviroc- (62 mg/kg, p.o.) exposed mice [n = 4 – 5 / group; Tat(-) in open bars, Tat(+) in stippled bars] that had Tat expressed for 48 h or 28 days (via doxycycline). Mice were administered saline (i.p.; white bars) or morphine (10 mg/kg, i.p., QD) acutely (cyan bars), repeatedly (4 consecutive days; repeated saline in yellow bars and repeated morphine in magenta bars), or were 24 h post repeated morphine treatment (gray bars). The dashed box indicates IL-10 as the only anti-inflammatory cytokine assessed that demonstrated Tat-potentiation with maraviroc amelioration. @ main effect of Tat [Tat(+) mice have greater expression than Tat(-) mice, irrespective of morphine or maraviroc condition]. † main effect of morphine (indicated morphine group has greater expression than the respective saline group, irrespective of Tat or maraviroc condition). * 3-way interaction for indicated groups to differ from vehicle-treated, saline-administered, Tat(-) controls. ‡ 3-way interaction for indicated group to differ from all other groups. § 3- way interaction for indicated groups to differ from respective vehicle-treated, Tat(-) control within the 24 h post morphine group. ^ 3-way interaction for indicated group be reduced compared to respective vehicle-treated Tat(+) group, $p < 0.05$.

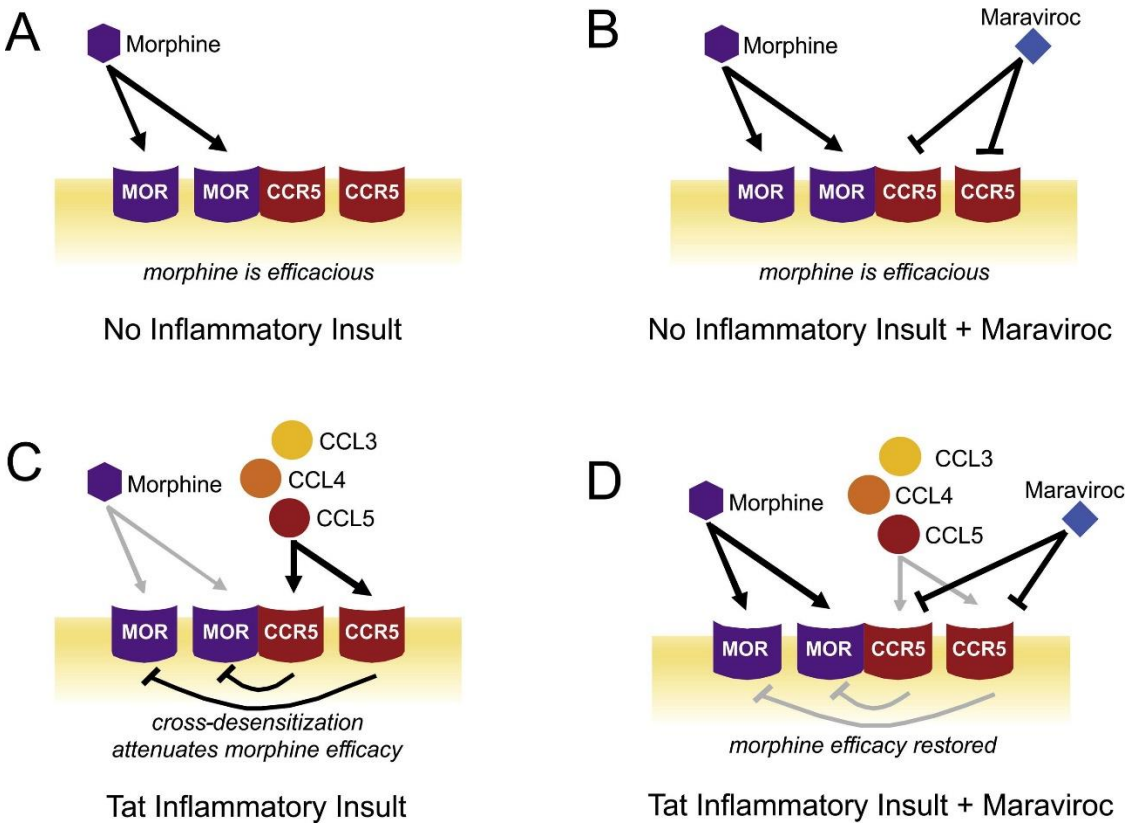


Figure 9: Proposed mechanism(s) of morphine/maraviroc interaction with or without HIV-1 Tat-induced neuroinflammation. (A) In the absence of Tat-mediated neuroinflammation [as modeled using (Tat-), control mice], morphine is behaviorally efficacious, partly via actions at mu opioid receptor (MOR) oligomers or MOR-CCR5 heteromers. MOR signaling may cross-desensitize CCR5 oligomers in this state (not depicted). (B) When no prior neuroinflammatory stimulus is present, the addition of maraviroc exerts little influence on the behavioral efficacy of morphine [as modeled using (Tat-), maraviroc-treated mice]. (C) When Tat-induced neuroinflammation is present [as modeled using Tat(+) mice], there are enhanced levels of β-chemokines that bind CCR5 and may cross-desensitize MORs. When morphine is administered in this state, its behavioral efficacy is attenuated. (D) The addition of maraviroc when neuroinflammation is present may block the ability of CCR5 to cross-desensitize MORs, thereby restoring morphine efficacy [as modeled using maraviroc-treated, Tat(+) mice].

4. Discussion

The overall hypotheses that HIV-1 Tat expression attenuates morphine potency, CCR5 antagonism reverses these effects, and withholding morphine for 24 h exacerbates Tat's effects on cytokines were upheld. The present data revealed even greater nuance in the behavioral response to morphine regimens than was anticipated. Consistent with prior reports (Fitting et al., 2012, 2016), HIV-1 Tat expression significantly attenuated the antinociceptive potency of acute morphine injection (2 – 64 mg/kg, i.p.) and significantly attenuated aspects of withdrawal among mice made tolerant via subcutaneous implant of a morphine pellet (75 mg). The present work further extends these findings to demonstrate that pretreatment with the CCR5 antagonist, maraviroc, blocks the effects of Tat on morphine tolerance and dependence behaviors (reinstating morphine potency in non-tolerant mice and restoring aspects of withdrawal symptomology in morphine tolerant mice). We further assessed the potential interactions of HIV-1 Tat and maraviroc on morphine reward via a CPP assay. Tat reduced the locomotor response associated with repeated, once-daily, morphine injections (10 mg/kg. i.p.) over 4 days of conditioning, but significantly potentiated (~3.5-fold) morphine-CPP after morphine was withheld for 24h. These results are congruent with prior findings of Tat-potentiated psychostimulant and ethanol reward (McLaughlin et al., 2014; Mediouni et al., 2015; Paris et al., 2014a,b) and a slower rate of extinction in HIV-1 transgenic rats contextually conditioned to morphine (Homji et al., 2012). Surprisingly, maraviroc further potentiated Tat's effects on morphine mediated locomotion and CPP (~5.7-fold), but exerted no influence on these behaviors when administered alone. Protein arrays revealed that withholding morphine for 24 h (commensurate with the timeframe of CPP assessment) markedly increased the levels of multiple categories of cytokines (proinflammatory, anti-inflammatory, and regulatory), suggesting a widespread dysregulation of immune function.

Importantly, maraviroc could block or attenuate the immunological consequences of terminating morphine administration across a number of cytokines. Tat-dependent elevations in the endogenous CCR5 ligands (CCL3 and CCL4) and CCL2 (whose activation is proposed to be downstream of CCR5-dependent Tat/opioid interactions; El-Hage et al., 2008), as well as other cytokines increased by Tat, were markedly attenuated by maraviroc.

The marked increase in a large number of cytokines that was observed 24 h after withholding repeated morphine (10 mg/kg/d) was unanticipated and may be contributed to by several sources. Although immune function is generally suppressed by sustained opioid exposure (Bayer et al., 1994; Rahim et al., 2003), it can transiently rebound followed by sustained immunosuppression after opioid withdrawal (Eisenstein et al., 2006; Rahim et al., 2004). Contextual cues alone (such as those used in CPP) alter immune responding once opiate conditioning is established (Hutson et al., 2017; Saurer et al., 2011), effects that involve the ventral striatum (Szczytkowski et al., 2011). Alternatively, chronic doxycycline treatment can produce alterations in the gut microbiome (Angelakis et al., 2014) which may influence immune responding and subsequent behavior. Interestingly, in this study we found that chronic doxycycline treatment upregulated some but not all chemokines and cytokines in dorsal striatum of both Tat(−) and Tat(+) mice (CCL5, IL-1α, IL-1β, IL-2, IL-6, and IL-13 were upregulated in chronic, compared to acute, doxycycline exposure). There is increasing evidence that microbial dysbiosis can influence many centrally mediated effects, including anxiety and mood (Cryan and Dinan, 2012; Foster et al., 2013). We have previously reported that HIV-1 Tat can induce bacterial translocation and enhance pro-inflammatory cytokines via TLR4 activation in the mouse colon (Guedia et al., 2016). Chronic morphine has also been reported to induce microbial dysbiosis and increase pro-inflammatory cytokines within the gut (Kang et al., 2017; Meng et al., 2013). In particular, morphine pellets have

been observed to induce sepsis (Eisenstein et al., 2006; Feng et al., 2005; Hilburger et al., 1997) which may be due, in part, to intestinal stasis. Given that HIV-1 Tat has been shown to increase the permeability of the blood-brain barrier (Leibrand et al., 2017), the presence of Tat may compound the central accumulation of systemic factors including cytokines. The present findings support previous literature demonstrating MOR and CCR5 interactions on HIV-1 Tat-mediated cellular outcomes and begin to reveal the related behavioral sequelae.

The comorbidity between opioid abuse and HIV is well established. The cellular/molecular interactions between opioids and HIV co-receptors are less well understood, but may exert important effects on chemokine function. Opioids modulate CCR5 expression and signaling in vitro. Morphine or methadone upregulates CCR5 mRNA and protein expression in murine BV-2 derived microglia (Bokhari et al., 2009). human astrocytes (Mahajan et al., 2005b), human lymphoid cell lines (Miyagi et al., 2000; Suzuki et al., 2002), or human monocyte-derived macrophages (Guo et al., 2002; Li et al., 2003) or microglia (Guo et al., 2002). These effects are functional and confer susceptibility to simian immunodeficiency virus (SIV; (Miyagi et al., 2000; Suzuki et al., 2002a,b)) or R5-tropic HIV (Guo et al., 2002; Li et al., 2003). Importantly, CCR5 upregulation and viremia has been shown to be attenuated with opioid receptor antagonists, such as naltrexone (Guo et al., 2002), naloxone (Mahajan et al., 2005b), or the quaternary opioid antagonist, methylnaltrexone (Ho et al., 2003). These effects are, at least partly, dependent on actions at MORs given that the highly selective MOR agonist, DAMGO, upregulates CCR5 and enhances R5-HIV viremia in vitro, an effect that is blocked by the MOR-selective antagonist, CTAP (Steele et al., 2003). In addition to altered CCR5 expression, MOR and CCR5 undergo heterologous cross-desensitization (Szabo et al., 2003, 2002; Zhang et al., 2004), which may occur through convergent signaling or via direct molecular interactions involving the formation of MOR-

CCR5 heteromers. Evidence for direct MOR-CCR5 dimeric/oligomeric interactions are partly based on findings that a bivalent ligand comprised naltrexone and maraviroc can reduce the infectivity of cultured human astrocytes (El-Hage et al., 2013; Yuan et al., 2013) and attenuate R5 HIV-mediated increases in CCL5, TNF-α, and IL-6 in human astrocytes (El-Hage et al., 2013). Divergent effects of the bivalent ligand were observed in cultured human microglia, which were much more dynamic in their response to morphine. Unlike astrocytes, microglia demonstrated a strong upregulation of CCR5 in response to the bivalent ligand in culture that may have accounted for the differential response between cell types (El-Hage et al., 2013). In other studies, treating a human lymphoid cell line with morphine and CCL4 (respective agonists for MOR and CCR5) increased morphine mediated upregulation of CCR5 protein expression (Suzuki et al., 2002c). Together, our data agree with prior findings (Szabo et al., 2003, 2002; Zhang et al., 2004) supporting the notion that opioids interact with CCR5 at multiple levels, from the regulation of CCR5 expression and signaling to potential actions at MOR-CCR5 heteromers and functional effects on viremia and neuroinflammation. These present findings begin to extend these data to behavioral dysfunction in a whole-animal model.

Actions at CCR5 may represent a critical point of convergence between the viral protein, Tat, and MORs. Apart from its role in HIV entry, CCR5 signaling is essential in macrophage and microglial chemoattraction, the homing of CD8+ T cells, and the pathogenesis of CNS inflammatory diseases (Martin-Blondel et al., 2016). Foremost, Tat/opioid interactions for neurotoxicity appear to be dependent on glial MORs. While Tat demonstrates neurotoxicity in co-cultured neurons from either wildtype mice or MOR knockout mice, these effects are potentiated by pretreatment with morphine only when neurons are co-cultured with wildtype glia (MOR-knockout glia do not potentiate toxicity; Zou et al., 2011). Second, Tat/opioid toxicity largely

involves downstream neuroinflammation. In murine progenitors, Tat and opioids have interactive effects on astroglial chemokine secretion (Hahn et al., 2010), CCL5 is often the predominant central chemokine upregulated in response to Tat, an effect that can be potentiated by chronic morphine (Dutta and Roy, 2015) and inactivating CCR5 with an antibody blocks the chemoattractive effects promoted by Tat (Hahn et al., 2010). Third, CCL5 activation appears to work in concert with downstream chemokine activation, particularly CCL2. In support, morphine stimulates production of CCL5 and CCL2 in cultured peripheral blood mononuclear cells (Wetzel et al., 2000) and Tat increases CCL2 and CCL5 production in murine primary astrocyte cultures; effects that are potentiated by co-application of morphine and reversed by MOR antagonists (such as β-FNA; El-Hage et al., 2005). Striatal infusion of Tat1-86 to mice significantly increases the proportion of CCL5 immunoreactive astroglia and co-administration of morphine and Tat significantly increases astrogliosis, microgliosis, the proportion of CCL2 immunofluorescent astrocytes/macrophages/microglia, and reactive nitrosative species co-localized to these cells (El-Hage et al., 2008a). These effects on murine striatum are obviated by CCL5 knockout (El-Hage et al., 2008a) or knockout of the CCL2 receptor (El-Hage et al., 2006). Findings in primary human astrocytes are supportive with morphine downregulating CCL2 and CCL4 mRNA and upregulating their receptors in a naloxone-dependent manner (Mahajan et al., 2005b). Thus, Tat and opioids acting at MORs appear to activate CCR5 and CCR2 signaling, increasing neuroinflammation and striatal neurotoxicity. These effects may contribute to the attenuated therapeutic efficacy and increased rewarding properties of morphine that we have observed herein.

We propose that MOR-CCR5 cross-desensitization may contribute to the behavioral pathology observed (Fig. 9). When HIV-1 Tat-mediated neuroinflammation was not present [as modeled using Tat(-) control mice], cytokine concentrations were at basal levels and morphine

exerted efficacious effects for tolerance, dependence, psychostimulation, and reward, presumably via actions partly mediated by MORs (Fig. 9A). The addition of maraviroc to Tat(-) mice did not influence morphine efficacy on these behavioral measures (Fig. 9B) consistent with in vitro reports wherein MOR-CCR5 bivalent ligands exert little influence when neuroinflammation is not present (Akgün et al., 2015; Portoghese et al., 2017). However, when a neuroinflammatory insult was first present [as modeled using Tat(+) mice], morphine efficacy was diminished on most measures; perhaps, due to endogenous MORs having already been cross-desensitized by activated CCR5 receptors (Fig. 9C). When maraviroc was introduced in the Tat inflamed state, morphine efficacy was restored on most measures and even potentiated on the measure of reward. We speculate that the addition of maraviroc in this state may relieve cross-desensitization, restoring morphine signaling through its endogenous receptors (Fig. 9D). In support, a bivalent ligand (MOR agonist/CCR5 antagonist) exerted a 3100-fold increase in potency when administered to LPS-inflamed mice, relative to noninflamed controls (Akgün et al., 2015; Portoghese et al., 2017). There was one behavioral exception to Tat's capacity to attenuate morphine efficacy and that was on the measure of reward prior to maraviroc. These divergent findings may involve the role of additional brain regions and opioid signaling beyond that involving CCR5 heterodimers (Shippenberg et al., 2009).

It must be noted that the timing of morphine and Tat-dependent interactions at CCR5 is likely to be critical in determining the nature of the outcome (see Fig. 9 in (Berman et al., 2006; Song et al., 2011)). The relative increases in cytokines seen with acute morphine and/or Tat exposure (for example, as in CCL3, CCL4, CCL5, CXCL1, IL-1α, IL- 9, and IL-10) were often smaller effects that may either be less biologically significant or may display peak activity earlier than the time examined. These effects were largely absent after sustained exposure suggesting that

the CNS adapts to sustained opiate or HIV-1 Tat insults via mechanisms involving drug tolerance (Eisenstein et al., 2006) or innate immune tolerance (Biswas SK, 2009; Cavaillon and Adib-Conquy, 2006), respectively. Future investigations should additionally assess the presence of endotoxemia which may contribute to these effects (Eisenstein et al., 2006), but may be masked by downregulation of the innate response.

The present work raises several clinical considerations. Seropositive patients within the first 100 d of HIV infection demonstrate positive correlations between circulating CCL2, CCL11, GM-CSF, IL-1α, IL-6, and lateral ventricular volume as well as IL-5, IL-10, and mean diffusivity within the caudate (Ragin et al., 2015). Herein, maraviroc exerted little influence over behavioral measures on its own, but attenuated the capacity for HIV-1 Tat to dampen morphine potency. Given that up to 40% of HIV-afflicted individuals experience distal polyneuropathies, headache, and additional chronic pain states (Keswani et al., 2002; Mirsattari et al., 1999) necessitating opioid medications for relief among a sizeable percentage (~23% of a 1400+ patient sample infected with HIV; Merlin et al., 2016), maraviroc may have potential benefits to improve opioid-based therapies. However, with chronic opioid use, the beneficial effects of maraviroc are less clear given that maraviroc was also associated with an increase in withdrawal symptomology and increased morphine reward. In addition, the neurotoxic actions of additional HIV proteins in the patient population, such as the HIV coat protein (gp120), may exert a divergent profile compared to that observed when HIV-1 Tat is expressed alone (Campbell et al., 2015; Maung et al., 2012; Mocchetti et al., 2013). The toxic effects of R5-tropic gp120 can be attenuated by morphine-mediated CCL5 upregulation, perhaps influenced by out-competition for CCR5 (Avdoshina et al., 2010). As such, future investigations may wish to target MOR-CCR5 bivalent strategies within the context of opioid dependency across a range of neuroAIDS models.

5. Conclusions

Several investigations using murine and human cell cultures have demonstrated neurotoxic interactions between the HIV-1 Tat, MORs, and CCR5. The present findings extend these data by investigating functional consequences of such interactions on morphine-mediated antinociception, tolerance, and reward in a murine model. Maraviroc blocked Tat's actions to attenuate the antinociceptive potency of acute morphine in nontolerant mice. Intriguingly, maraviroc also potentiated the Tat-induced increase of morphine-CPP, even while it reduced the levels of many inflammatory chemokines and cytokines in the striatum including β-chemokines. Thus, while maraviroc is widely appreciated for its role in blocking HIV entry, it may be considered for additional therapeutic roles related to pain control in HIV-infected patients; albeit, caution may be warranted for individuals that are opioid-dependent or at risk for such abuse.

CHAPTER 4

GAP JUNCTION INHIBITOR CARBENOXOLONE ATTENUATES THE DEVELOPMENT OF OPIOID ANTINOCICEPTIVE TOLERANCE

1. Introduction

In the clinic, the use of opioids is common for the management of acute and chronic pain. However, the development of tolerance to the analgesic effects of opioids, such as morphine, leads to the increase of drug needed to maintain analgesia. Increasing the dose of drug can lead to a greater chance of unwanted side effects. Tolerance to different effects of opioids occur at different rates, leading to increased risk of side effects and overdose (Hill et al., 2016; White and Irvine, 1999). Understanding the molecular and cellular mechanisms responsible for this phenomenon will lead to fewer deaths and potential new analgesics that do not lead to tolerance. A recent target of interest in understanding the development of tolerance is the gap junction.

Gap junctions are the channel-forming structures between the membranes of two adjoining cells. Gap junctions allow direct electrical communication between cells, hence they are also referred to as electrical synapses. Gap junction channels are formed by two hemichannels or connexons, one contributed by each cell at the synapse (Mccracken and Roberts, 2006; Söhl et al., 2005). Single hemichannels can exist on the cell membrane providing a pathway for exchange of intracellular components (including ions and ATP) with the extracellular solution. Such a role has been proposed for a specific type of gap junction called connexin 43 (Cx43). Its opening results in the release of glutamate and ATP from astrocytes, which has a major impact on glial-glial and glial-neuronal interactions. For example, gap junctions found only on neurons as well as only on glial cells have been found to affect the firing rate of neurons (Blenkinsop and Lang, 2006; Pannasch et al., 2011).

It has been suggested that gap junctions participate in particular mechanisms of the pain pathway. Researchers found that local administration of gap junction inhibitors into the dorsal horn blocked the inflammation-induced central pain sensitization without changing the baseline pain responses (Chiang et al., 2010). Recently, researchers have shown that blocking gap junctions intrathecally attenuated the development of morphine antinociceptive tolerance (Shen et al., 2014). Gap junctions may play a role in other chronic morphine related effects as well. It has been observed that the intracerebroventricular administration of the gap junction blocker, carbenoxolone significantly decreased naloxone-precipitated morphine withdrawal signs (Moradi et al., 2013). Systemic carbenoxolone blocked morphine-related colonic inflammation and constipation when administered intraperitoneally (Bhave et al., 2017). Carbenoxolone is used as a medication for the treatment of peptic ulcers and inflammation. Interestingly, a study concluded that negligible amounts of carbenoxolone cross the blood brain barrier; however, the researchers only examined the cerebrospinal fluid and such data may not be representative for the actual brain concentration (Leshchenko et al., 2006). Since carbenoxolone does not readily crossing the blood brain barrier, this suggest a peripheral site of action.

The objective of this study, therefore, was to test the hypothesis that carbenoxolone administered systemically would attenuate the development of morphine antinociceptive tolerance. We report here that the systemic administration of carbenoxolone during the exposure to morphine attenuates the development of antinociceptive tolerance to morphine. Further investigation of the pharmacokinetics reveal that less than 3% of carbenoxolone found in the blood was found in whole brain homogenates at several time points. Lastly, we observed whether in-vivo exposure of carbenoxolone would attenuate tolerance at the neuronal level in a cellular model

of isolated dorsal root ganglia neurons from adult mice and whole cell patch clamp electrophysiology

2. Materials and Methods

2.1. Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g were housed five to a cage in animal care quarters and maintained at 22 +/- 2°C on a 12-hour light-dark cycle. Food and water were available ad libitum. The mice were brought to the test room (22 +/- 2°C, 12-hour light-dark cycle), marked for identification, and allowed 18 hours to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the International Association for the Study of Pain (IASP).

5.2. Drugs and Chemicals

Morphine sulfate, 75-mg morphine pellets, and placebo pellets were obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD). Morphine sulfate was dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL). Carbenoxolone was obtained from Sigma-Aldrich Corporation (St Louis, MO, USA) and was dissolved in distilled water.

5.3. Antinociceptive Testing

Antinociception was assessed using the 52°C warm water tail withdrawal test performed according to Coderre and Rollman (1983). Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 seconds were used. Mice were gently wrapped in a cloth and the distal one-third of the tail was immersed in a water bath. The mice rapidly removed their tail from the bath at the first sign of discomfort and the tail-withdrawal latency was recorded. Tail-withdrawal latency was then assessed using a cumulative dosing procedure. Mice were injected with a starting dose of morphine and were tested for antinociception 20 min later. Mice that did not reach a 10 sec cut-off threshold received an additional cumulative dose of morphine and were retested. This process was repeated until the animals reached the cut-off value of the 10-second maximum cut-off time imposed to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (%MPE), which was calculated as: %MPE = [(test latency – control latency) / (10 – control latency)]* 100. Percent MPE was calculated for each mouse.

Antinociception was also assessed using the 56°C hot plate assay. The hot plate test was performed as described by Smith et. al. (2006). The mice were first placed on a Syscom Model 35D hot plate set at 56 °C to obtain baseline latencies before drug administration. The mice were observed for licking their hind-limb or jumping in response to the heat. The mice were tested again at the appropriate time after being administered test drugs. A 30 second cut-off was employed in order to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964).

5.4. Tolerance Studies

Prior to all behavioral testing, mice were acclimated to the testing room for 24 h. For assessments of morphine tolerance, mice received subcutaneous implants of placebo or morphine pellets. Isoflurane (2.5%) was used to anesthetize the mice after which the hair on the back of the neck was shaved. Povidone iodine (10%) and ethanol (70%) was used to clean the bare skin. A 1-cm transverse incision was made into the subcutaneous space toward the dorsal flan with sterile surgical instruments, and a placebo/ morphine pellet was placed distal to the incision. The incision was closed with sterile autoclips. Mice were allowed to recover in their home cages after the surgery and assessed every 48 h for signs of infection and weight loss. Five days later, mice were assessed for morphine tolerance in a warm-water tail-withdrawal assay. To test carbenoxolone's ability to attenuate opioid tolerance, carbenoxolone was administrated intraperitoneally once per day on days 3 & 4. This injection procedure was based off of a previous publication where prolonged exposure to morphine would induce inflammation starting at days 3 and 4 of morphine pellet implantation. Carbenoxolone administration on these days would prevent morphine induced inflammation (Bhave et al., 2017b).

5.5. Analysis of Carbenoxolone levels in Brain and Blood

Seven-point calibration curves of Carbenoxolone at concentrations of 10 -1000 ng/mL for blood and 10 – 1000 ng/kg for brain tissue homogenate and negative controls with or without internal standard (ISTD) were prepared in drug-free mouse blood and brain tissue with each analytical run. Carbenoxolone was extracted from blood and brain tissue homogenate using a modified Poklis et al method (Poklis et al., 2011). In brief, the ISTD, 100ng of 11-Nor-9-carboxy-Δ9-

tetrahydrocannabinol-d3 (THC-COOH-d3), was added to aliquots of 100 µL of blood or 400 µL of homogenized brain tissue calibrators, controls and samples. These samples were mixed and allowed to equilibrate. 1 mL of ice-cold acetonitrile was added drop by drop to each sample while vortex mixing. The samples were then centrifuged at 3500 rpm for 10 min. After centrifuging the samples were placed in –40°C freezer for at least 2 h. The top layer containing the acetonitrile was removed via a disposable glass pipette and placed in a clean test tube. Samples were dried using a Savant AES1000. The samples were reconstituted with 100 µL of acetonitrile and placed in auto sampler vials for analysis.

The Ultra performance liquid chromatography tandem mass spectrometer (UPLC-MS/MS) analysis of AEA, 2-AG, PEA OEA and arachidonic acid was performed on a Sciex 6500 QTRAP system with an IonDrive Turbo V source for TurbolonSpray® (Sciex, Ontario, Canada) attached to a Shimadzu UPLC system (Kyoto, Japan) controlled by Analyst software (Sciex, Ontario, Canada). Chromatographic separation of Carbenoxolone and the ISTD, THC-COOH-d3, was performed using a Zorbaz eclipse XDB-C18 column, 4.6 x 75 mm, 3.5 micron (Agilent Technologies, USA). The mobile phase contained water/methanol (10:90, v/v) with 0.1 mM ammonium formate and was delivered at a flow rate of 1 mL/min. The source temperature was set at 650°C, and curtain gas had a flow rate of 30 mL/min. The ionspray voltage was 5000 V, with the ion source gases 1 and 2 having flow rates of 60 mL/min. The acquisition mode used was multiple reaction monitoring (MRM). The following transition ions were monitored in negative mode: 569> 469 & 569> 425 for Carbenoxolone and 346> 302 & 346> 289 for THC-COOH-d3. The total run time for the analytical method was 3.5 minutes.

5.6. Isolation and Culture of Neurons from Adult Mouse Dorsal Root Ganglia

Mice were sacrificed by cervical dislocation. L5-S1 DRGs were immediately harvested under a dissecting microscope and placed in a dish containing cold (4 °C) Hanks' Balanced Salt Solution (HBSS, ThermoFisher Scientific, Waltham, MA). Ganglia were incubated (37 °C) for 18 min in HBSS with 15 U/ mL papain, washed, and incubated for 60 minutes in HBSS with 1.5 mg/mL *Clostridium histolyticum* collagenase. After incubation, ganglia were transferred to DMEM in a sterile 15mL conical, dissociated by triturating and centrifuged for 5 min at 1000 rpm. The supernatant was discarded and the pellet was re-suspended in neurobasal A media containing 1% fetal bovine serum, 1x B-27 supplement, 10 ng/mL GDNF, 2mM L-glutamine and 100 U/ml penicillin/streptomycin/amphotericin B (complete neuron media). Isolated cells were plated on laminin and poly-D-lysine-coated glass cover slips and maintained at 37°C in a humidified 5% CO2/air incubator.

5.7. Electrophysiology

Coverslips were transported to a microscope stage plate and continuously superfused with external physiologic saline solution (PSS) containing (in mM) 135 NaCl, 5.4 KCl, 0.33 NaH2PO4, 5 HEPES, 1 MgCl2, 2 CaCl2, and 5 glucose. A GΩ seal was achieved via pulled (Model P-97 Flaming/Brown Micropipette Puller, Sutter Instruments, CA) and fire-polished (2–4 MΩ) borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) filled with internal PSS containing (in mM) 100 L-aspartic acid (K salt), 30 KCl, 4.5 Na2ATP, 1 MgCl2, 10 HEPES, and 0.1 EGTA. DRG nociceptors being of the small, C and Aδ fiber types (Jin et al., 2013), only low capacitance (< 30 pF) neurons were selected for analysis. Standard patch clamp techniques were

performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and associated Clampex and Clampfit 10.2 software. Current clamp step protocols consisting of a 0 nA resting current and 0.01 nA steps from − 0.03 nA were employed to assess passive and active cell properties. Taking the derivative of the voltage with respect to time (dV/dt), threshold potentials were defined as the voltage at which dV/dt significantly deviated from zero in the course of an action potential uprise. For morphine tolerance studies, external PSS was supplement with 3 μ M morphine and threshold potentials recorded at 1 min intervals for 10 min. The maximal effect of morphine was recorded for each cell.

5.8. Statistical Analysis

Morphine dose-response curves were generated for calculation of ED_{50} values using least-squares linear regression analysis followed by calculation of 95% confidence limits (95% CL) by the method of Bliss (Bliss, 1967). All data are represented as mean ± standard error of the mean. Analyses were considered significant when $p < 0.05$. The V_t results were calculated by Student's paired t-test using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). For group comparisons, results were calculated by two-way ANOVA with Bonferroni post-hoc analyses and an alpha level set to 0.05. The results are expressed as mean value ± SEM.

3. Results

3.1. Attenuation of Antinociceptive Tolerance Development to Morphine by Carbenoxolone in Warm-Water Tail Withdrawal Assay

On the fifth day after either placebo or morphine pellet implantation, baseline latencies were taken prior to cumulative dosing regimen with morphine. In a 52°C water bath, cumulative morphine-dosing produced antinociception in the placebo pelleted mice commensurate with what

we have previously observed (Hull et al., 2013; Fig.1A). Using the 5-day tolerance model, morphine pellet implantation produced a significant rightward shift [morphine pellet/vehicle ED_{50} = 16.2 (95% CI: 13.3 –19.8) (Fig.1A). 12.5 mg/kg carbenoxolone did not significantly shift the morphine dose response curve following morphine pellet implantation [morphine pellet/12.5 mg/kg CBX ED_{50} = 12.9 (95% CI: 9.3 –17.9) (Fig.1B). The administration of a higher dose of carbenoxolone (25 mg/kg) significantly shifted the morphine dose response curve leftward, though still significantly different from acute morphine [morphine pellet/25 mg/kg CBX ED_{50} = 7.5 (95% CI: 5.8 – 9.6) (Fig.1C). 25 mg/kg carbenoxolone administered on days 3 and 4 of placebo pelleted mice did not significantly influence the acute morphine antinociception [placebo pellet/vehicle ED_{50} = 3.5 (95% CI: 3.0 – 4.2), placebo pellet/25 mg/kg CBX ED_{50} = 4.6 (95% CI: 4.1 – 5.2)] (Fig.1C). At 37.5 mg/kg carbenoxolone, the morphine dose response curve was not significantly different from the placebo pelleted + carbenoxolone treatment group while significantly different from the morphine pellet/vehicle group [morphine pellet/37.5 mg/kg CBX ED_{50} = 5.91 (95% CI: 4.8 – 7.3) (Fig.1D). Carbenoxolone administered on days 3 and 4 of placebo pelleted mice did not significantly influence the acute morphine antinociception [placebo pellet/vehicle ED_{50} = 3.5 (95% CI: 3.0 – 4.2), placebo pellet/37.5 mg/kg CBX ED_{50} = 4.8 (95% CI: 3.7 – 6.0)] (Fig.1D).

Carbenoxolone administered 30 minutes before the acute dosing regimen of morphine did not acutely reverse chronic morphine nor did it affect acute morphine antinociception [placebo pellet/vehicle ED_{50} = 3.2 (95% CI: 2.2 – 4.7), placebo pellet/25 mg/kg CBX ED_{50} = 5.0 (95% CI: 4.0 – 6.5), [morphine pellet/vehicle ED_{50} = 10.3 (95% CI: 7.8 –13.6), morphine pellet/25 mg/kg CBX ED_{50} =8.3 (95% CI: 6.2 – 11.3)] (Fig. 2.).

3.2. Attenuation of Antinociceptive Tolerance Development to Morphine by Carbenoxolone in Hot Plate Assay

To investigate the effect of carbenoxolone on supraspinal-mediated antinociception, we performed the hot plate rear hind paw lick assay. On the fifth day after either placebo or morphine pellet implantation, baseline latencies were taken prior cumulative dosing regimen of morphine. On a 56°C hot plate, cumulative morphine-dosing produced antinociception in the placebo pelleted mice [Placebo pellet/vehicle ED_{50} = 7.3 (95% CI: 5.8 – 9.2)]. Carbenoxolone administration on days 3 and 4 of placebo pellet implantation did not significantly shift the antinociceptive properties of morphine [Placebo pellet/25 mg/kg CBX ED_{50} = 7.5 (95% CI: 6.4 – 9.0)]. Exposure to chronic morphine through pellet implantation produced significant tolerance as indicated by a significant rightward shift [Morphine pellet/Vehicle ED_{50} =85.4 (95% CI 54.6 – 133.3)]. Carbenoxolone administration attenuated morphine antinociceptive tolerance in the hot-plate assay [Placebo pellet/25 mg/kg CBX ED_{50} = 35.1 95% CI: 25.8 – 47.6)] (Fig.3).

3.3. Carbenoxolone Does Not Readily Cross the Blood Brain Barrier

Although carbenoxolone was not observed in the cerebrospinal fluid in a previously published study, the possibility remained that carbenoxolone sequesters within brain tissue. Therefore**,** following an intraperitoneal injection of 25 mg/kg carbenoxolone, blood samples and whole brains were taken from mice. Figure 4 shows that the UPLC-MS/MS-determined carbenoxolone concentration in the blood samples at several time points. At the same time points, less than 3% of carbenoxolone levels found in the blood were observed in the brain at all time points tested (Fig. 4).

3.4. Morphine Tolerance Attenuated by In Vivo Carbenoloxone Treatment in Sensory Neurons from Dosrsal Root Ganglia.

To test whether the effects of carbenoxolone administration on morphine tolerance could be recapitulated in single cells, dorsal root ganglion (DRG) nociceptors were isolated from spinal levels supplying the lower alimentary canal (L5-S1). Candidate neurons were selected for their round spherical shape and small size. Active and passive properties of low capacitance (<30 pF) cells were measured from the DRG neurons of each group by whole-cell patch clamp techniques. Neurons from placebo pelleted mice and neurons from morphine pelleted mice treated with carbenoxolone responded to morphine challenge (3 μM) by a positive shift in the threshold potential indicating morphine-induced reduction in excitability. The threshold potential was determined from the derivative of the action potential (dV/dt) in the absence and presence of acute morphine challenge. The thresholds of neurons from morphine pelleted mice treated with vehicle did not respond to the morphine challenge, indicating the development of tolerance. (Fig. 5.). A number of cell properties did not significantly vary between treatment groups or between baseline and morphine exposure, including membrane capacitance, series resistance, input resistance, rheobases, and resting membrane potential (Table 3; Appendix). PP + SAL and MP + SAL groups have been previously published by our laboratory (Kang & Mischel et al., 2017).

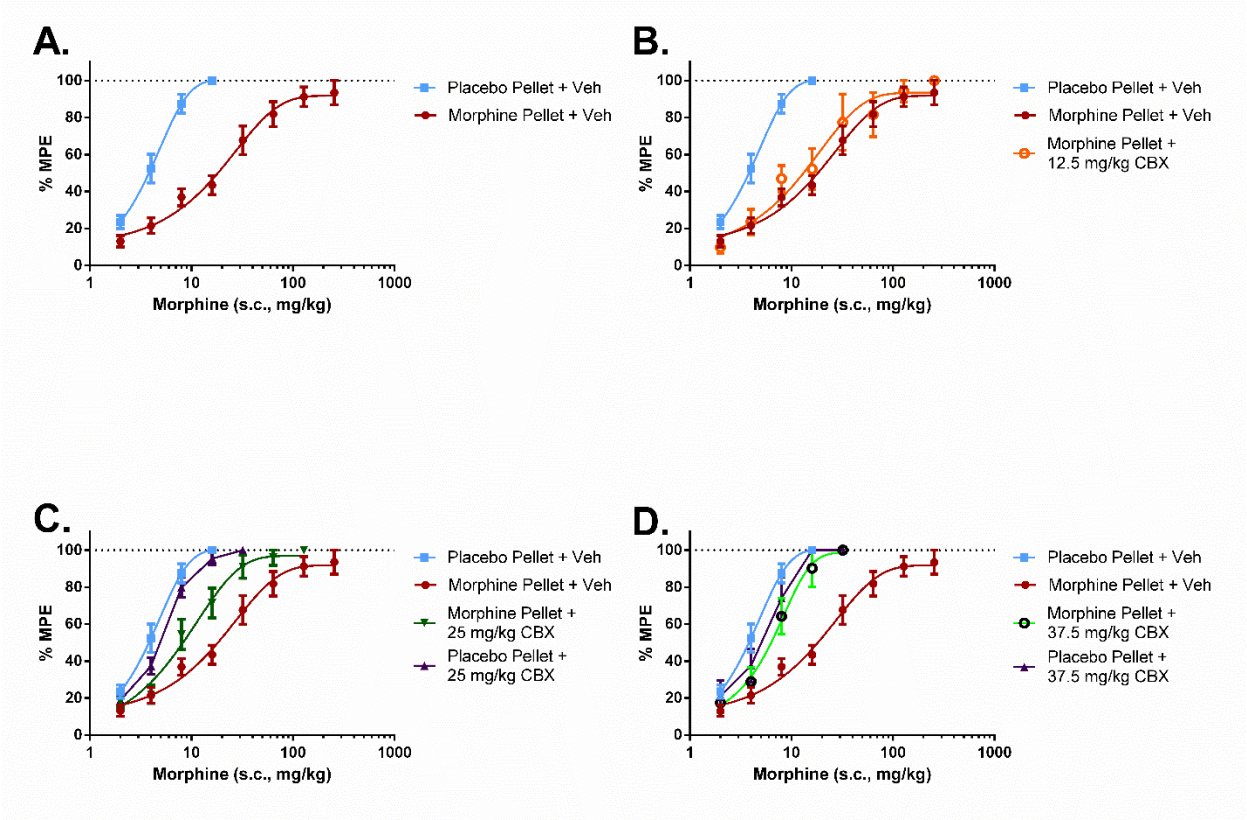


Fig. 1. Carbenoxolone attenuates development of morphine tolerance as measured in warm water tail withdrawal assay (A) 5 day morphine pellet implantation significantly shifted the ED_{50} for morphine to the right indicating tolerance (n = 15 / group). (B) 12.5 mg/kg Carbenoxolone administered 1x per day on days 3 & 4 (n = 5) did not significantly shift the morphine pellet ED_{50}. (C) 25 mg/kg administered 1x per day on days 3 & 4, carbenoxolone significantly shifted the morphine pellet curve leftward. (D) At the highest dose tested (37.5 mg/kg), carbenoxolone further significantly shifted the morphine pellet curve leftward (n = 5). Carbenoxolone administered to placebo pellet mice did not shift the acute morphine ED_{50}.

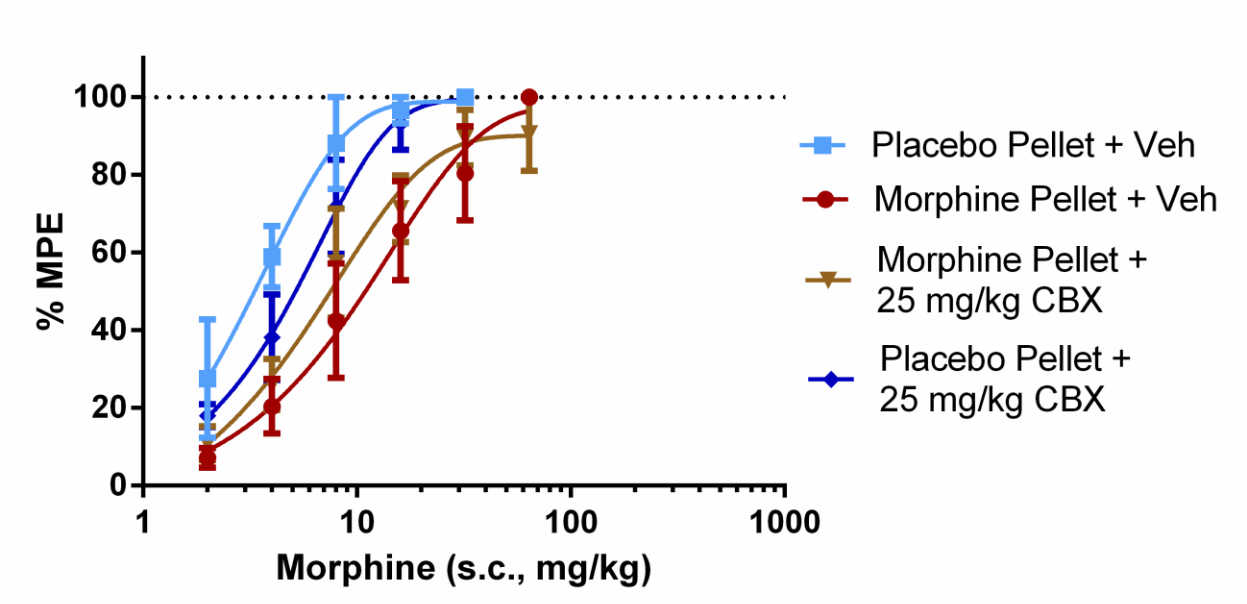


Fig. 2. Carbenoxolone does not acutely reverse morphine tolerance as measured in warm water tail withdrawal assay 5 day morphine pellet implantation significantly shifted the ED_{50} for morphine to the right indicating tolerance (n = 5 / group). Pretreatment of 25 mg/kg Carbenoxolone administered 30 min before challenge doses of morphine did not significantly shift the morphine pellet ED_{50}. Pretreatment of carbenoxolone administered to placebo pellet mice did not shift the acute morphine ED_{50}.

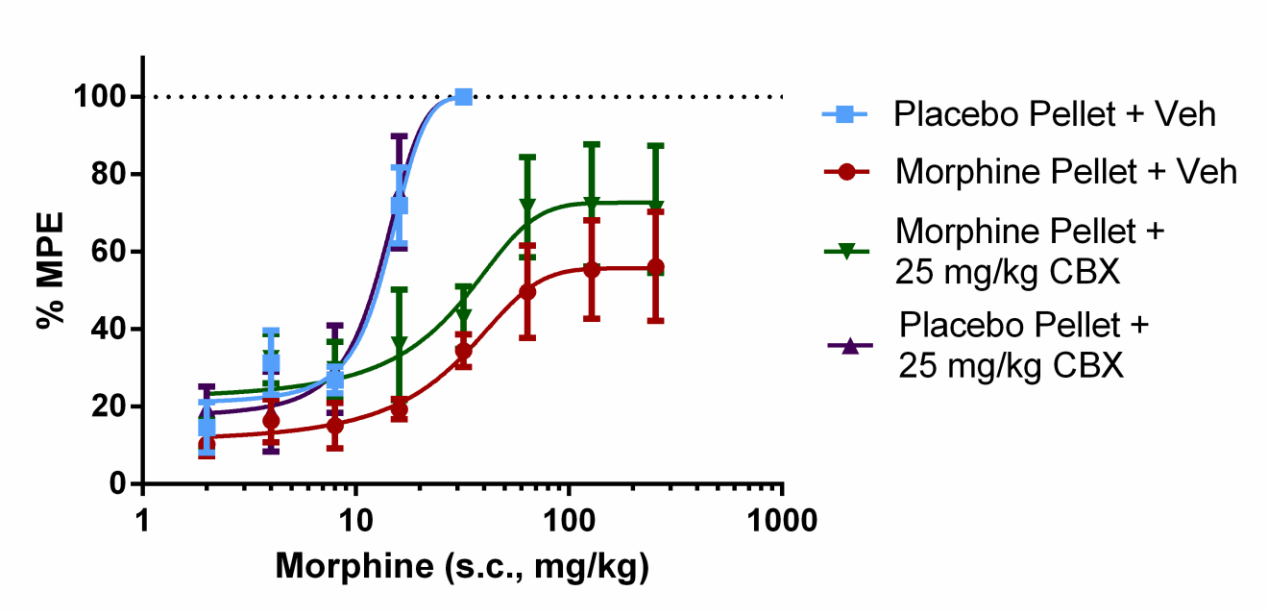


Fig. 3. Carbenoxolone attenuates development of morphine tolerance as measured in hot plate assay 5 day morphine pellet implantation significantly shifted the ED_{50} for morphine to the right indicating tolerance (n = 15 / group). 25 mg/kg carbenoxolone administered 1x per day on days 3 & 4 significantly shifted the morphine pellet curve leftward. Carbenoxolone administered to placebo pellet mice did not shift the acute morphine ED_{50}.

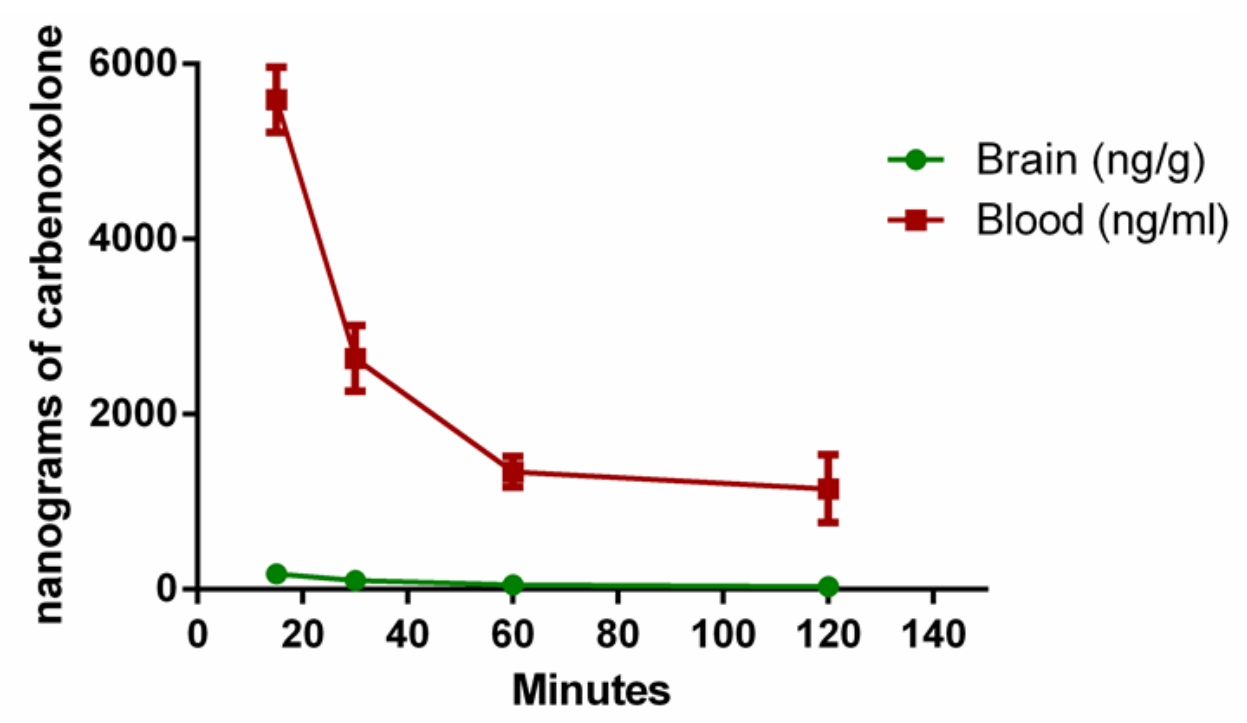


Fig. 4. Brain and Blood concentrations of Carbenoxolone (mean +/- SEM) were observed at different time points following a 25 mg/kg intraperitoneal injection in drug naïve mice. Less than 3% of carbenoxolone levels found in the blood were observed in the brain at all time points tested

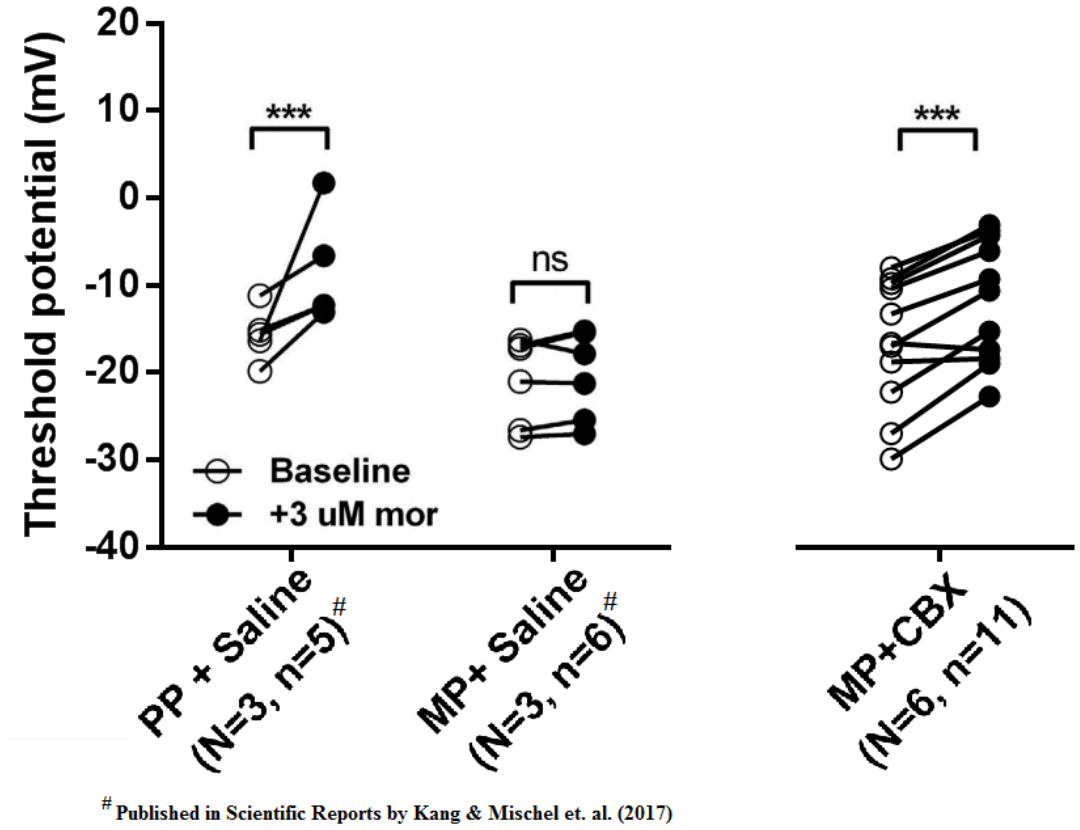


Fig. 5. CBX on DRG neurons isolated from MP mice Collective data from threshold potential (V_t) measurements before (open circle) and after (filled circle) morphine perfusion. Increases in V_t induced by 3μM morphine perfusion are prevented by chronic morphine exposure, but preserved alongside CBX treatment. #PP + SAL and MP + SAL groups have been previously published by our laboratory (Kang & Mischel et al., 2017). ns not significant, ***p<0.001

4. Discussion

Gap junctions are recognized as important contributors to intercellular communication. Through gap junctions flow second messengers, ions, and metabolites, generating waves of elevated calcium, sodium, and metabolic activity. Gap junctions exist on neurons but also have an important role in glial cell networks. Gap junctions consist of connexins, with the name of respective molecular weight. A number of studies indicate gap junctions having potential involvement in inflammatory cascades as well involvement in mechanisms in pain. For example, connexin 43 expression is increased in several neuropathic pain models including spinal cord injury, chronic constriction injury, and neuroinflammation (Haupt et al., 2007; Lee et al., 2005; O'Carroll et al., 2008). Administering gap junction inhibitors has been shown to attenuate neuropathic pain sensitization caused by chronic constriction injury (Jeon and Youn, 2015; Spataro et al., 2004). Gap junctions have also been shown to be involved in chronic morphine-induced effects including colon inflammation, dependence, and antinociceptive tolerance (Bhave et al., 2017b; Moradi et al., 2013; Shen et al., 2014). Astrocytic connexins in the spinal cord have been reported to be integral for the development of morphine antinociceptive tolerance. Carbenoxolone is a medication used to treat peptic ulcers in man and is also a known gap junction inhibitor. Studies in the past investigating gap junction function in neuropathic pain mechanisms or chronic morphine's effects concentrated on administering carbenoxolone intrathecally through intracerebroventricular injection (i.e. past the blood brain barrier).

In the present study, we observed carbenoxolone administered systemically attenuated the development of opioid antinociceptive tolerance. Through intraperitoneal injections during the exposure to chronic morphine, carbenoxolone was able to rescue morphine antinociceptive tolerance seen in the warm water tail withdrawal assay (primarily a spinal reflex) and in the hot

plate assay (primarily mediated by supraspinal mechanisms). Carbenoxolone did not affect acute morphine antinociception. Examining whether carbenoxolone administered right before the morphine challenge would affect tolerance, we observed that carbenoxolone was not able to acutely reverse morphine antinociceptive tolerance in the warm water tail withdrawal assay.

Carbenoxolone has been shown to mitigate central and peripheral effects caused by chronic exposure to morphine. However, the molecule of carbenoxolone is polar and relatively large which raises the question as to its ability to cross the blood brain barrier. One study examined carbenoxolone levels in cerebral spinal fluid (CSF) of rats after intraperitoneal injection. The researchers observed "negligible levels" (<1 μM) of carbenoxolone in CSF (Leshchenko et al., 2006). However, a major caveat of that study was that the drug potentially concentrated itself within brain tissue with little in the CSF. We developed a technique using UPLC-MS/MS in order to analyze carbenoxolone in whole brain tissue from mice. Less than 3% of the carbenoxolone levels found in blood was observed in the brain at any time point investigated. Whether the small amount of carbenoxolone observed in the brain tissue is biologically significant is unknown. However, the whole brain tissue was dissected and homogenized for this study. This dissection and homogenization would include some amount of blood vessels which may explain the small amount of carbenoxolone detected.

The evidence supporting carbenoxolone's inability to cross the blood brain barrier suggests a peripheral site of action. The dorsal root ganglia contain the cell bodies of primary afferent neurons that act as a "relay station" transmitting sensory information from the periphery into the CNS. DRGs sit in the periphery just outside the blood brain barrier alongside the spinal cord. It has been well established that MORs are expressed in DRG neurons and it has been suggested that peripheral mechanisms are important for the development of opioid antinociceptive tolerance.

Deletion of MOR specifically on afferent nociceptors eliminated antinociceptive tolerance in mice while antinociception was unaffected (Corder et al., 2017). This suggests that the analgesic effects of morphine are mediated centrally while peripheral effects are playing a role in tolerance development. In the DRGs, satellite glial cells (SGCs) completely wrap around the sensory neurons. This arrangement allows SGCs to regulate ion concentration in the extracellular space, recycle neurotransmitters, and facilitate non-synaptic communication between neurons and glia (Gu et al., 2010; Suadicani et al., 2010). The signaling that occurs between neurons and SGCs involve gap junctions and this communication can be interrupted by the use of a gap junction inhibitor such as carbenoxolone (Suadicani et al., 2010). We have since published that tolerance to opioids can be evaluated in DRGs (Jacob et al., 2018; Kang et al., 2017). With this model, we observed that tolerance developed to morphine on a neuronal level with the same 5-day morphine pellet in-vivo treatment as done with the tail withdrawal and hotplate assays. Furthermore, 25 mg/kg carbenoxolone administered on days 3 and 4 of chronic morphine exposure restored the neurons' ability to respond to a morphine challenge, indicating that tolerance did not develop. This suggests a possible peripheral mechanism by which carbenoxolone is able to exert its attenuation of antinociceptive tolerance effects.

Although these results are intriguing, additional issues need further investigation. The most important issue with interpreting the effects of gap junction blockers such as carbenoxolone is that in addition to gap junctions, they affect various ion channels, receptors and enzymes. Therefore, it would be important to compare the behavioral effects of different gap junction blockers with results obtained in knockout mice for specific types of gap junctions. In addition, carbenoxolone which has been shown to block gap junctions but also shown to block NMDA receptors and block calcium channels at higher concentrations (Chepkova et al., 2008; Vessey et al., 2004). These

targets have been implicated in opioid antinociceptive tolerance (Allen et al., 2000; Dogrul et al., 2005; Tiseo and Inturrisi, 1992; Trujillo and Akil, 1991). A study examining carbenoxolone metabolism noted that carbenoxolone hydrolysis in mammalian tissues does not appear to occur and most of carbenoxolone metabolism occurs due to the microflora of the stomach and small intestine (Iveson et al., 1971). This suggests that active metabolites should not be a concern from an intraperitoneal injection however further studies should examine carbenoxolone's metabolites, their possible effects, and ability to cross the blood brain barrier.

The notion that gap junctions may be involved in opioid tolerance has been suggested from previous studies. Initial work demonstrating that microglia and astrocyte activation occurs as a result of chronic morphine treatment has been followed by demonstration of chronic opioid induced upregulation of connexins in astrocytes alongside the development of tolerance. Administering a gap junction blocker intrathecally attenuated the development of antinociceptive tolerance (Shen et al., 2014). Our study shows that gap junctions playing an important role in the development of opioid antinociceptive tolerance while also suggesting a potential peripheral target. Further work is needed to determine whether gap junctions on neurons or glia cells are integral for the development of opioid tolerance and if a certain type of connexin plays a leading role in this effect.

CHAPTER 5

GENERAL DISCUSSION

Historically, the relief of moderate to severe pain was primarily managed by treatment utilizing opioid analgesic medications. However, hesitation exists to prescribe these medications due to the side effects that are associated with repeated use of these compounds. Repeated exposure to opioids can lead to tolerance to its desired effects, leading to a cycle of increasing doses accompanied by increased occurrence of side effects such as respiratory depression, constipation, dependence, in addition to addiction. This creates problems not only in clinical populations but also abusers as well. Clinically, opioids are often used in tandem with other medications and in different disease states. Outside the clinic, opioid abusers tend to be polydrug abusers, consuming a number of different substances often at the same time. In both cases, a variety of serious health complications can occur, many not fully understood. The objective of this dissertation was to investigate some of the complexities of chronic opioid exposure and how different disease states and medications may modulate the effects of chronic opioids. The studies described here lead to new understandings of what occurs on a behavioral and cellular level during repeated exposure to opioids.

In our first series of experiments, we investigated the increasing occurrence of benzodiazepine use alongside the prescription opioid epidemic. Benzodiazepines are among the most frequently prescribed medications in the world (Coach Jr., 1990). They are commonly prescribed for the treatment of anxiety disorders. Research suggests that the abuse liability of benzodiazepines is especially notable in recreational users of other drugs (Jones et al., 2012). Additionally, patients maintained on opioid agonists such as methadone and buprenorphine, as well as active heroin users, tend to abuse benzodiazepines (Barnas et al., 1992; Brands et al., 2008;

Darke et al., 1995, 1992; Kleber and Gold, 1978). One study investigating heroin users in Australia showed that 2 out of 3 heroin users reported nonmedical benzodiazepines use within the past year, and 91% reported a lifetime prevalence (Ross and Darke, 2000). Furthermore, opioid-dependent populations show a preference for particular benzodiazepines. One of the most commonly coabused benzodiazepine in these populations is diazepam (Bramness and Kornør, 2007; Du Pont, 1988; Iguchi et al., 1993). Whether the co-abuse of opioids and benzodiazepines stem from underlying psychiatric disorders (i.e. anxiety), aiding with dependence, or increasing the rewarding and reinforcing effects remains to be determined. However, studies have shown individuals may be using benzodiazepines to amplify the positive effects of opioids. For example, one study reported that 72% of methadone-maintained patients who were regular benzodiazepine users indicated that diazepam enhanced the effects of their daily methadone dose (Stitzer et al., 1981).

In 2016, the Food and Drug Administration issued a black-box warning about co-prescribing benzodiazepines and opioids. Among the data reviewed by the FDA, the agency concluded that from 2004 to 2011, the rate of emergency department visits involving non-medical use of both drug classes increased significantly, with overdose deaths involving both drug classes nearly tripling during that period. Studies have observed an increased effect of respiratory depression when benzodiazepines are administered intravenously (Carraro et al., 2009; Zacharias et al., 1996). It has yet to be determined how benzodiazepines may affect opioid tolerances, if it is simply additiveness or something more. Our observations suggest this phenomenon may not be simply due to the additive or synergistic of these CNS depressants but instead results from a reversal of opioid tolerance. Diazepam, at doses tested, did not induce any antinociceptive effects on their own nor did they potentiate acute doses of the commonly prescribed opioids oxycodone or hydrocodone. However, in an opioid tolerant state, diazepam rescued the antinociceptive effect

of both oxycodone and hydrocodone, enabling the opioids to produce antinociception at doses similar to those observed in a mouse acutely treated with morphine. This reversal of tolerance effect extends to locomotor tolerance as well. Commonly described as a depressant, diazepam was able to rescue the increased locomotor effects commonly seen by opioids administered to drug-naive mice.

Although not tested in this series of experiments, a previous publication from our laboratory showed similar reversal of tolerance for morphine, a partial μ-agonist like oxycodone and hydrocodone. That work showed that the reversal of tolerance effect of diazepam was blocked by the $GABA_A$ antagonist bicuculline, suggesting that diazepam is enacting these effects through $GABA_A$. Benzodiazepines act as positive allosteric modulators of the $GABA_A$ receptors. Benzodiazepines act to enhance the effects of GABA by increasing chloride flux and rate of channel opening. In terms of circuitry, it has been theorized that both benzodiazepines and opioids produce a hyperpolarization of GABA interneurons which causes a reduction in the release of GABA which results in the disinhibition of dopaminergic neurons and an increase in extracellular dopamine in areas such as the striatum (Tan et al., 2011). Opioid-induced locomotion is due to mu opioid receptor-mediated increases in striatal dopamine release (Johnson and Glick, 1993; Kalivas and Duffy, 1987; Piepponen et al., 1999). GABAergic interneurons also play an important role in opioid antinociception (Lau et al., 2014). This suggests the $GABA_A$ receptor as a potential site of action that should be further characterized.

People who suffer from addiction often have one or more accompanying medical issues including infectious diseases. Concurrent with the opioid abuse epidemic, almost 40,000 people were diagnosed with HIV infection in the United States in 2015 (CDC, 2016). Opioid abuse and the spread of HIV are intertwined public health epidemics. Worldwide, injection drug use (IDU)

accounts for ~30% of new HIV-1 infections while within the United States, over 3,500 new infections involved IDU in 2015 (WHO, 2016; CDC, 2016). Among non-addicts, HIV-related pain may necessitate opioid analgesics. These populations face special risks because a number of studies have uncovered evidence that opioid usage increases the progression of HIV-1 to AIDS and promotes neurocognitive impairment in humans and in non-human primates (Bell et al., 2006, 2002; Bokhari et al., 2011; Chuang et al., 2005; Donahoe et al., 1993; Kumar et al., 2006; R et al., 2004; Rivera et al., 2013). Furthermore, studies have shown that the HIV-1 regulatory protein Tat can be secreted from infected cells to exert direct and indirect neurotoxicity by promoting neuroinflammation (reviewed in King et al., 2006; Nath et al., 2002). Even with the recent innovative antiretrovirals that have made HIV treatable but not yet curable, Tat may sequester in regions of the brain to nearly undetectable levels and still cause neuroinflammation (Hellmuth et al., 2015). As previously mentioned, in vitro studies have shown that morphine exacerbates Tat effects to activate microglia, increase cytokine production, drive oxidative stress, increase intracellular calcium, and promote neurotoxicity. Morphine and Tat interactions may depend on μ opioid receptors (MORs) given that neurotoxic synergy is observed in co-cultures when mixed glia express MORs, but not when they are derived from MOR−/− mice (Zou et al., 2011). These data support the notion that glial MORs are critical for the indirect neurotoxic effects of Tat.

The proinflammatory effects of HIV-1 Tat at CCR5 have been reported to directly influence opioid sensitivity. In studies of opioid-mediated antinociception in rats, activation of CCR5 can rapidly desensitize μ-opioid-receptors (Chen et al., 2007). A bivalent ligand comprised of an opioid receptor antagonist (naltrexone) and CCR5 antagoinist maraviroc reduced the infectivity of human astrocytes when cultured with R5-tropic HIV (Arnatt et al., 2016; El-Hage et al., 2013; Yuan et al., 2013). These data suggest a dynamic relationship between MOR and CCR5

activation that may contribute to HIV pathology; however, the functional effects are poorly understood. As such, we investigated morphine tolerance, dependence, and reward in a transgenic murine model that conditionally expresses the proinflammatory HIV-1 regulatory protein, Tat. We demonstrated that pretreatment with the CCR5 antagonist, maraviroc, blocks the effects of Tat on morphine tolerance and dependence behaviors (reinstating morphine potency in non-tolerant mice and restoring aspects of withdrawal symptomology in morphine tolerant mice). Intriguingly, maraviroc also potentiated the Tat-induced increase of morphine-CPP, even while it reduced the levels of many inflammatory chemokines and cytokines in the striatum including β-chemokines. Interestingly, Tat was able to attenuate the antinociceptive properties of acute morphine in opioid-naïve mice. Maraviroc administered to Tat + mice rescued the antinociceptive properties of morphine. We proposed a possible model of cross-desensitization that may contribute to the behaviors observed. When a neuroinflammatory insult, in the form of Tat, is present, morphine potency is diminished on most measures, perhaps due to endogenous MORs becoming cross-sensitized by activated CCR5 receptors. When maraviroc is introduced to block the CCR5 receptors, we speculate that it may relieve the cross-sensitization of MORs, restoring morphine signaling. While cross-desensitization of MOR and CCR5 is fairly well documented in vitro and is seemingly due to phosphorylation of the receptors, the intracellular kinases responsible have yet to be determined. However in a 2005 study, researchers observed PKC playing an integral role in the cross-desensitization occurring among different chemokine receptors (including CCR5). It has been often reported that PKC play an important role in MOR desensitization in vitro and in both respiratory depressive and antinociceptive tolerance after repeated exposure to morphine and other opioids (Bailey et al., 2009, 2006; Hill et al., 2018; Hull et al., 2010; Jacob et al., 2018; Lin et al., 2012; Llorente et al., 2013; Smith et al., 1999). This suggests that activating CCR5 due to the Tat

inflammatory insult may be activating PKC downstream. This may preemptively desensitize MORs, behaviorally seeming like tolerance even though subjects are naïve to opioids. Future studies should examine whether Tat's attenuation of certain effects of morphine is due to intracellular processes involving PKC.

As mentioned previously, glial MORs appear to be critical for the indirect neurotoxic effects of Tat. Studies have shown that in astrocytes, significant changes in gene expression, apoptosis, glutamate metabolism, and blood brain barrier have been reported, suggesting that these cells play a key role in NeuroAIDS (Berman et al., 2016; Eugenin et al., 2011; Wang et al., 2004). A major component of astrocytes and other glial cells are gap junctions, which are channels that connect the cytoplasmic compartment a cell to other cells or into the extracellular space and allow electrical and secondary messenger based communication to occur. However, HIV infection of astrocytes results in opening of hemichannel and increases the amount of hemichannel present particularly connexin 43 (Berman et al., 2016). HIV-Tat protein has been implicated as directly promoting the increase in connexin upregulation, however a recent publication shows the mere exposure of HIV-Tat in the colon of rats causes an inflammatory cascade through connexin 43 upregulation going up the spinal cord into the brain with no detectable HIV-Tat in the CNS (Berman et al., 2016; Esposito et al., 2017). Blocking gap junctions using carbenoxolone significantly reduced HIV infection of human-derived pericytes (Cho et al., 2017). Another recent study shows that gap junctions are integral for HIV-induced toxicity (Malik et al., 2017). Upregulated gap junction expression and function, particularly connexin 43, has been associated with chronic exposure to morphine including morphine tolerance (Bhave et al., 2017a; Moradi et al., 2013; Shen et al., 2014). Therefore, we tested the hypothesis that carbenoxolone, a gap junction inhibitor, administered systemically would attenuate the development of morphine antinociceptive

tolerance. We observed that carbenoxolone was able to attenuate the development of morphine antinociceptive tolerance in a dose-dependent manner. Carbenoxolone given acutely before challenge doses of morphine did not affect morphine antinociception nor did it acutely reverse antinociceptive tolerance. In an attempt to identify a possible site of action where carbenoxolone may produce its effects, we demonstrated that carbenoxolone is not observed in brain tissue after intraperitoneal injection, suggesting an effect in the periphery. Subsequently, we observed that carbenoxolone administration in vivo during chronic exposure to morphine attenuated the development of tolerance in neurons isolated from dorsal root ganglion. Peripheral mechanisms for antinociceptive have been suggested in the past (Corder et al., 2017; Kang et al., 2017; Puig and Gutstein, 2017). Future studies should examine the location (neuronal or glial) and what type of gap junctions (i.e. connexin 43) are necessary for this effect to occur. This can be performed using specific connexin knockouts inducible on neurons or glial cells.

Collectively, the studies in this dissertation have increased our knowledge of the interactions between chronic opioids and different medications and disease states. The compounds diazepam, maraviroc and carbenoxolone highlight the complexities of how chronic opioid effects such as tolerance manifest and can be modulated. At the same time, these compounds teasingly point to mechanisms that may be shared by chronic opioid exposure. For instance, GABAergic interneurons play a pivotal in pain circuity as well as in opioid's analgesic effects from CNS areas such as the PAG and dorsal horn spinal cord (Lau et al., 2014). Timely activating $GABA_A$ receptors in DRGs after peripheral nerve injury has been shown to attenuate the development of neuropathic pain (Naik and Pathirathna, 2008). As previously shown, activating $GABA_A$ receptors reversed the development to opioid antinociceptive tolerance (Hull et al., 2013). Specifically where in the body this occurs should be examined in future studies. Chronic opioids are known to activate glial cells

including astrocytes, microglia, and satellite glia (Shen et al., 2014; Song and Zhao, 2001). Glia play an important role in the regulation of GABA reuptake (Allison et al., 2011). An integral component of glia activation and resulting communication with neurons are through gap junctions. We and others have shown that gap junctions appear to play a role in the development of opioid tolerance and other opioid mediated effects (Moradi et al., 2013; Shen et al., 2014). Glial cells are also important mediators of inflammation. Inflammatory insults including TNF-α, IL-1β, and IL-6 have been associated with chronic morphine exposure and with opioid tolerance (Shen et al., 2012). Inflammatory cytokines activating CCR5 can cross-desensitize MOR through a phosphorylation mechanism that, although experimental evidence is lacking, may involve PKC, a kinase that has been implicated in opioid tolerance. The interactions between these components warrant future experiments in order to elucidate the specific pathways by which opioids enact their acute and chronic effects.

With the overprescription of opioid analgesics as well as the availability of potent heroin, the opioid epidemic has grown to unprecedented heights in the past decade. Consequently, more people are chronically exposed to opioids. Studies on repeated exposure to opioids have been done for decades yet the mechanisms for certain phenomena such as tolerance are still not fully understood. Furthermore, different medications, such frequently prescribed benzodiazepines, or different disease states, such as HIV, have their own effects and interactions with chronic opioid exposure that are not fully examined. The present findings add to the body of literature by investigating the consequences of these interactions on opioid-mediated effects especially antinociceptive tolerance while highlighting potential components that play a role in opioid tolerances.

LIST OF REFERENCES

Abraham, W.C., 2008. Metaplasticity : tuning synapses and networks for plasticity. Nat. Rev. Neurosci. 9. doi:10.1038/nrn2356

Akgün, E., Javed, M., Lunzer, M., Powers, M., Sham, Y., Watanabe, Y., Portoghese, P., 2015. Inhibition of Inflammatory and Neuropathic Pain by Targeting a Mu Opioid Receptor/Chemokine Receptor5 Heteromer (MOR-CCR5). J Med Chem 8647–8657.

Al-Hasani, R., Bruchas, M.R., 2011. Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology 115, 1363–1381. doi:10.1097/ALN.0b013e318238bba6.Molecular

Allen, R.M., Dykstra, L.A., Hill, C., Carolina, N., 2000. Attenuation of mu-Opioid Tolerance and Cross-Tolerance by the Competitive N -Methyl- D -aspartate Receptor Antagonist LY235959 Is Related to Tolerance and Cross-Tolerance. J. Pharmacol. Exp. Ther. 295, 1012–1021.

Allison, D.W., Wilcox, R.S., Ellefsen, K.L., Askew, C.E., Hansen, D.M., Wilcox, J.D., Sandoval, S.S., Eggett, D.L., Yanagawa, Y., Steffensen, S.C., 2012. Mefloquine Effects on Ventral Tegmental Area Dopamine and GABA Neuron Inhibition: A Physiologic Role for Connexin-36 Gap Junctions. Synapse 65, 804–813. doi:10.1002/syn.20907.Mefloquine

Altun, A., Ozdemir, E., Yildirim, K., Gursoy, S., Durmus, N., Bagcivan, I., 2015. The effects of endocannabinoid receptor agonist anandamide and antagonist rimonabant on opioid analgesia and tolerance in rats. Gen Physiol Biophys 433–40.

Angelakis, E., Million, M., Kankoe, S., Lagie, r J., Armougom, F., Giorgi, R., Raoult, D., 2014.

Abnormal weight gain and gut microbiota modifications are side effects of long-term doxycycline and hydroxychloroquine treatment. Antimicrob Agents Chemother 3342–7.

Arnatt, C., Falls, B., Yuan, Y., Raborg, T., Masvekar, R., El-Hage, N., Selley, D., AV, N., Knapp PE, Hauser KF, Z.Y., 2016. Exploration of bivalent ligands targeting putative mu opioid receptor and chemokine receptor CCR5 dimerization. Bioorg Med Chem 5969–5987.

Avdoshina, V., Biggio, F., Palchik, G., Campbell, L., I, M., 2010. Morphine induces the release of CCL5 from astrocytes: potential neuroprotective mechanism against the HIV protein gp120. Glia 58, 1630–9.

Bailey, C.P., Llorente, J., Gabra, B.H., Smith, F.L., Dewey, W.L., Kelly, E., Henderson, G., 2009. Role of protein kinase C and ??-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. Eur. J. Neurosci. 29, 307–318. doi:10.1111/j.1460-9568.2008.06573.x

Bailey, C.P., Smith, F.L., Kelly, E., Dewey, W.L., Henderson, G., 2006. How important is protein kinase C in ??-opioid receptor desensitization and morphine tolerance? Trends Pharmacol. Sci. 27, 558–565. doi:10.1016/j.tips.2006.09.006

Barnas, C., Rossman, M., Roessler, H., Riemer, Y., Fleischhacker, W.W., 1992. Benzodiazepines and other psychotropic drugs abused by patients in a methadone maintenance program: familiarity and preference. J. Clin. Psychopharmacol. 12, 397–402.

Bayer, B., Brehio, R., Ding, X., Hernandez, M., 1994. Enhanced susceptibility of the immune system to stress in morphine-tolerant rats. Brain Behav Immun 8, 173–84.

Bell, J.E., Arango, J.-, Robertson, R., Brettle, R.P., Leen, C., Simmonds, P., 2002. HIV and drug

misuse in the edinburgh cohort. J. Acquir. Immune Defic. Syndomes.

Bell, J.E., Arango, J., Anthony, I.C., 2006. Neurobiology of Multiple Insults : HIV-1-Associated Brain Disorders in Those Who Use Illicit Drugs 182–191. doi:10.1007/s11481-006-9018-2

Bergman, S.A., Wyn, R.L., Williams, G., 1988. Diazepam enhances fentanyl and diminishes meperidine antinociception. Anesth Prog 35, 190–194.

Berman, J., Carson, M., Chang, L., Cox, B., Fox, H., Gonzalez, R., Hanson, G., Hauser, K., Ho, W., Hong, J., Major, E., Maragos, W., Masliah, E., McArthur, J., Miller, D., Nath, A., O'Callaghan, J., Persidsky, Y., Power, C., Rogers, T., 2006. NeuroAIDS, drug abuse, and inflammation: building collaborative research activities. J Neuroimmune Pharmacol 1, 351–399.

Berman, J.W., Carvallo, L., Buckner, C.M., Luers, A., Prevedel, L., Bennett, M. V, 2016. HIV-tat alters Connexin43 expression and trafficking in human astrocytes : role in NeuroAIDS. J. Neuroinflammation 1–11. doi:10.1186/s12974-016-0510-1

Bhave, S., Gade, A., Kang, M., Hauser, K.F., Dewey, W.L., Akbarali, H.I., 2017a. Connexin – purinergic signaling in enteric glia mediates the prolonged effect of morphine on constipation 31, 2649–2660. doi:10.1096/fj.201601068R

Bhave, S., Gade, A., Kang, M., Hauser, K.F., Dewey, W.L., Akbarali, H.I., 2017b. Connexin – purinergic signaling in enteric glia mediates the prolonged effect of morphine on constipation. FASEB J 31, 2649–2660. doi:10.1096/fj.201601068R

Biswas SK, L.-C.E., 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol 30, 475–487.

Blenkinsop, T.A., Lang, E.J., 2006. Block of Inferior Olive Gap Junctional Coupling Decreases Purkinje Cell Complex Spike Synchrony and Rhythmicity 26, 1739–1748. doi:10.1523/JNEUROSCI.3677-05.2006

Bliss, C.I., 1967. Statistics in Biology. McGraw-Hill Book Company, New York.

Bokhari, S., Yao, H., Bethel-Brown, C., Fuwang, P., Williams, R., Dhillon, N., Hegde, R., Kumar, A., Buch, S., 2009. Morphine enhances Tat-induced activation in murine microglia. J Neurovirol 15, 219–228.

Bokhari, S.M., Hegde, R., Callen, S., Yao, H., 2011. Morphine potentiates neuropathogenesis of SIV infection in rhesus macaques. J Neuroimmune Pharmacol 6, 626–639. doi:10.1007/s11481-011-9272-9.Morphine

Booss, J., Drake, A., Kerns, R., Ryan, B., Wasse, L., 2000. Pain as the 5 Th Vital Sign Toolkit.

Bramness, J.., Kornør, H., 2007. Benzodiazepine prescription for patients in opioid maintenance treatment in Norway. Drug Alcohol Depend 203–209.

Brands, B., Blake, J., Marsh, D., Sproule, B., Jeyapalan, R., Li, S., 2008. The impact of benzodiazepine use on methadone maintenance treatment outcomes. J. Addict. Dis. 37–48.

Brownstein, M.J., 1993. Review A brief history of opiates , opioid peptides , and opioid receptors 90, 5391–5393.

Bruce-Keller, A., Turchan-Cholewo, J., Smart, E., Geurin, T., Chauhan, A., Reid, R., Xu, R., Nath, A., Knapp, P., Hauser, K., 2008. Morphine causes rapid increases in glial activation and neuronal injury in the striatum of inducible HIV-1 Tat transgenic mice. Glia 56, 1414–1427.

Campbell, J.N., 1996. 1995 Presidential Address. Pain Forum 5, 85–88. doi:10.1016/S1082-3174(96)80076-6

Campbell, L., Avdoshina, V., Day, C., Lim, S., Mocchetti, I., 2015. Pharmacological induction of CCL5 in vivo prevents gp120-mediated neuronal injury. Neuropharmacology 92, 98–107.

Carraro, G., Russsi, E., Buechi, S., Bloch, K., 2009. Does oral alprazolam affect ventilation? A randomised, double-blind, placebo controlled trial. Psychopharmacology (Berl).

Cavaillon, J., Adib-Conquy, M., 2006. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care. 10, 233.

Center for Behavioral Health, 2016. Key substance use and mental health indicators in the United States: Results from the 2015 National Survey on Drug Use and Health.

Chang, K., Cooper, B., Hazum, E., Cuatrecasas, P., 1979. Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. Mol. Pharmacol. 91–104.

Chang, K., Cuatrecasas, P., 1979. Multiple opiate receptors. Enkephalins and morphine bind to receptors of different specificity. J Biol Chem 254, 2610–2618.

Chen, L.H., Hedegaard, H., Warner, M., 2014. Drug-poisoning Deaths Involving Opioid Analgesics : 1–8.

Chen, X., Geller, E., Rogers, T., Adler, M., 2007. Rapid heterologous desensitization of antinociceptive activity between mu or delta opioid receptors and chemokine receptors in rats. Drug Alcohol Depend 88, 36–41.

Chepkova, A.N., Sergeeva, O.A., Haas, H.L., 2008. Neuropharmacology Carbenoxolone impairs LTP and blocks NMDA receptors in murine hippocampus. Neuropharmacology 55, 139–147. doi:10.1016/j.neuropharm.2008.05.001

Chiang, C.Y., Li, Z., Dostrovsky, J.O., Sessle, B.J., 2010. Central sensitization in medullary dorsal horn involves gap junctions and hemichannels. Neuroreport 21, 233–237. doi:10.1097/WNR.0b013e328336eecb.Central

Cho, H.J., Kuo, A.M., Bertrand, L., Toborek, M., 2017. HIV Alters Gap Junction-Mediated Intercellular Communication in Human Brain Pericytes 10, 1–13. doi:10.3389/fnmol.2017.00410

Chuang, R., Suzuki, S., Chuang, T., Miyagi, T., Chuang, L., Doi, R., 2005. Opioids and the Progression of Simian AIDS. Front Biosci 10, 1666–1677.

Cicero, T.J., Ellis, M.S., Surratt, H.L., Kurtz, S.P., 2014. The Changing Face of Heroin Use in the United States. JAMAPsychiatry 71, 821–826. doi:10.1001/jamapsychiatry.2014.366

Coach Jr., W.F., 1990. Benzodiazepines: an overview. In: Epidemiologic Trends in Drug Abuse. Epidemiol. Trends Drug Abus.

Coderre, T., Rollman, G., 1983. Naloxone hyperalgesia and stress-induced analgesia in rats. Life Sci 32, 2139–2146.

Corder, G., Tawfik, V.L., Wang, D., Sypek, E.I., Low, S.A., Dickinson, J.R., Sotoudeh, C., Clark, J.D., Barres, A., Bohlen, C.J., Scherrer, G., 2017. Loss of μ-opioid receptor signaling in nociceptors, and not spinal microglia, abrogates morphine tolerance without disrupting analgesic efficacy. Nat Med 23, 164–173. doi:10.1038/nm.4262.Loss

Crawley, J., Paylor, R., 1997. proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Horm Behav 31, 197–211.

Cryan, J., Dinan, T., 20102. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13, 701–712.

Dalton, G.D., 2005. The Study of the Effect of Drugs of Abuse on Protein Kinase A Activity in Mouse Brain and Spinal Cord.

Dalvi, P., Sharma, H., Chinnappan, M., Sanderson, M., Alle, n J., Zeng, R., Choi, A., O'Brien-Ladner, A., Dhillon, N., 2016. Enhanced autophagy in pulmonary endothelial cells on exposureto HIV-Tat and morphine: Role in HIV-related pulmonary arterial hypertension. Autophagy 12, 2420–2438.

Darke, S.G., Hall, W., Ross, M., Wodak, A., 1992. Benzodiazepine use and HIV risk taking behavior among injecting drug users. Drug Alcohol Depend 31, 31–36.

Darke, S.G., Ross, J., Hall, W.., 1995. Benzodiazepine use among injecting heroin users. Med. J. Aust 645–647.

Daubresse, M., Chang, H., Yu, Y., Shah, N.D., Stafford, R.S., Stefan, P., Alexander, G.C., 2014. Ambulatory Diagnosis And Treatment Of Nonmalignant Pain In The United States, 2000–2010. Med Care 51, 1–18. doi:10.1097/MLR.0b013e3182a95d86.AMBULATORY

Diagnoses of HIV Infection in the United States and Dependent Areas , 2015, 2015. 27.

Dogrul, A., Bilsky, E.J., Ossipov, M.H., Lai, J., Porreca, F., 2005. Spinal L-Type Calcium Channel Blockade Abolishes Opioid-Induced Sensory Hypersensitivity and Antinociceptive

Tolerance. Anesth Analg 101, 1730–1735. doi:10.1213/01.ANE.0000184253.49849.B0

Donahoe, R., Byrd, L., McClure, H., Fultz, P., Brantley, M., Marsteller, F., Ansari, A., Wenzel, D., Aceto, M., 1993. Consequences of Opiate-Dependency in a Monkey Model of AIDS. Adv Exp Med Biol 21–28.

Du Pont, R.L., 1988. Abuse of benzodiazepines: the problems and the solutions. Am. J. Drug Alcohol Abuse 1–69.

Dumas, E.O., Pollack, G.M., 2008. Opioid Tolerance Development : A Pharmacokinetic / Pharmacodynamic Perspective 10. doi:10.1208/s12248-008-9056-1

Dutta, R., Roy, S., 2015. Chronic morphine and HIV-1 Tat promote differential central nervous system trafficking of CD3+ and Ly6C+ immune cells in a murine Streptococcus pneumoniae infection model. J Neuroinflammation 12, 120.

Eisenstein, T., Rahim, R., Feng, P., Thingalaya, N., Meissler, J., 2006. Effects of opioid tolerance and withdrawal on the immune system. J Neuroimmune Pharmacol 1, 237–249.

El-Hage, N., Bruce-Keller, A., Knapp, P., Hauser, K., 2008a. CCL5/RANTES gene deletion attenuates opioid-induced increases in glial CCL2/MCP-1 immunoreactivity and activation in HIV-1 Tat-exposed mice. J Neuroimmune Pharmacol 3, 275–285.

El-Hage, N., Bruce-Keller, A., Yakovleva, T., Bazov, I., Bakalkin, G., Knapp, P., Hauser, K., 2008b. Morphine exacerbates HIV-1 Tat-induced cytokine production in astrocytes through convergent effects on [Ca2+]i, NF-κB trafficking and transcription. PLoS One 3.

El-Hage, N., Dever, S., Podhaizer, E., Arnatt, C., Zhang, Y., KF, H., 2013. A novel bivalent HIV-1 entry inhibitor reveals fundamental differences in CCR5-μ-opioid receptor

interactions between human astroglia and microglia. AIDS 27, 2181–90.

El-Hage, N., Gurwell, J., Singh, I., Knapp, P., Nath, A., Hauser, K., 2005. Synergistic increases in intracellular Ca2+, and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. Glia 50, 91–106.

El-Hage, N., Wu, G., Ambati, J., Bruce-Keller, A., Knapp, P., Hauser, K., 2006. CCR2 mediates increases in glial activation caused by exposure to HIV-1 Tat and opiates. J Neuroimmunol 178.

Esposito, G., Capoccia, E., Gigli, S., Pesce, M., Bruzzese, E., Alessandro, A.D., Cirillo, C., Cerbo, A., Cuomo, R., Steardo, L., Sarnelli, G., 2017. HIV-1 Tat-induced diarrhea evokes an enteric glia-dependent neuroinflammatory response in the central nervous system. Sci. Rep. 1–11. doi:10.1038/s41598-017-05245-9

Eugenin, E., Clements, J., Zink, M., Berman, J., 2011. Human immunodeficiency virus infection of human astrocytes disrupts blood-brain barrier integrity by a gap junction-dependent mechanism. J. Neurosci.

Evans, C., Keith, D., Morrison, H., Magendzo, K., Edwards, R., 1992. Cloning of a delta opioid receptor by functional expression. Science (80-.). 1952–1955.

Feng, P., Meissler, J.J., Adler, M., Eisenstein, T., 2005. Morphine withdrawal sensitizes mice to lipopolysaccharide: elevated TNF-alpha and nitric oxide with decreased IL-12. J Neuroimmunol 164, 57–65.

Fitting, S., Ignatowska-Jankowska, B., Bull, C., Skoff, R., Lichtman, A., Wise, L., Fox, M., Su, J., Medina, A., Krahe, T., Knapp, P., Guido, W., Hauser, K., 2013. Synaptic dysfunction in

the hippocampus accompanies learning and memory deficits in human immunodeficiency virus type-1 Tat transgenic mice. Biol Psychiatry 73, 443–453.

Fitting, S., Knapp, P., Zou, S., Marks, W., Bowers, M., Akbarali, H., Hauser, K., n.d. Interactive HIV-1 Tat and morphine-induced synaptodendritic injury is triggered through focal disruptions in Na^+ influx, mitochondrial instability, and Ca^{2+} overload. J Neurosci 34.

Fitting, S., Scoggins, K., Xu, R., Dever, S., Knapp, P., Dewey, W., Hauser, K., 2012. Morphine efficacy is altered in conditional HIV-1 Tat transgenic mice. Eur J Pharmacol.

Fitting, S., Stevens, D., Khan, F., Scoggins, K., Enga, R., Beardsley, P., Knapp, P., Dewey, W., Hauser, K., 2016. Morphine Tolerance and Physical Dependence Are Altered in Conditional HIV-1 Tat Transgenic Mice. J Pharmacol Exp Ther.

Fitting, S., Zou, S., El-Hage, N., Suzuki, M., Paris, J., Schier, C., Rodríguez, J., Rodriguez, M., Knapp, P., Hauser, K., n.d. Opiate addiction therapies and HIV-1 Tat: interactive effects on glial $[Ca^{2+}]i$, oxyradical and neuroinflammatory chemokine production and correlative neurotoxicity. Curr HIV Res. 12, 424–434.

Fornasari, D., 2012. Pain pharmacology : focus on opioids. Clin. Cases Miner. Bone Metab. 165–168.

Foster, J., McVey Neufeld, K., 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36, 305–312.

Freireich, E., Gehan, E., Rall, D., Schmidt, L., Skipper, H., 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 219–244.

Gonek, M., Akbarali, H.I., Henderson, G., Dewey, W.L., 2017. Reversal of oxycodone and hydrocodone tolerance by diazepam. Brain Res. 1674, 84–90. doi:10.1016/j.brainres.2017.08.017

Gu, Y., Chen, Y., Zhang, X., Li, G., Wang, C.Y., Huang, L.-Y.M., 2010. Neuronal soma-satellite glial cell interactions in sensory ganglia and the participation of purinergic receptors. Neuron Glia Biol 6, 53–62. doi:10.1017/S1740925X10000116.Neuronal

Guedia, J., Brun, P., Bhave, S., Fitting, S., Kang, M., Dewey, W., Hauser, K., Akbarali, H., 2016. HIV-1 Tat exacerbates lipopolysaccharide-induced cytokine release via TLR4 signaling in the enteric nervous system. Sci Rep.

Guo, C., Li, Y., Tian, S., Wang, X., Douglas, S., Ho, W., 2002. Morphine enhances HIV infection of human blood mononuclear phagocytes through modulation of beta-chemokines and CCR5 receptor. J Investig Med. 50, 435–442.

Gupta, S., Knight, A., Gupta, S., Knapp, P., Hauser, K., Keller, J., Bruce-Kelle, r A., 2010. HIV-Tat elicits microglial glutamate release: role of NAPDH oxidase and the cystine-glutamate antiporter. Neurosci Lett 233–236.

Gurwell, J., Nath, A., Sun, Q., Zhang, J., Martin, K., Chen, Y., Hauser, K., n.d. Synergistic neurotoxicity of opioids and human immunodeficiency virus-1 Tat protein in striatal neurons in vitro. 2001 102, 555–563.

Haberstock-debic, H., Kim, K., Yu, Y.J., Zastrow, M. Von, 2005. Morphine Promotes Rapid , Arrestin-Dependent Endocytosis of □ -Opioid Receptors in Striatal Neurons 25, 7847–7857. doi:10.1523/JNEUROSCI.5045-04.2005

Haberstock-debic, H., Wein, M., Barrot, M., Colago, E.E.O., Rahman, Z., Neve, R.L., Pickel, V.M., Nestler, E.J., Zastrow, M. Von, Svingos, A.L., 2003. Morphine Acutely Regulates Opioid Receptor Trafficking Selectively in Dendrites of Nucleus Accumbens Neurons 23, 4324–4332.

Hahn, Y., Podhaizer, E., Farris, S., Miles, M., Hauser, K., Knapp, P., 2015. Effects of chronic HIV-1 Tat exposure in the CNS: heightened vulnerability of males versus females to changes in cell numbers, synaptic integrity, and behavior. Brain Struct Funct 605–623.

Hahn, Y., Podhaizer, E., Hauser, K., Knapp, P., 2012. HIV-1 alters neural and glial progenitor cell dynamics in the central nervous system: coordinated response to opiates during maturation. Glia 1871–1887.

Hahn, Y., Vo, P., Fitting, S., Block, M., Hauser, K., Knapp, P., 2010. β-Chemokine production by neural and glial progenitor cells is enhanced by HIV-1 Tat: effects on microglial migration. J Neurochem 97–109.

Harris, L.S., Pierson, A.K., 1964. Some Narcotic Antagonists In The Benzomorphan Series. J. Pharmacol. Exp. Ther. 143, 141–148.

Haupt, C., Witte, O., Frahm, C., 2007. Up-regulation of Connexin43 in the glial scar following photothrombotic ischemic injury. Mol Cell Neurosci 35, 89–99.

Hauser, K., Hahn, Y., Adjan, V., Zou, S., Buch, S., Nath, A., Bruce-Keller, A., Knapp, P., 2009. HIV-1 Tat and morphine have interactive effects on oligodendrocyte survival and morphology. Glia 194–206.

Hayhurst, C.J., Durieux, M., 2016. Differential Opioid Tolerance and Opioid-induced

Hyperalgesia. Anesthesiology 124.

Hellmuth, J., Valcour, V., Spudich, S., 2015. CNS reservoirs for HIV : implications for eradication. J. Virus Erad. 1, 67–71.

Herbein, G., Gras, G., Khan, K., Abbas, W., 2010. Macrophage signaling in HIV-1 infection. Retrovirology 7.

Hilburger, M., Adler, M., Truant, A., Meissler, J.J., Satishchandran, V., Rogers, T., Eisenstein, T., 1997. Morphine induces sepsis in mice. J Infect Dis 176, 183–188.

Hill, R., Dewey, W.L., Kelly, E., Henderson, G., 2018. Oxycodone-induced Tolerance to Respiratory Depression: Reversal by Ethanol, Pregabalin, and Protein Kinase C Inhibition. Br. J. Pharmacol.

Hill, R., Lyndon, A., Withey, S., Roberts, J., Kershaw, Y., MacLachlan, J., Lingford-Hughes, A., Kelly, E., Bailey, C., Hickman, M., Henderson, G., 2016. Ethanol Reversal of Tolerance to the Respiratory Depressant Effects of Morphine. Neuropsychopharmacology 41, 1–37. doi:10.1038/npp.2015.201

Ho, W., Guo, C., Yuan, C., Douglas, S., Moss, J., 2003. Methylnaltrexone antagonizes opioid-mediated enhancement of HIV infection of human blood mononuclear phagocytes. Pharmacol Exp Ther 307, 1158–62.

Homji, N., Vigorito, M., Chang, S., 2012. Morphine-induced conditioned place preference and associated behavioural plasticity in HIV-1 transgenic rats. Int Clin Exp Med. 105–123.

Hull, L.C., Gabra, B.H., Bailey, C.P., Henderson, G., Dewey, W.L., 2013. Reversal of morphine analgesic tolerance by ethanol in the mouse. J. Pharmacol. Exp. Ther. 345, 512–9.

doi:10.1124/jpet.112.202184

Hull, L.C., Llorente, J., Gabra, B.H., Smith, F.L., Kelly, E., Bailey, C., Henderson, G., Dewey, W.L., 2010. The Effect of Protein Kinase C and G Protein-Coupled Receptor Kinase Inhibition on Tolerance Induced by -Opioid Agonists of Different Efficacy. Pharmacology 332, 1127–1135. doi:10.1124/jpet.109.161455.ance

Hutson, L., Lebonville, C., Jones, M., Fuchs, R., Lysle, D., 2017. Interleukin-1 signaling in the basolateral amygdala is necessary for heroin-conditioned immunosuppression. Brain Behav Immun 62, 171–179.

Iguchi, M.Y., HAndelsman, L., Bickel, W.K., Griffiths, R.R., 1993. Benzodiazepine and sedative use abuse by methadone maintenance clients. Drug Alcohol Depend 32, 257–266.

Iveson, P., Lindijp, W.E., Parke, D. V, 1971. The Metabolism of Carbenoxolone in the Rat. Xenobiotica 1, 79–95.

Jacob, J.C., Poklis, J.L., Akbarali, H.I., Henderson, G., Dewey, W.L., Virginia, J.C.J., 2017. Ethanol Reversal of Tolerance to the Antinociceptive Effects of Oxycodone and Hydrocodone 45–52.

Jacob, J.C., Sakakibara, K., Mischel, R.A., Henderson, G., Dewey, W.L., Akbarali, H.I., 2018. Ethanol Reversal of Oxycodone Tolerance in Dorsal Root Ganglia Neurons. Mol. Pharmacol. doi:10.1124/mol.117.110775

Jeon, Y.H., Youn, D.H., 2015. Spinal Gap Junction Channels in Neuropathic Pain. Korean J. Pain 28, 231–235.

Jiménez-Velázquez, G., López-Muñoz, F.J., Fernández-Guasti, A., 2010. Parallel anxiolytic-like

and antinociceptive actions of diazepam in the anterior basolateral amygdala and dorsal periaqueductal gray. Brain Res. 1349, 11–20. doi:10.1016/j.brainres.2010.06.014

Jiménez-Velázquez, G., López-Muñoz, F.J., Fernández-Guasti, A., 2008. Participation of the GABA/benzodiazepine receptor and the NO-cyclicGMP pathway in the "antinociceptive-like effects" of diazepam. Pharmacol. Biochem. Behav. 91, 128–133. doi:10.1016/j.pbb.2008.06.021

Johnson, D.W., Glick, S.D., 1993. Dopamine Release and Metabolism in Nucleus Accumbens and Striatum of Morphine-Tolerant and Nontolerant Rats. Biochem. Pharmacol. Regimens, Pretreat. 46, 341–347.

Johnston, I.N., Milligan, E.D., Wieseler-frank, J., Frank, M.G., Zapata, V., Campisi, J., Langer, S., Martin, D., Green, P., Fleshner, M., Leinwand, L., Maier, S.F., Watkins, L.R., 2004. A Role for Proinflammatory Cytokines and Fractalkine in Analgesia , Tolerance , and Subsequent Pain Facilitation Induced by Chronic Intrathecal Morphine. J. Neurosci. 24, 7353–7365. doi:10.1523/JNEUROSCI.1850-04.2004

Jones, J.D., Mogali, S., Comer, S.D., 2012. Polydrug abuse: A review of opioid and benzodiazepine combination use. Drug Alcohol Depend. 125, 8–18. doi:10.1016/j.drugalcdep.2012.07.004.Polydrug

Kalivas, P.W., Duffy, P., 1987. Sensitization to Repeated Morphine Injection in the Rat : Possible Involvement of A10 Dopamine Neurons. J. Pharmacol. Exp. Ther. 204–212.

Kang, M., Mischel, R., Bhave, S., Komla, E., Cho, A., Huang, C., Dewey, W., Akbarali, H., 2017. The effect of gut microbiome on tolerance to morphine mediated antinociception in mice. Sci Rep.

Keswani, S., Pardo, C., Cherry, C., Hoke, A., McArthur, J., 2002. HIV-associated sensory neuropathies. AIDS.

Kieffer, B., Befort, K., Gaveriaux-Ruff, C., Hirth, C., 1992. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 12048–12052.

King, J., Eugenin, E., Buckner, C., Berman, J., 2006. HIV tat and neurotoxicity. Microbes Infect. 1347–1357.

Kleber, H.D., Gold, M.S., 1978. Use of psychotropic drugs in treatment of methadone maintained narcotic addict. New York Acad. Sci.

Kreek, M.J., Bart, G., Lilly, C., Laforge, K.S., Nielsen, D.A., 2005. Pharmacogenetics and Human Molecular Genetics of Opiate and Cocaine Addictions and Their Treatments 57, 1–26. doi:10.1124/pr.57.1.1.1

Kremer, M., Salvat, E., Muller, A., Yalcin, I., Barrot, M., 2016. Antidepressants and gabapentinoids in neuropathic pain. Neuroscience. 183–206.

Kumar, R., Orsoni, S., Norman, L., Verma, A., Tirado, G., Giavedoni, L., Staprans, S., Miller, G., Buch, S., A., K., 2006. Chronic morphine exposure causes pronounced virus replication in cerebral compartment and accelerated onset of AIDS in SIV/SHIV-infected Indian rhesus macaques. Virology 192–206.

Kwiatkowski, K., Piotrowska, A., Rojewska, E., Makuch, W., Jurga, A., Slusarczyk, J., Trojan, E., Basta-Kaim, A., Mika, J., 2016. Beneficial properties of maraviroc on neuropathic pain development and opioid effectiveness in rats. Prog Neuropsychopharmacol Biol Psychiatry

68–78.

Lau, B.K., Vaughan, C.W., Lau, B.K., Vaughan, C.W., 2014. Descending modulation of pain : The GABA disinhibition hypothesis of analgesia ScienceDirect Descending modulation of pain : the GABA disinhibition hypothesis of analgesia. Curr. Opin. Neurobiol. 29, 159–164. doi:10.1016/j.conb.2014.07.010

Lau, C.G., Zukin, R.S., 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Neuroscience 8, 413–427. doi:10.1038/nrn2153

Lee, I., Lindqvist, E., Kiehn, O., Widenfalk, J., Olson, L., 2005. Glial and neuronal connexin expression patterns in the rat spinal cord during development and following injury. J Comp Neurol.

Leibrand, C., Paris, J., Ghandour, M., Knapp, P., Kim, W., Hauser, K., McRae, M., 2017. HIV-1 Tat disrupts blood-brain barrier integrity and increases phagocytic perivascular macrophages and microglia in the dorsal striatum of transgenic mice. Neurosci Lett 136–143.

Leshchenko, Y., Likhodii, S., Yue, W., Burnham, W.M., Velazquez, J.L.P., 2006. Carbenoxolone does not cross the blood brain barrier: an HPLC study. BMC Neurosci. 3, 3–5. doi:10.1186/1471-2202-7-3

Li, F., Liu, L., Cheng, K., Chen, Z., Cheng, J., 2017. The Use of Stem Cell Therapy to Reverse Opioid Tolerance 0, 1–4. doi:10.1002/cpt.959

Li, W., Li, Y., Zhu, S., Ji, Q., Shu, Y., Zhang, L., Liu, J., 2015. Rosuvastatin attenuated the existing morphine tolerance in rats with L5 spinal nerve transection through inhibiting

activation of astrocytes. Neurosci. Lett. 584, 314–319. doi:10.1016/j.neulet.2014.11.003

Li, Y., Merrill, J., Mooney, K., Song, L., Wang, X., Guo, C., Savani, R., Metzger, D., Douglas, S., WZ, H., 2003. Morphine enhances HIV infection of neonatal macrophages. Pediatr Res. 282–288.

Lin, H.-Y., Law, P.-Y., Loh, H.H., 2012. Activation of protein kinase C (PKC)α or PKCε as an approach to increase morphine tolerance in respiratory depression and lethal overdose. J. Pharmacol. Exp. Ther. 341, 115–25. doi:10.1124/jpet.111.188235

Llorente, J., Withey, S., Rivero, G., Cunningham, M., Cooke, A., Saxena, K., McPherson, J., Oldfield, S., Dewey, W.L., Bailey, C.P., Kelly, E., Henderson, G., 2013. Ethanol reversal of cellular tolerance to morphine in rat locus coeruleus neurons. Mol. Pharmacol. 84, 252–60. doi:10.1124/mol.113.085936

Mahajan, S., Aalinkeel, R., Reynolds, J., Nair, B., Fernandez, S., Schwartz, S., Nair, M., n.d. Morphine exacerbates HIV-1 viral protein gp120 induced modulation of chemokine gene expression in U373 astrocytoma cells. Curr HIV Res 3, 277–288.

Mahajan, S., Schwartz, S., Aalinkeel, R., Chawda, R., Sykes, D., Nair, M., n.d. Morphine modulates chemokine gene regulation in normal human astrocytes. Clin Immunol 323–332.

Malik, S., Khalique, H., Buch, S., Seth, P., 2011. A growth factor attenuates HIV-1 Tat and morphine induced damage to human neurons: implication in HIV/AIDS-drug abuse cases. PLoS One.

Malik, S., Theis, M., Eugenin, E.A., 2017. Connexin43 Containing Gap Junction Channels Facilitate HIV Bystander Toxicity : Implications in NeuroHIV. Front. Mol. Neurosci. 10, 1–

10. doi:10.3389/fnmol.2017.00404

Malvar, J., Vaida, F., Sanders, C., Atkinson, J., Bohannon, W., Keltne, J., Robinson-Papp, J., Simpson, D., Marra, C., Clifford, D., Gelman, B., Fan, J., Grant, I., Ellis, R., 2015. Predictors of new-onset distal neuropathic pain in HIV-infected individuals in the era of combination antiretroviral therapy. Pain 731–739.

Mantegazza, P., Parenti, M., Tammiso, R., Vita, P., Zambotti, F., Zonta, N., 1982. MODIFICATION OF THE ANTINOCICEPTIVE EFFECT OF MORPHINE BY CENTRALLY ADMINISTERED DIAZEPAM AND MIDAZOLAM â€TM I. Br. J. Pharmacol. 75, 569–572.

Martin-Blondel, G., Brassat, D., Bauer, J., Lassmann, H., Liblau, R., 2016. CCR5 blockade for neuroinflammatory diseases--beyond control of HIV. Nat Rev Neurol 12, 95–105.

Martin, W., EAdes, C., Thompson, J., Huppler, R., Gilbert, P., 1976. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther.

Maung, R., Medders, K., Sejbuk, N., Desai, M., Russo, R., Kaul, M., 2012. Genetic knockouts suggest a critical role for HIV co-receptors in models of HIV gp120-induced brain injury. J Neuroimmune Pharmacol 7, 306–318.

Mccracken, C.B., Roberts, D.C.S., 2006. Neuronal Gap Junctions: Expression, Function , and Implications for Behavior 73, 125–151. doi:10.1016/S0074-7742(06)73004-5

McLaughlin, J., Ganno, M., Eans, S., Mizrachi, E., Paris, J., 2014. HIV-1 Tat protein exposure potentiates ethanol reward and reinstates extinguished ethanol-conditioned place preference.

Curr HIV Res 415–423.

Mediouni, S., Jablonski, J., Paris, J., Clementz, M., Thenin-Houssier, S., McLaughlin, J., Valente, S., 2015. Didehydro-cortistatin A inhibits HIV-1 Tat mediated neuroinflammation and prevents potentiation of cocaine reward in Tat transgenic mice. Curr HIV Res 13, 64–79.

Meier, B., 2003. Pain killer : a “wonder” drug’s trail of addiction and death.

Meng, F., Xie, G., Thompson, R., Mansour, A., Goldstein, A., Watson, S., Akil, H., 1993. Cloning and pharmacological characterization of a rat kappa opioid receptor. Proc Natl Acad Sci USA 90, 9954–9958.

Meng, J., Yu, H., Ma, J., Wang, J., Banerjee, S., Charboneau, R., Barke, R., Roy, S., 2013. Morphine induces bacterial translocation in mice by compromising intestinal barrier function in a TLR-dependent manner. PLoS One.

Merlin, J., Tamhane, A., Starrels, J., Kertesz, S., Saag, M., Cropsey, K., 2016. Factors Associated with Prescription of Opioids and Co-prescription of Sedating Medications in Individuals with HIV. AIDS 20, 687–698.

Mirsattari, S., Power, C., Nath, A., 1999. Primary headaches in HIV-infected patients. Headache 39, 3–10.

Miyagi, T., Chuang, L., Doi, R., Carlos, M., Torres, J., Chuang, R., 2000. Morphine induces gene expression of CCR5 in human CEMx174 lymphocytes. J Biol Chem 275.

Moatt, J., Souville, M., Escaffre, N., Obadia, Y., 1998. French general practitioners’ attitudes toward maintenance drug abuse treatment with buprenorphine. Addiction 1567–1575.

Mocchetti, I., Campbell, L., Harry, G., Avdoshina, V., 2013. When human immunodeficiency virus meets chemokines and microglia: neuroprotection or neurodegeneration? Neuroimmune Pharmacol 8, 118–131.

Moradi, S., Charkhpour, M., Ghavimi, H., Motahari, R., Ghaderi, M., 2013. Gap junction blockers : a potential approach to attenuate morphine withdrawal symptoms. J. Biomed. Sci. 20, 1. doi:10.1186/1423-0127-20-77

Naik, A.K., Pathirathna, S., 2008. GABAA receptor modulation in dorsal root ganglia in vivo affects chronic pain after nerve injury. Neuroscience 154, 1539–1553. doi:10.1016/j.neuroscience.2008.04.061

Nath, A., 2015. Eradication of human immunodeficiency virus from brain reservoirs. J Neurovirol 227–234.

Nath, A., Hause, K., Wojna, V., Booze, R., Maragos, W., Prendergast, M., Cass, W., Turchan, J., 2002. Molecular basis for interactions of HIV and drugs of abuse. J Acquir Immune Defic Syndr 31.

National Treatment Agency for Substance Misuse [NTA], 2007. Does the combined use of heroin or methadone and other substances increase the risk of overdose? Res. Brief. 27.

Neff, C., Ndolo, T., Tandon, A., Habu, Y., Akkina, R., 2010. Oral pre-exposure prophylaxis by antiretrovirals raltegravir and maraviroc protects against HIV-1 vaginal transmission in a humanized mouse model. PLoS One 5.

Nielsen, C.K., Ross, F.B., Lotfipour, S., Saini, K.S., Edwards, S.R., Smith, M.T., 2007. Oxycodone and morphine have distinctly different pharmacological profiles: Radioligand

binding and behavioural studies in two rat models of neuropathic pain. Pain 132, 289–300. doi:10.1016/j.pain.2007.03.022

O'Carroll, S., Alkadhi, M., Nicholson, L., Green, C., 2008. Connexin 43 mimetic peptides reduce swelling, astrogliosis, and neuronal cell death after spinal cord injury. Cell Commun Adhes 15, 27–42.

Pannasch, U., Vargová, L., Reingruber, J., Ezan, P., Holcman, D., Giaume, C., 2011. Astroglial networks scale synaptic activity and plasticity 108, 8467–8472. doi:10.1073/pnas.1016650108

Paris, J., Carey, A., Shay, C., Gomes, S., He, J., JP, M., n.d. Effects of conditional central expression of HIV-1 tat protein to potentiate cocaine-mediated psychostimulation and reward among male mice. Neuropsychopharmacology 39, 380–389.

Paris, J., Eans, S., Mizrachi, E., Reilley, K., Ganno, M., McLaughlin, J., 2013. Central administration of angiotensin IV rapidly enhances novel object recognition among mice. Neuropharmacology 70, 247–253.

Paris, J., Singh, H., Carey, A., JP, M., 2015. Exposure to HIV-1 Tat in brain impairs sensorimotor gating and activates microglia in limbic and extralimbic brain regions of male mice. Behav Brain Res 209–218.

Paris, J., Singh, H., Ganno, M., Jackson, P., McLaughlin, J., 2014. Anxiety-like behavior of mice produced by conditional central expression of the HIV-1 regulatory protein, Tat. Psychopharmacology (Berl). 2349–2360.

Pasternack, G., 2010. Molecular Insights Into μ Opioid Pharmacology: From the Clinic to the

Bench. Clin J Pain 26, 1–15. doi:10.1097/AJP.0b013e3181c49d2e.Molecular

Pasternak, G.W., 2018. Mu Opioid Pharmacology : 40 Years to the Promised Land, 1st ed, Advances in Pharmacology. Elsevier Inc. doi:10.1016/bs.apha.2017.09.006

Paulozzi, L.J., Jones, C.M., Mack, K.A., Rudd, R.A., 2011. CDC: Morbidity and Mortality Weekly Report 60.

Phillips, D.M., Chapman, C.R., 2000. JCAHO Pain Management Standards Are Unveiled 284.

Piepponen, T.P., Honkanen, A., Kivastik, T., Zharkovsky, A., Turtia, A., Mikkola, J.A. V, Ahtee, L., 1999. Involvement of Opioid mu 1 -Receptors in Opioid-Induced Acceleration of Striatal and Limbic Dopaminergic Transmission 63, 245–252.

Pinna, G., Agis-balboa, R.C., Zhubi, A., Matsumoto, K., Grayson, D.R., Costa, E., Guidotti, A., 2006. Imidazenil and diazepam increase locomotor activity in mice exposed to protracted social isolation 103, 4275–4280.

Poklis, J.L., Thompson, C.C., Long, K.A., Lichtman, A.H., Poklis, A., 2011. Disposition of Cannabichromene, Cannabidiol, and Δ9- Tetrahydrocannabinol and its Metabolites in Mouse Brain following Marijuana Inhalation Determined by High Performance Liquid Chromatography–Tandem Mass Spectrometry 34, 516–520.

Porreca, F., A, C., Ragga, R., Tallarida, R., 1982. Tolerance and cross-tolerance studies with morphine and ethylketocyclazocine. J Pharm Pharmacol.

Portoghese, P., Akgün, E., Lunzer, M., 2017. Heteromer Induction: An Approach to Unique Pharmacology? ACS Chem Neurosci 426–428.

Puig, S., Gutstein, H.B., 2017. A “ tail ” of opioid receptor variants. J. Clin. Invest. 127, 1221–

1224.

R, K., C, T., Y, Y., I, R., M, M., S, S., RM, D., E, K., Stephens, E., Kumar, A., 2004. No TitleModulation by morphine of viral set point in rhesus macaques infected with simian immunodeficiency virus and simian-human immunodeficiency virus. J Virol 11425–11428.

Rahim, R., Feng, P., Meissler, J., Rogers, T., Zhang, L., Adler, M., Eisenstein, T., 2004. Paradoxes of immunosuppression in mouse models of withdrawal. J Neuroimmunol 147, 114–120.

Rahim, R., Meissler, J., Zhang, L., Adler, M., Rogers, T., Eisenstein, T., 2003. Withdrawal from morphine in mice suppresses splenic macrophage function, cytokine production, and costimulatory molecules. J Neuroimmunol 16–27.

Rattan, A.K., McDonald, J.S., Tejwani, G.A., 1991. Differential effects of intrathecal midazolam on morphine-induced antinociception in the rat: role of spinal opioid receptors. Anesth Analg 73, 124–131.

Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J 22, 659–661.

Rivera, I., García, Y., Gangwani, M., Noel, R.J., Maldonado, L., Kumar, A., Rivera-Amill, V., 2013. Identification and molecular characterization of SIV Vpr R50G mutation associated with long term survival in SIV-infected morphine dependent and control macaques. Virology 144–151.

Rodgers, R.J., Randall, J.., 1987. Are the Analgesic Effects of Social Defeat Mediated by Benzodiazepine Receptors ? Physiol. Behav. 41, 279–289.

Rosland, J.H., Hunskanr, S., Hole, K., 1990. Diazepam Attenuates Morphine Antinociception Tes6Dependently i n Mice. Basic Clin. Pharmacol. Toxicol. 66, 382–386.

Ross, G., Gabra, B., Dewey, W., Akbarali, H., 2008. Morphine tolerance in the mouse ileum and colon. J Pharmacol Exp Ther 561–572.

Ross, J., Darke, S., 2000. The nature of benzodiazepine dependence among heroin users in Sydney, Australia. Addiction 95, 1785–1793.

Roux, P., Carrieri, M., Ville, s V, Dellamonica, P., Poizot-Martin, I., Ravaux, I., Spire, B., 2008. The impact of methadone or buprenorphine treatment and ongoing injection on highly active antiretroviral therapy (HAART) adherence: evidence from the MANIF2000 cohort study. Addiction 1828–1836.

Rudd, R.A., Seth, P., David, F., Scholl, L., 2016. Increases in Drug and Opioid-Involved Overdose Deaths — United States , 2010 – 2015 65, 2010–2015.

Sambamoorthi, U., Warner, L., Crystal, S., Walkup, J., 2000. Drug abuse, methadone treatment, and health services use among injection drug users with AIDS. Drug Alcohol Depend 77–89.

Saurer, T., Ijames, S., Carrigan, K., Lysle, D., 2008. Neuroimmune mechanisms of opioid mediated conditioned immunomodulation. Brain Behav Immun 22, 89–97.

Schier, C., Marks, W., Paris, J., Barbour, A., McLane, V., Maragos, W., McQuiston, A., Knapp, P., Hauser, K., 2017. Selective Vulnerability of Striatal D2 versus D1 Dopamine Receptor-Expressing Medium Spiny Neurons in HIV-1 Tat Transgenic Male Mice. J Neurosci 5758–5769.

Schulz, R., Wüster, M., Kreness, H., Herz, A., 1980. Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. Nature 285, 242–243.

Semenova, S., Kuzmin, A., Zvartau, E., 1995. Strain differences in the analgesic and reinforcing action of morphine in mice. Pharmacol Biochem Behav 17–21.

Shen, C.., Tsai, R.., Shih, M.., Lin, S.L., Tai, Y.., Chien, C.C., 2011. Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats. Anesth Analg 454–459.

Shen, N., Mo, L., Hu, F., Chen, P., Guo, R., Feng, J., 2014. A Novel Role of Spinal Astrocytic Connexin 43 : Mediating Morphine Antinociceptive Tolerance by Activation of NMDA Receptors and Inhibition of Glutamate Transporter-1 in Rats 20, 728–736. doi:10.1111/cns.12244

Shippenberg, T., Chefer, V., Thompson, A., 2009. Delta-opioid receptor antagonists prevent sensitization to the conditioned rewarding effects of morphine. Biol Psychiatry 65, 169–174.

Sierralta, F., Miranda, H.F., 1992. Analgesic effect of benzodiazepines and flumazenil. Gen. Pharmacol. 23, 739–742. doi:10.1016/0306-3623(92)90158-G

Smith, F.L., Gabra, B.H., Smith, P.A., Redwood, M.C., Dewey, W.L., 2007. Determination of the role of conventional , novel and atypical PKC isoforms in the expression of morphine tolerance in mice 127, 129–139. doi:10.1016/j.pain.2006.08.009

Smith, F.L., Javed, R.R., Smith, P.A., Dewey, W.L., Gabra, B.H., 2006. PKC and PKA inhibitors reinstate morphine-induced behaviors in morphine tolerant mice 54, 474–480.

doi:10.1016/j.phrs.2006.09.007

Smith, F.L., Lohmann, A.B., Dewey, W.L., 1999. Involvement of phospholipid signal transduction pathways in morphine tolerance in mice. Br. J. Pharmacol. 128, 220–6. doi:10.1038/sj.bjp.0702771

Söhl, G., Maxeiner, S., Willecke, K., 2005. Expression and Functions of Neuronal Gap Junctions. Nat. Rev. Neurosci. doi:10.1038/nrn1627

Song, C., Rahim, R., Davey, P., Bednar, F., Bardi, G., Zhang, L., Zhang, N., Oppenheim, J., Rodgers, T., 2011. Protein kinase Czeta mediates micro-opioid receptor-induced cross desensitization of chemokine receptor CCR5. J Biol Chem 20354–65.

Song, P., Zhao, Z., 2001. The involvement of glial cells the development of morphine tolerance. Neurosci Res.

Sorrell, M., Hauser, K., 2014. Ligand-gated purinergic receptors regulate HIV-1 Tat and morphine related neurotoxicity in primary mouse striatal neuron-glia co-cultures. J Neuroimmune Pharmacol. 9, 233–244.

Spataro, L.E., Sloane, E.M., Milligan, E.D., Wieseler-frank, J., Schoeniger, D., Jekich, B.M., Barrientos, R.M., Maier, S.F., Watkins, L.R., 2004. Spinal Gap Junctions: Potential Involvement in Pain Facilitation. J. Pain 5, 392–405. doi:10.1016/j.jpain.2004.06.006

Spyraki, C., Papadopoulou, Z., 1980. Effects of Diazepam-Infrasounds Combination on Locomotor Activity and Avoidance Behaviour of Rats 12, 767–771.

Stafford, K., Gomes, A.B., Shen, J., Yoburn, B.C., 2001. m -Opioid receptor downregulation contributes to opioid tolerance in vivo 69, 233–237.

Steele, A., Henderson, E., Rogers, T., 2003. Mu-opioid modulation of HIV-1 coreceptor expression and HIV-1 replication. Virology 99–107.

Stitzer, M., Griffiths, R., McLellan, A., Grabowski, J., JW, H., 1981. Diazepam use among methadone maintenance patients: Patterns and dosages. Drug Alcohol Depend 189–99.

Suadicani, S.O., Cherkas, P.S., Zuckerman, J., Smith, D.N., Spray, D.C., Hanani, M., Suadicani, S.O., Cherkas, P.S., Zuckerman, J., Smith, D.N., Spray, D.C., Hanani, M., 2010. Bidirectional calcium signaling between satellite glial cells and neurons in cultured mouse trigeminal ganglia. Neuron Glia Biol 43–51. doi:10.1017/S1740925X09990408

Suzuki, S., Carlos, M., Chuang, L., Torres, J., Doi, R., Chuang, R., n.d. Methadone induces CCR5 and promotes AIDS virus infection. FEBS Lett. 173–7.

Suzuki, S., Chuang, A., Chuang, L., Doi, R., Chuang, R., n.d. Morphine promotes simian acquired immunodeficiency syndrome virus replication in monkey peripheral mononuclear cells: induction of CC chemokine receptor 5 expression for virus entry. J Infect Dis. 1826–1829.

Szabo, I., Chen, X., Xin, L., Adler, M., Howard, O., Oppenheim, J., Rogers, T., 2002. Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. Proc Natl Acad Sci USA 10276–10281.

Szabo, I., Wetzel, M., Zhang, N., Steele, A., Kaminsky, D., Chen, C., Liu-Chen, L., Bednar, F., Henderson, E., Howard, O., Oppenheim, J., Rogers, T., 2003. Selective inactivation of CCR5 and decreased infectivity of R5 HIV-1 strains mediated by opioid-induced heterologous desensitization. J Leukoc Biol 1074–1082.

Szczytkowski, J., Fuchs, R., Lysle, D., 2011. Ventral tegmental area-basolateral amygdala nucleus accumbens shell neurocircuitry controls the expression of heroin-conditioned immunomodulation. J Neuroimmunol 237, 47–56.

Tan, K.R., Uwe, R., Lüscher, C., 2011. Hooked on benzodiazepines: GABAA receptor subtypes and addiction. Trends Neurosci 34, 188–197. doi:10.1016/j.tins.2011.01.004.Hooked

Thompson, R., Mansour, A., Akil, H., Watson, S., 1993. Cloning and pharmacological characterization of a rat mu opioid receptor. Neuron 11, 903–913.

Tiseo, P.J., Inturrisi, E., 1992. Attenuation and Reversal of Morphine Tolerance by the Competitive N-Methyl-D-Aspartate Receptor Antagonist ,. J. Pharmacol. Exp. Ther. 264, 1090–1096.

Trafton, J.A., Basbaum, A.I., 2004. [D-ALA2,N-MEPHE4,GLY-OL5] enkephalin-induced internalization of the mu Opioid receptor in the Spinal Cord of Morphine Tolerant Rats. Neuroscience 125, 541–543. doi:10.1016/j.neuroscience.2004.02.019

Trujillo, K.A., Akil, H., 1991. Inhibition of Morphine Tolerance and Dependence by the NMDA Receptor Antagonist MK-801. Science (80-.).

Tsao, P., Zastrow, M. Von, 2000. Downregulation of G protein-coupled receptors Patricia Tsao and Mark von Zastrow *. Curr. Opin. Neurobiol. 10, 365–369.

Turchan-Cholewo, J., Dimayuga, F., Gupta, S., Keller, J., Knapp, P., Hauser, KF, Bruce-Keller, A., 2009. No TitleMorphine and HIV-Tat increase microglial-free radical production and oxidative stress: possible role in cytokine regulation. J Neurochem 108, 202–215.

Vessey, J.P., Lalonde, M.R., Mizan, H.A., Welch, N.C., Kelly, M.E.M., Barnes, S., John, P.,

Lalonde, M.R., Mizan, H.A., Nicole, C., Kelly, M.E.M., Carbenoxolone, S.B., 2004. Carbenoxolone Inhibition of Voltage-Gated Ca Channels and Synaptic Transmission in the Retina. J Neurophysiol 1252–1256.

Wang, Z., Trillo-Pazos, G., Kim, S., Canki, M., Morgello, S., Sharer, L., Gelbard, H., Su, Z., Kang, D., Brooks, A., Fisher, P., Volsky, D., 2004. Effects of human immunodeficiency virus type 1 on astrocyte gene expression and function: potential role in neuropathogenesis. J. Neurovirol.

Wetzel, M., Steele, A., Eisenstein, T., Adler, M., Henderson, E., Rogers, T., 2000. μ-Opioid induction of monocyte chemoattractant protein-1, RANTES, and IFN-γ inducible protein-10 expression in human peripheral blood mononuclear cells. J Immunol 165, 6519–6524.

White, J.M., Irvine, R.J., 1999. Mechanisms of fatal opioid overdose. Addiction 94, 961–72. doi:10.1046/j.1360-0443.1999.9479612.x

Williams, J., Haller, V., Stevens, D., Welch, S., 2008. Decreased basal endogenous opioid levels in diabetic rodents: effects on morphine and delta-9-tetrahydrocannabinoid-induced antinociception. Eur J Pharmacol 78–86.

Williams, J.T., Ingram, S.L., Henderson, G., Chavkin, C., Zastrow, M. Von, Schulz, S., Koch, T., Evans, C.J., Christie, M.J., 2013. Regulation of m -Opioid Receptors : Desensitization , Phosphorylation , Internalization , and Tolerance 223–254.

Woody, G., Bruce, D., Korthuis, P., Chhatre, S., Poole, S., Hillhouse, M., Jacobs, P., Sorensen, J., Saxon, A., Metzger, D., Ling, W., 2014. HIV risk reduction with buprenorphine-naloxone or methadone: findings from a randomized trial. J Acquir Immune Defic Syndr 66, 288–293.

Yuan, Y., Arnatt, C., El-Hage, N., Dever, S., Jacob, J., Selley, D., Hauser, K., Zhang, Y., 2013. Bivalent Ligand Targeting the Putative Mu Opioid Receptor and Chemokine Receptor CCR5 Heterodimers: Binding Affinity versus Functional Activities. Medchemcomm. 4, 847–851.

Zacharias, M., Hunter, K., Parkinson, R., 1996. Respiratory effects of intravenous midazolam. New Zeal. Dent. J.

Zacny, J.P., Gutierrez, S., 2009. Within-subject comparison of the psychopharmacological profiles of oral hydrocodone and oxycodone combination products in non-drug-abusing volunteers 101, 107–114. doi:10.1016/j.drugalcdep.2008.11.013

Zhang, N., Rogers, T., Caterina, M., Oppenheim, J., 2004. Proinflammatory chemokines, such as C-C chemokine ligand 3, desensitize mu-opioid receptors on dorsal root ganglia neurons. J Immunol 174, 594–599.

Zhu, X., Sun, W., Li, X., Tan, S., Zhang, X., 2015. Effects of spatial memory on morphine CPP and locomotor sensitization in mice. Physiol Behav 149, 187–191.

Zilliox, L., 2017. Neuropathic Pain.

Zou, S., Fitting, S., Hahn, Y., Welch, S., El-Hage, N., Hauser, K., Knapp, P., 2011. Morphine potentiates neurodegenerative effects of HIV-1 Tat through actions at μ-opioid receptor expressing glia. Brain 134, 3616–3631.

Appendix A

Table 3: Active and Passive Cell Properties of DRG Neurons in Response to 3μM Morphine						
	PP + Saline#	**+ 3 uM Morphine#**	**MP + Saline#**	**+ 3 uM Morphine#**	**MP + CBX**	**+ 3 uM Morphine**
CMem (pF)	18.5 ± 1.6		15.8 ± 1.9		21.0 ± 1.7	
Rseries (MΩ)	8.6 ± 0.8		9.1 ± 0.8		10.8 ± 2.1	
VRest (mV)	−58.2 ± 1.6	−57.0 ± 2.1	−58.8 ± 2.8	−59.8 ± 2.8	−55.0 ± 1.4	−53.4 ± 1.3
AP VThresh (mV)	−13.2 ± 2.1	−3.4 ± 4.3***	−20.7 ± 2.3	−21.6 ± 3.2	−16.6 ± 2.2	−11.8± 2.1***
AP VPeak (mV)	51.6 ± 6.0	53.4 ± 5.6	43.3 ± 6.4	58.0 ± 3.4	56.1 ± 3.4	59.5 ± 6.3
Rheobase (nA)	0.15 ± 0.06	0.21 ± 0.07	0.05 ± 0.02	0.03 ± 0.01	0.23 ± 0.02	0.3 ± 0.05
RInput (MΩ)	479.4 ± 158.3	257.5 ± 62.8	422.8 ± 132.2	671.7 ± 41.67	413.9 ± 56.4	382.5 ± 63.4

Table 3. Changes in various parameters were analyzed at baseline (0 minutes) and 10 minutes following the application of an external solution containing 3μM morphine. Threshold potential (VThresh) was significantly reduced in response to morphine in all treatment groups except in the neurons isolated from the morphine pelleted mice. Membrane capacitance (CMem), resting membrane potential (VRest), peak action potential height (AP VPeak), rheobase, and input resistance (RInput) were all unaffected by 3 μM morphine. #PP + SAL and MP + SAL groups have been previously published by our laboratory (Kang & Mischel et al., 2017). ***p<0.001

Vita

Maciej Marcin Gonek was born on March 21st, 1991 in Bergen County, New Jersey and is an American and Polish citizen. He graduated from University of North Carolina at Chapel Hill with a Bachelor of Science in Psychology in 2013. He was accepted in Indiana University's Research Experience for Undergraduates (REU) program and investigated the role of the CB1 cannabinoid receptor in other drugs of abuse preference. Entering the graduate program at Virginia Commonwealth University in August 2013, he joined Dr. William L. Dewey's laboratory investigating the mechanisms of opioid tolerance. He served as the Vice President of the Pharmacology and Toxicology Student Organization from 2015-2016 and served as the Chair of the Medical Science section for Virginia Academy of Sciences from 2015-2017. He has taught undergraduates in the Biology of Drugs (BIOL-452) course in Fall 2017 at VCU.

Publications

Gonek M, McLane VD, Stevens DL, Lippold K, Akbarali HI, Knapp PE, Dewey WL, Hauser KF, Paris JJ. "CCR5 mediates HIV-1 Tat-induced neuroinflammation and influences morphine tolerance, dependence, and reward." 2017. Brain Behav Immun.

Gonek M, Akbarali HI, Henderson G, Dewey WL. "Reversal of oxycodone and hydrocodone tolerance by diazepam." 2017. Brain Res.

Marcus DJ, Henderson-Redmond AN, **Gonek M**, Zee ML, Farnsworth JC, Amin RA, Andrews MJ, Kampen JD, Davis B, Mackie K, Morgan DJ. "Mice expressing a "hyper-sensitive" form of the CB1 cannabinoid receptor (CB1) show modestly enhanced alcohol preference and consumption." 2017 PLoS One

Grim TW, Morales AJ, **Gonek MM**, Wiley JL, Thomas BF, Endres GW, Sim-Selley LJ, Selley DE, Negus SS, Lichtman AH. "Stratification of cannabinoid 1 receptor (CB1R) agonist efficacy: Manipulation of CB1R density through use of transgenic mice reveals congruence between in vivo and in vitro assays." 2016. J Pharmacol Exp Ther