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Ethanol Reversal of Oxycodone Tolerances

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

by

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Table of Contents

List of Tables	v
List of Figures	vi
List of Abbreviations	viii
Abstract	x
Chapter 1. Introduction	1
The History of Opioids	1
Opioid Receptors and Endogenous Opioid Ligands	4
Oxycodone and Morphine Metabolism.....	9
Reversal of Opiate Tolerances	10
Scope of Dissertation.....	13
Chapter 2. Ethanol Reversal of Tolerance to the Antinociceptive Effects of Oxycodone and Hydrocodone	14
Summary.....	14
Introduction.....	15
Materials and Methods.....	17
Results.....	21
Discussion	36
Chapter 3. The Effect of Ethanol on Oxycodone Tolerance in Dorsal Root Ganglia Neurons	41
Summary	41
Introduction.....	42

Chapter 3 ('ctd)	
Materials and Methods.....	44
Results.....	47
Discussion.....	69
Chapter 4. General Discussion.....	74
References.....	84
Appendix A.....	94
Vita.....	96

List of Tables

Table 1. Comparison of Tolerance Development and Ethanol Reversal of Various Opioid Compounds.	24
Table 2. Active and Passive Cell Properties of Dorsal Root Ganglia Neurons in Response to 3 μ M Oxycodone.	49

List of Figures

Figure 1. Structural Comparison of Morphine, Thebaine, Oxycodone and Hydrocodone	3
Figure 2. Ethanol Reversal of Oxycodone and Hydrocodone Tolerance	27
Figure 3. Intraperitoneal and Oral Ethanol Reversed Antinociceptive Tolerance to Subcutaneous Oxycodone	29
Figure 4. Oral Oxycodone Time Course: Antinociception vs. Brain Concentration.....	30
Figure 5. Comparing Subcutaneous and Oral Oxycodone: Equal Antinociceptive Effects of Oxycodone Were Not Due to Equal Brain Oxycodone Concentrations.	32
Figure 6. Acute and Chronic Oxycodone Brain Concentrations	34
Figure 7. Acute Oxycodone Reduced DRG Neuronal Activity.....	50
Figure 8. Response to Acute 3 μ M Oxycodone in DRG Neurons.....	51
Figure 9. The Effect of 3 μ M Oxycodone on Individual DRG Neuron Threshold Potentials.....	52
Figure 10. Co-administration of Naloxone Blocked the Acute Effects of Oxycodone	53
Figure 11. Response to 3 μ M Oxycodone in DRG Neurons Treated Overnight with 10 μ M Oxycodone	56
Figure 12. The Effect of 3 μ M Oxycodone on Individual DRG Neuron Threshold Potentials Following Overnight Incubation in 10 μ M Oxycodone	57
Figure 13. Tolerance Developed to Morphine in β -Arrestin 2 WT, but not KO DRG Neurons ...	58
Figure 14. Representative Traces: The Effect of Ethanol on the Response to 3 μ M Oxycodone in DRG Neurons Incubated Overnight with 10 μ M Oxycodone	62
Figure 15. The Effect of Acute Ethanol on Individual DRG Neuron Threshold Potentials Following Overnight Incubation in 10 μ M Oxycodone	63
Figure 16. Control Experiments: Threshold Potential Values in the Absence of Oxycodone	64

Figure 17. Representative Traces: The Effect of the PKC Inhibitor, Bis XI, on the Response to 3 μ M Oxycodone in DRG Neurons Incubated Overnight with 10 μ M Oxycodone.....65

Figure 18. The Effect of the PKC Inhibitor, Bis XI, on Individual DRG Neuron Threshold Potentials Following Overnight Incubation with 10 μ M Oxycodone.....66

Figure 19. Time-Dependent Change in Threshold Potentials to 3 μ M Oxycodone67

List of Abbreviations

AP	Action potential
BARR2	β arrestin 2
BBB	Blood-brain-barrier
CYP	Cytochrome P450 enzyme
DA	Degree of analgesia
DAMGO	([D-Ala ² , N-MePhe ⁴ , Gly-ol]-enkephalin)
DOR	δ -opioid receptor
DRG	Dorsal root ganglia
ED ₅₀	Effective Dose (half-maximal)
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
i.p.	Intraperitoneal injection
JNK	c-Jun N-terminal kinase
KO	Knockout
KOR	κ -opioid receptor
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MOR	μ -opioid receptor

MPE	Maximum possible effect
mRNA	messenger ribonucleic acid
PaG	Periaqueductal gray
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
qPCR	quantitative polymerase chain reaction
s.c.	Subcutaneous injection
WT	Wild type

Abstract

ETHANOL REVERSAL OF OXYCODONE TOLERANCES

By Joanna Caitlin Jacob, PhD

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

Virginia Commonwealth University, 2017

Mentor: William L. Dewey, PhD, Professor and Chair, Pharmacology and Toxicology

Oxycodone is a semi-synthetic opioid originally developed as a safer alternative to morphine. It is commonly prescribed for its pain-relieving effects, but has recently been implicated as a major underlying cause of the current opioid epidemic due to its clinical limitations that include tolerance, dependence and a high abuse liability. Simultaneous consumption of opioids and ethanol has been shown to increase the risk of overdose and death from opioids in opioid-tolerant individuals. We hypothesized that ethanol reversed opioid tolerance and previous studies showed that ethanol reversed morphine tolerance. This dissertation investigated whether ethanol reversed tolerance to other opioids in mice, primarily oxycodone. We found that tolerance developed to the antinociceptive effects of both oxycodone and hydrocodone, and that the same dose of ethanol (1 g/kg i.p.) reversed that tolerance. Oral

ethanol (2 g/kg) also effectively reversed oxycodone tolerance. Ethanol did not significantly alter either acute or chronic oxycodone brain concentrations, suggesting that the reversal effect was mediated by neuronal mechanisms. DRG neurons were isolated from adult mice and the effects of oxycodone were assessed using whole-cell patch clamp electrophysiology experiments. Oxycodone [3 μ M] acutely reduced neuronal excitability as measured by a shift in threshold potentials to a more positive value. DRG neurons incubated overnight with 10 μ M oxycodone did not respond to the 3 μ M oxycodone challenge, indicating tolerance developed within these neurons. To test if ethanol was reversing tolerance through neuronal mechanisms, we incubated DRG neurons overnight with 10 μ M oxycodone and applied 20mM ethanol to the media prior to recording. Tolerance was robustly reversed in these neurons, as indicated by a response to 3 μ M oxycodone. The PKC inhibitor, Bis XI, also reversed oxycodone tolerance.

In these studies we have clearly shown that tolerance develops to oxycodone in both the whole animal in an isolated neuronal preparation. In addition we have shown that the tolerance produced in these two preparations was reversed by ethanol at blood levels similar to those seen in humans. Further we have also included preliminary data that suggest that this reversal of oxycodone tolerance by ethanol may well be due to its actions on PKC.

Chapter 1

Introduction

I. The History of Opioids

It is widely reported that the earliest evidence suggesting human knowledge and use of opioid substances was among the ancient Sumerians, where records of cultivated opium poppy plants (*Papaver somniferum*) date back to 3400 B.C. They described the opium poppy as “hul gil”, or the “joy plant”, due to the sticky latex material that seeps from within the seedpod if scored prior to blooming (Brownstein, 1993). Contained within that latex, known as opium, reside many alkaloid compounds called opiates. In 1805, a German pharmacist named Friedrich Sertürner reported the isolation of the first active alkaloid compound from opium. Originally named “morphium” after the Greek god of dreams, Morpheus, it is better known today as morphine, and is the most abundant opiate found within opium. In fact, it can contribute anywhere from 8 – 14% of the dry opium latex weight (Kapoor, 1996).

Morphine was first injected in the mid 1800s following the invention of the hypodermic needle and syringe, quickly gaining popularity among physicians for use in minor surgical procedures and postoperative pain. It was quite unreliable when given orally, however, and morphine was increasingly being recognized as having an abuse liability similar to opium itself, along with safety concerns due to side effects. This prompted chemists to search for safer opioid compounds that were efficacious, but non-addicting. Their efforts led to the generation of many semi-synthetic opioid compounds that were based on natural opium compounds (such as morphine) as a structural backbone. In 1917, oxycodone was synthesized, followed shortly by hydrocodone, which was reported in 1920. Oxycodone and hydrocodone are considered “opiate”

compounds, since they are derived from, and are structurally similar to, the naturally occurring opium compounds, thebaine and morphine (Figure 1). “Opioid” compounds 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).

Decades passed without much attention directed toward either oxycodone or hydrocodone, until the mid-1990s. Oxycodone was thrust into the limelight when Purdue Pharma L.P. began marketing it as Oxycontin®, claiming it was a long-acting, highly efficacious and safer opiate compound for the relief of acute and chronic pain. These efforts were corroborated by the American Academy of Pain Medicine (AAPM), which endorsed the use of opioids for non-cancerous chronic pain in 1997 (Haddox *et al.*, 1997). Sales of oxycodone increased by 866% over the next ten years, both due to the AAPM endorsement, and also due to the American Pain Society simultaneously introducing the idea in 1996 that pain is the “fifth vital sign”. In addition, this objective (that pain is the fifth vital sign) was adopted in 2000 by the Veterans Health Administration as part of its national pain management strategy (Booss *et al.*, 2000). Sales of other opioids also skyrocketed, with hydrocodone sales up 280% between 1997 and 2007 (Kaplan, 2015). The implementation of prescription opioid use for pain management was well intentioned. However by 2012, it was reported that individuals in the United States consumed more narcotic medications “gram for gram” than any other nation worldwide (Manchikanti *et al.*, 2012). The unfortunate reality was that the primary accomplishment of these efforts to manage the pain crisis instead provided the basis for the current opioid epidemic, which was first recognized by the CDC in 2011.

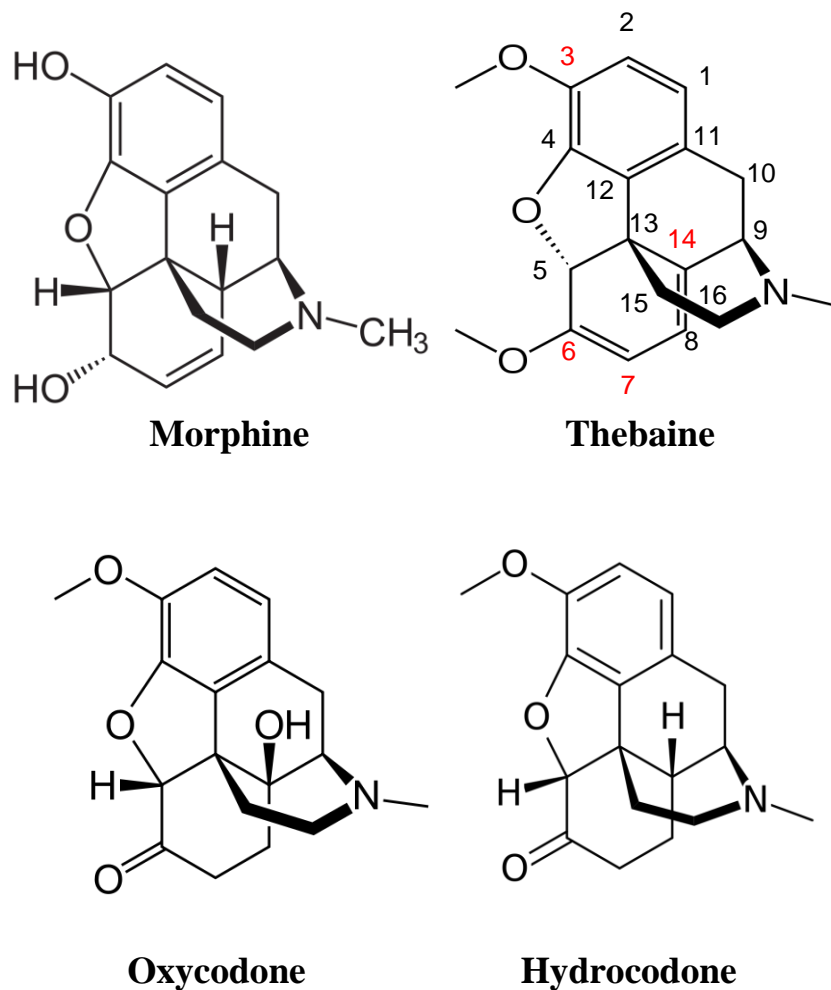


Figure 1. Structural Comparison of Morphine, Thebaine, Oxycodone and Hydrocodone.

Morphine and thebaine are natural compounds contained within the opium poppy plant (*Papaver somniferum*). Oxycodone and hydrocodone are semi-synthetic opioid compounds, consisting of a thebaine backbone with slight structural changes (see red numbered carbons). Oxycodone and hydrocodone each exhibit a loss of the double bond between carbon 6 & 7, and the exchange of a ketone for the ether at carbon 6. Oxycodone contains an additional hydroxy group on carbon 14.

II. Opioid Receptors and Endogenous Opioid Ligands

By the mid 1960s, it was readily hypothesized that not only one, but perhaps multiple opiate receptor types existed due to findings with the various opioid agonists, antagonists and mixed agonist-antagonist compounds available at the time (Brownstein, 1993). Within the following decade, three research groups (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973) independently demonstrated via radiolabeled ligand binding techniques, that there were stereospecific opiate binding sites within the central nervous system of rodents and guinea pigs. Shortly thereafter, further studies in human and primate brain tissues provided evidence that opiate receptor expression and distribution was heterogeneous (Hiller *et al.*, 1973; Kuhar *et al.*, 1973). These discoveries led to the hypothesis that the receptors were targets of endogenous ligands, which gained credence after it was shown that not only did stress from a foot shock induce analgesia in rats, but it was partially blocked by the antagonist, naloxone (Akil, Madden, *et al.*, 1976). The same group tested analgesia following focal electrical stimulation in the brain of rats using the radiant tail flick test as the noxious stimulus. In this study, it was shown that the focal stimulation produced a measurable “degree of analgesia” (DA) to the radiant heat stimulus, and 1 mg/kg naloxone reduced the DA from 100% to 62% (Akil, Mayer, *et al.*, 1976). The results from these studies strongly suggested the existence of endogenous ligands within the brain.

Endogenous opioid ligands were discovered almost immediately, and all are peptide compounds. These peptide compounds can be organized into one of three families: the enkephalins (Hughes *et al.*, 1975), the endorphins (Bradbury *et al.*, 1976; Li *et al.*, 1976) and the dynorphins (Goldstein *et al.*, 1981). Each of these endogenous opioids are products of larger precursor peptides: proenkephalin, proopiomelanocortin and prodynorphin, respectively.

Upwards of twenty endogenous opioid ligand candidates have been identified. There is even evidence to suggest mammalian tissues can synthesize morphine and codeine, with the highest concentrations detected in the spinal cord and adrenal glands of rats (Donnerer *et al.*, 1987). The presence of morphine had also been found in the skin of toads (Oka *et al.*, 1985), suggesting multiple classes of vertebrates may have some ability to synthesize morphine endogenously. Despite the possibility that vertebrates are capable of synthesizing morphine, the concentrations are quite low, and thus the activation and regulation of the endogenous opioid system is considered to be mediated primarily by the release of the endogenous peptide ligands described above.

Given that multiple endogenous peptides had been discovered, it was proposed that more than one opiate receptor existed. Conclusive evidence for the presence of distinct opioid receptors in the central nervous system was first shown in a chronic spinal dog model (Martin WR *et al.*, 1976). Morphine, ketocyclazocine and SKF-10,047 were the three prototypical ligands used to distinguish between various neurophysiological and behavioral properties resulting in the description of the μ , κ and σ opioid receptors, respectively. The acute actions of all three ligands were blocked by “the pure antagonist” naltrexone, confirming that these were agonist compounds. Selective affinity of these ligands for their respective receptor was inferred by establishing that withdrawal symptoms in morphine-treated dogs were suppressed only by morphine, and not by ketocyclazocine. Additionally, ketocyclazocine did not precipitate withdrawal in chronic morphine-treated dogs, leading the authors to conclude that ketocyclazocine had little affinity for the μ receptor.

Today, the general consensus is that the σ receptor is not an opioid receptor after all. There is, however, a third opioid receptor known as the δ receptor. It wasn't until the enkephalins

were discovered that then led the authors to search for the specific opioid receptor(s) to which these new opioid peptide ligands bound preferentially. Comparisons of the inhibitory effects of opioid peptides on the guinea pig ileum and the mouse vas deferens led to the conclusion that the receptor populations present in these tissues were not identical, and that the actions in the vas deferens were mediated by the δ opioid receptor (Lord *et al.*, 1977). Using selective radiolabeled ligands (Chang and Cuatrecasas, 1979; Chang *et al.*, 1979) and cross-tolerance studies (Schulz *et al.*, 1980; Porreca *et al.*, 1982), the presence of μ (MOR), κ (KOR) and δ (DOR) opioid receptors were clearly demonstrated, and determined to be the predominant three opioid receptor types. These findings were ultimately confirmed when various laboratories successfully cloned each of the receptor subtypes (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Meng *et al.*, 1993; Thompson *et al.*, 1993).

Though there are characteristics that distinguish these receptors from one another, several similarities are shared amongst them. All three receptor types belong to the superfamily of G-protein coupled receptors (GPCR), which in total includes over 1,000 members (Wess, 1997). GPCRs represent the largest class of receptors localized to the cell surface in the mammalian genome and exhibit a hallmark characteristic of seven hydrophobic transmembrane-spanning domains with an extracellular NH₂ terminus and an intracellular COOH terminus. Hydrophilic intracellular and extracellular loops connect the domains to one another. Homology is preserved across the three receptors throughout the transmembrane domains and particularly in the third intracellular loop, which contains the binding site for the G_{i/o} G-protein α subunits (Mansour *et al.*, 1995). It has been shown that the primary role of the G α_i subunit inhibits adenylyl cyclase (Kurose *et al.*, 1983), while the G α_o subunit inhibits voltage-gated Ca²⁺ channels (Hescheler *et al.*, 1987). It has also been shown that both the G α_i and the G α_o subunits contribute directly to

the activation of inwardly-rectifying K⁺ channels in the plasma membrane of neurons (Hescheler *et al.*, 1987) and in cardiac atrial cells (Pfaffinger *et al.*, 1985) without acting through second messengers. Collectively, these actions have been shown to produce a net effect resulting in reduced membrane excitability, particularly following activation of central μ opioid receptors (Neumaier *et al.*, 1988).

Opioid receptors are expressed all throughout the body, however distribution and localization varies. Peripherally, MOR, DOR and KOR can all be found on several tissues including the small and large intestines, adrenal glands, lungs, kidneys, spleen, and on the reproductive organs of both sexes: ovaries and uterus in females and testis in males (Wittert *et al.*, 1996). Interestingly, from that same study, it was also determined that MORs are absent in the stomach, while KORs are absent in the liver of rats.

All three opioid receptor types are also dispersed throughout the central nervous system (Mansour *et al.*, 1995). In some regions, binding localization diverged from mRNA expression, suggesting that opioid receptors can undergo receptor transport to specific presynaptic terminal locations. Regions such as the nucleus accumbens, caudate putamen, amygdala and locus coeruleus contain high levels of all three opioid receptors (Mansour *et al.*, 1994; Arvidsson *et al.*, 1995). MOR and DOR are expressed generally throughout the cortical regions, while KOR is restricted to the entorhinal cortex. The antinociceptive effects of opioids are primarily mediated by μ receptors in the periaqueductal gray (PaG), the spinal cord and in the dorsal root ganglia, however, κ and δ receptors have also been shown to play a role at these spinal and supraspinal levels.

Opioid agonists elicit multiple effects throughout the body, which is not surprising given the vast receptor distribution in peripheral and central sites. The degree of physiological response

following an acute administration depends on many factors, including efficacy, dose and whether the compound is brain-penetrant. For many opioid agonists, however, chronic administration *in vivo* or prolonged exposure *in vitro* leads to tolerance. Tolerance to opioids is intriguing in that it occurs to some, but not all, of the elicited effects. Antinociception, respiratory depression and euphoria are all opioid effects subject to tolerance development, while miosis (excessive pupillary constriction) and inhibition of colonic transit are not. Furthermore, tolerance develops to these various effects at different rates. The fact that tolerance develops to the euphoric effects earlier than the respiratory depressive effects has led many experts to speculate that this discrepancy in tolerance development may be responsible for a number of opioid overdose deaths.

Due to the multiplicity of opioid tolerance expression, it is sometimes discussed in its plural form, as opioid *tolerances*. Currently, multiple mechanisms underlying these opioid tolerances have been identified, and though extensively studied, none are fully understood. Given that opiate receptors belong to the GPCR family, they are all generally susceptible to the classic mechanisms of GPCR tolerance: desensitization, internalization and degradation (Ferguson *et al.*, 1998; Lefkowitz, 1998). The recruitment of various key intracellular proteins such as PKC, GRK, JNK and β -arrestin (1 & 2) each contribute to specific signaling pathways that determine the extent to which desensitization/internalization/degradation takes place (Williams *et al.*, 2013). There are at least two factors that predominantly determine a receptor's fate: 1) the efficacy of the agonist bound, and 2) the cell type on which the receptor is expressed. It is thought that different opioid agonists initiate different receptor conformations upon binding, contributing not only to the efficacy observed, but also the intracellular proteins recruited. Depending on which intracellular proteins are engaged, opioid agonists will vary in their receptor

internalization patterns. The μ opioid receptor is readily internalized when bound by high-efficacy agonists such as DAMGO and etorphine, but partial agonists, such as morphine, do not lead to internalization (Keith *et al.*, 1996, 1998). Tolerance still develops following chronic morphine however, and often involves receptor desensitization, rather than internalization (Keith *et al.*, 1996; Whistler and von Zastrow, 1998).

Oxycodone's influence on μ receptor internalization is just as complicated. In transfected HEK-293 cells, oxycodone acted similarly to morphine in that it did not recruit the GRK-arrestin complex (McPherson *et al.*, 2010), but dissimilarly in that it efficiently evoked μ receptor internalization (Melief *et al.*, 2010). Alternatively, oxycodone was shown to be incapable of evoking μ receptor internalization or desensitization in locus coeruleus neurons (Arttamangkul *et al.*, 2008). In support of that finding, a separate group demonstrated similar results in the spinal cord of mice, where etorphine significantly reduced the μ opioid receptor density (as measured by B_{\max}), but oxycodone did not (Pawar *et al.*, 2007). This discrepancy could certainly be due to the fact that one study focused on *in vitro* approaches using a stably-transfected cell line, while the other was investigating neurons *ex vivo*. Differences such as transfected versus endogenously expressed μ receptors, along with the presence or absence of specific intracellular proteins, could have contributed to the conflicting results reported between the two cell types.

III. Oxycodone and Morphine Metabolism

It is well known that opiates are primarily metabolized in the liver, though enzymes are present in the brain and kidneys, which further contribute to metabolite production. Oxycodone is metabolized in the liver by CYP (cytochrome P450) enzymes. CYP2D6 generates an active metabolite, oxymorphone, from oxycodone, while CYP3A4 generates the main metabolite, noroxycodone (Klimas *et al.*, 2013). These two metabolites can further be metabolized into

noroxymorphone. For a while, the hypothesis stood that these metabolites contributed to oxycodone's antinociceptive effects (Lemberg *et al.*, 2006). Affinity for the μ receptor has been estimated to be up to 60 times greater in the case of oxymorphone compared to oxycodone (Volpe *et al.*, 2011), which had some predicting oxymorphone was the major analgesic compound following oxycodone administration. Two pharmacokinetic findings argue against that possibility; the first is that oxymorphone does not readily cross the blood-brain-barrier (BBB), and secondly, plasma levels do not indicate high circulating concentrations (Lalovic *et al.*, 2006). Additionally, it was recently demonstrated that the antinociceptive properties of oxycodone are most likely mediated by itself (i.e. the parent compound), rather than an active metabolite, due to the negligible contributions produced by either noroxymorphone or oxymorphone toward analgesia (Klimas *et al.*, 2013).

Morphine metabolism is quite different from oxycodone, undergoing glucuronidation rather than CYP-mediated degradation in the liver and brain of both man and rodents (Wahlström *et al.*, 1986; Wahlström, Pacifici, *et al.*, 1988; Wahlström, Winblad, *et al.*, 1988). The primary metabolites of morphine in man are morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), while in rodents only M3G is produced. M3G has essentially no affinity for μ receptors and does not bind to them. M6G however, has high affinity for μ receptors and demonstrated greater antinociceptive activity over morphine, when injected directly into mice (Shimomura *et al.*, 1971). The M6G metabolite of morphine may contribute to longer-lasting antinociceptive effects in man, but morphine itself elicits the antinociceptive effects in rodents.

IV. Reversal of Opiate Tolerances

The tolerance that develops to the antinociceptive and respiratory depressive effects of opiates is susceptible to reversal by a variety of agents. Just as is the case for tolerance

development, none of the mechanisms underlying tolerance reversal are completely understood. To date, reversal of tolerance to the euphoric effects of opiates has not been studied, while studies investigating reversal of tolerance to other opiate effects such as enhanced locomotor activity and inhibition of the GI tract have produced inconclusive results (B. David, K. Lippold, J. Jacob, unpublished observations). Thus far, ethanol, diazepam, and a multitude of PLC, PKC and PKA inhibitors have been reported to reverse morphine tolerances (Smith *et al.*, 1999, 2006; Javed *et al.*, 2004; Hull *et al.*, 2013; Llorente *et al.*, 2013; Hill *et al.*, 2016; Withey *et al.*, 2017). Perhaps the most straightforward mechanism of antinociceptive reversal is through benzodiazepines (i.e. diazepam) given that there is a known target: GABA_A receptors. When the GABA_A inhibitor, bicuculline, was administered it fully blocked diazepam's ability to reverse morphine antinociceptive tolerance, demonstrating that as expected, diazepam's effects were mediated entirely through the GABA_A receptor (Hull *et al.*, 2013). Whether the reversal was due to a direct alteration of GABA_A receptor function or of GABA release has yet to be determined.

The PKA inhibitor, KT-5720, has also been reported to reverse antinociceptive tolerance to morphine, both in the radiant tail flick test and in the hot plate test (Bernstein and Welch, 1997; Javed *et al.*, 2004). Interestingly, in order to completely reverse tolerance to the hypothermic effects of morphine, inhibitors of both PKA and PKC were required. The degree of tolerance to hypothermia was 5 times greater than the degree of tolerance observed to the antinociceptive effects within that study, which may partially explain the need for both types of inhibitors. There is also evidence that the hypothermic effects of opioids may involve contributions from all three opioid receptors, not just actions through the μ receptor (Baker and Meert, 2002).

The majority of studies investigating reversal of morphine tolerance have focused on PKC, using a variety of PKC inhibitors. The inhibitors Gö-6976, Gö-7874, Bisindolylmaleimide I HCl, and sangivamycin have each been reported to reverse morphine antinociceptive tolerance (Smith *et al.*, 1999; Javed *et al.*, 2004; Hull *et al.*, 2010). Gö-6976 also effectively reversed tolerance to morphine in locus coeruleus neurons (Llorente *et al.*, 2013). Expanding on that finding, it was recently reported that the tolerance to morphine's respiratory depressive effects was reversed *in vivo* in mice using tamoxifen and calphostin C, two non-selective yet brain-penetrant PKC inhibitors (Withey *et al.*, 2017). Interestingly, selective inhibitors of PLC have also led to the reversal of morphine tolerance, further suggesting a role of PKC and IP₃ in morphine tolerance expression (Smith *et al.*, 1999).

Not surprisingly, the studies investigating ethanol reversal of morphine tolerance have all conclusively shown that reversal occurred without conclusively determining an underlying mechanism. In the same study where diazepam was tested, the reversal effects of ethanol were partially blocked when either the GABA_A inhibitor bicuculline, or the GABA_B inhibitor, phaclofen was administered. The reversal effect of ethanol on morphine tolerance was fully blocked only after these two inhibitors were administered together (Hull *et al.*, 2013). From this study came the proposed mechanism that ethanol reversed morphine antinociceptive tolerance via actions on both GABA_A and GABA_B receptors. A study investigating ethanol reversal of tolerance to morphine's respiratory depressive effects *in vivo* did not test GABA receptor inhibitors, and instead proposed another mechanism involving an interaction with PKC (Hill *et al.*, 2016). In locus coeruleus neurons, 20 mM ethanol reversed morphine tolerance, but the protein phosphatase inhibitor, okadaic acid, blocked that reversal effect. Furthermore, the authors showed that the reversal of morphine tolerance by ethanol was unaffected by the collective

inhibition of L-type Ca^{2+} channels and GABA_A , glycine, and NMDA receptors (Llorente *et al.*, 2013).

V. Scope of Dissertation and Aims

The overall goal of this study was to investigate ethanol reversal of tolerance to prescription opiates other than morphine, mainly, oxycodone. From these studies, we can begin to understand if and how ethanol interacts with signaling pathways underlying tolerance to multiple opioid compounds. Our findings provide important public health information not only because we are discovering more about how tolerance to opioids works, but also because ethanol and oxycodone are commonly used and abused substances today.

The first aim was to characterize the effect of ethanol reversal of oxycodone and hydrocodone tolerance in the whole animal. The second aim was to determine if ethanol elicited its effects by altering the metabolism or distribution of oxycodone. The third aim was to determine if tolerance to oxycodone was measurable in isolated dorsal root ganglia neurons, and if ethanol could reverse that tolerance.

Chapter 2

Ethanol Reversal of Tolerance to the Antinociceptive Effects of Oxycodone and Hydrocodone

This chapter has been accepted for publication in the Journal of Pharmacology and Experimental Therapeutics (Joanna C. Jacob, Justin L. Poklis, Hamid I. Akbarali, Graeme Henderson and William L. Dewey (2017) “Ethanol Reversal of Tolerance to the Antinociceptive Effects of Oxycodone and Hydrocodone”).

I. Summary

This study compared the development of tolerance and its reversal by ethanol of two orally bioavailable prescription opioids, oxycodone and hydrocodone, to that of morphine. Oxycodone (s.c) was significantly more potent in the mouse tail withdrawal assay than either morphine or hydrocodone. Oxycodone was also significantly more potent in this assay than hydrocodone when administered orally. Tolerance was seen following chronic subcutaneous administration of each of the three drugs and by the chronic administration of oral oxycodone, but not following the chronic oral administration of hydrocodone. Ethanol (1 g/kg i.p.) significantly reversed the tolerance that developed to the subcutaneous administration of each of the three opioids. It took twice as much ethanol when given orally to reverse the tolerance to oxycodone. We investigated whether the tolerance observed to oxycodone and its reversal by ethanol were due to bio-dispositional changes or were reflecting a true neuronal tolerance. As expected, a relationship between brain oxycodone concentrations and activity in the tail immersion test existed following administration of acute oral oxycodone. Following chronic treatment, brain oxycodone concentrations were significantly lower than acute concentrations. Oral ethanol (2 g/kg) reversed the tolerance to chronic oxycodone, but did not alter brain

concentrations of either acute or chronic oxycodone. These studies show that there is a metabolic component of tolerance to oxycodone, however the reversal of that tolerance by ethanol is not due to an alteration of the bio-disposition of oxycodone, but rather is neuronal in nature.

II. Introduction

Prescription opioids such as oxycodone were responsible for over half of the reported 28,000 opioid overdose deaths in 2014 (CDC, 2016). Individuals who abuse prescription opioids often use other substances leading to poly-drug abuse (Ogbu *et al.*, 2015). Ethanol is one of the most commonly co-abused drugs by opioid users, despite the long-standing warning that ethanol and opioids pose a significant health risk when taken together (Karch and Drummer, 2001; Oliver *et al.*, 2007). Multiple post-mortem analyses have shown that individuals who consumed opioids such as heroin along with alcohol died from blood opioid concentrations measuring significantly below than those who died from an opioid without alcohol consumption (Darke and Hall, 2003). Additionally, a separate study that specifically investigated oxycodone-related overdoses reported that deaths resulting from the combined intake of oxycodone and ethanol were ruled to be exclusively accidental, rather than intentional (Thompson *et al.*, 2008). While it is possible that a general lack of awareness exists among opioid users regarding the dangers related to co- consumption of ethanol with opioids, it is likely that these individuals experience some enhancement of opioid effects when used together, leading to riskier drug taking behavior to offset the tolerance(s) developed to the opioids. Post-mortem studies reported findings from blood levels extracted from peripheral sites such as the femoral artery and heart blood. Collection of post- mortem blood is easy to obtain and samples are reliably quantified, however it is important to recognize that the lethal event during opioid overdose is respiratory depression – a centrally-mediated effect controlled primarily in the brainstem where opioid receptor density

is quite high (Delfs *et al.*, 1994; Satoh and Minami, 1995). One hypothesis relating to the increased lethality of oxycodone when ethanol was also detected in presumed opioid-tolerant individuals, is that ethanol is altering the kinetics of oxycodone. Therefore, it was important to investigate if in fact the distribution and concentration of oxycodone in the brain is altered by ethanol administration.

Ethanol is also known to reverse various tolerances to morphine, including the antinociceptive and respiratory depressive effects, which may be explained by mechanisms that involve PKC and GABA_A and GABA_B receptor signaling (Hull *et al.*, 2013; Hill *et al.*, 2016). Additionally, recent evidence has shown that ethanol was unable to reverse the respiratory depressive tolerance to methadone, suggesting that certain opioids may be more susceptible to ethanol's reversal effects than others (Withey *et al.*, 2017). Our goal for the studies presented here was to compare the effects of ethanol reversal as seen previously in morphine tolerant mice to those of mice made tolerant to two other commonly abused opioids, oxycodone and hydrocodone, to determine if they are more "morphine-like" or more "methadone-like" in regards to their interaction with ethanol.

These drugs, like morphine and heroin, have been shown to exert their analgesic and respiratory depressive effects through similar pathways and mechanisms involving the μ -opioid receptor. There are a number of unique properties belonging to oxycodone and hydrocodone, however, that set them apart from morphine beyond slight structural differences, such as varying degrees of oral bioavailability and their primary enzymatic degradation pathways (Reisine and Pasternak, 1996; Kolesnikov *et al.*, 2003). Discrepancies exist in the literature regarding which opioid receptor mediates oxycodone's antinociceptive effects, with studies carried out in rats supporting a primary role of the kappa receptor based on in vitro binding studies and behavioral

assessments (Nielsen *et al.*, 2007). Studies utilizing *in vitro* and *in vivo* approaches in mice however, showed the μ -opioid receptor is the primary receptor type that is preferentially bound and activated by oxycodone (Yoburn *et al.*, 1995). Additionally, it was shown that in the tail flick assay, neither NorBNI nor naltrindole, the κ opioid receptor and δ opioid receptor antagonists respectively, were able to block the antinociceptive properties of oxycodone (Beardsley *et al.*, 2004). Given the differences underlying the pharmacodynamics and pharmacokinetics of these opioid compounds, it was of interest to determine if ethanol reversed analgesic tolerance to both of these compounds, and to what degree each of the compounds were susceptible to ethanol's reversal effects.

III. Materials and Methods

Drugs and Chemicals. Morphine sulfate, Oxycodone HCl, Hydrocodone bitartrate and 75-mg morphine pellets and placebo pellets were obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD). Morphine sulfate, Oxycodone HCl and Hydrocodone bitartrate were each dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL). Ethanol was obtained from AAPER Ethanol and Chemical Co. (Shelbyville, KY) and was diluted with pyrogen-free isotonic saline.

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 $22 \pm 2^\circ\text{C}$ on a 12-hour light- dark cycle. Food and water were available *ad libitum*. The mice were brought to the test room ($22 \pm 2^\circ\text{C}$, 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).

Tail Immersion Test. The warm-water tail immersion test was performed using a water bath with the temperature stabilized at $56 \pm 0.1^{\circ}\text{C}$ (Coderre and Rollman, 1983). Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time between 2 to 4 seconds were used. Test latencies were assessed 20 minutes following opioid treatment, with a 10-second maximum cut-off time utilized to prevent tissue damage. Antinociception was quantified as the percentage of maximum possible effect (%MPE), which was calculated as: $\%MPE = [(test\ latency - control\ latency) / (10 - control\ latency)] \times 100$. Percent MPE was calculated for each mouse using at least six mice per dose of drug (Harris and Pierson, 1964).

Acute Dose Response Curves in Tail Immersion Test: Oxycodone, Hydrocodone and Morphine.

Male Swiss Webster mice were weighed and baseline tail withdrawal latencies were recorded as described above. Oxycodone was administered subcutaneously at doses of 0.25, 0.5, 1.0, 1.25 and 1.5 mg/kg and mice were returned to their home cage. Hydrocodone was administered subcutaneously at doses of 1.0, 3.0, 5.0 and 6.0 mg/kg and mice were returned to their home cage. Morphine was administered subcutaneously at doses of 2.0, 4.0, 8.0, and 16 mg/kg and mice were returned to their home cage. After a 20-minute pretreatment period, mice were re-tested for tail withdrawal latencies to assess antinociceptive effects and to conduct dose-response curves.

Single-Day Tolerance Model. Antinociceptive tolerance to oxycodone and hydrocodone was developed as follows. Mice were injected subcutaneously (s.c.) once every hour (for a total of seven injections) with the respective ED80 dose of each opioid corresponding to the tail

immersion test, 1.25 mg/kg for oxycodone and 5.0 mg/kg for hydrocodone, and saline in control mice. One hour after the final injection, mice were administered 1 g/kg ethanol or 0.9% saline vehicle by intraperitoneal (i.p.) injection and 30 minutes later were challenged with various subcutaneous doses of oxycodone (ascending log₂ doses from 0.25 – 4.0 mg/kg) or hydrocodone (ascending log₂ doses from 1 – 16 mg/kg) to construct dose-response curves for calculation of ED₅₀ values and potency ratios.

4-day Tolerance Model. Tolerance to oral oxycodone or hydrocodone was developed using a twice-daily gavage method whereby animals were administered 64 mg/kg oxycodone, or 128 mg/kg hydrocodone, in the morning and again in the evening, with at least 8 hours separating the two gavage events. Animals were weighed on day 1, 3 and 5 (test day) and dosing was adjusted accordingly. The evening gavage administration on day 4 was the final maintenance dose animals received prior to test and challenge treatments on day 5. Drug dose was calculated for 0.1cc/10g body weight administration with 0.9% physiological saline as the vehicle. All mice had continued access to ad libitum food and water throughout the dosing paradigm and remained group-housed in their home cages.

Oral Oxycodone Time Course. A time course study was conducted to assess the antinociceptive effects of oral oxycodone following a single gavage of 16 mg/kg oxycodone at the following time points: 5, 10, 20, 30, 60, 120, 240 and 480 minutes. Here, 5 mice were repeatedly tested to determine average %MPE at each time point. Utilizing the same time points and dose of oxycodone, we repeated the time course study to assess brain oxycodone concentrations. An N of 5 mice per time point was utilized for the brain concentration analysis.

Reagents for GC/MS Analysis. The primary reference materials of oxycodone and oxycodone-d₆

were purchased from Cerilliant Corporation (Round Rock, Texas) as metabolic solutions. The chloroform, deionized (DI) water, hydroxamine hydrochloride (HCL), 2-propanol, sodium bicarbonate and sodium carbonate were purchased from Fisher Scientific (Hanover Park, Illinois). BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) + 10% TMCS (Trimethylchlorosilane) was purchased from Regis Technologies (Morton Grove, Illinois).

Sample Extraction. Quantitative analysis of morphine and oxycodone was based upon a previously described method (Broussard et al., 1997; Wolf and Poklis, 1997). This method is routinely performed in our laboratory for the analysis of opiates in blood and tissue samples. Pre-extraction preparation was unnecessary for the whole blood specimens. Whole brain tissues specimens were diluted as 1 part tissue to 3 parts deionized water (v/v) and homogenized. Matched matrix five-point calibration curves containing opiates of interest were prepared at 20-1000 ng/mL for blood or 20-1000 ng/g for tissue, along with blank and double blank controls. 10 μ L of internal standard (ISTD) consisting of 10 μ g/mL (100 ng total) of oxycodone-d₆ was added to 1.0 mL or 1.0 g aliquots of calibrators, controls and specimens, except the double blank control. 10% hydroxamine HCL (200 μ l) was added to each sample. They were then mixed and heated at 30°C for 30 min. Samples were then cooled and 1 mL of saturated carbonate/bicarbonate buffer (1:1, N:N, pH 9.5) and 2 mL of chloroform:2-propanol (8:2) were added. Samples were mixed for 5 min and then centrifuged at 2500 rpm for 5 min. The top aqueous layer was aspirated and the organic layer was transferred to a clean test tube and evaporated to dryness at 40°C under a constant stream of nitrogen. BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) + 10% TMCS (Trimethylchlorosilane) (50 μ L) was added and the samples were heated for 30 min at 70°C. The samples were then placed in auto-sampler vials for gas chromatography mass spectrometry (GC/MS) analysis.

Instrumental Analysis. The GC/MS analysis was performed with a Hewlett-Packard 6890 with a split/splitless injection port attached to Hewlett-Packard model 5793A mass selective detector (MSD) with a 7683 autosampler. The chromatographic separation was performed on an Agilent (Santa Clara, California) HP-1 12 m x 0.2mm x 0.33 μ m analytical column with the injection temperature set to 170°C in run in pulsed splitless mode. The initial oven temperature was 170°C and was held for 1.0 min., then heated at 10°C/min. to 280°C. The total run time was 12 min. The quantification and qualifying ions monitored for oxycodone were 269, 459 and 474 m/z, and for oxycodone-d₆ 465 and 480 m/z. A linear regression of the ratio of the peak area counts of quantification ion of ISTD versus concentration was used to construct the calibration curves.

Data Analysis. Opioid dose response curves were conducted for calculation of ED₅₀ values by the method of Bliss (1967), utilizing least-square linear regression analysis followed by calculation of 95% confidence limits (Bliss, 1967). For all other statistical analyses, GraphPad Prism 5 was used (GraphPad Software, Inc., La Jolla, CA). All data are represented as mean \pm standard error of the mean (SEM). A One-way ANOVA with Tukey's post-hoc analysis was utilized when comparing changes across three or more groups over a single factor. Statistical differences between only two groups of data were analyzed using Student's two-tailed unpaired t-test. Significant differences were considered when $P < 0.05$.

IV. Results

Acute Effects of Oxycodone and Hydrocodone. To test acute antinociceptive properties, oxycodone or hydrocodone was administered subcutaneously and assessed in the warm water tail withdrawal test. Tail withdrawal latencies dose-dependently increased, reaching a ceiling effect at 1.5 mg/kg for oxycodone and at 6.0 mg/kg for hydrocodone, where all animals exhibited an

MPE of 100%. The ED₅₀ value for acute oxycodone was calculated to be 0.84 mg/kg (0.68 – 1.04). The ED₅₀ value for hydrocodone was calculated to be 3.95 mg/kg (2.40 – 6.52). Morphine was administered subcutaneously with all mice reaching 100% MPE at 8 mg/kg. The ED₅₀ was calculated to be 3.94 mg/kg (3.55 – 4.36). As predicted, we found that oxycodone was more potent than hydrocodone, which was equally as potent as morphine when administered subcutaneously in mice. We then compared the potencies of oxycodone and hydrocodone when given orally, due to their reliable oral bioavailability. Acutely, oxycodone produced an oral ED₅₀ of 9.29 mg/kg (7.18 – 12.02), ten times its subcutaneous ED₅₀ value. In subsequent experiments comparing chronic saline treated (i.e. control) versus chronic oxycodone treated mice, a similar ED₅₀ value of 8.29 mg/kg (6.12 – 11.52) was obtained in the control mice, indicating acute oral oxycodone ED₅₀ values are reliably reproducible (Table 1). Hydrocodone orally produced an ED₅₀ value also nearing ten times the subcutaneous value, equaling 38.79 mg/kg (29.21 – 51.53) (Table 1).

Tolerance Developed to Oxycodone and Hydrocodone Following Repeated Administration.

Oxycodone Tolerance: Chronic injections of s.c. saline prior to s.c. oxycodone challenge doses yielded a dose response curve of oxycodone with an ED₅₀ value of 0.90 mg/kg (0.72 - 1.12), reproducing what was observed in our acute dose response experiments, suggesting no adverse effects of handling or repeated vehicle injections on oxycodone's antinociceptive effect. In mice repeatedly administered s.c. oxycodone prior to receiving s.c. oxycodone challenge doses, the ED₅₀ value was 1.70 mg/kg (1.42 - 2.03), significantly shifted to the right compared to the mice that received chronic saline, indicating tolerance was observed (Figure 2A). We further characterized oxycodone tolerance development by investigating the response to repeated oral oxycodone. A modified protocol with repeated exposure across 4 days, rather than repeated

exposure within a single day, was utilized for this experiment. We found a significant shift to the right in response to chronic oral oxycodone with an ED₅₀ value of 33.41 mg/kg (25.50 – 44.17) compared to the acute ED₅₀ value of 8.29 mg/kg (6.12 -11.52) reported above, demonstrating that these mice were tolerant to the antinociceptive effects of orally administered oxycodone.

Hydrocodone Tolerance: Chronic s.c. injections of saline followed by acute challenge doses of s.c. hydrocodone generated a dose response curve with an ED₅₀ value of 3.92 mg/kg (3.26 - 4.71) (Figure 2B) in mice, similar to what was observed in previous acute dose response experiments as stated above. The ED₅₀ value significantly shifted to the right in animals chronically injected with hydrocodone prior to receiving the challenge injections, equaling 9.01 mg/kg (6.44 – 12.62) and indicating tolerance developed in these mice. We also investigated the development of tolerance to oral hydrocodone and utilized a 4-day protocol similar to what was used for our chronic oral oxycodone studies. We observed an increase in the oral ED₅₀ value, equaling 55.92 mg/kg (40.63 – 76.96), however, the confidence limits overlap that of the acute oral ED₅₀ value (38.79 mg/kg (29.21 – 51.53)), suggesting that complete tolerance was not observed in the oral dosing study (Table 1). In a follow-up study, we increased the maintenance dose to 256 mg/kg po and still did not observe tolerance to the antinociceptive effects of hydrocodone (data not shown).

Table 1. Comparison of Tolerance Development and Ethanol Reversal of Various Opioid Compounds.

ED₅₀s and 95% confidence limits were calculated under acute, chronic and chronic + ethanol conditions in mice. All opioids tested produced antinociceptive tolerance when repeatedly administered subcutaneously. Oxycodone and hydrocodone were also evaluated for oral antinociceptive potencies and tolerance development. Only oral oxycodone produced significant tolerance to itself after repeated administration. Asterisk * denotes a significant shift from acute ED₅₀ values, determined by confidence limits that no longer overlap.

	Morphine s.c.	Oxycodone s.c.	Oxycodone p.o.	Hydrocodone s.c.	Hydrocodone p.o.
Acute ED ₅₀	4.8 mg/kg (2.5 – 5.7)	0.89 mg/kg (0.72 – 1.12)	8.29mg/kg (6.12 – 11.52)	3.92 mg/kg (3.26 – 4.71)	38.79 mg/kg (29.21 – 51.53)
Chronic ED ₅₀	19.9 mg/kg* (14.8 – 29.1)	1.70 mg/kg* (1.42 – 2.03)	33.41 mg/kg * (25.50 – 44.17)	9.01 mg/kg * (6.44 – 12.62)	55.92 mg/kg (40.63 – 76.96)
Chronic + 1 g/kg Ethanol ip ED ₅₀	5.2 mg/kg (4.9 – 5.5)	1.02 mg/kg (0.77 – 1.37)	35.52 mg/kg (20.19 – 59.03)	4.73 mg/kg (3.51 – 6.38)	N/A

Reversal of Oxycodone and Hydrocodone Antinociceptive Tolerance by Ethanol (i.p.). We tested ethanol (1 g/kg, i.p.) administration in mice repeatedly administered oxycodone or hydrocodone. Previously, our lab determined that a dose of 1g/kg ethanol was inert in the warm water tail withdrawal test, but fully reversed morphine tolerance in mice (Hull *et al.*, 2013). ED50 values were calculated from the resulting dose response curves and compared across three conditions: chronic saline followed by saline and acute opioid challenge, chronic opioid followed by saline and opioid challenge, and chronic opioid followed by ethanol and opioid challenge. A single injection of ethanol (1 g/kg, i.p.) reversed antinociceptive tolerance to both oxycodone and hydrocodone (Figure 2A and 2B) as shown by the restoration of the ED50 values which closely resembled values observed in the acute dose response experiments (Table 1). These results add to our previous findings that ethanol reversed the analgesic tolerance to morphine, suggesting that ethanol may be interfering with a pathway common to some, but not all, opioids.

Reversal of Oxycodone Antinociceptive Tolerance by Oral Ethanol. Mice were assessed for oxycodone tolerance and reversal by po ethanol in addition to ip ethanol, again utilizing the single- day tolerance paradigm. Mice that were repeatedly injected with s.c. oxycodone and received a saline gavage displayed tolerance to the antinociceptive effects in response to an oxycodone challenge injection (1.25 mg/kg, s.c.) as shown by a significantly lower %MPE ($10.25\% \pm 2.71$) ($P < 0.05$, One-way ANOVA) when compared to acute oxycodone controls ($50.98\% \pm 11.95$). Mice that were repeatedly injected with s.c. oxycodone but received a 2 g/kg po ethanol gavage prior to receiving the challenge s.c. oxycodone injection continued to respond to oxycodone, displaying antinociceptive responses similar to that of both acute oxycodone treated mice and oxycodone-treated mice that received 1 g/kg ethanol i.p. The %MPE values

observed in both of these chronic oxycodone plus ethanol treatment groups displayed significantly higher %MPE values compared to that of the chronic oxycodone plus saline treatment group, $P < 0.05$ (2 g/kg ethanol po, $63.90\% \pm 16.88$) and $P < 0.01$ (1 g/kg ethanol ip, $71.49\% \pm 11.83$), (One-way ANOVA) (Figure 3). These data indicate that both i.p. and p.o. ethanol reversed oxycodone tolerance.

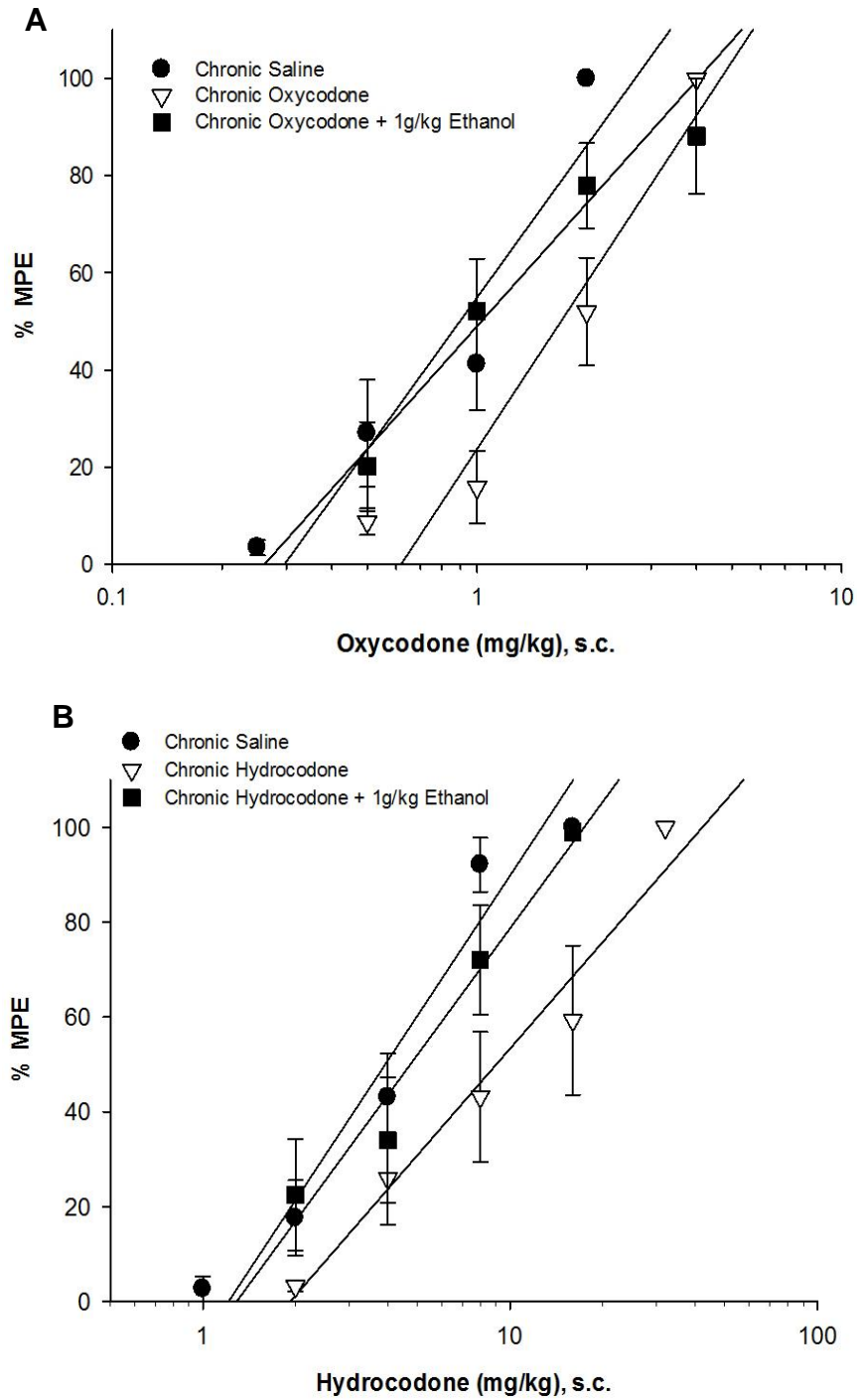


Figure 2. Ethanol Reversal of Oxycodone and Hydrocodone Tolerance. Ethanol fully reversed both oxycodone (A) and hydrocodone (B) tolerance at a dose of 1g/kg. Each data point is represented by a minimum of five mice and represented as mean \pm SEM. Animals were

injected once hourly with either saline or an ED₈₀ dose of oxycodone or hydrocodone s.c. for six hours, followed by an i.p. injection of ethanol (1g/kg) or saline one hour later. 30 minutes later, various challenge doses of oxycodone or hydrocodone were injected s.c. to construct dose response curves and generate ED₅₀ values.

Pharmacokinetic Studies¹ Acute Oral Oxycodone Time Course: Antinociception and Brain

Concentrations. Oxycodone (16 mg/kg, p.o.) was detectable in the brain at the earliest time point measured of 5 minutes, averaging 80.2 ng/g, while antinociception was marginal at 10.14% MPE on average. Peak brain concentrations were observed at 20 and 30 minutes following oxycodone administration, 153.58 ng/g and 153.24 ng/g respectively. Brain concentrations at these times were significantly higher than concentrations detected at 120 minutes ($P < 0.05$) and 480 minutes ($P < 0.001$) (One-way ANOVA). Significant antinociception was detected at the 20, 30 and 60 minute time points but not at the 5 minute time point ($P < 0.05$ (20 min) and $P < 0.001$ (30, 60 min), One-way ANOVA). Peak antinociception, measured as 100% MPE, was not detected until 30 minutes following administration, and persisted until the 60 minute point. Antinociception at 20 ($P < 0.05$), 30, and 60 minutes following oxycodone was significantly higher than 480 minutes ($P < 0.0001$), with the 30 and 60 minute time points also significantly higher than at 240 minutes ($P < 0.001$, One-way ANOVA) Brain oxycodone concentrations at 60 minutes however were markedly lower, measuring on average closer to the 5 and 10 minute time points, though the antinociceptive effects were vastly greater. Notably, oxycodone was not detectable in any of the brain tissue samples tested 480 minutes (8 hours) after a single gavage and there was no antinociception at this time (Figure 4).

¹ Corresponding blood oxycodone concentrations following acute or chronic oxycodone administration with and without the presence of ethanol can be found in Appendix A.

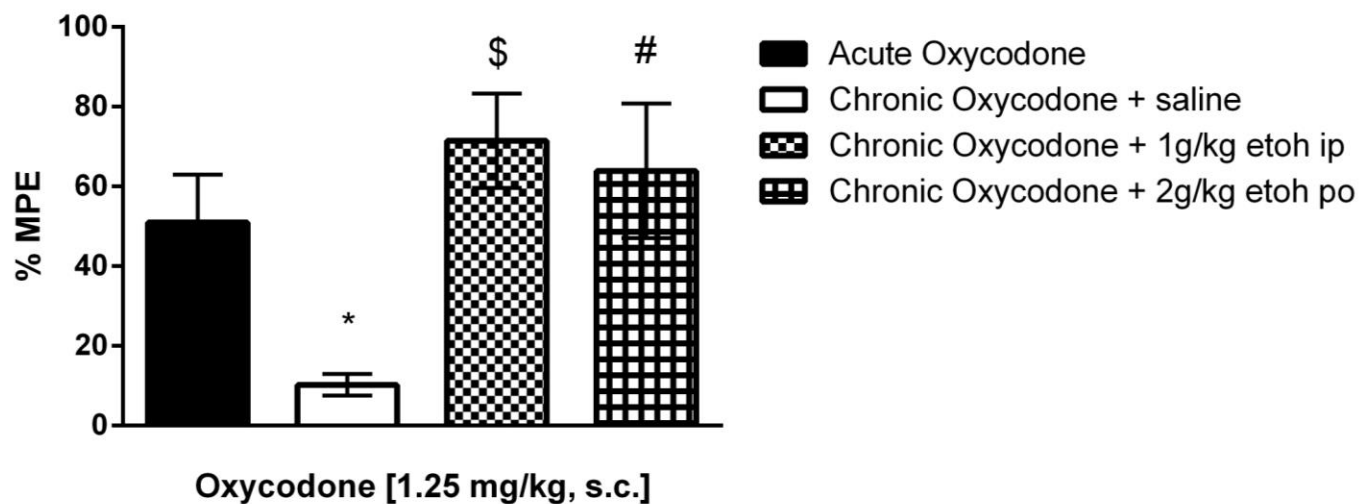


Figure 3. Intraperitoneal and Oral Ethanol Reversed Antinociceptive Tolerance to Subcutaneous Oxycodone. Mice were chronically injected with s.c. oxycodone (1.25 mg/kg) or saline hourly for 6 hours and treated with saline, i.p ethanol (1 g/kg) or po ethanol (2 g/kg) prior to receiving a challenge injection of 1.25 mg/kg oxycodone. Antinociception was assessed using the tail immersion assay where significant tolerance was displayed in mice chronically treated with oxycodone and no ethanol, compared to the acute oxycodone treatment group (* $P < 0.05$, One-way ANOVA). Two additional groups of mice were treated with repeated injections of oxycodone, but received either an ip injection of 1 g/kg ethanol or a gavage of 2 g/kg ethanol 30 minutes prior to receiving an oxycodone challenge injection. Ethanol treatment in these mice reversed tolerance development to oxycodone as seen by a restored response to the antinociceptive effects of oxycodone. Chronic oxycodone treated mice given either ip ethanol (\$ $P < 0.01$) or oral ethanol (# $P < 0.05$) displayed significantly greater antinociceptive effects in response to a challenge injection of oxycodone compared to chronic oxycodone mice given saline prior to an oxycodone challenge (One-way ANOVA). All groups are represented by a minimum of 5 mice with data shown as mean \pm SEM.

Tail Flick Latency vs Brain Concentration: 16 mg/kg Oral Oxycodone

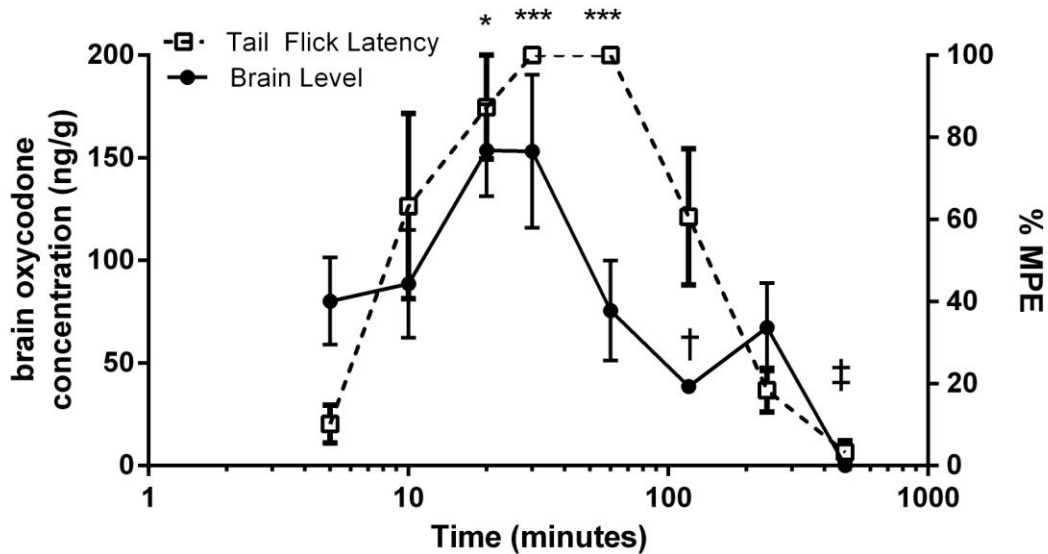


Figure 4. Oral Oxycodone Time Course: Antinociception and Brain Concentration. After a single administration of 16 mg/kg oxycodone po, brain oxycodone concentrations were plotted against oxycodone’s antinociceptive effect in the warm water tail withdrawal assay at various time points ranging from 5 – 480 minutes. All data points represent mean \pm SEM from a minimum of 5 mice. Brain oxycodone concentrations increased during the first 20 minutes where a plateau was observed until 30 minutes. Antinociception was slower to reach 100% MPE, which was not observed until 30 minutes and persisted until 60 minutes. Brain oxycodone concentrations at 60 minutes were much lower, measuring closer to the 5-minute time point, despite maximum antinociception. Significant observations for brain oxycodone concentrations were noted at 120 ($\dagger P < 0.05$) and 480 ($\ddagger P < 0.001$) minute time points (one-way ANOVA), where concentrations were lower than all other time points. Antinociception was significantly higher at 20 ($*P < 0.05$), 30 and 60 minutes ($***P < 0.001$) compared to 5 minutes. Antinociception at 30 and 60 minutes was significantly higher compared to 240 ($P < 0.001$) and 480 minutes ($P < 0.0001$).

Brain Oxycodone Concentrations Did Not Directly Correlate With Antinociception. We compared brain concentrations of oxycodone when given orally versus subcutaneously using equianalgesic doses. 1.25 mg/kg s.c. and 16 mg/kg p.o. oxycodone both produced near 80% MPE. 1.25 mg/kg s.c. oxycodone produced 74.48% MPE while 16 mg/kg po oxycodone produced 77.71% MPE. Brain oxycodone concentrations averaged 348.89 ng/g following subcutaneous administration, significantly higher than oral oxycodone concentrations which averaged 114.1 ng/g ($p < 0.0001$, Student's two-tailed unpaired t-test). Our findings indicate that the antinociceptive effects of oxycodone are not directly correlated with brain oxycodone concentrations (Figure 5).

Acute Brain Concentrations of Oxycodone After Oral Administration. Oxycodone was administered orally in ascending log₂ doses ranging from 16 to 64 mg/kg to assess a dose response relationship for brain concentrations. Brain concentrations showed an average of 114.1 ng/g (N = 10), 312.4 ng/g (N = 5), and 731.2 ng/g (N = 14) 20 minutes following administration of 16, 32 and 64 mg/kg oxycodone respectively. Significant differences were detected between the 64 mg/kg and 16 mg/kg doses ($P < 0.01$, One-way ANOVA). These data revealed a general relationship between dose of oral oxycodone and brain oxycodone concentrations.

Brain Concentration of Oral Oxycodone After Repeated Administration. The development of oral oxycodone tolerance was carried out over 4 days via twice daily gavage administrations of 64 mg/kg oxycodone. Mice were challenged on day 5 with a single gavage of 16 mg/kg oxycodone. Brain concentrations 20 minutes following the challenge gavage showed an average of 28.92 ng/g (N = 13). These concentrations were significantly lower than seen after acute 16 mg/kg at the 20 minute time point ($P < 0.0001$) (Figure 6A).

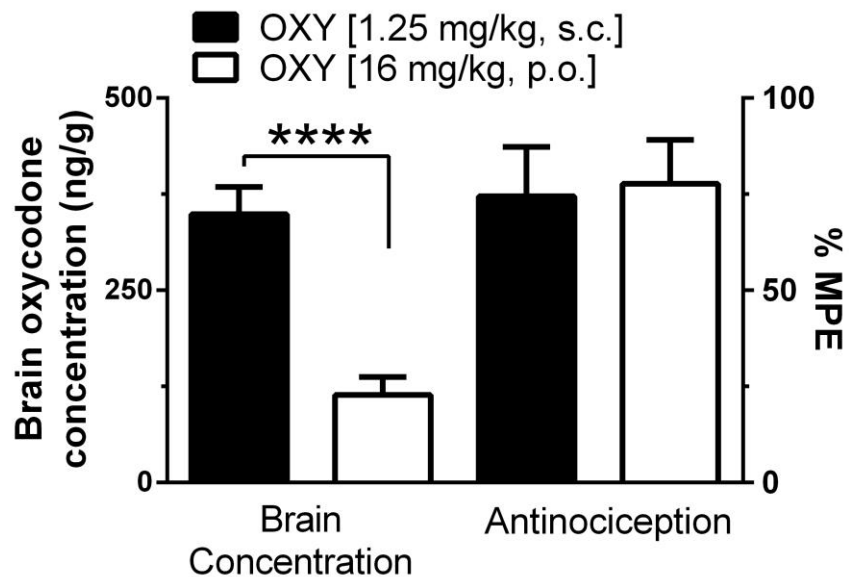
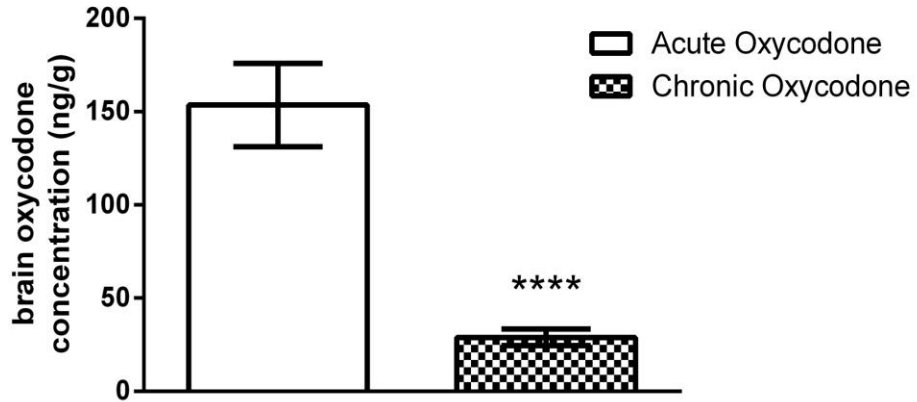


Figure 5. Comparing Subcutaneous and Oral Oxycodone: Equal Antinociceptive Effects of Oxycodone Were Not Due to Equal Brain Oxycodone Concentrations. Mice were injected or gavaged with the respective ED₈₀ dose of oxycodone, 1.25 mg/kg s.c. and 16 mg/kg po. Both doses produced equal antinociception in mice; however, brain oxycodone concentrations significantly differed, with much higher concentrations detected after subcutaneous administration ($p < 0.0001$, student's unpaired two-tailed t-test). A minimum of 5 mice were used for each dose tested, with bars representing means \pm SEM.

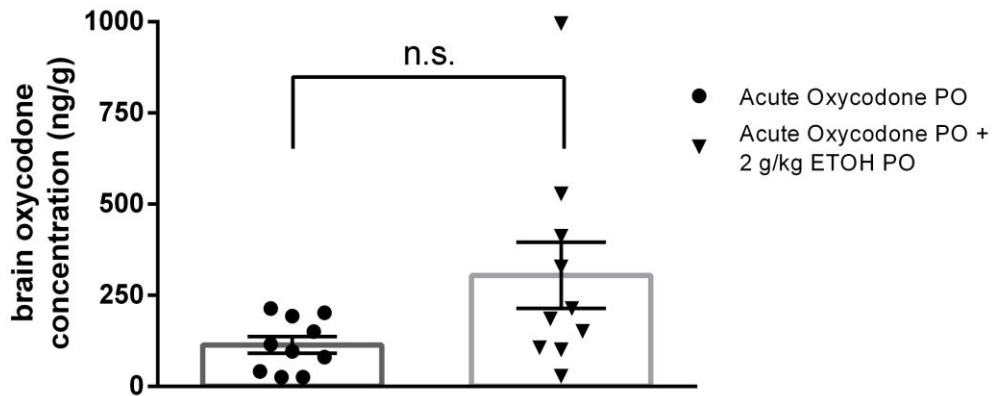
The Effect of Oral Ethanol on Brain Concentrations of Acute Oral Oxycodone. To determine if ethanol alters acute oxycodone brain concentrations, mice were pretreated with 2 g/kg ethanol 30 minutes prior to receiving a gavage of 16 mg/kg oxycodone. Mice were sacrificed 20 minutes following oxycodone administration and brain samples were collected and processed immediately thereafter. After the oral administration of 2 g/kg ethanol, brain concentrations averaged 304.7 ± 90.8 ng/g. Brain oxycodone concentrations were not significantly altered by 2 g/kg acute ethanol as compared to acute oxycodone alone ($P > 0.05$, Student's two-tailed t-test) (Figure 6B).

The Effect of Ethanol on Chronic Oxycodone Brain Concentrations. To determine if ethanol altered chronic oxycodone brain concentrations, mice were repeatedly administered oxycodone twice daily for four days and pretreated on day 5 with a gavage of 2 g/kg ethanol 30 minutes prior to receiving a challenge gavage of 16 mg/kg oxycodone. Mice were sacrificed 20 minutes following oxycodone administration and brain samples were collected and processed immediately thereafter. Brain oxycodone concentrations following chronic oxycodone administration and the acute administration of 2 g/kg ethanol equaled 26.13 ± 3.45 ng/g ($N = 8$) (Figure 6C). Chronic oxycodone concentrations measured in the presence of ethanol were consistent with the chronic oxycodone samples measured in the absence of ethanol, providing supporting evidence that acute ethanol did not alter chronic oxycodone brain concentrations ($P > 0.05$, unpaired Student's two- tailed t-test).

A



B



C

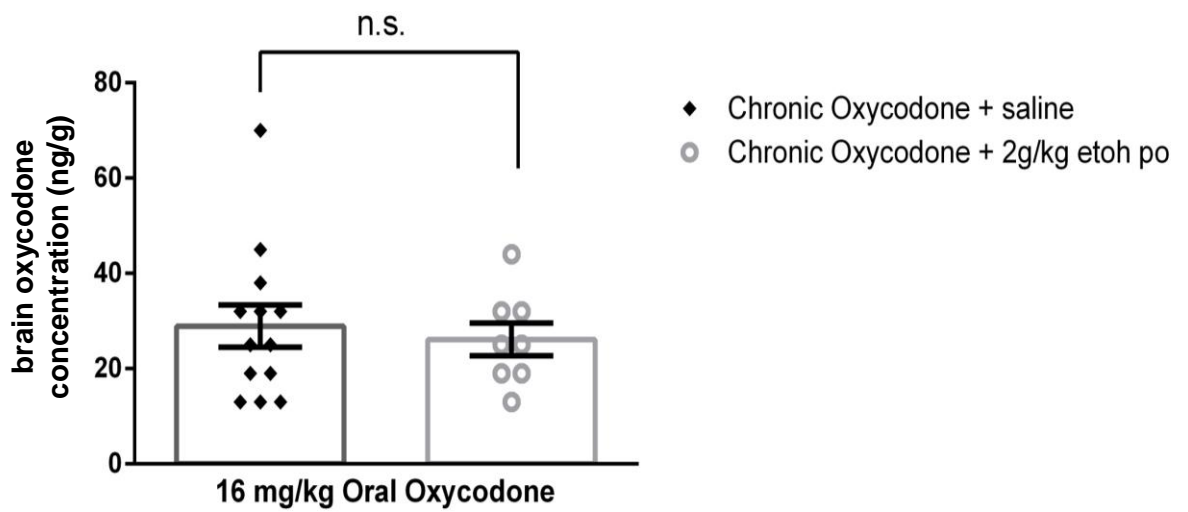


Figure 6. Acute and Chronic Oxycodone Brain Concentrations. (A) Oxycodone brain concentrations 20 minutes following a challenge gavage of 16 mg/kg oxycodone in mice either naïve to oxycodone or chronically treated with 64 mg/kg b.i.d. for four days. Acute concentrations represent the mean \pm SEM of 13 mice. Brain oxycodone concentrations detected 20 minutes following the oxycodone challenge were significantly lower in mice chronically treated with oxycodone compared to that of acutely treated mice (**** $P < 0.001$, Student's two-tailed unpaired t-test). (B) The effects of 2 g/kg ethanol were assessed against acute oxycodone brain levels. Each bar represents the mean \pm SEM of at least 10 mice. Ethanol did not have a significant effect ($P > 0.05$, Student's unpaired two-tailed t-test) and both groups displayed similar brain oxycodone levels. (C) The effects of 2 g/kg ethanol were assessed against chronic oxycodone brain levels. Ethanol did not have a significant effect ($P > 0.05$, Student's unpaired two tailed t-test) and both groups displayed similar brain oxycodone levels in response to a challenge oxycodone gavage following chronic oxycodone treatment. Each bar represents the mean \pm SEM of at least 8 mice.

V. Discussion

Oxycodone and hydrocodone are two of the most commonly prescribed opioids for the relief of pain despite their untoward side effects including tolerance development and abuse liability. Presently, an opioid abuse epidemic exists, in part, due to numerous individuals becoming dependent on prescription opioids, who then switch to heroin. When alcohol is consumed simultaneously with heroin, the risk of overdose and death increases. This could be due to one drug potentiating or adding to the depressant effects of the other, or due to the reversal of the tolerances that have developed to the opioid. We have previously shown that ethanol reversed morphine tolerance. The goal of this study was to compare the acute potency, propensity to produce tolerance, and assess reversal of that tolerance to oxycodone and hydrocodone by ethanol. A second goal was to elucidate if the tolerance was reversed due to an alteration of pharmacokinetic parameters.

Characterization of the Development of Oxycodone and Hydrocodone Antinociceptive Tolerance and the Effect of Ethanol on that Tolerance. Using a single-day injection schedule, mice were made tolerant to either oxycodone or hydrocodone. We found a 2-fold rightward shift in ED₅₀ values in mice repeatedly injected with oxycodone. The doses we used produced similar levels of antinociception as seen in other studies, though a different outbred mouse strain (ICR) was used (Beardsley *et al.*, 2004; Minami *et al.*, 2009). Similarly, we found a 2-fold rightward shift in ED₅₀ values in mice repeatedly injected with hydrocodone. Interestingly, our acute ED₅₀ values for hydrocodone are between values reported by two others studies that used the radiant heat tail flick assay rather than warm-water tail immersion. Kolesnikov *et al.*, (2003) found an ED₅₀ value of 1.37 mg/kg in Swiss Webster mice bred by a different vendor, while

Navani and Yoburn (2013), calculated an ED₅₀ value of 11 mg/kg in CD-1 mice.

Ethanol was similarly effective in reversing the antinociceptive tolerance to both oxycodone and hydrocodone when administered at 1 g/kg i.p. In previous studies, this dose was also effective at reversing analgesic tolerance and respiratory depressive tolerance to morphine (Hull *et al.*, 2013; Hill *et al.*, 2016). Further, 20mM in vitro ethanol reversed *ex vivo* morphine tolerance in the locus coeruleus of rats, suggesting that low-to-moderate ethanol doses reverse tolerance without eliciting effects acutely (Llorente *et al.*, 2013).

Pharmacokinetic Analysis of Acute Oxycodone Time Course. We investigated the relationship between brain oxycodone concentrations and antinociceptive effects in mice. Previously, we have shown that there is a correlation between brain morphine concentrations and tail flick latencies (Patrick *et al.*, 1975, 1978). Oxycodone was detected in brain tissue 5 minutes after the oral administration of 16 mg/kg, but antinociception was minimal. The concentration continued to increase until 20 minutes post gavage, where it remained steady for at least ten more minutes, indicating peak concentrations for this dose of oxycodone were present in the brain. Significant antinociception was observed 20 minutes following oxycodone administration, with maximal or near maximal effects lasting from 20 to 60 minutes. Given that oxycodone was administered orally, it is not surprising that peak concentrations were not detected sooner. Additionally, mice were not food restricted in these studies, and gastric emptying time could have altered or delayed the time to which oxycodone was actually absorbed and distributed through the liver and to the brain. In addition, at 60 minutes, oxycodone concentrations were lower, though not significantly, as compared to peak concentrations at 20 and 30 minutes. Between 30 minutes and 60 minutes, there was a 50% decrease in oxycodone concentrations. The antinociceptive effects between 30 and 60 minutes however, did not change and continued

to produce 100% MPE. This discrepancy between brain concentrations of oxycodone and the antinociceptive effect measured likely suggests the presence of an active metabolite, however our study did not investigate which metabolite(s) contributed to our observation. Oxycodone was not detected in the brain 8 hours after mice were dosed and no antinociceptive effects were observed.

Brain Concentrations of Oxycodone After Chronic Administration. In chronic oxycodone treated-mice, brain oxycodone concentrations following a challenge gavage of 16 mg/kg were significantly lower compared to those observed in brains of mice that only received a single administration of the same dose of oxycodone. The effect was consistent, as demonstrated by the minimal variability between sample values, suggesting a well-regulated mechanism underlies oxycodone tolerance and metabolism. It is possible that while initial variances in individual response are likely to occur upon acute exposure, highly regulated signaling events and selectively activated enzymatic pathways lead to more structured biological responses upon repeated exposure. These data also provide further insight into the potential mechanisms underlying oxycodone tolerance. There could be a significant upregulation of degradative enzymes leading to the development of metabolic tolerance, and warrants further investigation into this possible explanation. Additionally, the reduction in brain oxycodone concentrations after repeated administration could be a result of increased P-glycoprotein (P-gp) activity, a chaperone protein that actively transports drug molecules across the blood brain barrier. P-gp is well characterized in its effects on opioid agonists (Dagenais *et al.*, 2004), yet there are opposing reports regarding P-gp's actions on oxycodone. One study showed P-gp's ATP-ase activity was dose-dependently increased by acute oxycodone and was upregulated after chronic oxycodone in rats (Hassan *et al.*, 2007), however, another study showed that the P-gp inhibitor, PSC833, had

no effect on oxycodone's ability to enter and remain in the brain in rats (Bostrom *et al.*, 2005). The contributions of the latter study's findings are difficult to interpret given that PSC833 has been shown to be less specific than previously thought (Mayer *et al.*, 1997; Cvetkovic *et al.*, 1999).

Acute and Chronic Oxycodone Pharmacokinetics Unaffected by Ethanol Co-administration in Mice. One of our main objectives in this study was to better characterize the effect of doses of ethanol that are moderately intoxicating in humans on acute and chronic brain oxycodone concentrations in mice. In our acute ethanol and acute oxycodone study we found that oral ethanol at a dose of 2 g/kg did not significantly alter oxycodone brain concentrations. It was of utmost importance to evaluate the effects of ethanol on brain oxycodone concentrations in chronic oxycodone treated mice in order to address the primary health concern of poly-drug abuse leading to opioid overdose. We tested 2 g/kg oral ethanol on chronic oxycodone brain concentrations. Somewhat unexpectedly, we observed that 2 g/kg ethanol did not significantly alter oxycodone brain concentrations. These results in brain tissue differ from blood results from multiple human post-mortem analyses, where the co-detection of ethanol corresponded with significantly lower opioid levels in the blood compared to those where only opioids were detected at time of death (Kerr *et al.*, 2007; Darke, 2011). These data do however agree with and add to the previous observations in Hill *et al* (2016) where 0.3 g/kg ethanol ip did not alter morphine brain or plasma concentrations in mice while still reversing the tolerance to respiratory depression, suggesting that ethanol is not working through mechanisms that alter the kinetics of either of these opioids. Furthermore, our findings were the result of acute ethanol effects on oxycodone brain concentrations, whereas repeated ethanol treatments might alter those findings. The effects of repeated ethanol have been tested on morphine behavioral responses and [³H]-

dihydromorphine binding in mice, where a change in affinity for striatal opioid receptors was observed after ethanol feeding (Tabakoff *et al.*, 1981). Clearly, ethanol effects on opioid blood and brain concentrations in mice are dependent on treatment regimen and the specific opioid compound tested.

These studies show that there is a metabolic component underlying oxycodone tolerance, yet our results suggest the reversal of that tolerance by ethanol is not due to an alteration of the bio-disposition of oxycodone. We therefore conclude that ethanol reversal of oxycodone tolerance is mediated by specific neuronal mechanisms, and future experiments will be conducted to address this finding.

The data gathered in chapter 2 suggested that reversal of tolerance to oxycodone in the whole animal was neuronal in nature. To confirm whether tolerance to oxycodone and reversal by ethanol occurs at the neuronal level, we utilized the cellular model of isolated dorsal root ganglia neurons from adult mice and whole cell patch clamp electrophysiology in chapter 3.

Chapter 3

The Effect of Ethanol on Oxycodone Tolerance in Dorsal Root Ganglia Neurons

I. Summary

Oxycodone is a semi-synthetic opioid compound that is widely prescribed, used and abused today, and has a well-established role in shaping the current opioid epidemic. Previously we have shown that tolerance develops to the antinociceptive and respiratory depressive effects of oxycodone in mice, and that a moderate dose of acute ethanol or a PKC inhibitor reversed that tolerance. To investigate further if tolerance was occurring through neuronal mechanisms, our aims for this study were threefold: 1) to assess the effects of acute oxycodone in isolated dorsal root ganglia (DRG) neurons, 2) determine if tolerance occurred within the neuronal cell body, and 3) if the tolerance was reversed by either ethanol or a PKC inhibitor. We found that 3 μM oxycodone reduced neuronal excitability, as measured by an increase in threshold potential and reduced action potential amplitude, without eliciting measurable changes in the resting membrane potential. Overnight exposure to 10 μM oxycodone was sufficient to produce tolerance in DRG neurons when exposed to a 3 μM oxycodone challenge. Oxycodone tolerance was reversed by the acute application of either ethanol (20mM) or the PKC inhibitor, Bisindolylmaleimide XI (Bis XI), in a single DRG neuron, when a challenge of 3 μM oxycodone once again reduced neuronal excitability. Through these studies, we conclude that oxycodone acutely reduced neuronal excitability, tolerance developed to this effect, and that reversal of tolerance occurred at the level of a single neuron, suggesting that reversal of oxycodone tolerance by either ethanol or Bis XI involves neuronal mechanisms.

II. Introduction

Oxycodone is a semi-synthetic opioid that has potent analgesic properties. It was initially recommended to physicians under the pretense that it was a highly effective opioid with reduced abuse liability and tolerance development. As we now know, oxycodone contributed substantially to the current opioid epidemic, unmasking its high abuse potential along with dependence and tolerance development. Many individuals who became dependent on oxycodone have switched to abusing heroin for reasons including increased policing and prosecution of oxycodone distribution and possession, as well as decreasing prices and increased purity of available heroin on the street. Opioid abusers are often poly-drug abusers, with ethanol being the most widely co-abused substance among these individuals. We have previously reported that ethanol reversed various morphine tolerances *in vivo* and *in vitro* (Hull *et al.*, 2013; Hill *et al.*, 2016). In addition, we recently reported that ethanol also effectively reversed oxycodone antinociceptive tolerance *in vivo* (Jacob *et al.*, 2017). It has also been reported that inhibitors of PKC reversed tolerance to the antinociceptive (Javed *et al.*, 2004; Smith *et al.*, 2007) and respiratory depressive effects of morphine, but not methadone (Withey *et al.*, 2017). Today, oxycodone is still prescribed in various formulations, such as Oxycontin® and Percocet®, supporting the fact that it is critical to further our understanding of the interactions that occur when ethanol or other drugs known to inhibit PKC, such as Tamoxifen, are consumed with oxycodone.

Evidence supporting the hypothesis that spinal mechanisms may mediate tolerance to opioids was introduced over twenty years ago (Gutstein and Trujillo, 1993). Until recently however, tolerance has primarily been described and studied as a central phenomenon, with little attention directed toward potential peripheral contributions. There has been renewed interest in

peripheral mechanisms of opioid tolerance, with a recent study suggesting that while the analgesic effects of morphine are mediated centrally, the signaling mechanisms underlying tolerance are distinctly different, and occur primarily outside the brain and spinal cord (Corder *et al.*, 2017). This conclusion was based, in part, on the observation that following the administration of a peripherally restricted opioid antagonist prevented the initiation of opioid tolerance, but did not affect the antinociceptive effects in mice. A recent review covering the aforementioned study along with other related studies collectively suggested presynaptic μ opioid receptors in afferent nociceptors are necessary for the development of tolerance to opioids (Puig and Gutstein, 2017).

We demonstrated that the acute effects of morphine, initially on enteric neurons (Smith *et al.*, 2012), followed soon after on dorsal root ganglia (DRG) neurons (Ross *et al.*, 2012), result in an increase in threshold potential, a decrease in action potential amplitude, and a greater proportion of inactivated sodium channels at resting membrane potential, as indicated by a shift to the left in the inactivation curve suggesting reduced sodium channel availability. We subsequently showed that tolerance to morphine can be evaluated in dorsal root ganglia (DRG) neurons, by looking directly at electrophysiological measures of neuronal excitability (Kang *et al.*, 2017). Given that we now have a reproducible model to evaluate neuronal tolerance, we hypothesized that oxycodone would produce similar responses to those induced by morphine. Also, by utilizing the notion that the dorsal root ganglia serve as a relay station between the external nociceptive stimuli and the central nervous system, we further evaluated the argument that tolerance to opioids involves peripheral μ opioid receptors by demonstrating that tolerance occurs within the afferent cell bodies themselves.

The studies described in this paper were designed to assess the acute effects of oxycodone on dorsal root ganglia neurons and to determine if tolerance developed to those effects. We then evaluated whether ethanol reversed the tolerance to oxycodone in these isolated neurons, as it did in the whole animal (Jacob *et al.*, 2017). We further investigated if the reversal of oxycodone tolerance in this assay by ethanol was due to an interaction of the opioid and ethanol on PKC.

III. Materials and Methods

Drugs and Chemicals

Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS) and fetal bovine serum were purchased from Gibco (Grand Island, NY). Papain was purchased from Worthington Biochemical Corporation (Lakewood, NJ). B27 supplement, L-glutamate, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Glial cell line-derived neurotrophic factor (GDNF) was purchased from Neuromics (Edina, MN). Glass cover slips were purchased from ThermoFisher Scientific (Waltham, MA). Laminin was purchased from BD Biosciences (San Jose, CA) and poly-D-lysine was purchased from MP Biomedicals (Solon, OH). 24-well cell culture dishes were purchased from CELLTREAT (Pepperell, MA). Collagenase from *Clostridium histolyticum*, magnesium chloride (MgCl₂), calcium chloride (CaCl₂), NaCl, KCl, HEPES, EGTA, sodium dihydrogen phosphate (NaH₂PO₄), glucose, ATP disodium salt, K-aspartic acid, potassium hydroxide (KOH) and sodium hydroxide (NaOH) were purchased from Sigma Aldrich (St. Louis, MO). Ethanol was obtained from AAPER Ethanol and Chemical Co. (Shelbyville, KY). Oxycodone HCl was obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD) and dissolved in ddH₂O.

Bisindolylmaleimide XI, HCl (Bis XI) and Naloxone were purchased from (Sigma Aldrich Co., St. Louis, MO).

Animals

Adult male C57/BL6 mice, 25-30 g and at least 6 weeks of age, were purchased from ENVIGO (Frederick, MD). β -arrestin2 wild type and knockout male mice (25-30 g) were obtained from Dr. Lefkowitz (Duke University, Durham, NC). All animals were housed up to five per cage in animal care quarters and maintained at $22\pm 2^{\circ}\text{C}$ on a 12 hour light-dark cycle. Access to food and water was available ad libitum. 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).

Isolation and Culture of Primary Cells from Adult Mouse Dorsal Root Ganglia

DRGs from the adult mouse were prepared as described recently (Ross *et al.*, 2012). Mice were sacrificed via CO_2 followed by cervical dislocation. L5-S1 DRGs were immediately harvested under a dissecting microscope and placed in a dish containing HBSS. Papain [15 U/ml] was then added to the dish and incubated for 18 minutes (min) at 37°C . Subsequently, ganglia were transferred to a separate dish containing HBSS and 1.5 mg/ml collagenase from *Clostridium histolyticum* and incubated for 60 min at 37°C . 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. Where indicated, isolated neurons were exposed to 10 μM oxycodone in complete neuron media for 18-24 hours (hr) prior to whole-cell patch-clamp experiments.

Electrophysiology

Patch micropipettes were pulled from 1.5/0.84 OD/ID (mm) borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) on a Flaming/Brown Micropipette puller P97 (Sutter Instruments, Novato, CA) and fire polished. Initial pipette resistances were 2–4 MΩ when filled with filtered internal solution containing (in mM): 100 L-aspartic acid (K⁺ salt), 30 KCl, 4.5 Na₂ATP, 1 MgCl₂, 10 HEPES, and 0.1 EGTA (pH adjusted to 7.2 with KOH). Current-clamp experiments were conducted by transporting coverslips containing adherent DRG neurons to a microscope stage plate and superfusing with HEPES-buffered external solution containing (in mM): 135 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 5 HEPES, 1 MgCl₂, 2 CaCl₂, and 5 glucose (pH adjusted to 7.4 with NaOH). Because small-diameter neurons correspond to nociceptive Aδ fiber and C-type neurons, only small neurons (<30 pF capacitance, C_m) were used (C_m = 16.06 ± 0.64 pF, n = 64) (Abraira and Ginty, 2013; Barabas *et al.*, 2014). Whole cell current-clamp recordings were made at room temperature using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), with a set protocol consisting of 0.01 nA steps beginning at -0.03 nA to assess both active and passive cell properties. Values reported did not reflect corrected junction potentials. Pulse generation and data acquisition were achieved with Clampex and Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA). Action potential (AP) first derivatives were determined using the differential function in Clampfit software and plotted as the first derivative of voltage with respect to time (dV/dT). Threshold potentials were defined as the voltage at which dV/dt deviated significantly from zero during the course of an action potential uprise.

Assessment of acute oxycodone effects began after a 2-3 min equilibration period, where an external solution containing 3 μM oxycodone solution was then superfused over neurons. Threshold potentials were determined from the first-derivatives of current-clamp recordings measured at 1 min intervals for 10 min following oxycodone exposure. The difference between threshold potential values at 0 and 10 min was calculated for each cell. Tolerance to oxycodone was assessed in an identical manner in cells that had been incubated overnight in media containing 10 μM oxycodone. The effect of ethanol on oxycodone tolerance was assessed by incubating cells overnight in 10 μM oxycodone followed by the addition of ethanol to the media (20mM [final]) for 50 min immediately preceding whole-cell patch clamp recording. Bis XI was added to the pre-filtered internal pipette solution at a final concentration of 100 nM to investigate the role of PKC inhibition on oxycodone tolerance. Neurons were treated overnight with 10 μM oxycodone and exposed to 100 nM Bis XI immediately upon membrane breakthrough. Neurons were then superfused with external solution containing 3 μM oxycodone. In all experiments, “N” represents the total number of mice and “n” represents the total number of cells within each group from which recordings were obtained.

Data Analysis

Statistical differences were calculated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Within-subject comparisons were analyzed via Student’s paired t-test. 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 \pm SEM.

IV. Results

Acute Oxycodone Reduced Neuronal Excitability. It has been established that DRG neurons isolated from adult rats and mice express opioid receptors (Coggeshall *et al.*, 1997; Wang *et al.*,

2010; Stein, 2013) and studies from our own lab have shown various effects of morphine on DRG neurons isolated from adult mice (Ross *et al.*, 2012; Kang *et al.*, 2017). However, the effects of oxycodone on DRG neuronal excitability have yet to be investigated. Candidate neurons were selected for their round spherical shape and small size. As shown in Figure 7, representative traces demonstrate the initiation of action potentials at rheobase (designated in red, Figure 7A) and the determination of threshold potential values from the calculated dV/dt plots before and after the application of 3 μM oxycodone (Figure 7B). Action potentials were recorded at rheobase every minute for ten minutes in 3 μM oxycodone external solution. Extrapolated threshold potential values gradually increased to more positive potentials as exposure to oxycodone continued, with a noticeable increase beginning 5 min post oxycodone application (Figure 8). Continuous superfusion of the 3 μM oxycodone bath for 10 min led to a statistically significant increase in threshold potential ($P < 0.01$, $N = 7$, $n = 12$) (Figure 9) and a decrease in action potential amplitude ($P < 0.05$) (Table 2). This concentration of oxycodone was the minimal effective concentration to elicit acute opioid effects as determined by the two factors listed above, as a 10 min exposure to 1 μM oxycodone did not result in significantly increased threshold potentials (data not shown). Other cell parameters such as peak action potential height, input resistance, resting membrane potential and rheobase were measured and analyzed, but no statistically significant changes were found after an acute 3 μM oxycodone challenge (Table 2).

To determine if the acute effects of oxycodone on neuronal excitability were mediated by opiate receptors, we co-perfused external solution over neurons that contained 3 μM oxycodone and 300 nM naloxone. The addition of naloxone prevented the increase in threshold potentials observed after acute oxycodone alone ($N = 2$, $n = 4$, $P > 0.05$) (Figure 10).

Table 2. Active and Passive Cell Properties of Dorsal Root Ganglia Neurons in Response to 3 μ M Oxycodone.

Changes in various parameters were analyzed at baseline (0 minutes) and 10 minutes following the application of an external solution containing 3 μ M oxycodone. Threshold potential (VThresh) and action potential amplitude (AP Amp) were significantly reduced in response to oxycodone in all treatment groups except that which only received 10 μ M oxycodone overnight. Membrane capacitance (CMem), resting membrane potential (VRest), peak action potential height (AP VPeak), rheobase and input resistance (RInput) were all unaffected by 3 μ M oxycodone. Asterisks * (P < 0.05) and ** (P < 0.01) denote statistically significant differences determined by Student's one-tailed paired t-test.

	Control	+ 3 μ M Oxy	10 μ M Oxy	+ 3 μ M Oxy	10 μ M Oxy + EtOH	+ 3 μ M Oxy	10 μ M Oxy + Bis XI	+ 3 μ M Oxy
CMem (pF)	17.31 \pm 1.47		13.45 \pm 2.01		13.38 \pm 1.15		19.26 \pm 1.66	
VRest (mV)	-48.53 \pm 1.50	-46.15 \pm 2.59	-49.58 \pm 3.85	-43.54 \pm 2.26	-48.78 \pm 1.77	-46.94 \pm 1.95	-45.59 \pm 1.81	-39.85 \pm 2.61
AP VThresh (mV)	-17.13 \pm 1.43	-12.55 \pm 1.20**	-23.77 \pm 3.79	-23.18 \pm 3.72	-14.74 \pm 1.59	-10.81 \pm 2.17**	-14.33 \pm 1.67	-9.66 \pm 1.73*
AP VPeak (mV)	64.46 \pm 1.67	60.50 \pm 4.19	62.50 \pm 6.79	63.70 \pm 6.61	61.33 \pm 2.78	56.19 \pm 4.04	62.76 \pm 2.86	64.19 \pm 2.17
AP Amp (mV)	81.59 \pm 1.78	73.26 \pm 4.74*	87.86 \pm 9.17	86.88 \pm 10.01	76.07 \pm 3.36	67.00 \pm 5.21*	77.09 \pm 2.82	73.85 \pm 3.03
Rheobase (nA)	0.04 \pm 0.01	0.06 \pm 0.01	0.02 \pm .002	0.03 \pm 0.02	0.05 \pm 0.01	0.03 \pm 0.01	0.05 \pm 0.01	0.04 \pm 0.01
RInput (M Ω)	1440.0 \pm 222.98	1160.56 \pm 244.40	1600.0 \pm 110.47	1346.67 \pm 442.95	1241.39 \pm 197.73	1197.92 \pm 181.20	1093.33 \pm 118.03	782.08 \pm 118.88

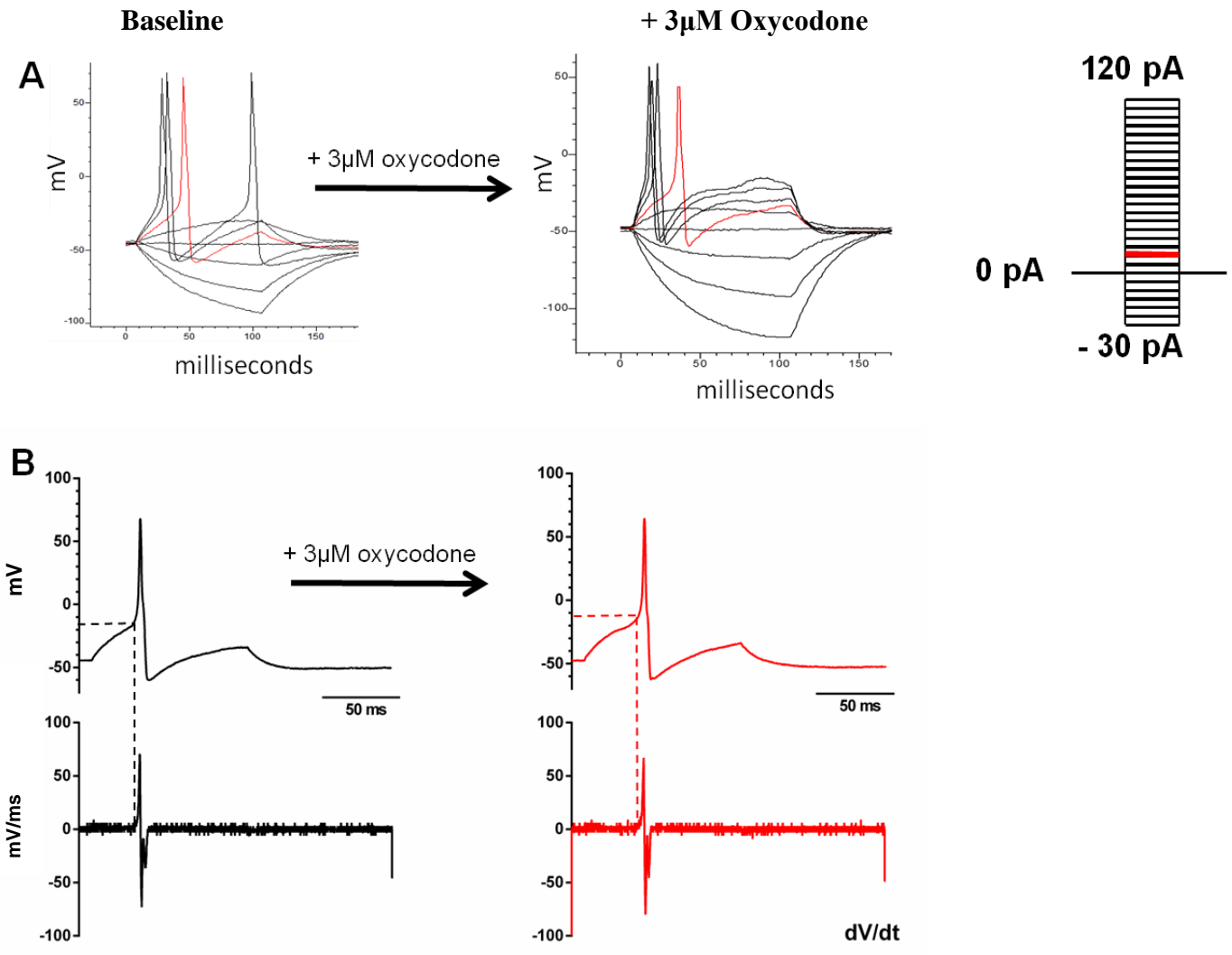


Figure 7. Acute Oxycodone Reduced DRG Neuronal Activity. (A) Raw Traces representing neuronal activity before (left panel) and after (right panel) 3 μ M oxycodone, with the associated protocol (top right) showing stepwise increase in current injection corresponding to the initiation of action potentials. (B) Upper panel: Action potential at rheobase (left panel) before and after 3 μ M oxycodone (right panel). Bottom panel: Derivative (dV/dt) of the action potential traces. Dotted line indicates threshold potential value determined from the change in dV/dt.

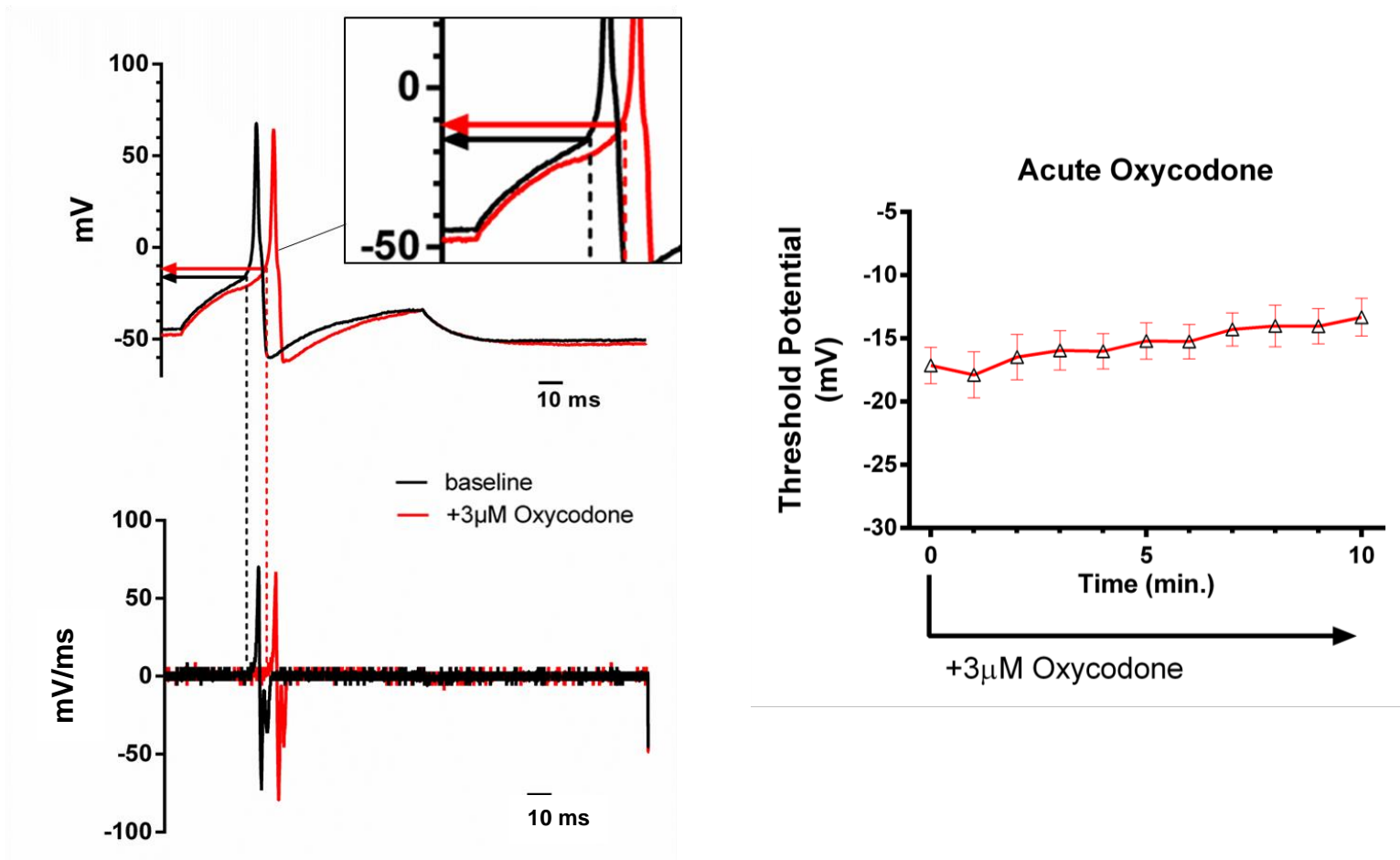


Figure 8. Response to Acute 3 μ M Oxycodone in DRG Neurons. Representative Traces (top left panel) showing action potentials at rheobase before (black) and after (red) 3 μ M oxycodone. Dotted lines represent threshold potential values as determined by dV/dt traces (bottom left panel). Neurons grown overnight in untreated media responded to an acute challenge of 3 μ M oxycodone, as seen by the increase in threshold potential over a period of ten minutes (right panel).

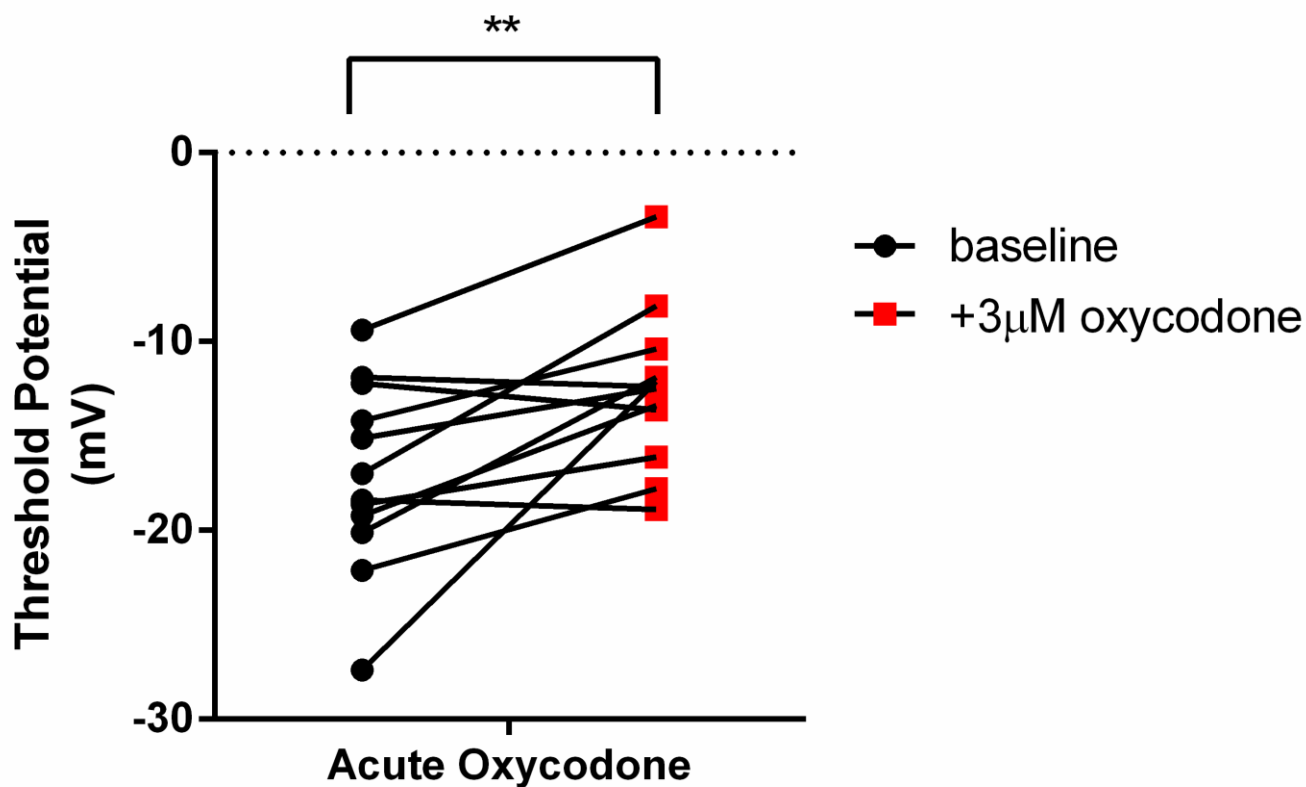


Figure 9. The Effect of 3 μ M Oxycodone on Individual DRG Neuron Threshold Potentials.

Data represent individual changes in threshold potentials before (●) and 10 minutes after (■) oxycodone exposure. DRG neurons were incubated overnight in untreated media and exposed to 3 μ M oxycodone for 10 min (N = 7, n = 12). A significant increase in threshold potential values was observed following acute oxycodone exposure (**P < 0.01, Student's one-way paired t-test).

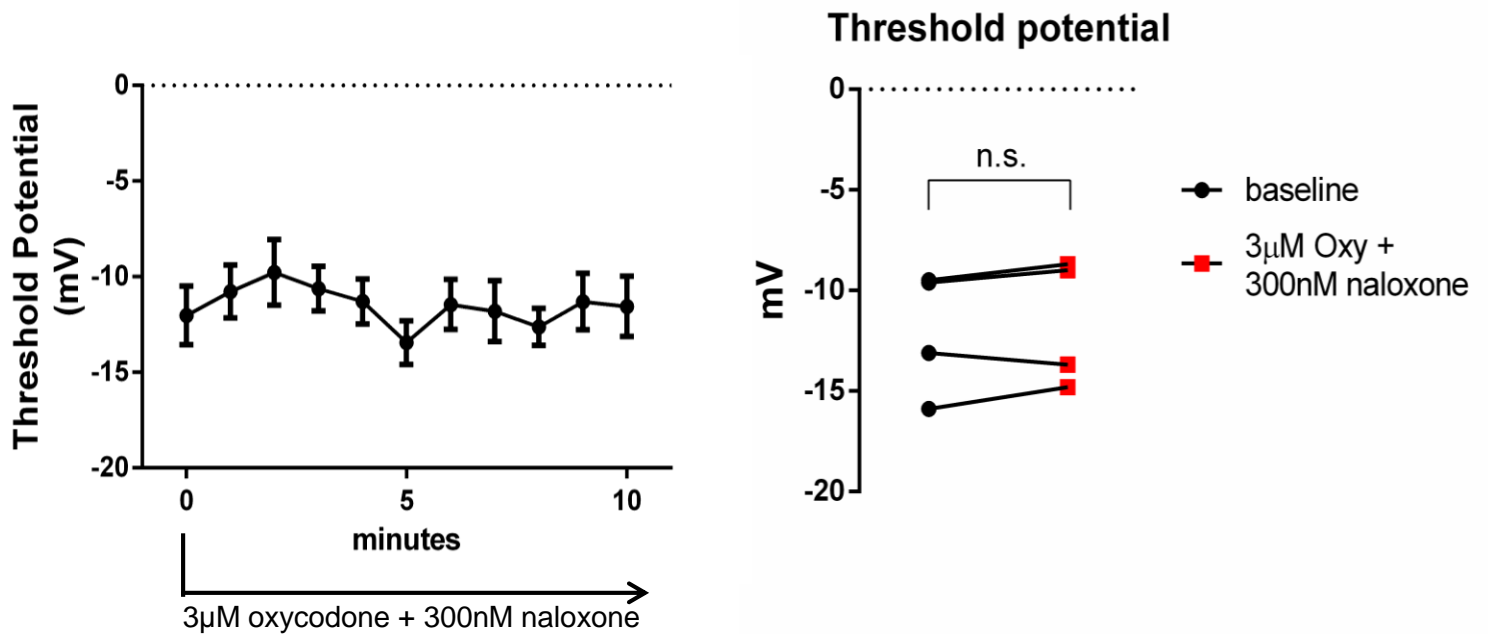


Figure 10. Co-administration of Naloxone Blocked the Acute Effects of Oxycodone. DRG neurons were perfused with external solution containing 3 μ M oxycodone and 300 nM naloxone for ten minutes. Naloxone prevented the shift in threshold potentials to a more positive value induced by 3 μ M oxycodone ($P > 0.05$, Student's one way paired t-test, $N = 2$, $n = 4$). Data are shown as mean \pm SEM in the left panel and as individual points in the right panel.

Overnight Exposure to Oxycodone Led to Tolerance. Previously, we have shown tolerance to morphine in DRG neurons isolated from mice implanted with morphine pellets for 5 days (Ross *et al.*, 2012; Kang *et al.*, 2017). Here we tested the hypothesis that prolonged exposure to oxycodone *in vitro* would lead to a tolerant phenotype. Neurons (N = 4, n = 6) were incubated overnight (minimum 18 hr) with 10 μ M oxycodone prior to being moved to the microscope stage plate containing an external solution with no drug treatment. Baseline threshold potentials were lower, on average, than in cells not treated with oxycodone, indicating a more excitable state was induced simply from prolonged oxycodone exposure (Figure 11). Tolerance occurred since these cells did not respond to a 3 μ M oxycodone challenge continuously applied over 10 min, as threshold potentials did not shift from baseline values ($P > 0.05$) (Figure 12) and amplitude remained the same (Table 2).

To validate this model of tolerance, we tested the development of tolerance in DRG neurons isolated from β -arrestin 2 (β -arr2) WT and KO mice (Figure 13). The response to 3 μ M morphine was absent in WT neurons incubated overnight with 10 μ M morphine, as observed by the lack of an increase in threshold potentials (Figure 13A) (N = 4, n = 6). Neurons isolated from β -arr2 KO mice however, responded to a 3 μ M morphine challenge the following day, as indicated by a significant shift in threshold potential values (Figure 13b, $P < 0.05$; N = 4, n = 6). Morphine is well known to recruit β -arrestin 2, a scaffolding protein thought to contribute to tolerance mechanisms (Bohn *et al.*, 2000; Raehal and Bohn, 2011; Williams *et al.*, 2013). Neurons isolated from mice lacking β -arrestin 2 continued to respond to a 3 μ M morphine challenge following exposure to 10 μ M morphine overnight and did not exhibit signs of tolerance to morphine. However in WT neurons, where β -arrestin 2 was functional, overnight incubation in 10 μ M morphine prevented the response to 3 μ M morphine challenge on day 2,

indicating tolerance developed within those neurons. Based on these results, we are confident that true cellular tolerance to oxycodone and to morphine developed *in vitro* within a single DRG neuron following overnight exposure to 10 μ M of either opioid.

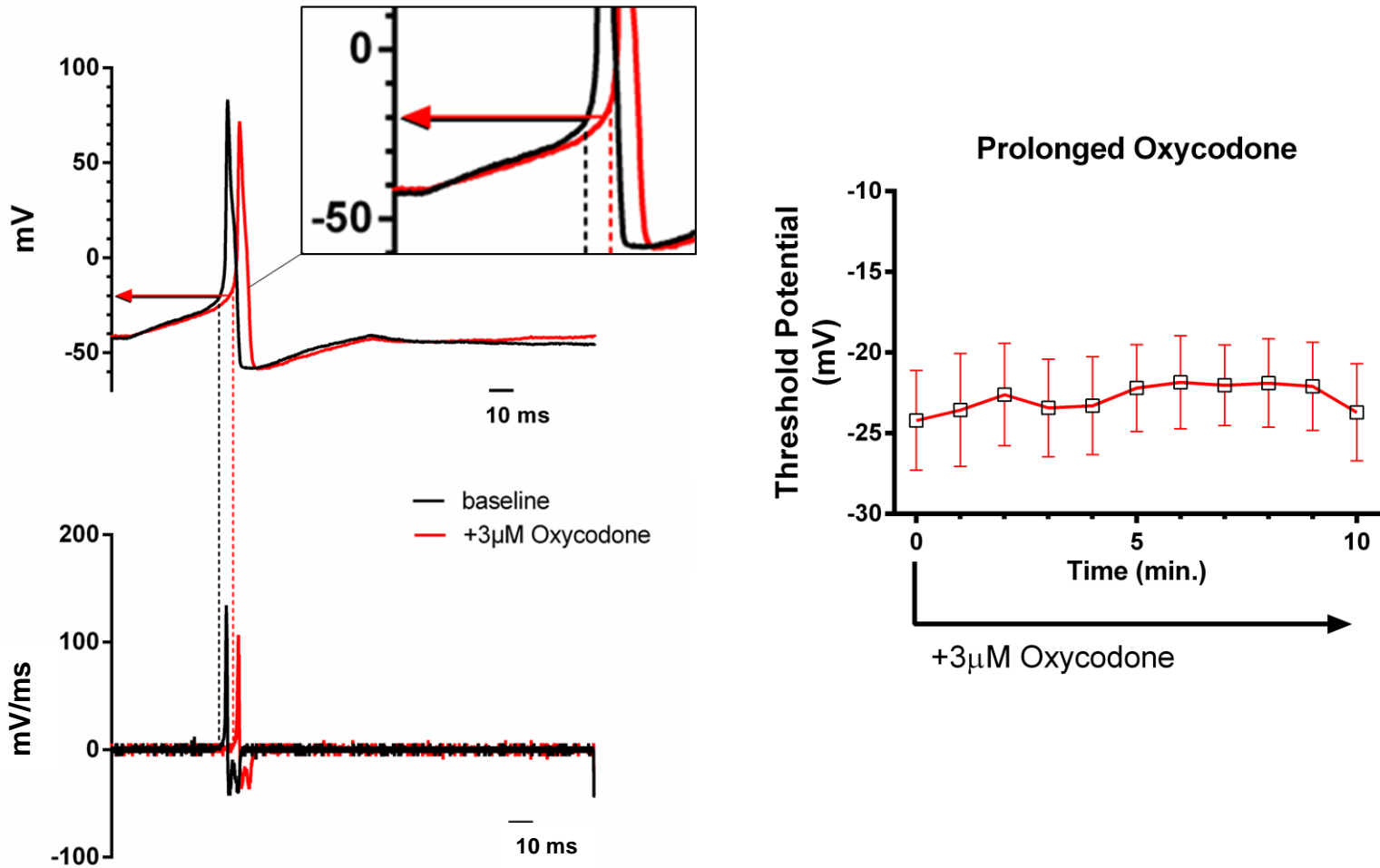


Figure 11. Response to 3 μ M Oxycodone in DRG Neurons Treated Overnight with 10 μ M Oxycodone. Representative Traces (top left panel) showing action potentials at rheobase before (black) and after (red) 3 μ M oxycodone in DRG neurons following an overnight treatment with 10 μ M oxycodone. Dotted lines represent threshold potential values as determined by dV/dt traces (bottom left panel). Neurons grown overnight in media treated with 10 μ M oxycodone did not show a statistically significant shift in threshold potential response to a 3 μ M oxycodone challenge (right panel).

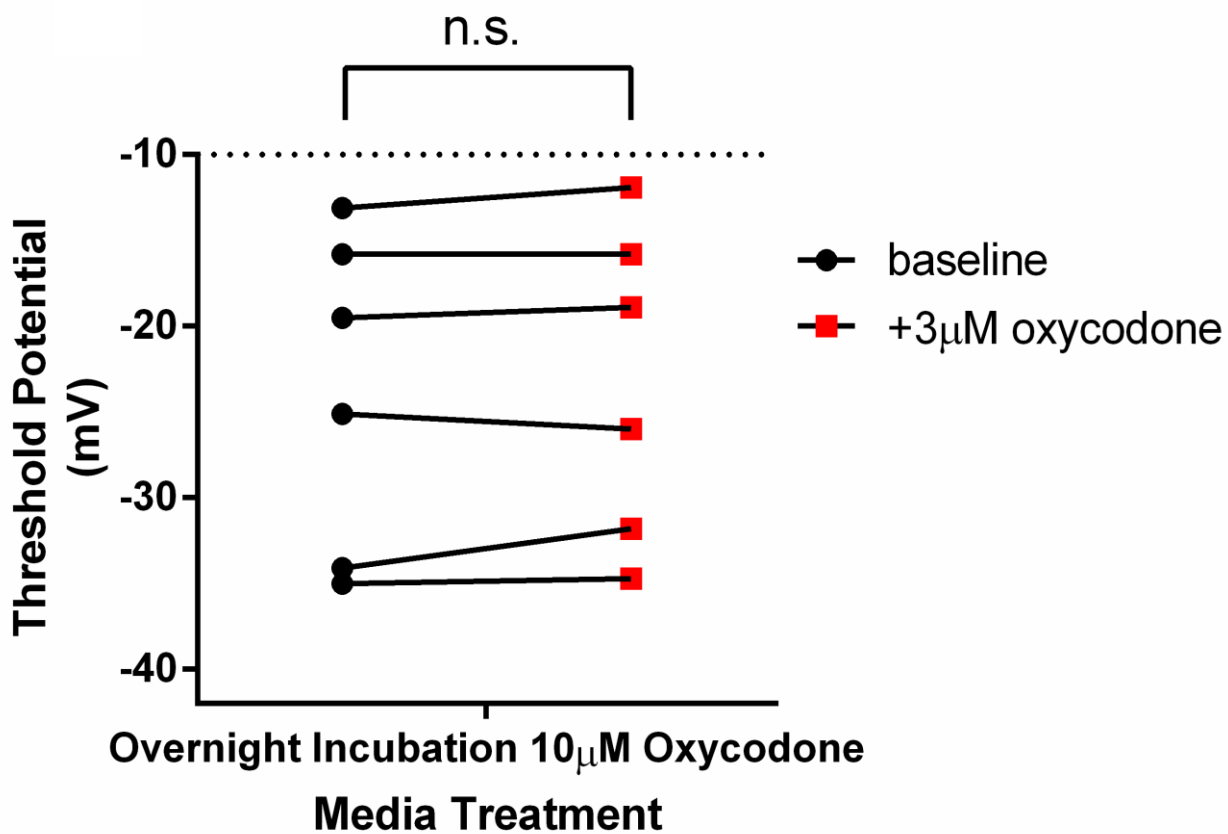


Figure 12. The Effect of 3µM Oxycodone on Individual DRG Neuron Threshold Potentials Following Overnight Incubation in 10µM Oxycodone. Data represent individual changes in threshold potentials before (●) and 10 minutes after (■) oxycodone exposure. DRG neurons were incubated overnight in media treated with 10µM oxycodone and exposed to a challenge bath containing 3µM oxycodone for 10 minutes (N = 4, n = 6). No significant increases in threshold potential values were observed following the challenge oxycodone exposure, indicating that tolerance to oxycodone had developed in these DRG neurons (P > 0.05, Student's one-way paired t-test).

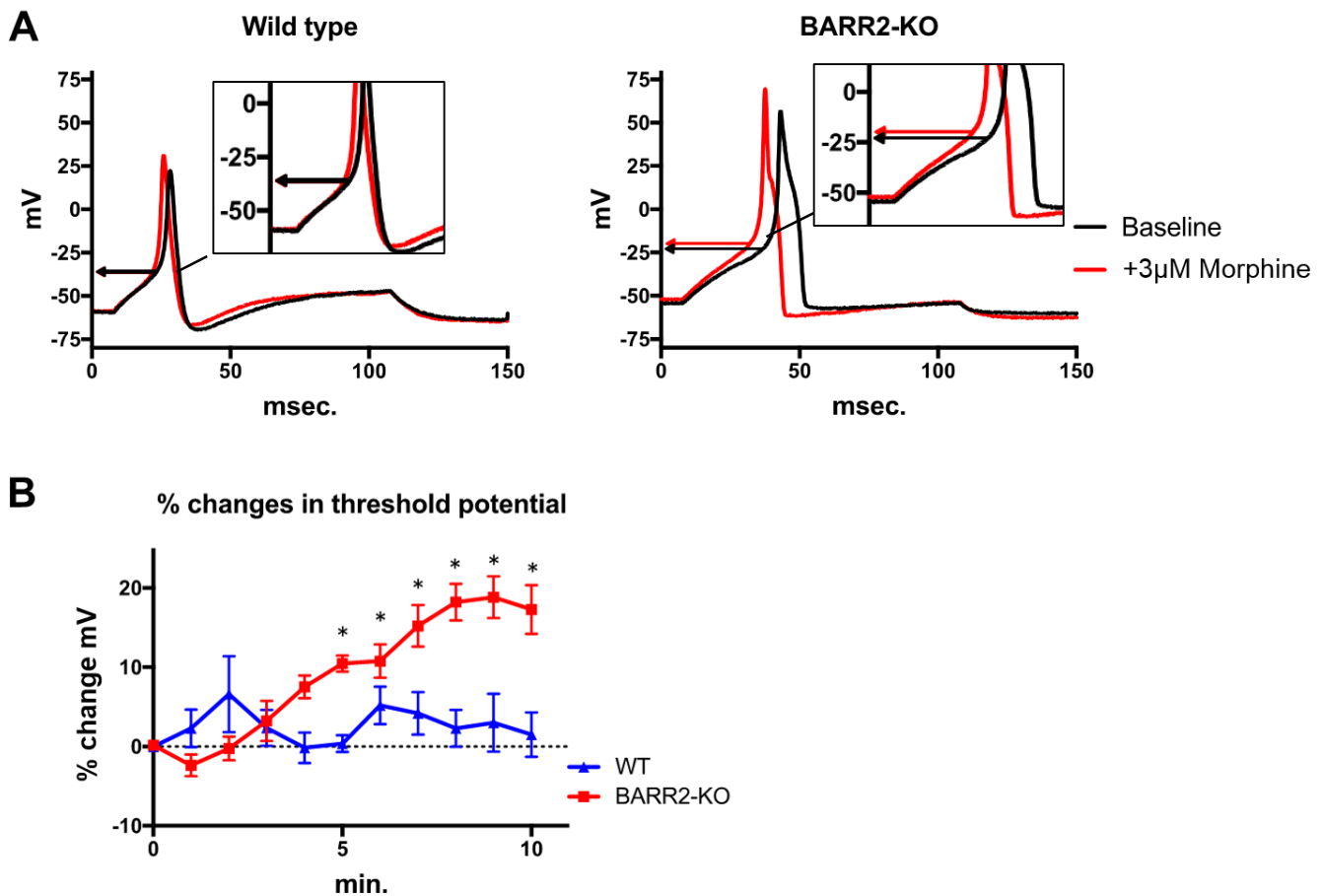


Figure 13. Tolerance Developed to Morphine in β -Arrestin 2 WT, but not KO DRG Neurons². Threshold potential values increased in DRG neurons incubated overnight with 10µM morphine from BARR2-KO mice, but not WT, by external solution containing 3µM morphine. In BARR2-KO neurons (A, right), threshold potential was clearly increased in contrast to WT (A, left). Increases were observed in threshold potential over time in BARR2-KO, but not WT DRG neurons following Morphine [3µM] (B). Each group represents the mean \pm SEM (BARR2-KO: N=4, n=6, WT: N=4, n=6). Statistically significant differences in mean threshold potentials were detected at 5-10 minutes compared to baseline values in BARR2-KO DRG neurons, as determined by Two-way ANOVA with repeated measures, followed by Bonferroni's post-hoc test, significance at *P < 0.05.

² The data for this figure were generated by Dr. Kensuke Sakakibara, who is an author on the manuscript from which this chapter was generated.

Acute Ethanol Reversed Oxycodone Tolerance. Based on the evidence that tolerance to oxycodone occurred following overnight exposure, we tested the hypothesis that acute exposure to ethanol would reverse that tolerance. Following an overnight incubation in 10 μ M oxycodone, ethanol [20mM] was added directly to the DRG neuron coverslips while still present in growth media containing 10 μ M oxycodone. A period of 50 minutes elapsed prior to transferring the coverslip to the microscope stage plate. This timing directly corresponded to the amount of time ethanol was given to reverse oxycodone tolerance *in vivo* (Jacob *et al.*, 2017). Neurons (N = 8, n = 13) were initially exposed to an external bath solution without any drug treatments, and baseline recordings were obtained. Interestingly, we no longer observed the heightened excitability at baseline that was noted in the cells exposed only to 10 μ M oxycodone overnight. Cells were then exposed to a bath containing 3 μ M oxycodone for 10 minutes. Threshold potential values increased in response ($P < 0.05$), showing similar shifts observed under acute oxycodone conditions (Figure 14). Action potential amplitude height was also significantly decreased ($P < 0.05$) (Table 2). These cells no longer displayed a tolerant phenotype, and the response to oxycodone indicated that acute exposure to ethanol reversed tolerance within a single neuron (Figure 15). The same 50-minute 20 mM ethanol treatment had no effect on DRG neurons when assessed for changes in excitability over a 10-minute time period (Figure 16), suggesting the effects observed in the neurons exposed to prolonged oxycodone followed by ethanol were due specifically to oxycodone.

The Investigation of the Mechanism of Ethanol Reversal Using an Inhibitor of PKC.

Inhibition of PKC Rapidly Reversed Oxycodone Tolerance. Various PKC inhibitors have also been reported to reverse tolerance to both the antinociceptive effects and the respiratory depressive effects of morphine *in vivo* (Smith *et al.*, 2003; Javed *et al.*, 2004; Hull *et al.*, 2010; Withey *et al.*, 2017). Additionally, we have implicated PKC inhibition in ethanol reversal of morphine tolerance *in vitro* in locus coeruleus neurons (Llorente *et al.*, 2013). We tested the hypothesis that inhibiting PKC would also reverse oxycodone tolerance. Neurons (N = 7, n = 8) incubated overnight with 10 μ M oxycodone were transferred to the microscope stage plate and submerged into an untreated external bath solution. In these studies, the PKC inhibitor, Bisindolylmaleimide XI, HCl (Bis XI) was added to the internal pipette solution at a concentration of 100nM. Baseline recordings showed these neurons had similar threshold potentials to untreated neurons, suggesting that the inhibition of PKC eliminated the hyperexcitability state observed in neurons exposed only to 10 μ M oxycodone (Table 2). Upon exposure to a 3 μ M oxycodone bath challenge over 10 minutes, these neurons containing 100nM Bis XI internally showed a robust decrease in excitability (Figure 17). Threshold potential values were shifted significantly by 3 μ M oxycodone to a more positive potential (P < 0.05, Two-way ANOVA) (Figure 18). Action potential amplitude was reduced on average, however the p-value was just outside the pre-determined significance level (P = 0.06, Student's one-way paired t-test) (Table 2). Significance would likely be reached if the sample size was increased. The onset of the response time to an oxycodone challenge was similar to that observed in neurons representing either acute oxycodone conditions or neurons treated with 10 μ M oxycodone and ethanol. The rate at which oxycodone tolerance reversal occurred in the experiments with Bis XI suggests that the mechanism(s) underlying reversal by PKC inhibition are rapid. Furthermore,

the percent change in threshold potential values from baseline to 10 minutes that resulted from exposure to 3 μ M oxycodone was significantly different compared to control DRG neurons that did not receive drug, or the DRG neurons incubated with 10 μ M oxycodone overnight (Figure 19). This was observed in DRG neurons acutely treated with oxycodone, as well as those incubated overnight with oxycodone but pretreated with ethanol. From these data we concluded that tolerance developed to oxycodone and was measurable at the level of the neuronal cell body, and that this tolerance was reversed by the direct application of either ethanol or the PKC inhibitor, Bis XI, to those neurons.

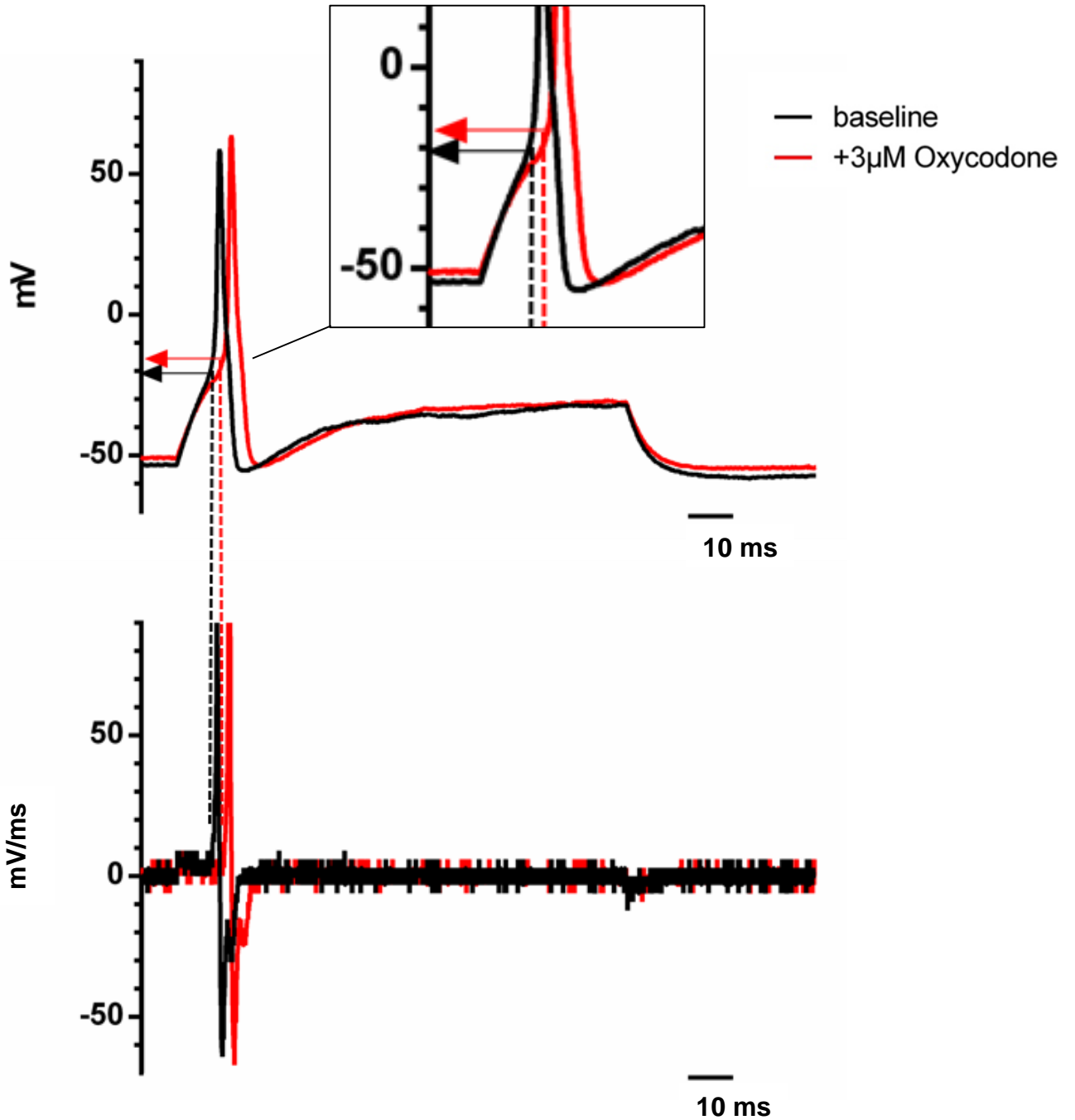


Figure 14. Representative Traces: The Effect of Ethanol on the Response to 3 μ M Oxycodone in DRG Neurons Incubated Overnight with 10 μ M Oxycodone. Oxycodone tolerance was reversed by a 50 minute pretreatment of 20 mM ethanol as indicated by the shift in threshold potential values following a 3 μ M oxycodone challenge.

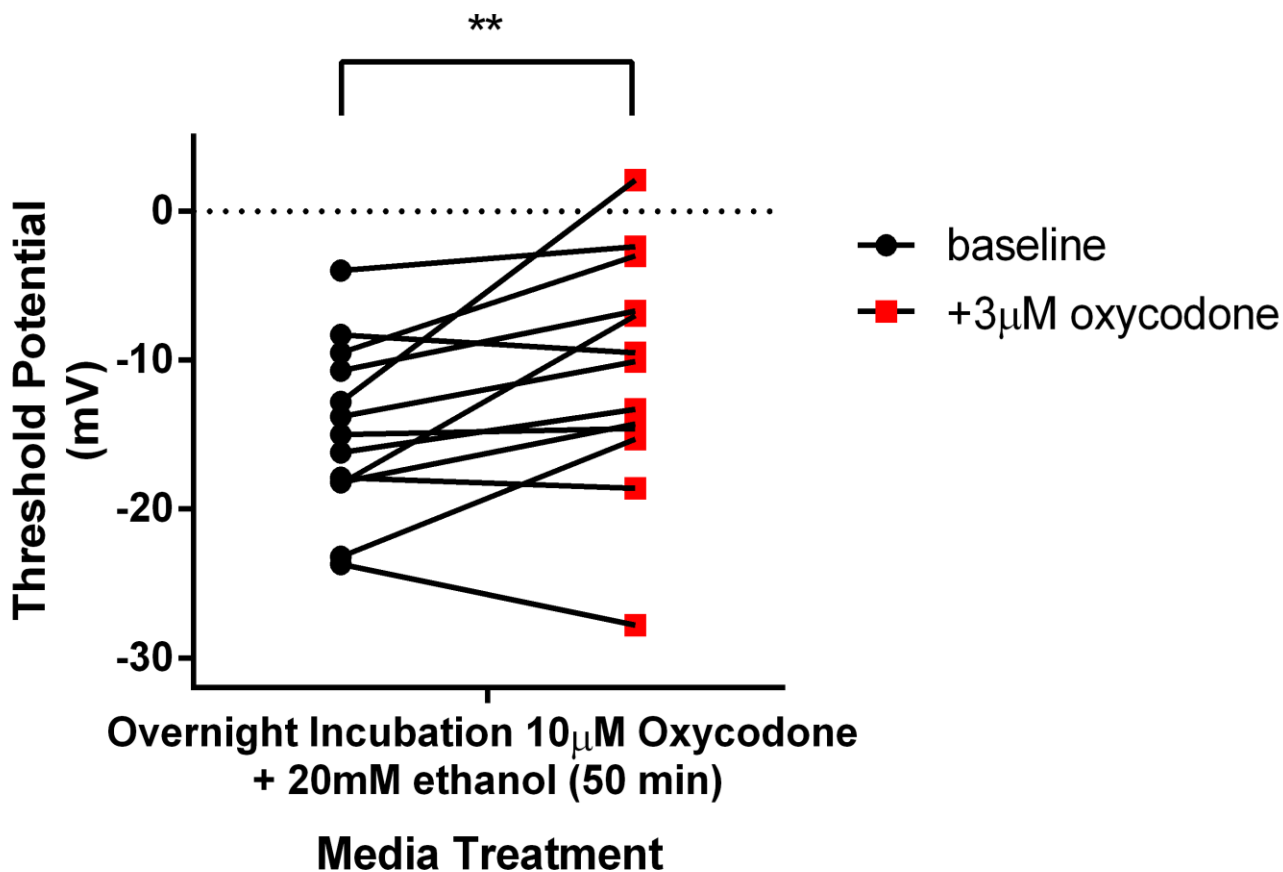


Figure 15. The Effect of Acute Ethanol on Individual DRG Neuron Threshold Potentials Following Overnight Incubation in 10 μM Oxycodone. Data represent individual changes in threshold potentials before (●) and 10 minutes after (■) oxycodone exposure. DRG neurons were incubated overnight in media treated with 10 μM oxycodone and exposed to a 50-minute pretreatment of 20 mM ethanol in the media prior to being moved to the microscope stage. A challenge bath solution containing 3 μM oxycodone was perfused over the DRG neurons for a 10-minute recording period (N = 8, n = 12). Significant increases in threshold potential values were observed following the challenge oxycodone exposure, indicating tolerance to oxycodone had been reversed by ethanol in these DRG neurons (** P < 0.01, Student's one-way paired t-test).

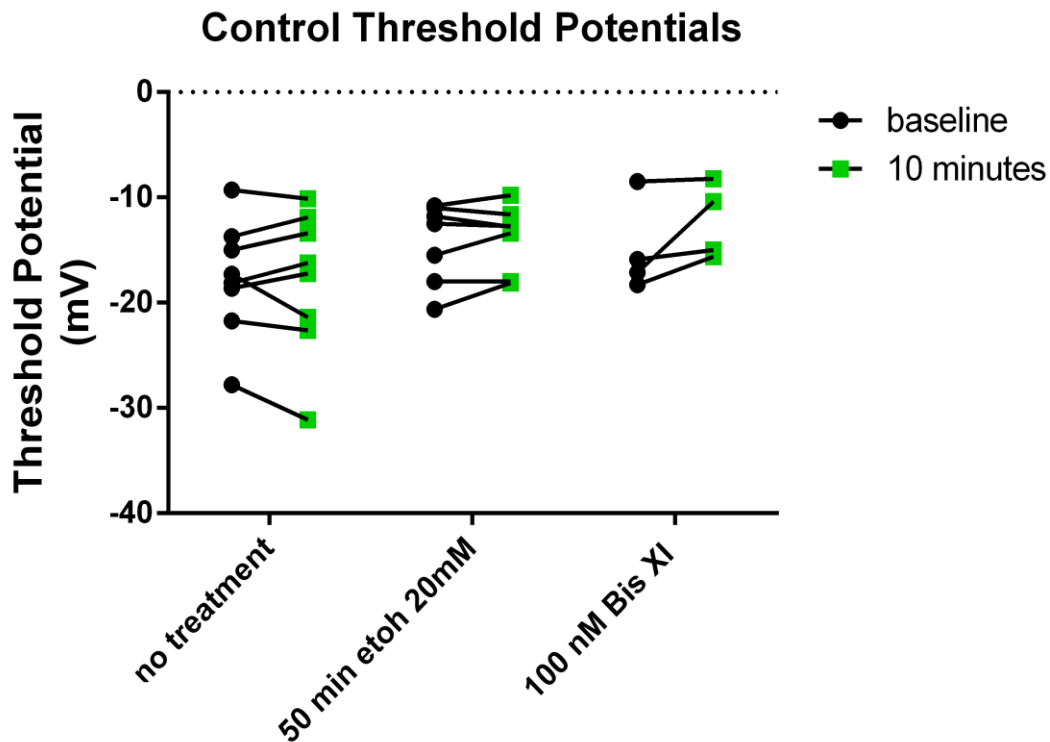


Figure 16. Control Experiments: Threshold Potential Values in the Absence of Oxycodone.

Threshold potential values are displayed in DRG neurons grown overnight in untreated media and exposed to no drug treatment (left), a 50-minute pretreatment with 20 mM ethanol (middle), or to 100 nM Bis XI via the internal pipette solution (right). Data represent individual changes in threshold potentials at time 0 (●) and 10 minutes (■) following application of external solution without any oxycodone treatment. Statistical significance was considered if $P < 0.05$ following analysis by Two-way ANOVA (repeated measures) with Bonferroni's post-hoc test. No significant differences were detected between time 0 and time 10 minutes in these control groups, nor were there differences detected between groups.

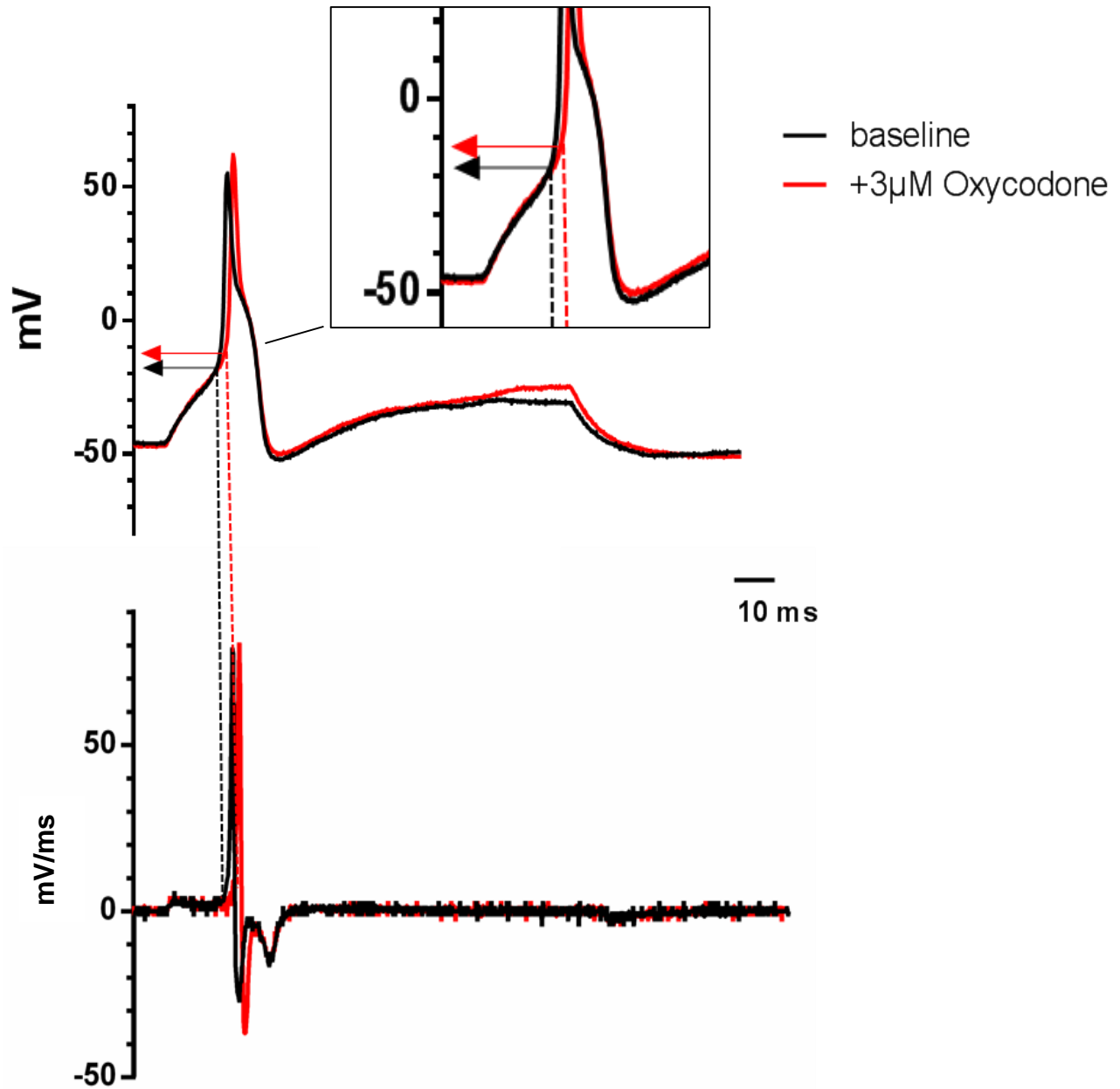


Figure 17. Representative Traces: The Effect of the PKC Inhibitor, Bis XI, on the Response to 3 μ M Oxycodone in DRG Neurons Incubated Overnight with 10 μ M Oxycodone.

Oxycodone tolerance was significantly reversed by 100 nM Bis XI, contained in the internal pipette solution, as indicated by the shift in threshold potential values following a 3 μ M oxycodone challenge.

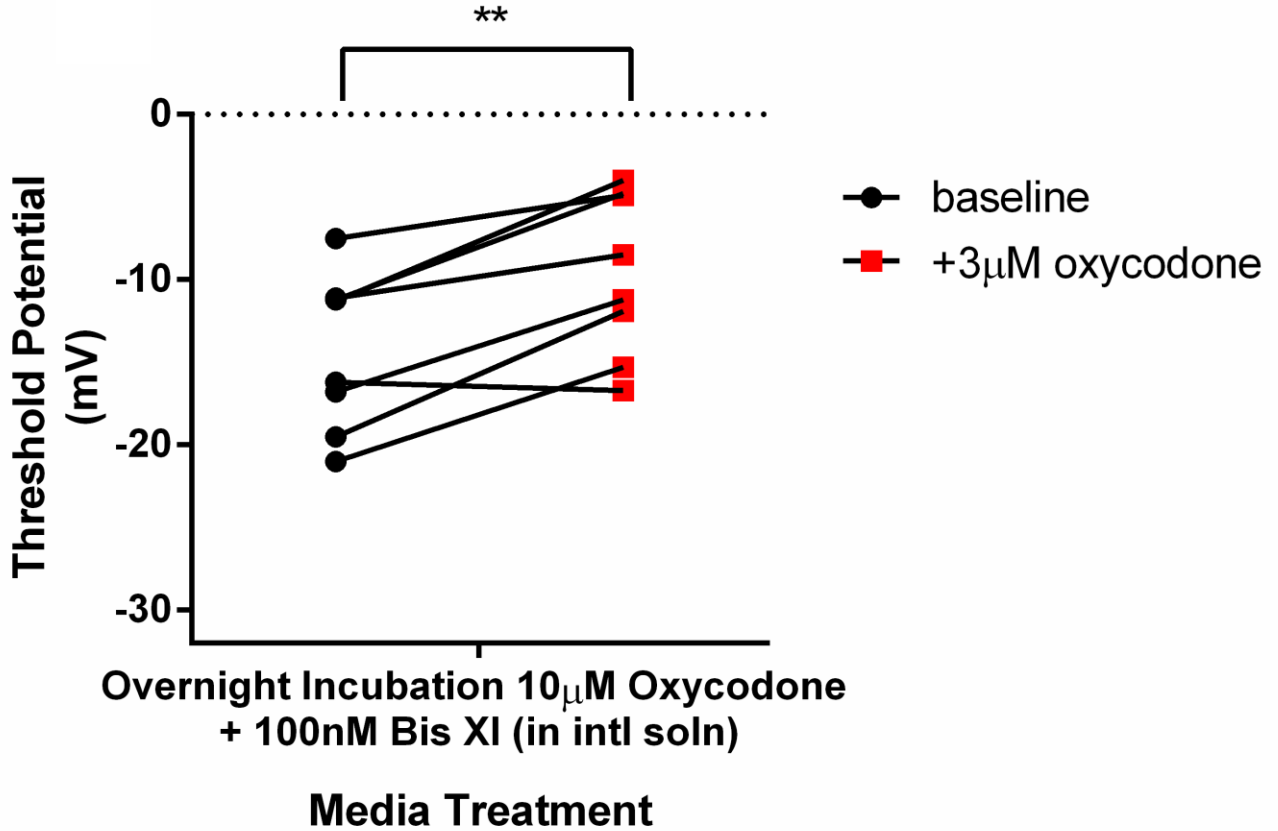


Figure 18. The Effect of the PKC Inhibitor, Bis XI, on Individual DRG Neuron Threshold Potentials Following Overnight Incubation in 10 μM Oxycodone. Data represent individual changes in threshold potentials before (●) and 10 minutes after (■) oxycodone exposure. DRG neurons were incubated overnight in media treated with 10 μM oxycodone and exposed to 100nM Bis XI via the internal pipette solution. A challenge bath solution containing 3 μM oxycodone was perfused over the DRG neurons for a 10-minute recording period (N = 3, n = 8). Significant increases in threshold potential values were observed following the challenge oxycodone exposure, indicating tolerance to oxycodone had been reversed by Bis XI in these DRG neurons (** P < 0.01, Student's one-way paired t-test).

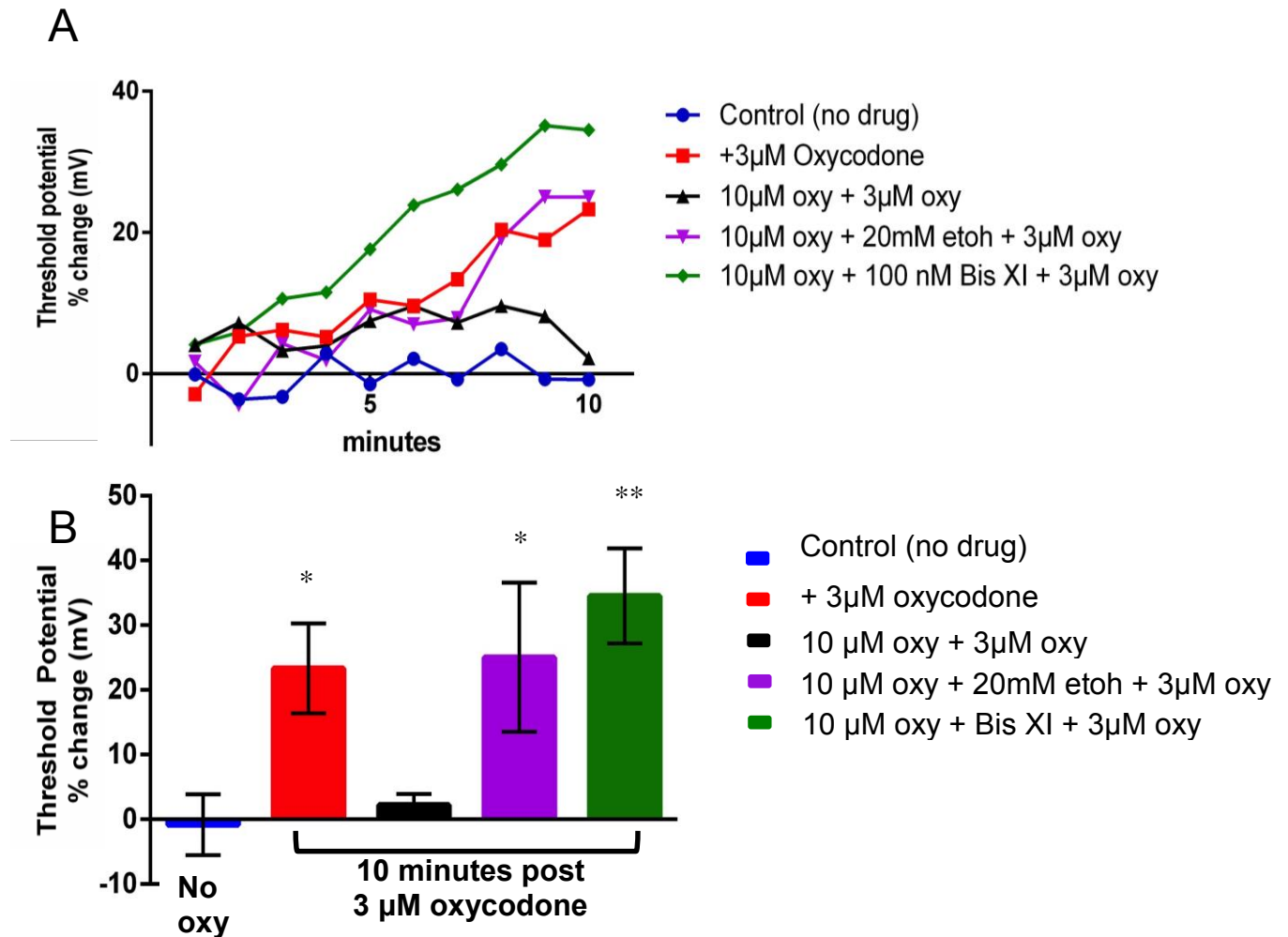


Figure 19. Time-Dependent Change in Threshold Potentials to 3 μ M Oxycodone.

A) Threshold potentials were determined by dV/dt of action potentials every minute for 10 minutes. Data are presented as percent change from baseline values. Blue line (upper panel) and bar (lower panel) represent threshold potentials in the absence of oxycodone. B) Bar graph showing percent change at the 10-minute time point for each group. Data were calculated and analyzed using Two-way ANOVA with Bonferroni's post hoc test. Threshold potentials increased in response to an external bath solution containing 3 μ M oxycodone in untreated DRG neurons (\square , red) ($P < 0.05$; $N = 7$, $n = 12$), and in DRG neurons pretreated with 10 μ M oxycodone that were exposed to 20mM ethanol (∇ , purple) ($P < 0.05$; $N = 8$, $n = 12$) or 100nM

Bis XI (\diamond , green) ($P < 0.01$; $N = 3$, $n = 8$). Overnight treatment with 10 μM oxycodone (Δ , black) led to tolerance to the effects of 3 μM oxycodone ($P > 0.05$; $N = 4$, $n = 6$). No changes in threshold potential were observed over the 10 minute recording time in control neurons (\circ , blue) that were not exposed to oxycodone ($P > 0.05$; $N = 6$, $n = 8$).

V. Discussion

Our studies described here represent the first reported findings of a variety of oxycodone effects in DRG neurons. Opioids, including oxycodone, are primarily thought to elicit their antinociceptive effects through both central and peripheral mechanisms, however tolerance to that effect has predominantly been studied with a focus on mechanisms within the central nervous system (Patrick *et al.*, 1975; Hull *et al.*, 2010). Dorsal root ganglia sit in the periphery, adjacent to the spinal cord, just outside the blood-brain-barrier. Here we show that tolerance developed to the acute effects of oxycodone at the level of a single DRG neuron. Our subsequent aim investigated whether ethanol or a PKC inhibitor could reverse tolerance to oxycodone in DRG neurons, as they each have previously been shown to reverse various morphine tolerances both *in vivo* and *in vitro* (Bailey *et al.*, 2009; Hull *et al.*, 2010, 2013; Llorente *et al.*, 2013; Hill *et al.*, 2016). We found that neurons exposed to either ethanol or the PKC inhibitor, Bis XI, responded to 3 μM oxycodone despite a prior overnight exposure to oxycodone, indicating tolerance to oxycodone had been reversed.

The Effect of Acute Oxycodone on Dorsal Root Ganglia Neuron Excitability.

We found an acute effect of 3 μM oxycodone on threshold potential and action potential amplitude height. These two characteristics have previously been reported to change following the acute application of morphine on DRG neurons, suggesting that oxycodone acts like morphine and likely works through similar mechanisms at the cellular level (Kang *et al.*, 2017). We believe these data are the first reported effects of oxycodone on murine DRG neuronal excitability. Other cell parameters such as resting membrane potential and input resistance were not affected by acute oxycodone, which agrees with published morphine studies in both central and peripheral neurons (Siggins and Zieglgänsberger, 1981; Ross *et al.*, 2012). One explanation

for the reduced excitability following application of morphine, and now oxycodone, is the reduction in available sodium channels. Only one other study has attempted to investigate the role of oxycodone on sodium channels, where tetrodotoxin-resistant sodium channels (TTX-R Na) were specifically evaluated (Osawa *et al.*, 2007). It was found that oxycodone continued to reduce the maximum sodium current output in rat DRG neurons pre-treated with tetrodotoxin (TTX), and that it was mediated via opioid receptor independent pathway(s) since naloxone did not prevent the reduced current elicited by oxycodone. Furthermore, much higher concentrations of oxycodone were utilized than in our studies, ranging from 10 μ M to 10 mM. We observed, however, that when 300 nM naloxone was co-perfused with 3 μ M oxycodone, the acute response to oxycodone was blocked. This suggests that in DRG neurons, when both TTX-sensitive and TTX-resistant sodium channels are available, neuronal excitability is affected by acute oxycodone in an opioid-receptor dependent manner. Further studies using selective antagonists for each of the three opiate receptors (μ , δ and κ) will need to be conducted to determine which receptor, or receptors, mediate oxycodone's effects on DRG neurons.

The Effects of Prolonged Exposure to Oxycodone.

Tolerance to oxycodone at the single-cell level using DRG neurons has not been shown prior to our studies presented here. We developed a model of *in vitro* tolerance by incubating neurons in media containing 10 μ M oxycodone for at least 18 hours. When challenged with 3 μ M oxycodone the following day, these neurons showed no response, but did display a hyper-excitable baseline threshold value in comparison to untreated neurons. This has been reported by others following prolonged morphine treatment in DRG neurons and is thought to be due, in part, to a shift in TTX-resistant sodium channels (Chen *et al.*, 2012; Ross *et al.*, 2012). We have not evaluated if this is the case in oxycodone-treated DRG neurons, but we hypothesize that

oxycodone is activating similar mechanisms based on the analogous findings. Additionally, the fact that tolerance was observed in isolated DRGs suggests that reduced antinociceptive responses *in vivo* following repeated oxycodone treatment could be influenced not only by tolerant μ -opioid receptors in central neuron populations but also those expressed in peripheral neuronal populations.

Reversal of Tolerance to Oxycodone by Ethanol or a PKC Inhibitor

Our data showed a robust reversal of oxycodone tolerance by a low-to-moderate concentration of ethanol [20 mM] in DRG neurons, suggesting that the mechanisms of ethanol reversal occur via intracellular signaling pathways independent of pharmacokinetic influences or neuronal signaling networks. Most states enforce a legal driving limit for blood alcohol concentrations to 0.08% or lower, which corresponds to approximately 17 mM ethanol (Miller, 2013), suggesting that the reversal of tolerance does not require consumption of excessive amounts of alcohol or extremely high blood alcohol concentrations. This concentration of ethanol was also sufficient to reverse morphine tolerance in locus coeruleus neurons (Llorente *et al.*, 2013), indicating that the mechanisms underlying the reversal of opioid tolerance are functional in multiple neuronal populations.

To our knowledge, the effect of inhibiting PKC or its specific isoforms on oxycodone tolerance has not been investigated in DRG neurons. By applying the PKC inhibitor Bis XI in the internal pipette solution, we were able to demonstrate that inhibiting PKC in neurons exposed to oxycodone overnight led to a rapid reversal of tolerance, due to the continued response to a 3 μ M oxycodone challenge. Based on published IC_{50} calculations, this concentration of Bis XI [100nM] selectively inhibits two isoforms: the conventional isoform, PKC α , and the novel isoform, PKC ϵ , with a 10-fold selectivity for PKC α over PKC ϵ (Wilkinson *et al.*, 1993). These

isoforms have previously been identified as having important roles in maintaining opioid tolerance and implicated to have potential interactions with ethanol (Bailey *et al.*, 2006, 2009; Smith *et al.*, 2007; Wilkie *et al.*, 2007). This data highlights the fact that like morphine, oxycodone tolerance mechanism(s) at the neuronal level contain a PKC-mediated portion and can be reversed by direct and selective inhibition of PKC α and ϵ isoforms. Furthermore, this data suggests that ethanol reversal could also involve a PKC-mediated mechanism.

Though it has only been reported in animals tolerant to morphine, there exists an interesting difference between ethanol and PKC inhibitors when each served as a reversal agent. PKC inhibition alone can restore a response to circulating morphine released from a pellet without a further opioid challenge administration (Smith *et al.*, 2006), whereas in the case of ethanol, reversal of tolerance has only been reported in studies where a subsequent morphine challenge injection was administered (Hull *et al.*, 2013; Hill *et al.*, 2016). PKC has been reported to have effects on sodium channels directly. It has been shown that inhibiting PKC reduced maximal conductance of TTX-resistant sodium channels, possibly due to a reduction in channel density (Gold *et al.*, 1998). It could be inferred that inhibition of PKC influences neuronal excitability in two ways: 1) modulation of sodium channels directly and 2) reduced phosphorylation at the μ -opioid receptor. It is less clear with ethanol whether there are any direct influences on sodium channels, and though it has been suggested to play a role in dephosphorylation of μ -opioid receptors, the proposed mechanism by which ethanol works to accomplish this involves PKC. These differences suggest that although ethanol and PKC inhibitors have each been reported to effectively and completely reverse both morphine and oxycodone tolerance in mice *in vivo* and *in vitro*, the underlying mechanisms may be related, but not identical.

Our conclusions from these studies indicate that oxycodone similarly reduced dorsal root ganglia neuronal excitability with the same potency as reported for morphine, and that prolonged exposure led to tolerance at the level of a single neuron. The onset of these acute observations occurred within minutes and was opioid receptor dependent. We found that concentrations of ethanol, equivalent to moderately intoxicating doses in humans, completely reversed oxycodone tolerance. Additionally, we showed that reversal of oxycodone tolerance is in part mediated by inhibition of PKC. Collectively these studies provide additional insight into the reversal mechanisms of oxycodone tolerance by ethanol, and that this phenomenon is neither limited to central neuron populations, nor require external influences such as pharmacokinetic factors or intact neuronal networks.

Chapter 4

General Discussion

Opioids exist in a very delicate balance in today's society. They serve a necessary medicinal role in the alleviation of pain, coughing and diarrhea. However the tolerance, dependence and, too often, addiction that is associated with repeated use of these compounds creates a major societal problem. Oxycodone is one example of an opioid compound whose contributions to society are both beneficial and destructive. There is no doubt that oxycodone has provided, and continues to provide, pain relief to those who need it most, yet it is also increasingly recognized today just how deeply rooted oxycodone is in the foundation of the current opioid epidemic. When opioids such as oxycodone are consumed in tandem with other substances, particularly known substances of abuse, a variety of serious health concerns arise. One such substance is ethanol. Though legal and socially accepted, it can be detrimental to one's health if misused or combined with certain drugs, especially other depressants. Overall, the objective of this dissertation was to investigate the ability of ethanol to reverse tolerance to common opiates other than morphine, with a primary interest in oxycodone. The studies described here advance our knowledge regarding ethanol's interaction with acute and chronic oxycodone, which had not previously been investigated. In addition, these experiments have progressed our understanding of tolerance development to oxycodone at the metabolic and cellular levels.

Oxycodone is a potent antinociceptive compound in mice, as it is in man. Our acute studies produced ED₅₀ values that agree with previously published ED₅₀ values, suggesting we have a reproducible model of assessing oxycodone antinociception in mice (Pawar *et al.*, 2007;

Minami *et al.*, 2009). With repeated administration, tolerance developed to this effect, where higher doses were required to produce antinociception. We also tested the antinociceptive effects of hydrocodone. Hydrocodone was similar in potency to morphine, and less potent than oxycodone in mice. In humans however, the potency relationship between these two compounds depends on the specific effect being measured. Hydrocodone and oxycodone have been shown to be equally potent when used as an analgesic in the emergency room (Marco *et al.*, 2005), but oxycodone was shown to be more potent at eliciting other opioid effects such as miosis in non-dependent individuals (Zacny and Gutierrez, 2009). The fact that oxycodone and hydrocodone are equally potent in humans, but not in mice, could result from the type of pain stimuli elicited (thermal vs. inflammation/broken bones) as well as species differences underlying the pharmacokinetic events following administration. Regardless of the acute differences in potency detected in our model, tolerance developed to hydrocodone's antinociceptive effects following repeated injections, just as observed with oxycodone. Furthermore, the degree of tolerance observed was similar between the two compounds.

Based on data from post-mortem investigations, it was hypothesized that ethanol may reverse certain opiate-induced tolerances, and recent pre-clinical studies have demonstrated that ethanol does reverse antinociceptive and respiratory depressive tolerance to morphine (Hull *et al.*, 2013; Hill *et al.*, 2016; Withey *et al.*, 2017). The data presented in this dissertation have expanded our knowledge about the interaction between ethanol and prescription opiates. Ethanol (1 g/kg i.p.) did not significantly alter the acute antinociceptive effects of either oxycodone or hydrocodone. This was the first line of evidence to suggest that the interaction between ethanol and opioids is not the result of additive effects. The next series of experiments addressed the effect of ethanol in animals tolerant to either oxycodone or hydrocodone. In these studies, when

ethanol (1 g/kg i.p.) was given thirty minutes prior to a challenge opiate injection, tolerance to oxycodone or hydrocodone was reversed. The ED₅₀ values obtained from this treatment group closely resembled that of acutely-treated oxycodone mice, and were significantly different from mice that received repeated oxycodone and saline (i.e. no ethanol). From these experiments, we concluded that ethanol was capable of reversing tolerance to not just morphine, but also subcutaneously administered oxycodone and hydrocodone. Furthermore, the same dose of ethanol was effective in fully reversing the tolerance to each. This was a critical observation for a few reasons. The first implication is that tolerance to opiates that are classified as partial μ -agonists (such as morphine, oxycodone and hydrocodone), are each susceptible to reversal by ethanol. Second, even though these compounds differed in potency acutely, the tolerance that developed to each was reversed by the same dose of ethanol. Third, the ethanol dose that completely reversed tolerance in these mice is estimated to reflect relatively low-to-moderate blood alcohol levels in humans. Thus, the predicted blood ethanol levels necessary to reverse opiate tolerance could realistically be reached with only a few drinks in a single sitting. Given this reality, individuals who consume hydrocodone or oxycodone repeatedly are at risk for an interaction with ethanol if they were to drink. Our data suggest that co-consumption of ethanol following chronic exposure to morphine, oxycodone, or hydrocodone is not safe and poses significant health risks.

The behavioral observations we gathered demonstrated that ethanol effectively reversed tolerance to the antinociceptive effects of oxycodone and hydrocodone. The following set of experiments were designed to uncover potential mechanisms by which ethanol was eliciting its effects. While the complete picture is likely complicated, this set of experiments set out to address basic pharmacokinetic influences that could be responsible for ethanol's reversal effect

on oxycodone tolerance. These experiments were necessary because oxycodone brain concentrations had not been measured in mice, nor had they been measured in animals exposed to ethanol simultaneously. Additionally, post-mortem data from heroin or oxycodone-related overdose deaths showed the blood opioid concentrations were significantly lower in individuals who also consumed ethanol (Darke and Hall, 2003; Thompson *et al.*, 2008). Our first experiment aimed to assess what the brain concentrations of oxycodone were following an acute oral administration and across time. We found that oxycodone, when given orally, still enters the brain quite rapidly, with detectable concentrations present at just 5 minutes post administration. Peak levels were observed after 20-30 minutes, and oxycodone was no longer detectable in the brain after 8 hours. These data were correlated with the timing of antinociceptive effects elicited by oxycodone, supporting the hypothesis that the antinociception is likely mediated in part by oxycodone's actions at receptors located supraspinally within the central nervous system.

Interestingly, mice that were chronically administered oxycodone and were tolerant to oxycodone's antinociceptive effects showed significantly lower brain oxycodone concentrations following a challenge administration compared to an acutely-treated mouse. This finding provides new evidence that tolerance to oxycodone includes a metabolic component. Whether this is a direct effect of the upregulation of CYP enzymes or through changes in the ability for oxycodone to enter and remain in the brain has not yet been investigated. Further experiments such as measuring mRNA levels of specific CYP enzymes via qPCR could be conducted to begin answering these lingering questions.

Importantly, we found that ethanol did not alter acute or chronic brain oxycodone concentrations. The data were particularly convincing for chronic oxycodone, where the brain oxycodone concentrations detected in mice given ethanol were nearly identical to those that only

received chronic oxycodone. From this observation, we concluded that ethanol reversal of oxycodone tolerance is not due to a pharmacokinetic event. This further supported our hypothesis that reversal by ethanol is a phenomenon that involves neuronal mechanisms.

Given that our behavioral studies focused on tolerance to and reversal of the antinociceptive properties of oxycodone, we determined the best model to test our hypotheses at the cellular level was dorsal root ganglia neurons isolated from adult mice. These ganglia sit adjacent to the spinal cord and include neurons such as C and A δ nociceptive fibers. The neurons contain projections from the periphery into the spinal cord itself, with the soma located within the ganglion. Because of this morphology, they are often thought of as relay stations between the periphery and the central nervous system. Most of the neurons contained within the dorsal root ganglia are known to express μ opioid receptors, making them a fitting model for our studies. Furthermore, these neurons can be readily used for whole-cell patch clamp electrophysiology experiments.

DRGs have been used as a model to study the effects of morphine on neuronal excitability, but similar studies had not been conducted for oxycodone. The experiments described in chapter 3 provide the first line of evidence documenting that oxycodone reduced neuronal activity directly, and that it was mediated through opioid receptors. We went on to develop a model of *in vitro* tolerance, by incubating neurons in media containing 10 μ M oxycodone overnight. The fact that these neurons exhibited tolerance to oxycodone suggests that *in vivo* tolerance likely involves μ receptors located both within the central nervous system as well as peripheral sites, such as the DRGs. There is recent evidence to suggest that antinociceptive tolerance may be primarily influenced by peripherally located μ receptors, since the peripherally restricted antagonist methylnaltrexone was able to block antinociceptive

tolerance to morphine (Corder *et al.*, 2017). From these studies, we have shown that tolerance to oxycodone is due in part to lower total brain concentrations, as well as a loss in effective signaling through μ opioid receptors located on DRG neurons. Further experiments are needed to determine if the reduction in oxycodone's ability to decrease neuronal excitability is mediated by the desensitization or internalization of μ opioid receptors following prolonged oxycodone exposure. Collectively, these observed cellular and metabolic changes help explain our behavioral observations that repeated oxycodone administration led to antinociceptive tolerance to oxycodone's effects in the whole animal.

Once we conclusively showed that tolerance developed to oxycodone at the neuronal level, it was imperative to test the effect of ethanol in those neurons. Given our findings that ethanol did not alter chronic oxycodone brain concentrations, our hypothesis was that ethanol reversed tolerance to oxycodone through neuronal mechanisms. We found that 20mM ethanol did in fact reverse tolerance to oxycodone within a single neuron. This concentration of ethanol was similar to that of the estimated 17mM ethanol concentration that equals the legal driving limit in the United States (i.e. 0.08% blood alcohol concentration). This is a critical finding because this suggests that at the neuronal level, ethanol reversed oxycodone tolerance at a concentration that would be considered at most moderately-intoxicating in humans. Furthermore, these data support our behavioral observations that tolerance to oxycodone's antinociceptive effects was reversed by 1 g/kg ethanol, which is estimated to be moderately intoxicating as well. Collectively this data supports the notion that an opioid-dependent individual could easily put themselves at further risk of overdose if they also consume a few alcoholic drinks.

The reversal of morphine tolerance by ethanol has been hypothesized to involve PKC in locus coeruleus neurons (Llorente *et al.*, 2013), which play a role in the regulation of respiration.

Additionally, various inhibitors of PKC have reversed tolerance to the antinociceptive effects of morphine. To date, the involvement of PKC in oxycodone-induced tolerances has not been investigated, nor has PKC been investigated in combination with oxycodone and ethanol. We chose to use a selective PKC inhibitor, Bis XI, to determine if the tolerance to oxycodone in DRG neurons involved a PKC component. Tolerance to oxycodone was reversed within minutes of being exposed to 100nM Bis XI. Since the Bis XI was applied via the internal pipette solution, the results strongly indicate that reversal of oxycodone tolerance involves intracellular (i.e. neuronal) mechanisms that are PKC-dependent. Further experiments will need to be done to determine if ethanol itself is acting as a PKC inhibitor, and whether specific PKC isoforms are preferentially inhibited by ethanol. Based on IC_{50} calculations, the concentration of Bis XI that we used [100nM] suggests that the conventional isoform, PKC α , and the novel isoform, PKC ϵ , are involved. Our findings agree with prior research where both of these isoforms were specifically shown to play a role in tolerance to morphine's antinociceptive and respiratory depressive effects (Newton *et al.*, 2007; Smith *et al.*, 2007; Bailey *et al.*, 2009; Lin *et al.*, 2012). Our data suggests that these isoforms are likely involved in the mechanisms underlying tolerance to oxycodone, as well as morphine. Interestingly, PKC ϵ and PKC α have both been reported to interact with ethanol (Gordon *et al.*, 1997; Olive *et al.*, 2000). Transgenic knockout mouse models are available for each of these isoforms and could be used in future experiments to determine if one or both isoforms are important for ethanol's reversal effects of oxycodone tolerance.

It is interesting to note that much of the work investigating "reversal of tolerance" with PKC inhibitors was attempting to unmask signaling molecules that were necessary for the development of tolerance to morphine. The approach was clever and quite informative given the

available selective inhibitors. The goal of these studies however, was not to investigate “reversal” itself. In contrast, our studies *were* designed to study the reversal phenomenon induced by ethanol, and to investigate further the potential mechanisms by which ethanol acted. It is likely that many signaling molecules, including the PKC isoforms described above, play a role in both the development of tolerance and ethanol’s ability to reverse tolerance. However, the exact mechanism(s) by which tolerance develops to either morphine or oxycodone may not be identical to the exact mechanism(s) by which ethanol reverses that tolerance.

There could be different mechanisms involved depending on the specific neuronal populations that are being studied and the particular opiate effect(s) they mediate. It was hypothesized and recently demonstrated that the development of tolerance to the antinociceptive effects of opiates occurs at a different rate than that of the respiratory depressive effects, with tolerance to antinociception occurring faster (White and Irvine, 1999; Hill *et al.*, 2016). Additionally, it is hypothesized that tolerance to euphoria also develops faster than respiratory depressive effects, but is only supported by anecdotal evidence thus far, since the pre-clinical models of “reward” or euphoria are not well accepted. Given these discrepancies, it is possible that the mechanisms underlying tolerance to each of these effects are not identical. Furthermore, the rate at which the development of tolerance to various opiate effects occurs is significantly altered by periods of dependence and abstinence. Individuals who have been dependent on opiates and then enter a period of abstinence report that their tolerance to the euphoric and antinociceptive effects of opiates returns fairly quickly. Perhaps there is some cellular “memory” mechanism by which the receptors rapidly desensitize and reinstate tolerance shortly following relapse from abstinence. It has been noted that heroin overdose deaths occurred at a greater rate in individuals who relapsed after having been abstinent for a period of time, and who also drank

alcohol, suggesting that ethanol may be even more lethal when used in combination with opiates in these relapsing individuals (Warner-Smith *et al.*, 2001; Hickman *et al.*, 2008). This has not been studied in a pre-clinical model and warrants further investigation.

Collectively, the work from this dissertation has increased our knowledge regarding the interaction of ethanol with commonly prescribed opiates. At the level of the whole animal we showed that antinociceptive tolerance developed to oxycodone or hydrocodone following repeated administration. We then found that the same dose of ethanol (1 g/kg i.p.) that reversed morphine antinociceptive tolerance fully reversed tolerance to oxycodone (s.c.) and hydrocodone (s.c.) as well. Narrowing our focus to oxycodone, we explored the possibility that ethanol elicited its reversal effects via pharmacokinetic interactions. From those studies, we discovered that brain oxycodone concentrations were much lower following chronic administration than after an acute administration, and this fact likely contributes to the mechanism of tolerance to oxycodone. When we investigated if ethanol altered either acute or chronic oxycodone brain levels, we found that ethanol did not have a significant effect, which indicated that the reversal of tolerance by ethanol to the antinociceptive effects of oxycodone was not due to pharmacokinetic events. This supported our hypothesis that tolerance to oxycodone and reversal by ethanol is mediated by neuronal mechanisms. To show that oxycodone could alter neuronal activity directly, we acutely applied oxycodone to isolated DRG neurons, where we observed a reduction in overall excitability as measured by whole-cell patch clamp electrophysiology. Following overnight incubation with oxycodone, DRG neurons demonstrated tolerance to oxycodone when exposed to a bath solution containing a challenge concentration of oxycodone. This tolerance was robustly reversed by either a 50 minute pretreatment with 20mM ethanol (which matched our whole-animal exposure time), or when exposed to a PKC inhibitor applied via the internal

pipette solution. These findings strongly support our hypothesis by providing direct evidence that tolerance to oxycodone and reversal by ethanol requires neuronal mechanisms that are mediated by intracellular signaling molecules. The interaction between ethanol and inhibitors of PKC warrant future experiments in order to elucidate the specific neuronal mechanisms that are involved in the reversal of oxycodone tolerance.

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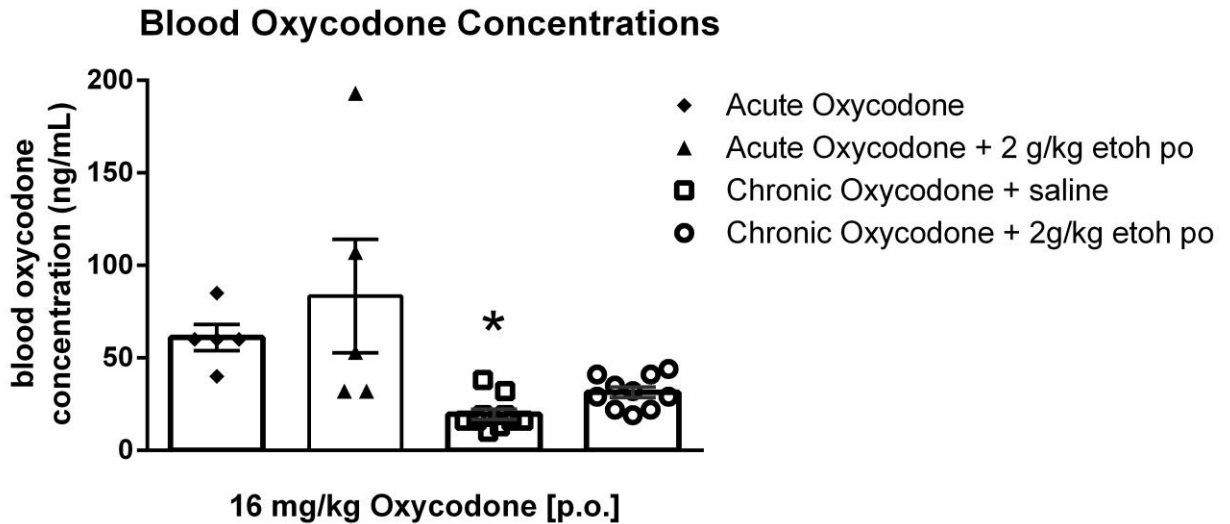
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Appendix A



* $P < 0.05$ vs. Acute Oxycodone, One-way ANOVA, Dunnett's post-hoc
 $N \geq 5$ mice/group

Oxycodone concentrations in the blood were assessed following acute or chronic administration of oxycodone in mice, in the presence and absence of ethanol. Under acute conditions, mice were given a gavage of saline or ethanol (2 g/kg, PO) 30 min prior to receiving a single gavage of oxycodone (16 mg/kg, PO). Mice were sacrificed 20 min following the acute oxycodone gavage and whole blood samples were immediately collected in tubes containing an anticoagulant agent. To assess chronic oxycodone blood concentrations, mice were gavaged twice daily with oxycodone (64 mg/kg, PO) for four days and on day 5 received a saline or ethanol (2 g/kg, PO) gavage 30 min prior to receiving a challenge gavage of oxycodone (16 mg/kg, PO). Mice were sacrificed 20 min following the challenge oxycodone gavage and whole blood was immediately collected in tubes containing an anticoagulant agent.

Pre-extraction preparation was unnecessary for the whole blood specimens, and oxycodone was extracted directly from whole blood. A matched matrix five-point calibration curve containing oxycodone was prepared at 20-1000 ng/mL for blood. The samples were placed in auto-sampler vials for gas chromatography mass spectrometry (GC/MS) analysis.

Acute oxycodone blood concentrations measured 61.0 ng/ml. In mice that also received ethanol, oxycodone blood concentrations measured 83.4 ng/ml. Acute ethanol did not significantly increase acute oxycodone blood concentrations ($P > 0.05$). Following chronic oxycodone administration, blood oxycodone concentrations were significantly lower compared to acutely treated mice (19.5 ng/ml, $P < 0.05$, One-way ANOVA, Dunnett's post hoc). Chronic oxycodone treated mice that received ethanol prior to receiving a challenge oxycodone administration also showed lower blood oxycodone concentrations compared to acutely-treated mice (31.4 ng/ml), though the differences were not significant ($P = 0.17$). These data showed that blood oxycodone concentrations were significantly lower following chronic oxycodone treatment and that ethanol had no effect on either acute or chronic oxycodone blood concentrations. Furthermore these data corroborate the findings for oxycodone brain concentrations under these same conditions. Together, these data suggest that ethanol reversal of oxycodone tolerance is not due to any pharmacokinetic alterations of oxycodone concentrations in either the blood or brain tissue.

Vita

Joanna Caitlin “Jacy” Jacob was born on February 5, 1987 in Richmond, Virginia and is an American citizen. She graduated Cum Laude from Virginia Polytechnic Institute and State University (Virginia Tech) in Blacksburg, Virginia, with a Bachelor of Science in Animal and Poultry Science in 2009. In 2013, she graduated from Virginia Commonwealth University in Richmond, Virginia, with a Master of Science in Pharmacology and Toxicology. She served as President of the Pharmacology and Toxicology Student Organization from 2014-2015 and served as the Editor of the Medical Sciences section for Virginia Academy of Sciences from 2012-2015.