DRUG-SPECIFIC DIFFERENCES AMONG THE OPIOID ANALGESICS HYDROCODONE, OXYCODONE AND MORPHINE

A Dissertation

by

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ABSTRACT

Opioids have been utilized for millennia for analgesia, but are accompanied by numerous adverse effects in addition to their pain relief abilities. These include tolerance, opioid-induced hyperalgesia, the propensity for abuse, and discrepancies regarding their ability to treat chronic pain. Despite efforts to find non-opioid alternatives, opioids remain the gold standard in spite of their drawbacks. Historically, both prescription and research of opioids have been guided by the assumption that analgesic properties and adverse associated effects are mediated by the same mechanisms and therefore are inseparable. Relatively recent discoveries regarding ligand-directed signaling at the opioid receptors have called this assumption into question, indicating that similar opioid analgesics may exhibit drug-specific differences in antinociceptive function and adverse effects. The current work investigated this hypothesis by examining three commonly prescribed and abused opioids, oxycodone, hydrocodone and morphine, for drug-specific differences in their antinociceptive potency and ability to prevent injury-induced hyperalgesia, their behavioral effects on the reward-related D2 signaling system, and molecular & gene expression changes.

First, a mouse burn-injury model was developed that produced significant nociception which worsened across 28 days. Then, using this model, opioids were examined for drug-specific differences in their antinociceptive potency to treat burn pain, their ability to prevent/treat injury-induced long-term hyperalgesia, and correlations between antinociceptive potency and hyperalgesia prevention.

It was found that burn injury pain *per se* reduced the antinociceptive potency of opioids, but no drug-specific differences existed in potency. However, drug-specific differences did exist in the ability to prevent hyperalgesia development. When examined on the individual level, it was found that greater early pain relief led to reduced long-term pain outcomes.

In addition, drug-specific differences were shown to exist in the ability to increase behavioral sensitivity of the D2DR system in both the absence and presence of burn pain, to alter intracellular signaling in both the absence and presence of D2DR agonism, and to alter gene expression levels.

These findings provide evidence of wide-spread drug-specific differences between common opioid analgesics that can carry clinically relevant implications for pain treatment, chronic pain outcomes, addiction, and other long-term outcomes resulting from opioid exposure.

DEDICATION

This work is dedicated to my mother, Janet Sue (Kneezel) Emery. She never saw her son graduate college, but she never doubted that he'd go on to make her proud. I hope I have, and will.

And to my father, Ronald Columbus Emery. Dad could tell from a young age that I was inclined toward intellectual pursuits, and he always strongly encouraged it. Some of my fondest, and earliest memories, are of my father's bookshelf; of exploring used books in the college bookstores in Carbondale; of wandering the stacks of the Public Library branches and the University Library in Lexington, and stopping at bookstores every roadtrip we took. Although money was tight, my family never told me 'no' if I wanted a book.

> "…Just remember that all of the people in the world haven't had the advantages that you've had."
>
> —F. Scott Fitzgerald, "The Great Gatsby"

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All other work for the dissertation was completed by the student, under the advisement of Shoshana Eitan of the Department of Psychology and the Texas A&M Institute for Neuroscience.

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The contents are solely the responsibility of the authors and do not necessarily represent the official views of the College of Liberal Arts or Texas A&M University.

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CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

Opioids are a ubiquitous class of drugs which are routinely prescribed to alleviate moderate-to-severe pain (Volkow et al., 2011). They are unique among analgesics for their ability to act at both central and peripheral sites to silence pain signals. Unfortunately, opioids also interact indirectly with the dopaminergic reward system in the brain, giving them highly rewarding and euphoric effects and making them liable for dependence, abuse, and addiction. Because of their propensity to be abused, physicians and researchers have long been searching for effective and less risky replacements for opioids. Despite this, opioids remain the gold standard for analgesia, and are the metric by which all other analgesic options are measured.

Due to the relative failure to find effective opioid replacements, and in light of emerging evidence of the phenomenon of ligand-directed signaling (Urban et al., 2007; Pradhan et al., 2012), research has turned back to examining opioids in the hope that drug-specific differences in mechanism or function may be found amongst them which may be exploited for enhanced analgesic function, reduced risk of unwanted side effects including addiction, or both.

Prior research on the opioids was performed under the assumption that agonists for receptors (such as opioid analgesics for the opioid receptors) differ from one another in terms of pharmacokinetic and pharmacodynamic considerations such as absorption, distribution and elimination rates, receptor binding and dissociation rates, and so forth.

However, it was believed that, once bound to a receptor, all agonists for that receptor functioned identically, activating the same intracellular responses. That is to say receptors existed in what was essentially a quantal, binary state and agonists acted to switch this state (Kenakin, 2011).

A common metaphor for this is the 'lock and key' model. Many keys (agonists) may fit and open the same lock (receptor). For the opioid receptors this can include the keys meant to open them (endogenous agonists) as well as keys which happen by sheer accident of similar construction to fit (exogenous opioid drugs). Mother Nature is brilliant in many ways, but a decent locksmith she is not. However, so the conventional wisdom says, whatever key was being used, the lock could only be in one of two states; locked (not signaling) or open (signaling).

Because of this, physicians have long been taught that the analgesic (and negative) effects of opioids can be made equivalent by adjusting dosages and administration rates (and routes) to compensate for the pharmacokinetic and pharmacodynamic differences. Likewise, researchers have focused much of their efforts on characterizing the effects of the archetypal opioid, morphine, assuming that these effects can be similarly extrapolated to other opioids by adjusting for pharmacological differences.

This bias in the research can easily be observed in the existing literature. A search of the PubMed database for the term "morphine" returns 53,356 hits at the time of writing, with 1,425 new publications in 2016. In comparison, "oxycodone" returns only 2,967 papers, with 285 published in 2016, and "hydrocodone" returns a mere 954

publications, 91 from 2016, indicating that the body of literature on hydrocodone is less than 2% that of morphine (at least, as archived in this database).

This is especially surprising considering that hydrocodone combination products are the most prescribed drug in the United States (Von Korff et al., 2008; Center for Medicare and Medicaid Services, 2016). It is possible that prescription rates for hydrocodone products are so high, and research into the effects of hydrocodone so sparse, are both due to the common belief that hydrocodone is a 'weak' opioid. Opioid drugs have previously been subdivided into weak and strong opioids, based upon their affinity for the μ-receptor. It has been the prevailing opinion that 'weak' opioids are perhaps less likely to foster addiction and other adverse side effects such as respiratory depression, but are less effective analgesics, incapable of adequately managing more severe pain. In contrast, 'strong' opioids such as oxycodone or morphine have been recommended for use in more severe pain situations, but their use is tempered by the belief that their adverse risks, including the propensity for abuse, are similarly increased (WHO, 1990).

However, recent findings using a variety of GPCR model systems including the opioid receptors have demonstrated that this simplistic view of ligand-receptor interaction is incorrect, or at least incomplete. Different ligands for the same receptor interact with the receptor in different ways, which in turn alter the nature and balance of the responses elicited by the receptor's activation. This phenomenon is referred to by multiple different names, including but not limited to biased agonism, ligand-directed signaling, and functional selectivity (Galandrin et al., 2007). In the case of opioids, this

implies that distinct opioid analgesic drugs may elicit very different intracellular responses, and therefore may ultimately have very different outcomes on behavior, including analgesic ability, addiction liability, risk for acute negative side effects such as respiratory depression, and risk for precipitation of psychological disorders. In addition, it implies that the pain-relieving properties of these compounds, as well as their associated risks and side effects, may not be dependent solely on their receptor binding affinity, therefore rendering the distinction between 'weak' and 'strong' opioids less definitive than previously thought, and resulting in a need to adjust prescribing guidelines to account for the fact that some 'weak' opioids may in fact be superior under certain circumstances to 'strong' opioids.

The knowledge that opioid analgesic drugs may have different behavioral effects, coupled with the dearth of research regarding the behavioral and molecular effects of widely used and abused opioids, highlights the need for studies which examine drug-specific differences among opioid drugs as compared to morphine. The set of experiments performed here seek to elucidate these differences and add to the body of knowledge regarding drug-specific differences among opioids in a variety of clinically relevant paradigms including pain and addiction-related models.

CHAPTER II

GENERAL METHODS

1. Subjects

Adolescent (PND 21 on arrival) C57BL/6 male mice (Harlan/Envigo Houston) were used for all experiments. For longer running experiments, namely the burn experiments where mice were examined for 28 days post-injury, the mice ran the entirety of adolescence and nearly reached adulthood (PND 60) but were not examined in adulthood. The choice of adolescents in this proposed work was directed by multiple observations in the literature, including high rates of burn injuries in adolescents, high rates of non-medical use of opioids in teenagers, and enhanced sensitivity of key addiction-related pathways in adolescents compared to adults (Benes et al., 2000; PATS, 2009; SAMSHA, 2011; Hofford et al., 2012; Johnston et al., 2014; ABA, 2016a). Mice were acclimated to the colony for a minimum of 5 days before the start of any experiment. Mice received food and water *ad libitum* and were housed in a temperature-controlled (21 ± 2 °C) vivarium on a 12:00 hour light/dark cycle with the lights on at 7:30 a.m. and off at 7:30 p.m. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals after receiving the approval of Texas A&M University's Institutional Animal Care and Use Committee.

2. Housing

All mice were group-housed with age-matched peers, litter-mates whenever possible, 3 or 4 per cage. All mice within a cage were exposed to the same treatment conditions (i.e. there were no mixed-treatment cages).

3. Drugs

Morphine sulfate, hydrocodone bitartrate, oxycodone hydrochloride, quinpirole hydrochloride, ketamine hydrochloride and sodium pentobarbital were purchased from Sigma (St. Louis, MO) or Spectrum Chemical (New Brunswick, NJ). Xylazine and silver sulfadiazine were provided by the Texas A&M University Comparative Medicine Program veterinary pharmacy.

4. Drug Injection Paradigm

All drugs were dissolved in their respective vehicle (which was sterile 0.9% saline unless otherwise noted) to a volume of 10 mL/kg for each drug dose. A total of 3 doses of opioids, 10, 20 or 80 mg/kg, were used throughout the following studies. These doses were calculated for salt concentrations for all drugs. For oxycodone, these doses roughly correspond to 9, 18, and 72 mg/kg free base respectively. For hydrocodone, these doses roughly correspond to 6.5, 13, and 52 mg/kg free base. For morphine, these doses roughly correspond to 7.5, 15 and 60 mg/kg free base. These doses are equianalgesic across the 3 opioids, as confirmed by our previous studies (Emery et al., 2015) as well as by the data presented herein. All opioids were administered via gavage

(p.o.) using a 24G x 1 inch, stainless-steel needle ending in a ball (Fine Science Tools, Inc.). All other drugs were administered intraperitoneally (i.p.). Frequency and duration of injection regimens differed from experiment to experiment, and are noted for each in their respective specific experimental procedures.

CHAPTER III

EXPERIMENT 1: DEVELOPMENT AND VALIDATION OF A BURN INJURY MODEL IN THE MOUSE*

1. Background

Burn injury is common, accounting for approximately 486,000 emergency room visits and 40,000 hospitalizations in the US in 2016 (ABA, 2016b). Burn injuries are known to be very painful, and analgesic treatment is often necessary to manage both background pain and procedural pain during the healing process. In addition to this acute, inflammatory pain early after injury, one of the most common and severe long-term consequences of burn injury is the development of chronic and/or neuropathic pain (Summer et al., 2007). It is thought that this type of pain arises from the development of central sensitization and other chronic, maladaptive alterations in intracellular signaling pathways (Woolf and Mannion, 1999; Woolf, 2011), which could be targeted for more efficacious pain treatments and development of preventative interventions.

Despite the common use of opioids to treat pain in burn patients, burn pain is notoriously resistant to treatment. Burn pain patients often require opioid doses much greater than standard dosing recommendations to provide adequate analgesia (Patterson

*Part of this chapter is reprinted with permission from "Burn injury decreases the antinociceptive effects of opioids" by Emery MA, Bates MLS, Wellman PJ, and Eitan S Behavioural Pharmacology, 28(4): 285-293. Copyright [2017] Wolters Kluwer Health, Inc; and "Hydrocodone, but neither morphine nor oxycodone, is effective in suppressing burn-induced mechanical allodynia in the uninjured foot contralateral to the burn" by Emery MA, Bates MLS, Wellman PJ, and Eitan S (2017) Journal of Burn Care and Research, 38(5): 319-326. Copyright [2017] The American Burn Association.

et al., 2004; Wiechman Askay et al., 2009), and even so, often report that their pain is not entirely managed (Latarjet, 2002; Wiechman Askay et al., 2009).

Historically, it has been the opinion of the medical community at large that opioids are mostly ineffective for treating neuropathic pain (Arner and Meyerson, 1988), and therefore should not be considered a first-line treatment option, due to the increased risk of addiction outweighing the minimal analgesic benefit (Dworkin et al., 2010). However, recent findings indicate that this lack of effect of "opioids" may be due to the prior research bias toward using morphine as a model opioid, as other opioids besides morphine are significantly more efficacious to treat neuropathic pain symptoms (Gimbel et al., 2003; Agarwal et al., 2007; Salpeter et al., 2013).

Drug-specific differences in the ability of opioids to manage burn pain during the early phase following treatment, as well as differences in their ability to prevent or treat the development of chronic, neuropathic pain in burn sufferers, has not previously been well explored. Differences, if they are found to exist, could influence the treatment of burn pain as well as the development of novel pharmaceutical compounds which capitalize on features of existing drugs which are found to be more efficacious.

In order to explore potential differences between opioids in their ability to manage burn pain, a reliable, experimental model of burn-induced pain was needed in an animal model for whom opioid pharmacology is already well established. Necessary features of this model include robust exhibition of acute, inflammatory nociception, as well as the continued experience of pain following tissue healing, a defining feature of chronic pain.

2. Experimental Procedure

Subjects

Adolescent (PND 28-29 at beginning of experiment) C57BL/6 mice were used in this experiment. Approximately 15 animals per group were used for this experiment, for a total of 33 animals.

Burn Injury

Mice were anesthetized using ketamine/xylazine (100 mg/kg, 10 mg/kg respectively) and a burn injury was induced by holding the dorsal portion of the right hindpaw pressed against a prefabricated plastic template with a 4.5 mm × 5.0 mm window. The exposed surface of the hindpaw (through the window) was then immersed into a hot water bath (85 °C) for 5 seconds. A sham injury was induced by immersing the dorsal part of the right hindpaw into a water bath at 37 °C for 5 seconds using the same method. This burn injury is modified from that developed by Wang et al. (2005) and was adapted by the author for use in mice. Silver sulfadiazine cream (1% USP) was applied to the burn twice daily until the wound healed. Sham animals did not have antibiotic cream applied, but did have their right hindpaw touched to mimic the handling procedure.

Mechanical Sensitivity Test

Mice were placed in a Plexiglas cylinder (D: 3 inches x H: 6 inches) atop a mesh platform made of aluminum window screen, with 1 mm^2 holes. The mechanical

allodynia test was performed as described by Wang et al. (2005). Von Frey filaments were applied to the plantar surface of the hind paw (of both feet) five times or until a withdrawal response was observed at least once. The response was defined as a rapid withdrawal of the paw, with toes curling, from the mesh. If no response was observed after 5 applications of the fiber, the next stiffer fiber was applied to the same paw until a response was evoked. If a response was observed, a less stiff fiber was applied until no response was observed over 5 trials. This method was repeated until a threshold was determined where one fiber evoked a response, but the next finer filament did not. The finest filament that produced a response was recorded for that trial.

All mice were examined for their baseline response to the von Frey mechanical allodynia test the day before receiving a burn injury. This pre-injury baseline reactivity was compared across conditions, to ensure no pre-existing differences were present before the burn. The mice were retested on days 4, 7, 11, 14, 21, and 28 after the burn. This design is presented schematically in Figure 1.

On each of the post-injury testing days, the mice were assessed twice. First, in the morning, their mechanical reactivity in the burn-injured and uninjured feet were examined, to assess their level of pain reactivity in the absence of drug. Then the animals received their morning dose of saline and were re-examined for their pain reactivity 1 hour after administration. This second pain reactivity test was compared to that day's pre-injection reactivity test in order to control for the effects of the injection and/or repeated von Frey testing on reactivity thresholds.

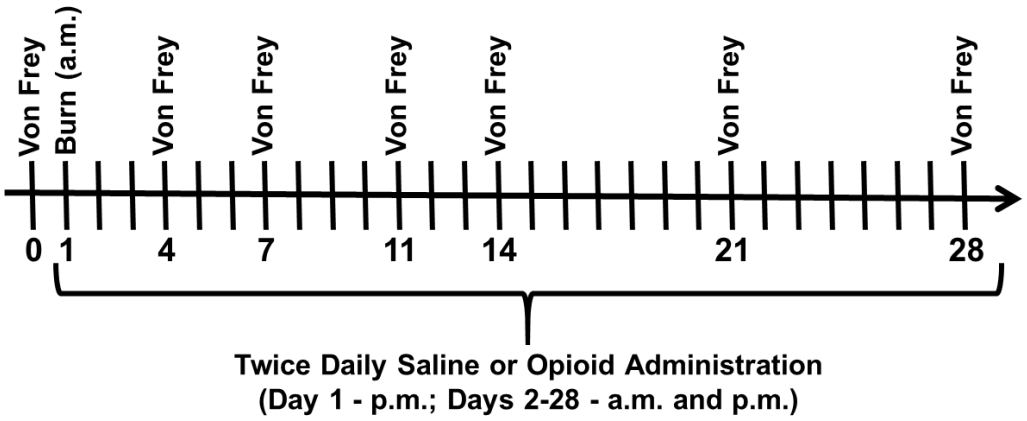

Figure 1. Timeline of burn injury experiment. Animals were treated with saline (Experiments 1-5) or opioids (Experiments 2-5).

Drug Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were administered saline at a volume of 10 mL/kg body weight. Mice received saline twice daily (a.m. and p.m.) for 28 days, beginning the evening of the day of injury.

Statistical Analysis

The difference between post-treatment and pre-treatment pain sensitivity threshold for saline-injected animals was calculated as described above. Data was analyzed using a mixed model ANOVA (SPSS Statistics 20, Somers, NY) in a 3 (injury)

x 7 (timepoint) design. Post hoc contrasts were computed using Bonferroni procedure. Differences with p-values of less than .05 were defined as statistically significant.

3. Results

Development of Pain/Nociception Following Burn Injury

To verify that pain is developed in the injured and uninjured foot following burn injury, we compared the daily pre-treatment pain sensitivity thresholds between the uninjured left feet of burn-injured animals treated with saline, the burned right feet of these animals, and saline-treated shams (Figure 2). Two-way repeated ANOVA revealed a significant main effect of injury ($F(2, 53) = 55.36$, $p < .0001$), a significant main effect of day ($F(6, 318) = 25.16$, $p < .0001$), and a significant interaction between day and injury ($F(12, 318) = 11.95$, $p < .0001$).

Bonferroni post-hoc comparison revealed that mechanical sensitivity thresholds in the burned foot of burn-injured animals was significantly lower than sham-injured animals at D4 after the injury and continued to decrease throughout the course of the 28-day study. Additionally, mechanical sensitivity thresholds in the uninjured foot of burn-injured animals was significantly lower than sham-injured animals at D21 and D28 post-injury. There were no significant differences in pre-injury, baseline pain sensitivity thresholds among the groups. These data are presented in Figure 2.

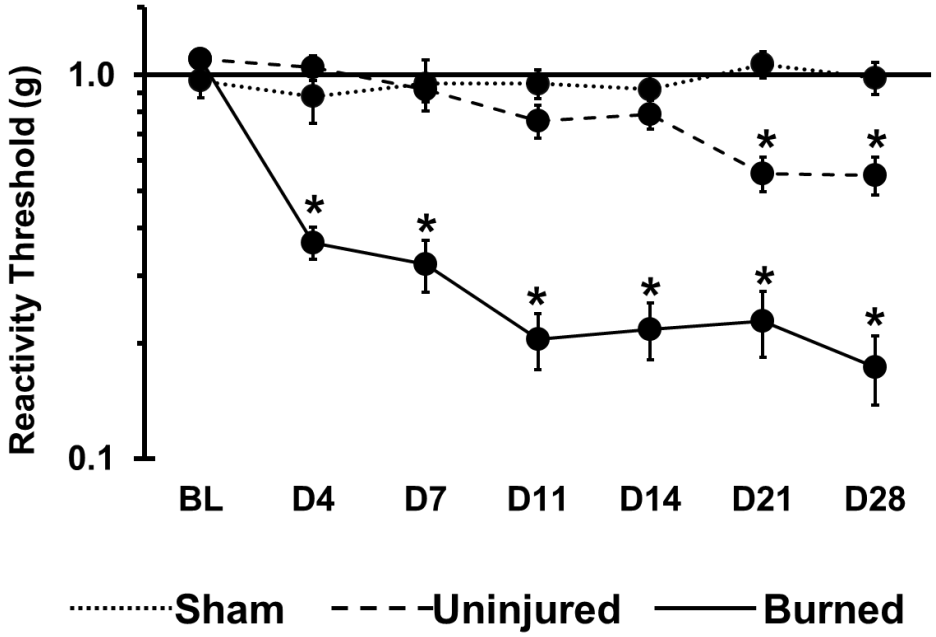

Figure 2. Development of burn-induced pain in saline-treated animals. Following the burn injury and in the absence of opioid treatment, animals develop robust mechanical hyperalgesia in the burned foot by Day 4 which worsens throughout the 28 days of the test. By Day 21, mechanical hyperalgesia has also emerged in the uninjured foot. * Significantly different ($p < .01$) from sham-injured animals. Results are presented as mean ± SEM.

Neither Injection nor Repeated Testing Affected Pain Response

Following daily pretreatment mechanical threshold testing, animals were administered saline via gavage infusion, and were retested 1 hour later. The difference between each animal's daily post-injection measure and their daily pre-treatment threshold was calculated. A posttest-pretest value of zero (0) indicates no change in sensitivity threshold between that day's pretest and posttest recording. This was done to examine whether a) repeated daily measurement of mechanical sensitivity thresholds and/or b) the injection procedure *per se* altered mechanical thresholds, as the presence of

either of these effects would significantly interfere with the interpretation of data gathered in the experiments which followed (Chapters IV-VII).

In saline-treated animals, mixed-model ANOVA revealed no main effect of injury ($F(2, 53) = 3.01$, $p = .058$, n.s.), no main effect of day ($F(5, 265) = 0.71$, $p = .618$, n.s.), and no significant interactions between the main effects ($F(10, 265) = 1.02$, $p = .429$, n.s.). These data are presented in Figure 3.

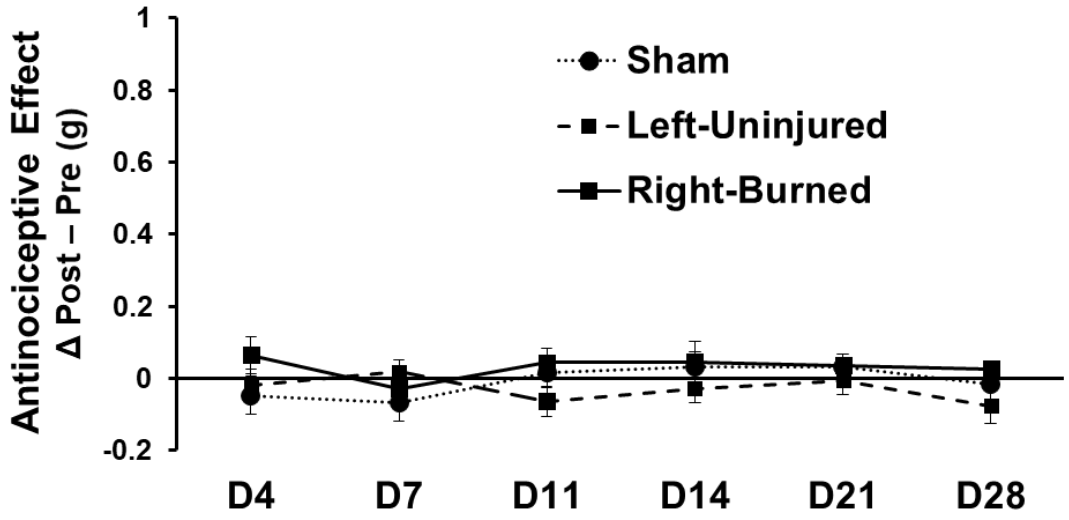

Figure 3. No effects of saline injection or daily retest on pain threshold. Results are presented as mean ± SEM.

4. Discussion

The current definitions of pain, and their operationalization in animal research, are not yet well established and are at times contradictory, due to lasting impacts of

historical definitions which are no longer officially accepted but remain commonly used. Although the International Academy for the Study of Pain (IASP) have reasonably precise definitions for hyperalgesia, allodynia, chronic pain, and neuropathic pain, these definitions are human-centric and tend to rely heavily on physicians' clinical assessments and are informed by the patient's history.

For the purposes of research, the IASP has recently released revised definitions of these terms, which help to characterize them physiologically rather than based on subjective perception (Sandkühler, 2009). Under the new definitions, hyperalgesia is characterized as a pain state above normal levels, resulting from either lowered thresholds to evoke responses in pain fibers or from increased pain fiber reactivity once thresholds are exceeded, or both. Importantly, hyperalgesia by definition must involve high-threshold pain sensory fibers. In contrast, allodynia is defined as pain or nociceptive behavioral responses resulting from the activation of low-threshold sensory fibers in the absence of activation of nociceptive fibers. Under this definition, any ambiguous aberrant pain mechanism is to be considered hyperalgesia (i.e. aberrant pain can only be defined as allodynia if it can be conclusively demonstrated that nociceptive fibers are *not* involved in the effect).

This redefinition is very helpful for precision in the study of pain and nociception mechanisms, but has added a heavy burden to animal researchers attempting to study 'neuropathic-like pain', which has previously been characterized by the presence of allodynia following injury, not hyperalgesia.

For the purposes of the following discussion and the experiments which follow, the current IASP definitions of allodynia and hyperalgesia are being utilized. Although the von Frey filaments test, typically considered a measure of allodynia, was employed, no electrophysiological confirmation was made to ensure pain-sensing C-fibers were *not* being engaged. Therefore, alterations in reactivity threshold are termed 'hyperalgesia', in keeping with IASP guidelines. However, because of the ubiquity of the von Frey test in the literature as a characterization of neuropathic-like pain, it should be noted that the aberrant pain here observed may be neuropathic in nature. This likelihood is further supported by the findings of (Chang et al., 2010), who employed a highly comparable burn injury model in rats, observed a highly similar pattern of aberrant pain response in the von Frey test to the current findings, and electrophysiologically confirmed that the observed responses were indeed neuropathic in nature.

In the absence of treatment with antinociceptive drugs, the burn injury employed in this model caused significant degrees of nociception to develop in the burned limb by Day 4 post-injury. This nociception not only persisted, but worsened across the 28 days of the study, well past the point of tissue healing as observed in previous studies using comparable injury models (Wang et al., 2005; Chang et al., 2010). In addition to the hyperalgesia in the burned limb, mechanical hyperalgesia also presented in the contralateral limb, beginning 3 weeks after the burn injury. This again mirrors the findings of (Chang et al., 2010) who concluded that this was most likely due to central sensitization within the spinal cord.

It can be concluded that the hot-water burn injury model here presented results in significant and severe acute pain, as characterized by large reductions in the sensitivity threshold necessary to elicit a withdrawal response in the von Frey filament test. Furthermore, this increased sensitivity not only persists, but progressively worsens, across 28 days after the injury. The scientific and medical literature is divided regarding exactly when (or even *if*) pain conclusively switches from 'acute' to 'chronic'. However, in the case of pain resulting from a traumatic injury, a common and (relatively) uncontroversial definition is that the pain can be considered 'chronic' when it persists beyond the time necessary for the trauma to heal. Because the burn trauma injury in this model, as well as comparable models using rats, heals fully (or as fully as it is going to, with moderate degrees of scarring) within the first 14 days following injury, it can be reasonably stated that the pain which persists (and worsens) following this healing is a model for 'chronic' pain. Despite the fact that the exact origin of the pain is unknown, it appears unconnected to tissue damage or acute inflammation.

The fact that the pain worsens following healing, rather than resolving or even plateauing, is additional, albeit indirect, evidence that this models chronic-like pain. This progressive worsening of pain over time parallels what is seen in the clinical pain population, as well as other, established animal models of chronic and neuropathic pain. In fact, the persistence or worsening of pain (as opposed to its resolution) is often considered an establishing criterion that must be met in animal models of neuropathic pain.

Further, the emergence of mechanical hyperalgesia (decreased response threshold in the von Frey test) in the contralateral limb, emerging only after complete healing of the initial injury, reinforces the idea that the pain observed late in the study (i.e. at the 3 and 4 week mark) is resulting from pathological processes unconnected to the initial tissue damage.

Thus, it can be concluded that the hot water burn injury model here described results in a pain model suitable for the further investigation of opioid-specific differences in pain treatment and the development of injury-induced hyperalgesia.

CHAPTER IV

EXPERIMENT 2: BURN INJURY DECREASES THE ANTINOCICEPTIVE POTENCY OF OPIOIDS*

1. Background

Burn injuries are known to be very painful, both due to high levels of background pain and the necessity of painful treatment procedures including wound debriding and bandage changes. Opioids are commonly utilized to treat this pain (Alencar de Castro et al., 2013). However, burn pain patients often require opioid doses much greater than standard dosing recommendations to provide adequate analgesia (Patterson et al., 2004; Wiechman Askay et al., 2009), and even so, often continue to report that their pain is not entirely managed (Latarjet, 2002; Wiechman Askay et al., 2009).

Burn injury is known to induce a complex inflammatory state which is relatively unique from other forms of inflammatory pain (Farina et al., 2013; McIntyre et al., 2016; Xu et al., 2016), and to alter levels of immunomodulatory cytokines and prostaglandins (He et al., 2001; Strong et al., 2001; Schwacha et al., 2002; Luo et al., 2005). Several of the pathways and injury-related responses involved in burn injury are known to interact with the antinociceptive effects of opioids (McIntyre et al., 2016). Further, it is known that a generally pro-inflammatory state is associated with reduced opioid analgesic

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efficacy (Shavit et al., 2005; Nicotra et al., 2012; Bai et al., 2014; Pilat et al., 2016; Thomas et al., 2015). Based on this knowledge, it is likely that the experience of a burn injury and the molecular alterations which such an injury precipitates causes a reduction in opioid antinociceptive potency.

Further, many of the signaling pathways implicated in this effect also play a role in central sensitization to pain, which could account for the reports of developed pain in parts of the body far from the injury site in burn patients (Woolf, 2011), such that reductions in opioid antinociception may also be observed in distal tissues.

Lastly, opioids exert ligand-directed effects on intracellular signaling pathways (Pradhan et al., 2012). In multiple cases, the effector molecules which are differentially affected by different opioids have also been demonstrated to be involved in nociception, including β-arrestin, activation of which appears to antagonize opioid antinociception (Bohn et al., 1999; Raehal et al., 2005; DeWire et al., 2013); JNK, which has been shown to be crucial in burn pain, morphine antinociceptive tolerance development, and central sensitization in the spinal cord (Alexander et al., 2004; Kam et al., 2004; Shahabi et al., 2006; Chen and Sommer, 2009; Mittal et al., 2012); TRPV1 channels, crucial for the experience of pain (Chen et al., 2008; Rowan et al., 2014b; Rowan et al., 2014a); and P38-MAPK, implicated in antinociceptive tolerance (Ballard-Croft et al., 2002; Alexander et al., 2004; Cui et al., 2006; Chen et al., 2008).

The known ligand-specific alterations by opioids of effector molecules which influence nociception implies that burn injury may reduce the antinociceptive potency of opioids to different degrees, in a drug-specific manner.

In this experiment, I examined the hypothesis that the experience of burn injury reduces the antinociceptive potency of opioids, which accounts for the reports of much higher-than-expected doses being needed to provide analgesia in burn patients, both in the secondary injury site near the wound (i.e. in the burned foot), and in sites distal from the injury (i.e. the contralateral, uninjured foot).

2. Experimental Procedure

Subjects

Adolescent (PND 28-29 at beginning of experiment) C57BL/6 mice were used in this experiment. Approximately 10-15 animals per group were used for this experiment, for a total of 181 animals.

Burn Injury

Burn or sham injury was induced as described above (Experiment 1).

Mechanical Sensitivity Test

Mice were examined for their mechanical sensitivities using the von Frey filaments test as described above (Experiment 1).

All mice were examined for their baseline response to the von Frey mechanical allodynia test the day before receiving a burn injury. This pre-injury baseline reactivity was compared across conditions, to ensure no pre-existing differences were present

before the burn. The mice were retested on days 4, 7, 11, 14, 21, and 28 after the burn. This design is presented schematically in Figure 1. On each of these testing days, the mice were assessed twice. First, in the morning, their mechanical reactivity in the burn-injured and uninjured feet were examined, to assess their level of pain reactivity in the absence of drug (i.e. ~12 h after their last dose of opioid analgesics). Then the animals received their morning dose of opioids (or saline) and were re-examined for their pain reactivity 1 hour after opioid administration. Opioid analgesic potency was defined as the degree of change in reactivity threshold pre-drug to post-drug, daily (i.e. post-drug reactivity threshold minus pre-drug reactivity threshold).

Drug Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were administered saline, hydrocodone, oxycodone, or morphine. All opioids were given at either 20 or 40 mg/kg doses. Mice received opioids (or saline) twice daily (a.m. and p.m.) for 28 days, beginning the evening of the day of injury.

Group Assignment

Animals were randomly assigned to receive either a sham or burn injury. After injury, animals were further randomly assigned to one of 4 drug treatment conditions (saline, morphine, oxycodone, or hydrocodone) and one of 2 drug dose conditions (20 mg/kg or 40 mg/kg for each of the 3 opioids) for a total of 14 groups.

Statistical Analysis

The antinociceptive potency of the various drugs were calculated as described above. For each opioid dose (20 and 40 mg/kg), potency was collapsed across all drugs and data were analyzed using mixed-model ANOVAs with between-group factors of injury (sham, burned, uninjured, sham-saline), and a within-group factor of time (day). This analysis was then repeated for each individual opioid. Please note that the 'saline' group to which the groups are being compared is the same data presented in Chapter III and graphed in Figure 3 for sham-injured, saline-treated animals. Post hoc contrasts were computed using Bonferroni procedure. Differences with p-values of less than .05 were defined as statistically significant.

3. Results

Antinociceptive Potency Across All Opioids

The effect of burn-injury on the antinociceptive potency of the 20 mg/kg dose of all opioids is shown in Figure 4 A. Two-way mixed model ANOVA revealed a significant main effect of injury condition ($F(3, 132) = 79.384$, $p < .001$), no effect of day ($F(5, 660) = 1.462$, $p = .18$, n.s.), and no interaction between day and injury ($F(15, 605) = 1.462$, $p = 114$, n.s.).

Bonferroni post-hoc comparison revealed that across the entire study, the antinociceptive potency of opioids were significantly different across all injury conditions, in the rank-order such that opioids were most to least potent in sham-injured animals, the burned foot of burned animals, and the uninjured foot of burned animals,

respectively. Opioids provided significant antinociceptive potency in all these conditions relative to sham-injured animals treated with saline.

When analyzed by day, opioids were more potent in the sham animals than any other group, on days 7 and 21. Opioids were equally potent in the burned foot of injured animals as in sham-injured animals on days 4, 11, 14, and 28. Notably, across the entire study, the pain-relieving effects were significantly greater in the burned foot of the burn-injured animals than the uninjured foot. Opioids were more potent in the burned foot of injured animals than in the contralateral, uninjured foot on all days post-injury. The potency of opioids in the uninjured foot of burn-injured animals was quite low, and was indistinguishable from saline treatment on days 4, 14, 21, and 28. Moreover, across the entire study, the antinociceptive effects were significantly greater in the sham animals than the left (uninjured) feet of the burn-injured animals, even on days where the 20 mg/kg dose produced significant antinociceptive effect on the left.

The effect of burn-injury on the antinociceptive effects of the 40 mg/kg dose of all opioids is shown in Figure 4 B. Two-way mixed model ANOVA revealed a significant main effect of injury condition ($F(3, 121) = 68.937$, $p < .001$), a significant main effect of day ($F(5, 605) = 2.775$, $p < .01$), and a significant interaction between day and injury ($F(15, 605) = 3.751$, $p < .001$).

Bonferroni post-hoc comparison revealed that across the entire study, the antinociceptive effects were significantly greater in the sham animals than both the burned and non-injured foot of the burn-injured animals. Additionally, on days 7-21, the

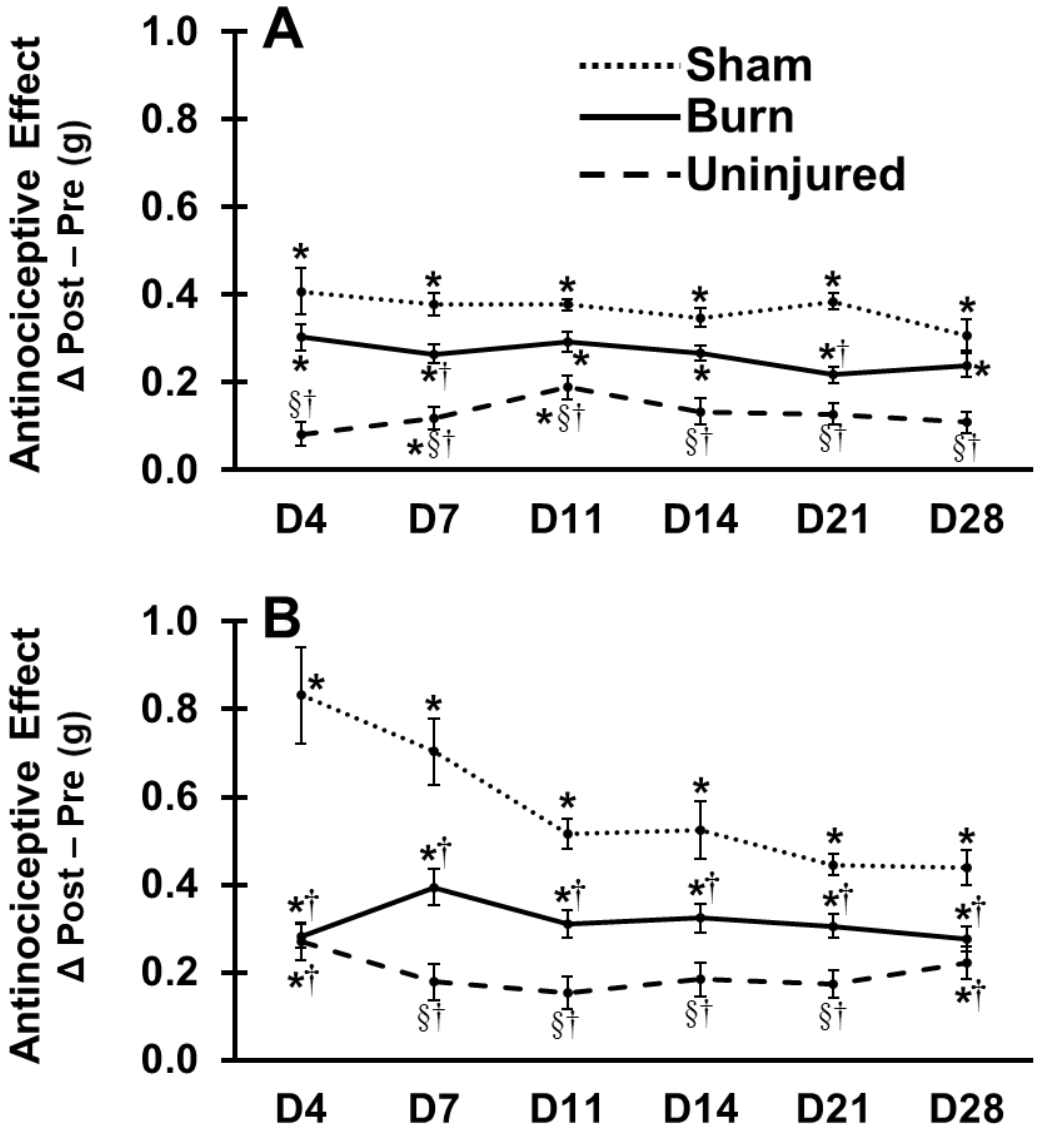

Figure 4. The effect of burn injury on the antinociceptive effects of opioids. A. 20 mg/kg. B. 40 mg/kg. * Significantly different ($p < .05$) from saline-treated animals (Figure 3). † Significantly different ($p < .05$) from sham animals. § Significantly different ($p < .05$) from burned foot (marked only for burn-uninjured foot for clarity). Results are presented as mean ± SEM.

antinociceptive effects were also significantly greater in the right (burned) feet compared to the left (uninjured) feet of the burn-injured animals. In the uninjured foot of burned animals, opioid treatment only produced antinociceptive effect greater than saline on days 4 and 28 of the study.

Hydrocodone

The effect of burn-injury on the antinociceptive effects of hydrocodone is shown in Figure 6. Two-way mixed model ANOVA of the 20 mg/kg dose (Figure 5 A) revealed a significant main effect of injury condition ($F(3, 47) = 78.806$, $p < .001$), no effect of day ($F(5, 235) = .491$, $p = .783$, n.s.), and no interaction between day and injury ($F(15, 235) = 1.395$, $p = .15$, n.s.).

Two-way mixed model ANOVA of the 40 mg/kg dose (Figure 5 B) revealed a significant main effect of injury condition ($F(3, 49) = 47.882$, $p < .001$), a significant effect of day ($F(5, 245) = 2.561$, $p = .028$), and a significant interaction between day and injury ($F(15, 235) = 3.325$, $p < .001$).

Bonferroni post-hoc analysis revealed that across the study, antinociceptive potency of both 20 mg/kg and 40 mg/kg hydrocodone doses were significantly different in all injury conditions (sham, burned foot, uninjured foot), and hydrocodone at both doses provided significant antinociception as compared to saline. When analyzing by day, Bonferroni post-hoc comparison revealed that across the entire study the antinociceptive effect of hydrocodone was significantly greater in the sham animals than the uninjured feet of the burn-injured animals, with the exception of days 14 and 28 for

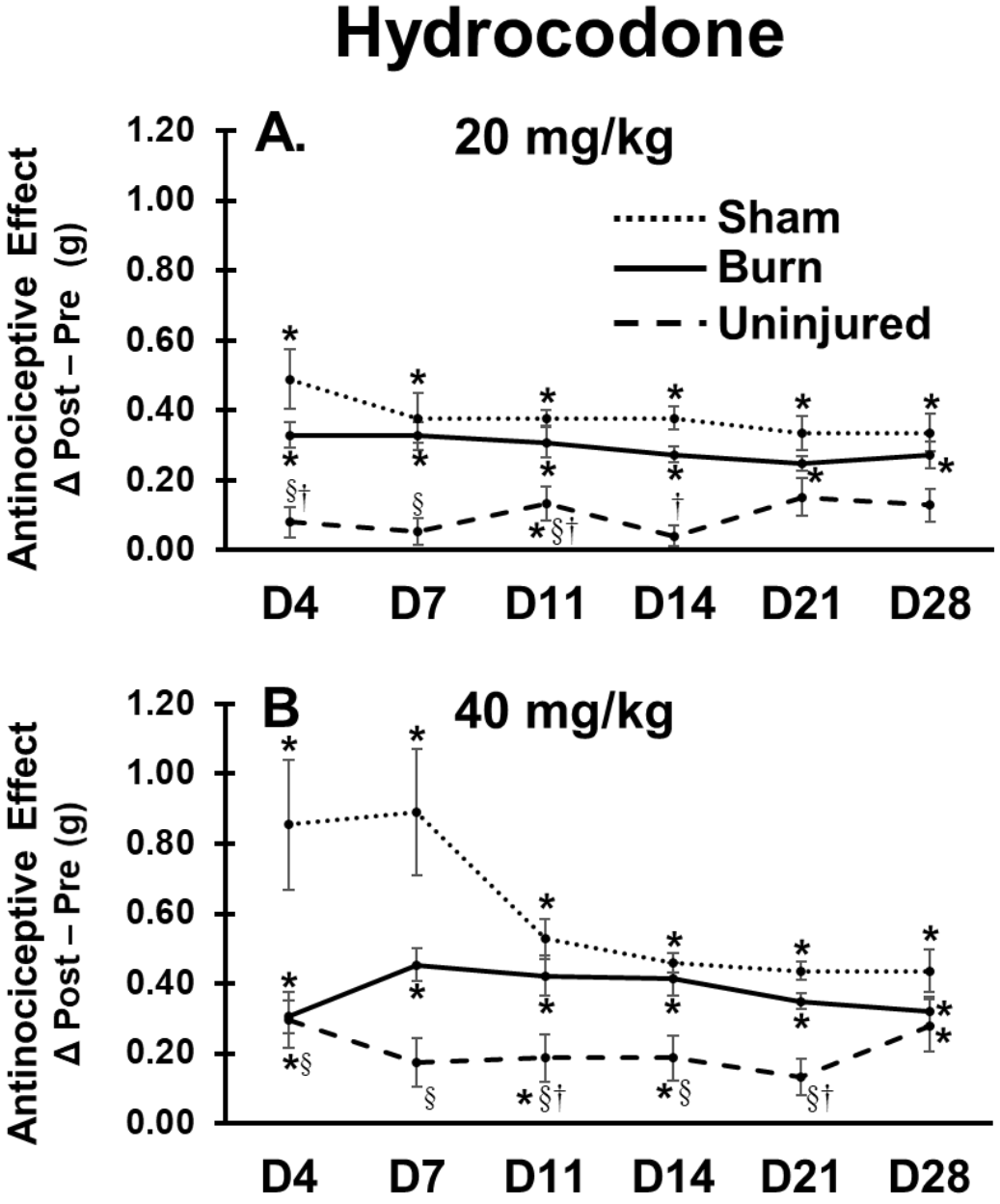

Figure 5. The effect of burn injury on the antinociceptive effects of hydrocodone. A. 20 mg/kg. B. 40 mg/kg. * Significantly different ($p < .05$) from saline-treated animals (Figure 3). † Significantly different ($p < .05$) from sham animals. § Significantly different ($p < .05$) from burned foot (marked only for burn-uninjured foot for clarity). Results are presented as mean ± SEM.

the 40 mg/kg dose. The antinociceptive effect of hydrocodone was significantly greater in the sham animals than the burned feet only on day 14 for the 20 mg/kg dose, as well as on days 4, 7, and 21 for the 40 mg/kg dose. Notably, the pain-relieving effect was significantly greater in the burned feet as compared to the uninjured feet on days 4-11 for the 20 mg/kg dose, and on days 11 and 21 for the 40 mg/kg dose.

Oxycodone

The effect of burn-injury on the antinociceptive effects of oxycodone is shown in Figure 7. Two-way mixed model ANOVA of the 20 mg/kg dose (Figure 6 A) revealed a significant main effect of injury condition ($F(3, 50) = 34.879$, $p < .001$), no effect of day ($F(5, 250) = 1.91$, $p = .093$, n.s.), and no interaction between day and injury ($F(15, 250) = .940$, $p = .52$, n.s.).

Two-way mixed model ANOVA of the 40 mg/kg dose (Figure 6 B) revealed a significant main effect of injury condition ($F(3, 45) = 28.345$, $p < .001$), a significant effect of day ($F(5, 225) = 2.271$, $p = .048$), but no interaction between day and injury ($F(15, 225) = 1.695$, $p = .053$, n.s.).

Bonferroni post-hoc analysis revealed that across the study, antinociceptive potency of the 20 mg/kg dose was significantly different in all injury conditions (sham, burned foot, uninjured foot), and oxycodone at 20 mg/kg provided significant antinociception as compared to saline. The antinociceptive potency of the 40 mg/kg dose of oxycodone was significantly higher in the sham-injured condition, but oxycodone

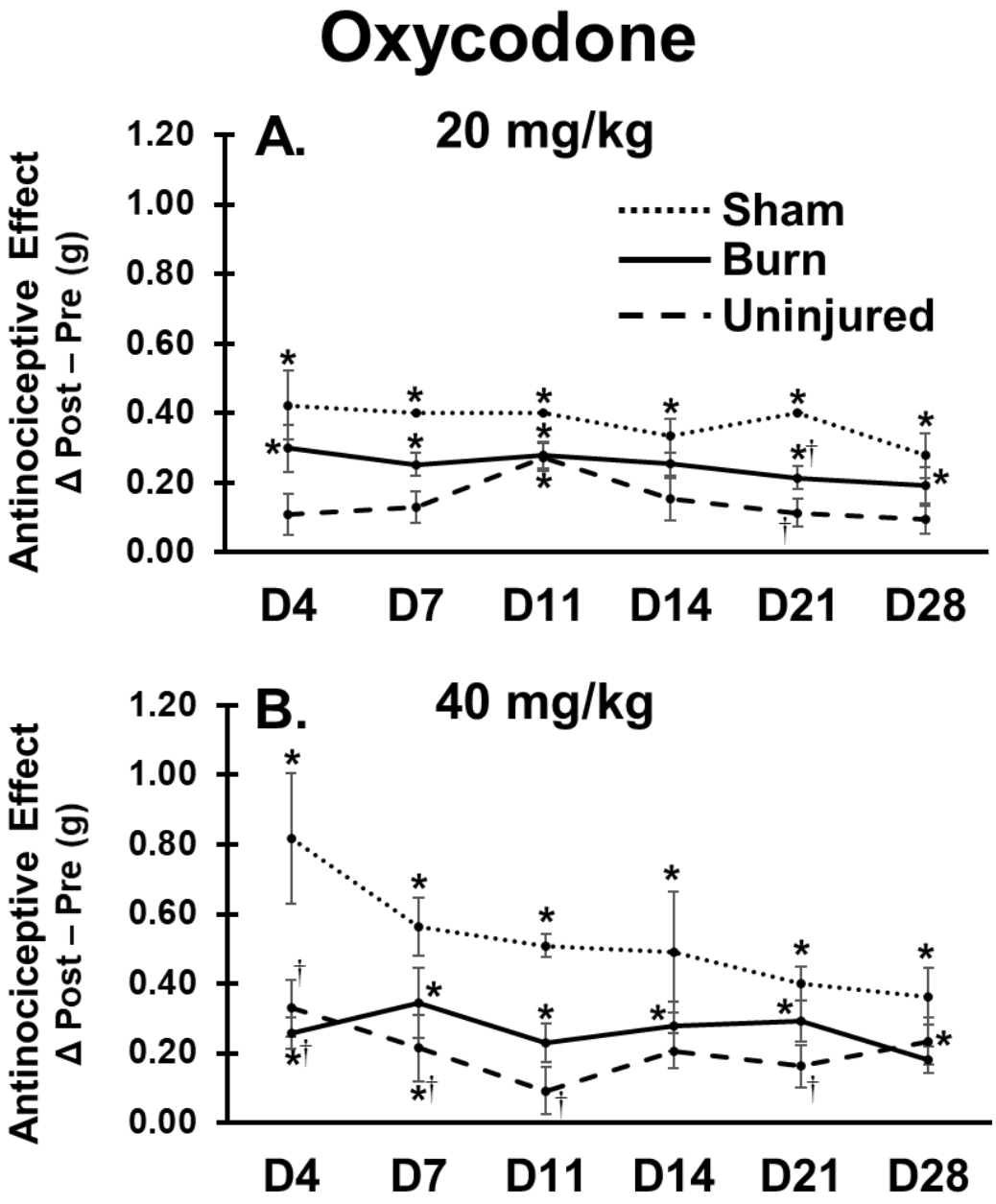

Figure 6. The effect of burn injury on the antinociceptive effects of oxycodone. A. 20 mg/kg. B. 40 mg/kg. * Significantly different ($p < .05$) from saline-treated animals (Figure 3). † Significantly different ($p < .05$) from sham animals. § Significantly different ($p < .05$) from burned foot (marked only for burn-uninjured foot for clarity). Results are presented as mean ± SEM.

potency in the burned and uninjured feet were not significantly different. Despite this, oxycodone at 40 mg/kg provided significant antinociception as compared to saline.

When analyzing by day, Bonferroni post-hoc comparison revealed that the antinociceptive effect of oxycodone was significantly greater in the sham animals than the uninjured feet of the burn-injured animals on days 4, 7, and 21 for the 20 mg/kg dose, and days 4, 7, 11, and 21 for the 40 mg/kg dose. The antinociceptive effect of oxycodone was significantly greater in the sham animals than the burned feet on days 14 and 21 for the 20 mg/kg dose, as well as on days 4, 11, and 14 for the 40 mg/kg dose. There were no significant differences in the antinociceptive effect of oxycodone between the burned and uninjured feet of the burn-injured animals.

Morphine

The effect of burn-injury on the antinociceptive effects of morphine is shown in Figure 8. Two-way mixed model ANOVA of the 20 mg/kg dose (Figure 7 A) revealed a significant main effect of injury condition ($F(3, 51) = 32.676$, $p < .001$), no effect of day ($F(5, 255) = 1.14$, $p = .34$, n.s.), and no interaction between day and injury ($F(15, 255) = .953$, $p = .51$, n.s.).

Two-way mixed model ANOVA of the 40 mg/kg dose (Figure 7 B) revealed a significant main effect of injury condition ($F(3, 43) = 49.048$, $p < .001$), no effect of day ($F(5, 215) = .625$, $p = .681$, n.s.), and no interaction between day and injury ($F(15, 215) = 1.241$, $p = .243$, n.s.).

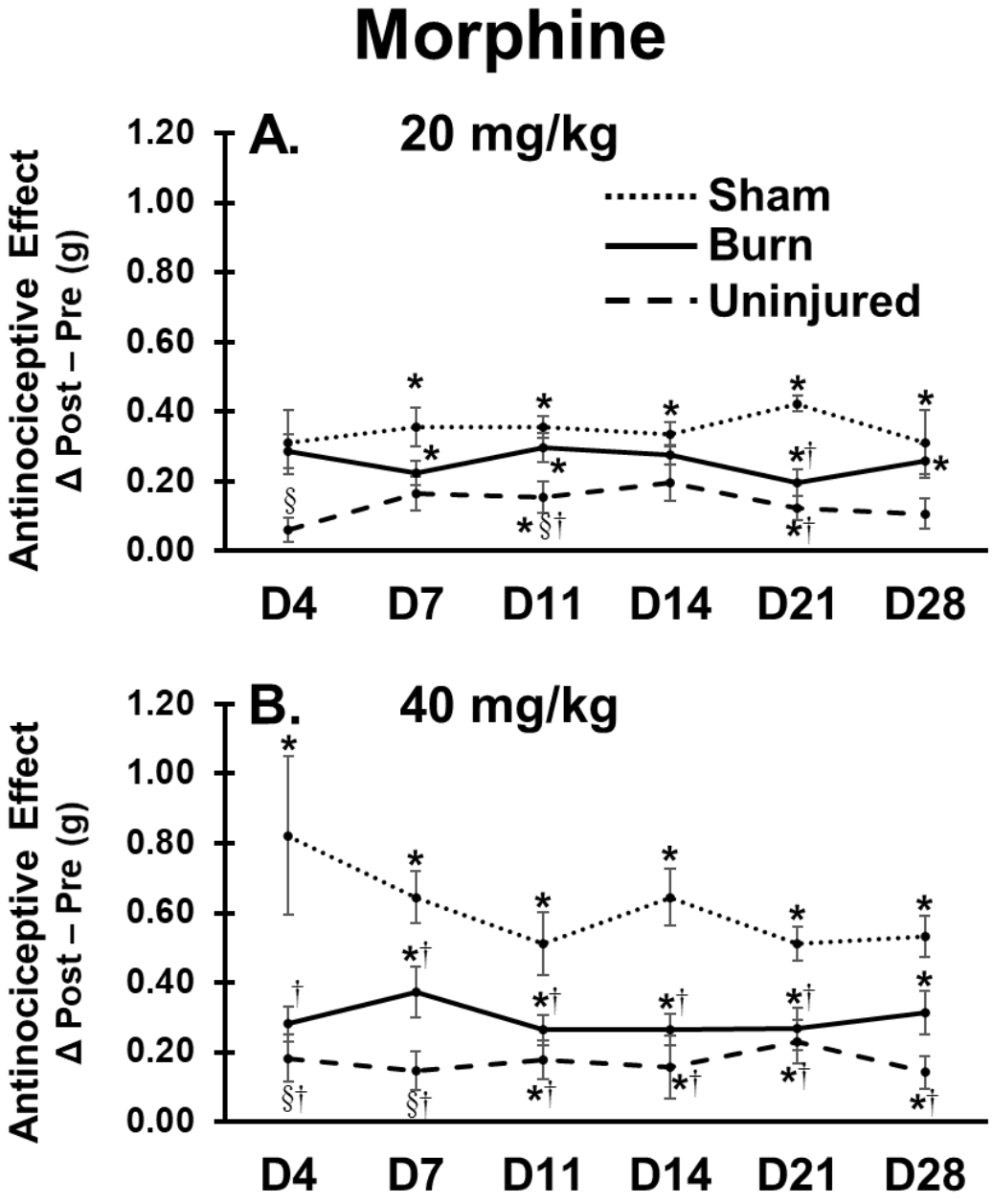

Figure 7. The effect of burn injury on the antinociceptive effects of morphine. A. 20 mg/kg. B. 40 mg/kg. * Significantly different ($p < .05$) from saline-treated animals (Figure 3). † Significantly different ($p < .05$) from sham animals. § Significantly different ($p < .05$) from burned foot (marked only for burn-uninjured foot for clarity). Results are presented as mean ± SEM.

Bonferroni post-hoc analysis revealed that across the study, antinociceptive potency of the 20 mg/kg dose was not significantly different between sham animals and the potency observed in the burned foot of injured animals. However, both of these were higher than the potency observed in the uninjured foot. Bonferroni post-hoc analysis revealed that across the study, antinociceptive potency of the 40 mg/kg dose was significantly higher in the sham-injured condition, but that morphine potency in the burned and uninjured feet were not significantly different. Despite these exceptions, morphine at both doses provided significant antinociception as compared to saline in all conditions.

When analyzing across days, Bonferroni post-hoc comparison revealed that across the entire study the antinociceptive effect of the 40 mg/kg dose of morphine was significantly greater in the sham animals than both the burned and uninjured feet of the burn-injured animals. The antinociceptive effect of the 20 mg/kg dose was significantly greater in the sham animals than the uninjured feet of the burn-injured animals on days 4, 7, 11, and 21, and was greater in the sham animals than the burned feet on day 21. Notably, the pain-relieving effect was significantly greater in the burned feet as compared to the uninjured feet on days 4-11 for the 20 mg/kg dose, and on days 4 and 7 for the 40 mg/kg dose.

4. Discussion

The results of this experiment indicate that the experience of burn injury *per se* reduces the antinociceptive potency of opioids. While all opioids were superior to

treatment with saline, all three drugs at both 20 mg/kg and 40 mg/kg doses were less potent in animals with a burn injury than in animals with a sham injury. This finding may help explain the observation that burn patients often report pain despite being treated with opioid doses in excess of standard dosing guidelines. It may be that opioids are less potent in these individuals, and therefore doses which would be expected to provide relief are not strong enough to do so.

It would be reasonably assumed that pain would be greater in the injured foot than the uninjured one, and therefore that this reduction would be greater near the site of injury, due to the heightened sensitivity of wounded tissue. Interestingly, and contrary to expectations, this reduction in antinociceptive potency was greater in the contralateral, uninjured foot of burned animals than in the burned foot. On most days during the experiment, antinociceptive potency was reduced so profoundly in the uninjured foot that treatment with opioids of either dose provided no greater antinociceptive benefit than treatment with saline.

It was also observed that burn injury reduced the antinociceptive potencies of opioids by equivalent degrees; no drug-specific differences were observed in burn-induced antinociceptive reductions. This implies that, most likely, whatever mechanisms underlie the reduced potency of opioids following burn injury, these mechanisms are general, as opposed to drug-specific, and shared by at least the 3 opioids examined here. Indeed, this conclusion helps narrow and refine the list of potential mechanisms, as mechanisms which differ between these drugs, such as receptor internalization (sometimes exhibited by oxycodone but not by morphine) or desensitization without

internalization (displayed by morphine and the oxycodone metabolite oxymorphone, but not oxycodone) (Keith et al., 1996; Van Bockstaele and Commons, 2001; Arttamangkul et al., 2008; Virk and Williams, 2008; Melief et al., 2010) are not likely to mediate the reduced antinociceptive potency of opioids following burn injury. It should be noted that the ability of oxycodone to induce receptor desensitization and/or internalization is highly dependent upon the model (Williams et al., 2013).

It may be that the molecular mechanisms underlying the observed reduction in opioid potency following a burn injury are partially antagonized by signals released by the inflammation and tissue damage associated with the burn wound. These counteractive signals may be reduced or absent in distal tissues due to the absence of inflammation in that tissue, resulting in overall greater reductions in opioid potency in distal, non-injured tissue.

Multiple potential mechanisms by which burn injury could alter the potency of opioids are indicated by the literature. Burn injury results in a significant amount of inflammation (Rowan et al., 2015). In severe cases, a systemic inflammatory response syndrome (SIRS) can be developed (Farina et al., 2013; Xu et al., 2016). Inflammatory signals have been demonstrated to modulate opioid pharmacology (Shavit et al., 2005; Hutchinson et al., 2008b; Hutchinson et al., 2008a; Narita et al., 2008a; Berta et al., 2013; Bai et al., 2014; Bao et al., 2014; Pilat et al., 2015; Pilat et al., 2016). Thus, these findings may be due to alterations in opioid activity because of the heightened inflammatory state following burn injury.

Burn injury, like any large-scale traumatic injury, also profoundly alters the functional state of the organism's immune system. Burn-induced alterations in immune system function result in modulation in the secretion of cytokines and prostaglandins (He et al., 2001; Strong et al., 2001; Schwacha et al., 2002; Luo et al., 2005). Additionally, recent studies highlight the involvement of non-neuronal mechanisms, such as Toll-like receptors on immune cells, in the antinociceptive effect of opioids (reviewed in (Thomas et al., 2015) and (Nicotra et al., 2012)). Thus, the findings in this study involving the uninjured foot might be explained by burn-induced changes in interactions between opioid and immune signaling.

However, these mechanisms seem to fail to account for the greater reduction in potency in uninjured tissues. Indeed, if the reduction in opioid potency were directly related to inflammation or immune response, one would again expect the reduction to be greater in the injured tissue where inflammation and immune reactivity are heightened. Burn injury has been demonstrated to alter the expression levels of receptor, effector, and signaling molecules within the ipsilateral side of spinal cord's dorsal horn (Wang et al., 2011a; Song et al., 2014). Alterations were observed in the expression levels of NR1 subunit of the N-methyl-d-aspartate (NMDA) receptor as well as of multiple effector and signaling molecules such as Akt, protein kinase C, nitric oxide synthase, and glycogen synthase kinase-3β. The pain relieving effects of opioids could be manifested at the spinal and supraspinal levels, as well as peripherally (Stein and Zöllner, 2009; Sehgal et al., 2011). Thus, changes in expression levels of receptors and effector molecules within

the area of injury and/or spinal cord is likely to explain the reduction in the antinociceptive effectiveness of opioid following burn trauma.

Opioid receptors activate P38 MAPK and JNK in what appears to be a β-Arr2-dependent manner (Bruchas et al., 2006). β-Arr2 is also thought to scaffold and inactivate Akt (Beaulieu et al., 2005), and β-Arr2 is known to be a crucial player in the reduction of opioid analgesia, as mice with β-Arr2 knocked out demonstrate significantly enhanced morphine analgesia (Bohn et al., 1999).

Interestingly, burn injury has been demonstrated to reduce Akt phosphorylation (Sugita et al., 2005). Decreased pAkt in turn results in increased activation of P38 MAPK (Rane et al., 2010). Therefore, β-Arr2-mediated reductions in analgesic effect of opioids may be due to inactivation of Akt, activation of P38 MAPK/JNK or both. It may be the case that in this experiment, unilateral burn injury causes an overall imbalance in P38 MAPK, JNK, and Akt activity in the spinal cord, likely in a β-Arr2-dependent manner, which in turn causes a reduction in opioid analgesia. This reduction may be due to reduced opioid signaling via Akt and P38 MAPK, P38-mediated increases in opioid receptor internalization, or (most likely) both concurrently. This proposed mechanism has the advantage of explaining why this effect is more pronounced in the contralateral side, vs the injured side, and this is due to a lack of buffering countereffects that are present in the injured side.

CHAPTER V

EXPERIMENT 3: OPIOIDS DO NOT EXHIBIT DRUG-SPECIFIC DIFFERENCES IN THE ABILITY TO TREAT BURN PAIN*

1. Background

The previous finding (Chapter IV) demonstrated that the experience of burn injury and the accompanying pain decreases the antinociceptive potency of the three opioids. In addition, it demonstrated that this reduction in potency occurred to equivalent degrees for all three drugs, indicating that there are no drug-specific differences in sensitivity to whatever mechanism drives this reduced antinociception. However, it remained possible that the opioids, despite showing equivalent *reductions* in antinociception, may have differential degrees of potency to provide pain relief.

Opioids have been shown to have drug-specific differences in their ability to activate the μ-opioid receptor, largely responsible for analgesia, in the case of bone cancer pain (Nakamura et al., 2013). In addition, these three compounds display slight differences in their binding affinity ratios for the three opioid receptors, which could potentially impact analgesic function. As previously discussed (Chapter IV), opioids have been demonstrated to exhibit drug-specific differences in pain-related signaling

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pathways, including β-arrestin, JNK, various MAPK-family molecules, and others (Bohn et al., 1999; Ballard-Croft et al., 2002; Alexander et al., 2004; Kam et al., 2004; Raehal et al., 2005; Cui et al., 2006; Shahabi et al., 2006; Chen et al., 2008; Chen and Sommer, 2009; Mittal et al., 2012; DeWire et al., 2013).

In light of these pharmacokinetic and pharmacodynamic differences among opioids, it is possible that different opioid analgesics may have differential degrees of antinociceptive potency, despite equivalent degrees of potency reduction in the presence of a pain state. If this is the case, some opioids may therefore provide superior antinociception following a burn injury as compared to others. This experiment tests the hypothesis that various opioids may have differential antinociceptive potencies, either in pain-free animals or in the presence of burn pain.

2. Experimental Procedure

Subjects

Adolescent (PND 28-29 at beginning of experiment) C57BL/6 mice were used in this experiment. Approximately 10-15 animals per group were used for this experiment, for a total of 181 animals.

Burn Injury

Burn or sham injury was induced as described above (Experiment 1).

Mechanical Sensitivity Test

The mechanical allodynia test was performed as described above (Chapter IV).

Drug Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were administered saline, hydrocodone, oxycodone, or morphine. All opioids were given at either 20 or 40 mg/kg doses. Mice received opioids (or saline) twice daily (a.m. and p.m.) for 28 days, beginning the evening of the day of injury.

Group Assignment

Animals were randomly assigned to receive either a sham or burn injury. After injury, animals were further randomly assigned to one of 4 drug treatment conditions (saline, morphine, oxycodone, or hydrocodone) and one of 2 drug dose conditions (20 mg/kg or 40 mg/kg for each of the 3 opioids) for a total of 14 groups.

Statistical Analysis

The antinociceptive potency of the various drugs were calculated as described above in Chapter IV. For each dose (20 and 40 mg/kg) and each injury condition (sham, burned foot, uninjured foot), data was analyzed using a mixed-model ANOVA (SPSS Statistics 20, Somers, NY) in a 3 (drug) x 6 (day) design. Post hoc contrasts were computed using Bonferroni procedure. Differences with p-values of less than .05 were defined as statistically significant.

3. Results

No Drug-Specific Differences Are Present in Opioid Antinociceptive Potency Following Burn

In sham animals, injured (right) and uninjured (left) feet of burned animals, (Figure 8), morphine, oxycodone, and hydrocodone had similar antinociceptive effects.

Shams

At the 20 mg/kg dose, two-way mixed model ANOVA revealed no main effect of opioid ($F(2, 24) = .598$, $p = .558$, n.s.), no effect of day ($F(5, 120) = 1.6$, $p = .166$, n.s.), and no significant interaction between day and drug ($F(10, 120) = .019$, $p = .577$, n.s.) This finding indicates that all 3 opioids resulted in equivalent degrees of antinociception (Figure 8 A).

At the 40 mg/kg dose, two-way mixed model ANOVA revealed no main effect of opioid ($F(2, 28) = .983$, $p = .387$, n.s.), a significant main effect of day ($F(5, 140) = 5.742$, $p < .001$), but no significant interaction between day and drug ($F(10, 140) = .59$, $p = .82$, n.s.). Bonferroni post-hoc analysis indicated that all opioids were significantly more potent at D4 than D21 or D28, and more potent at D7 than at D28. This indicates the development of analgesic tolerance and/or opioid-induced hyperalgesia (OIH) at this dose in the sham-injured animals. However, all 3 opioids resulted in equivalent degrees

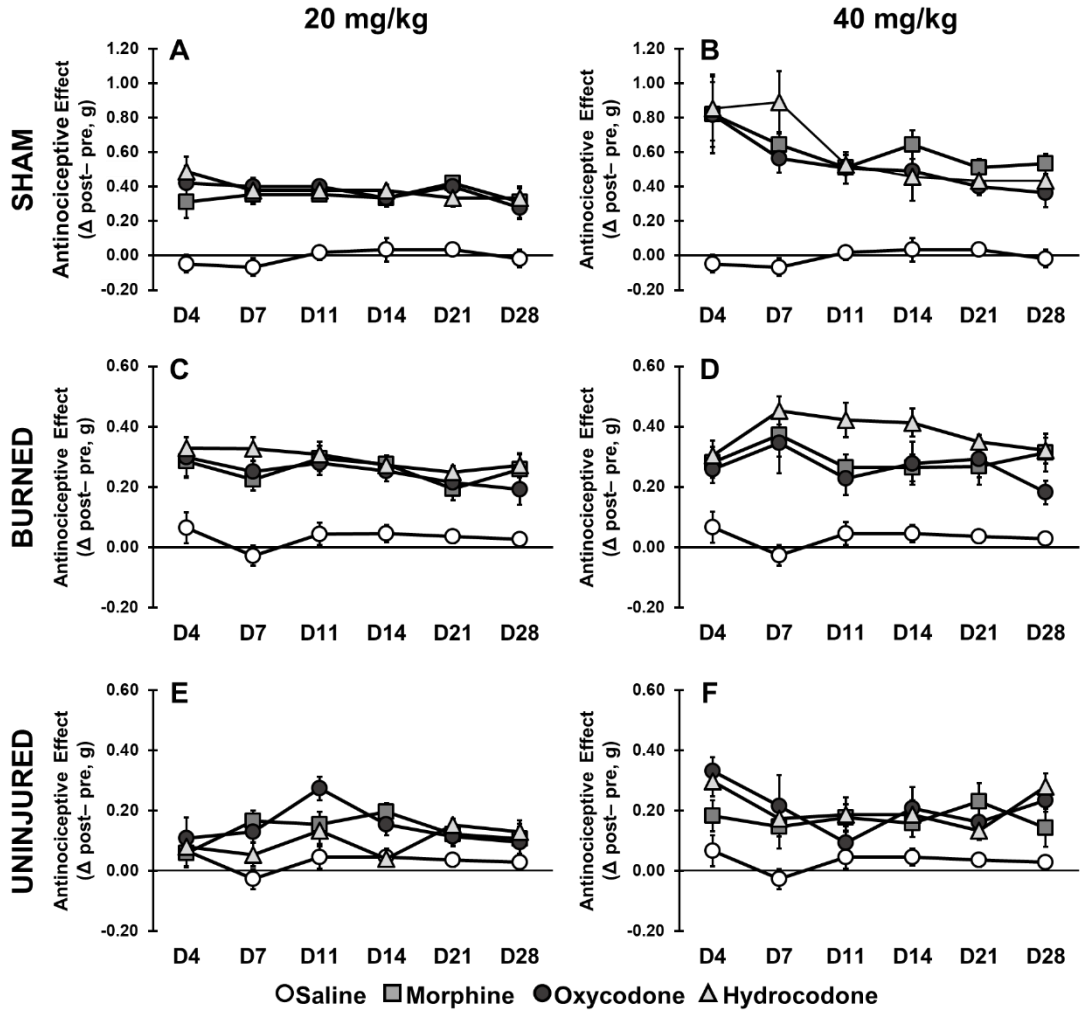

Figure 8. Drug-specific comparison of antinociceptive potency across time. Shown are sham animals (A, B), the burned foot of injured animals (C, D) and the uninjured foot of burned animals (E, F) at both 20 mg/kg dose (A, C, E) and 40 mg/kg dose of opioids (B, D, F). The three opioids all exhibited comparable antinociceptive potency to one another within each dose and injury condition. Results are presented as mean ± SEM.

of antinociceptive potency as well as equivalent rates of tolerance/OIH development (Figure 8 B).

Injured Foot of Burn-Injured Animals

At the 20 mg/kg dose of opioids, two-way mixed model ANOVA revealed no main effect of opioid ($F(2, 45) = .662$, $p = .521$, n.s.), a significant effect of day ($F(5, 225) = 2.674$, $p = .023$), but no significant interaction between day and drug ($F(10, 225) = .515$, $p = .878$, n.s.). Bonferroni post-hoc analysis indicated that opioid antinociceptive potency was significantly reduced at D21 as compared to D4 and D11, but no other differences were observed (Figure 8 C).

At the 40 mg/kg dose, two-way mixed model ANOVA revealed no main effect of opioid ($F(2, 38) = 2.478$, $p = .097$, n.s.), a significant main effect of day ($F(5, 190) = 3.097$, $p < .01$), but no significant interaction between day and drug ($F(10, 190) = .977$, $p = .465$, n.s.). The main effect of day indicates the development of analgesic tolerance and/or OIH. However differences among specific days were not detected by Bonferroni post-hoc test. Moreover, Bonferroni post-hoc analysis indicated no differences in opioid antinociceptive potency. Visually it appears that hydrocodone has slightly increased potency relative to the other opioids. However it did not reach statistical significance in the ANOVA nor in the Bonferroni post-hoc test (Figure 8 D).

Uninjured Foot of Burn-Injured Animals

At the 20 mg/kg dose of opioids, two-way mixed model ANOVA revealed no main effect of opioid ($F(2, 46) = 1.755$, $p = .184$, n.s.), no effect of day ($F(5, 230) = 1.837$, $p = .107$, n.s.), and no significant interaction between day and drug ($F(10, 230) = 1.339$, $p = .211$, n.s., Figure 8 E). Similarly, at the 40 mg/kg dose, two-way mixed model

ANOVA revealed no main effect of opioid ($F(2, 38) = .327$, $p = .723$, n.s.), no effect of day ($F(5, 190) = 1.405$, $p < .224$, n.s.), and no significant interaction between day and drug ($F(10, 190) = .807$, $p = .622$, n.s., Figure 8 F). This indicates that all 3 opioids at both doses had equivalent degrees of antinociceptive potencies.

4. Discussion

The observation of comparable potency between these 3 drugs in the burned animals indicates that, contrary to my initial hypothesis, no drug-specific differences exist in the antinociceptive potency of opioids when used to treat burn pain. Although burn injury reduced antinociceptive potency for all 3 opioids, no differences were observed in antinociceptive potency between the opioids, in either sham animals or burned animals.

The finding that the 3 opioids displayed comparable potency in the sham animals across the 28 days of the experiment re-confirms and, importantly, extends our finding that demonstrated acute equianalgesia between these three drugs in the tail withdrawal task (Emery et al., 2015). The present findings demonstrate that the drugs remain equianalgesic for at least 28 days when administered twice daily. In addition, while tolerance developed in the sham animals treated with the higher, 40 mg/kg dose, this tolerance developed at equivalent rates for all 3 opioids.

These findings also demonstrate that, while on-board, oxycodone, hydrocodone, and morphine provide equivalent levels of analgesia/antinociception. This is somewhat surprising, considering the common assumption that hydrocodone is considered to be a pharmacologically ‘weaker’ opioid than oxycodone or morphine, which are considered

to be high affinity μ-opioid receptor agonists and therefore ‘strong’ opioids, as defined by the World Health Organization (WHO, 1990; Reddy et al., 2014). This result indicates that affinity for the μ-opioid receptor is not the only determinant of opioid analgesic potency. In addition, this result calls into question the common treatment protocols which recommend the use of ‘strong’ opioids in most cases of moderate-to-severe pain, and contra-indicate the use of ‘weak’ opioids (i.e. low-affinity agonists) out of fear that they may foster abuse while failing to adequately manage pain. In contrast, these current results indicate that ‘weak’ opioids such as hydrocodone can provide equivalent pain relief, with potentially fewer adverse consequences.

CHAPTER VI

EXPERIMENT 4: DIFFERENTIAL PREVENTION OF THE DEVELOPMENT OF BURN-INDUCED MECHANICAL HYPERALGESIA*

1. Background

As previously discussed (Chapter III), in addition to high levels of inflammatory pain, one of the most common and severe long-term consequences of burn injury is the development of chronic and/or neuropathic pain (Summer et al., 2007). Many of the same pathways responsible for acute pain and development of antinociceptive tolerance are implicated in the development of chronic pain (Guo et al., 2009; Sotgiu et al., 2009; Hervera et al., 2012; Manassero et al., 2012; Mittal et al., 2012; Sanna et al., 2014; Marcus et al., 2015; Pilat et al., 2015).

In addition to observed (Nakamura et al., 2013) and expected drug-specific differences in acute antinociception, opioids have been demonstrated to have drug-specific effects in their effectiveness to treat neuropathic pain in both animal models (Arner and Meyerson, 1988; Tsai et al., 2000; Suzuki et al., 2007; Narita et al., 2008b) and humans (Arner and Meyerson, 1988; Gimbel et al., 2003; Agarwal et al., 2007).

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*Part of this chapter is reprinted with permission from "Hydrocodone is more effective than morphine or oxycodone in suppressing the development of burn-induced mechanical allodynia" by Emery MA, Bates MLS, Wellman PJ, and Eitan S (2017) Pain Medicine, 18(11): 2170-2180. Copyright [2017] American Academy of Pain Medicine; and "Hydrocodone, but neither morphine nor oxycodone, is effective in suppressing burn-induced mechanical allodynia in the uninjured foot contralateral to the burn" by Emery MA, Bates MLS, Wellman PJ, and Eitan S (2017) Journal of Burn Care and Research, 38(5): 319-326. Copyright [2017] The American Burn Association.

Using a similar burn injury paradigm as the one utilized here, Chang et al. (2010) produced a long-lasting, bilateral, and gradually worsening mechanical allodynia that persisted after wound healing (which occurred at 1 week in their model) and which was characterized electrophysiologically as neuropathic pain.

In this experiment, I utilized the burn model in mice developed as described in Chapter III, which as noted exhibited a similar pain development trajectory as that characterized by Chang et al. (2010). Then, drug-specific differences in the ability of opioids to block development of chronic, neuropathic-like pain, defined here and based upon Chang et al. (2010) as continued or worsening mechanical allodynia after wound healing, in both the injured and non-injured sides of the body, was examined.

2. Experimental Procedure

Subjects

Adolescent (PND 28-29 at beginning of experiment) C57BL/6 mice were used in this experiment. The animals were housed as described in the general methods. Approximately 10-15 animals per group were used for this experiment, for a total of 181 animals.

Burn Injury

Burn or sham injury was induced as described above (Experiment 1).

Mechanical Sensitivity Test

All mice were examined for their baseline response to the von Frey mechanical allodynia test the day before receiving a burn injury. The mice were retested on days 4, 7, 11, 14, 21, and 28 after the burn. On each of these testing days, the mechanical reactivity in the burn-injured and uninjured feet were examined before drug treatment, to assess their level of pain reactivity in the absence of drug (i.e. ~12 h after their last dose of opioid analgesics). The degree of mechanical reactivity in the pre-drug tests were used as a measure for the development of burn-induced hyperalgesia.

Drug Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were administered saline, hydrocodone, oxycodone, or morphine. All opioids were given at either 20 or 40 mg/kg doses. Mice received opioids (or saline) twice daily (a.m. and p.m.) for 28 days, beginning the evening of the day of injury.

Group Assignment

Animals were randomly assigned to receive either a sham or burn injury. After injury, animals were further randomly assigned to one of 4 drug treatment conditions (saline, morphine, oxycodone, or hydrocodone) and one of 2 drug dose conditions (20 mg/kg or 40 mg/kg for each of the 3 opioids) for a total of 14 groups.

Statistical Analysis

The pre-treatment thresholds for each drug and each side (left/uninjured and right/injured) were analyzed using a mixed model ANOVA in a 6 (injury_treatment) x 7 (timepoint) design for a total of 6 individual tests (see Figure 9). Post hoc contrasts were computed using Bonferroni procedure. Differences with p-values of less than .05 were defined as statistically significant.

3. Results

Hydrocodone

In the injured foot, two-way repeated ANOVA revealed a significant main effect of injury ($F(5, 78) = 44.22$, $p < .0001$), a significant main effect of day ($F(6, 468) = 67.58$, $p < .0001$), and a significant interaction between day and injury ($F(6, 468) = 9.26$, $p < .0001$) (Figure 9 A).

Bonferroni post-hoc comparison revealed that the Burn-Hydro-40 animals had a small but significantly higher baseline pain sensitivity threshold than the Sham-Saline animals. However, their baseline did not significantly differ than the Burn-Saline animals, or any of the other experimental groups. Hydrocodone resulted in only minimal OIH (Figure 9 A). Specifically, a significant decrease in pain sensitivity threshold was observed in the Sham-Hydro-20 vs. Sham-Saline animals on day 21 post -injury. This was not observed in the Sham-Hydro-40 animals. Rather, a small but significant increase in pain sensitivity thresholds was observed in the Sham-Hydro-40 animals on day 4 post-injury. Hydrocodone was very effective to prevent the development of burn-induced

mechanical allodynia (Figure 9 A and B). Bonferroni post-hoc comparison revealed that, compared to the Burn-Saline animals, the Burn-Hydro-40 animals had significantly decreased pain sensitivity (i.e. increased pain sensitivity thresholds) starting at 7 days post-injury and continued up through the end of the 28-day study. The Burn-Hydro-20 animals had significantly decreased pain sensitivity only on days 11 and 28 post-injury.

In the uninjured foot, three-way repeated ANOVA revealed a significant main effect of dose ($F(2, 78) = 16.47$, $p < .0001$), a significant main effect of day ($F(6, 468) = 23.51$, $p < .0001$), a significant interaction between day and injury ($F(6, 468) = 3.67$, $p < .0001$), a significant interaction between day and dose ($F(12, 468) = 2.38$, $p < .01$), and a significant interaction between day, injury, and dose ($F(12, 468) = 2.78$, $p < .001$). (Figure 9 B).

Bonferroni post hoc comparison revealed that the Uninj-Hydro-40 animals had a small but significantly higher baseline pain sensitivity threshold than the Sham-Saline animals. However, their baseline did not significantly differ from the Uninj-Saline animals or any of the other experimental groups. Hydrocodone was effective in minimizing the development of burn-induced mechanical allodynia (Figure 9 B). Bonferroni post hoc comparison revealed that, compared with the Uninj-Saline animals, the Uninj-Hydro-40 animals had significantly decreased pain sensitivity (ie, increased pain sensitivity thresholds) starting at 11 days postinjury and continued up through day 21. The Uninj-Hydro-20 animals did not significantly differ in their pain sensitivity from the Uninj-Saline animals.

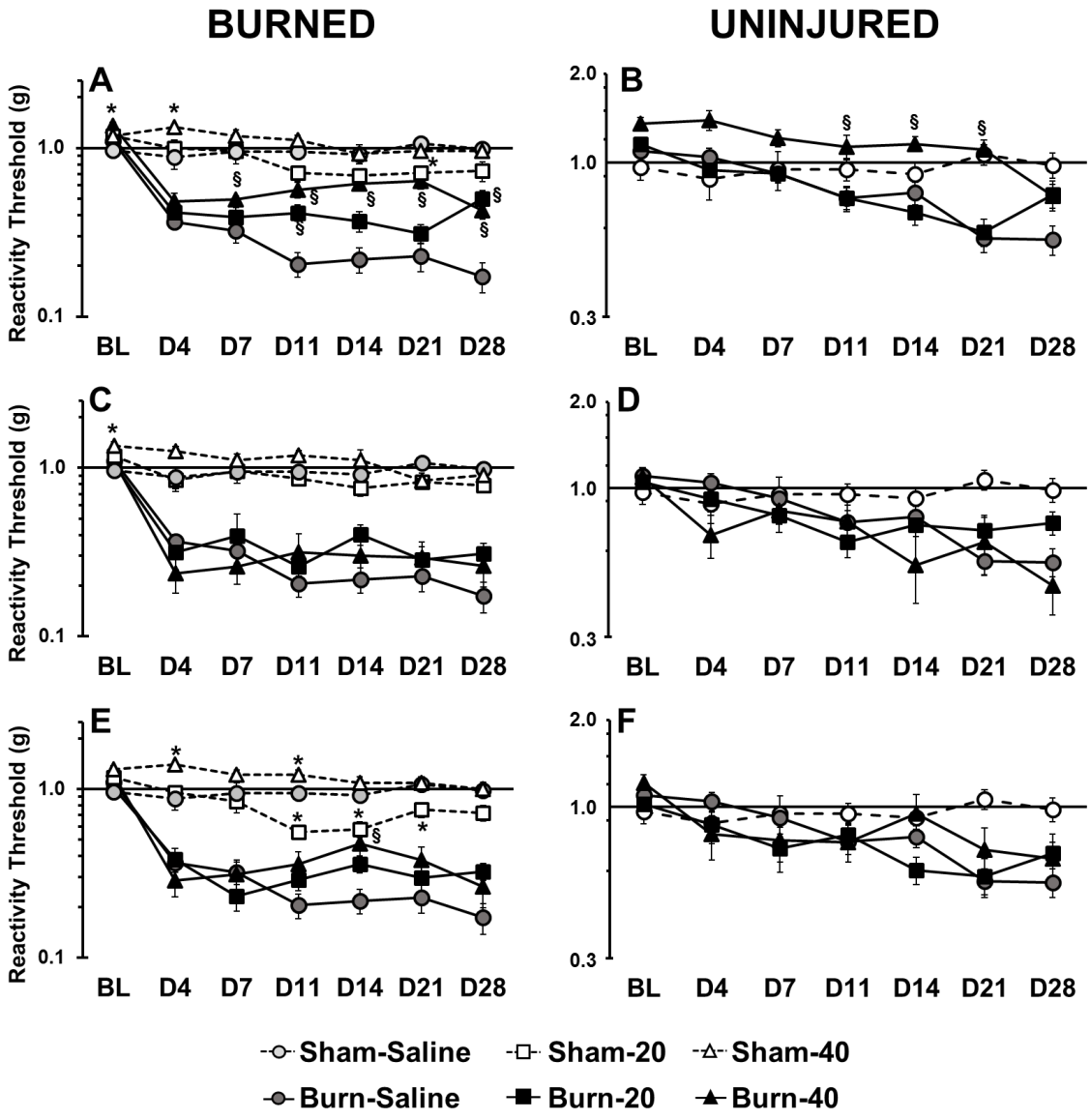

Figure 9. Effects of opioids on the development of burn-induced hyperalgesia. Sham/burn-20 and sham/burn-40 indicate injury type and opioid dose (20 mg/kg, 40 mg/kg) respectively. A, B. Hydrocodone. C, D Oxycodone. E, F. Morphine. * Significantly different ($p < .05$) from sham-saline animals. § Significantly different ($p < .05$) from burn-saline animals. Results are presented as mean ± SEM. Please note the use of different scales, due to significantly less severe overall hyperalgesia in the uninjured foot.

Oxycodone

In the injured foot, two-way repeated ANOVA revealed a significant main effect of injury ($F(5, 78) = 48.96$, $p < .0001$), a significant main effect of day ($F(6, 468) = 51.10$, $p < .0001$), and a significant interaction between day and injury ($F(6, 468) = 5.60$, $p < .0001$). Bonferroni post-hoc comparison revealed that the Sham-Oxy-40 animals had a small but significantly higher baseline pain sensitivity threshold than the Sham-Saline animals (Figure 9 C). OIH was not developed in the sham animals at either of the doses (Figure 9 C). Importantly, oxycodone had no significant effects on burn-induced mechanical allodynia at any timepoint (Figure 9 C). Bonferroni post-hoc comparison revealed that neither of the doses significantly changed pain sensitivity threshold at any given time as compared to Burn-Saline animals.

In the uninjured foot, three-way repeated ANOVA revealed a significant main effect of injury ($F(1, 78) = 17.58$, $p < .0001$), a significant main effect of day ($F(6, 468) = 17.99$, $p < .0001$), a significant interaction between injury and dose ($F(2, 78) = 3.43$, $p < .05$), a significant interaction between day and injury ($F(12, 468) = 2.13$, $p < .05$), and a significant interaction between day, injury, and dose ($F(12, 468) = 3.60$, $p < .0001$). Bonferroni post-hoc comparison revealed no significant differences in baseline pain sensitivity threshold between the Sham-Saline, Uninj-Saline, Uninj-Oxy-20, and Uninj-Oxy-40 animals. Importantly, oxycodone had no significant effects on secondary burn-induced mechanical allodynia at any timepoint (Figure 9 D). Bonferroni post-hoc comparison revealed that neither of the doses significantly changed pain sensitivity threshold at any given time as compared to Uninj-Saline animals.

Morphine

In the burn-injured foot, two-way repeated ANOVA revealed a significant main effect of injury ($F(5, 76) = 47.84$, $p < .0001$), a significant main effect of day ($F(6, 456) = 78.62$, $p < .0001$), and a significant interaction between day and injury ($F(6, 456) = 9.52$, $p < .0001$). Bonferroni post-hoc comparison revealed no significant differences in baseline pain thresholds. OIH was developed in the Sham-Mor-20, but not the Sham-Mor-40, animals (Figure 9 E). Specifically, on days 11, 14, and 21 post-injury a significant decrease in pain sensitivity threshold was observed in the Sham-Mor-20 animals vs. Sham-Saline animals. In contrast, small but significant increases in pain sensitivity thresholds were observed on days 4 and 11 post-injury in the Sham-Mor-40 animals. Morphine had minimal effects on severity of burn-induced mechanical allodynia (Figure 9 E). Bonferroni post-hoc comparison revealed that the Burn-Mor-20 animals did not significantly differ in pain sensitivity threshold from the Burn-Saline animals. The Burn-Mor-40 animals had significantly reduced pain sensitivity (i.e. increased pain sensitivity threshold) only on day 14.

In the uninjured foot, three-way repeated ANOVA revealed a significant main effect of injury ($F(1, 76) = 10.54$, $p < .01$), a significant main effect of dose ($F(2, 76) = 5.61$, $p < .01$), a significant main effect of day ($F(6, 456) = 20.40$, $p < .0001$), a significant interaction between day and injury ($F(6, 456) = 3.61$, $p < .01$), and a significant interaction between day, injury, and dose ($F(12, 462) = 4.51$, $p < .0001$). Bonferroni post-hoc comparison revealed no significant differences in baseline pain

sensitivity threshold. Importantly, morphine had no significant effects on secondary burn-induced mechanical allodynia at any timepoint (Figure 9 F). Bonferroni post-hoc comparison revealed that neither of the doses significantly changed pain sensitivity threshold at any given time as compared to thresholds in the uninjured foot of burn animals treated with saline.

4. Discussion

In the burned foot, hydrocodone at both doses resulted in significant reductions in development of injury-induced hyperalgesia, with animals receiving high-dose hydrocodone (40 mg/kg) being statistically indistinguishable from sham-injured animals receiving hydrocodone. Burn-injured animals receiving oxycodone showed no reductions in hyperalgesia development at either dose, compared to burned animals treated with saline. Burned animals treated with morphine showed modest but minimal reductions in injury-induced hyperalgesia, in a dose-dependent manner.

In the uninjured foot of burned animals, hydrocodone at a high dose (40 mg/kg) significantly improved hyperalgesia development, but the low dose (20 mg/kg) did not. Oxycodone and morphine resulted in no significantly better outcome in the uninjured foot than saline, at either dose. Additionally, morphine treatment in sham animals resulted in the development of minor but statistically significant OIH.

The finding that morphine and oxycodone are ineffective at treating chronic pain is unsurprising and well supported by the literature. Morphine is notoriously ineffective at relieving neuropathic pain in both humans and animals (Arner and Meyerson, 1988;

Bian et al., 1995; Mao et al., 1995). Indeed, a recent study demonstrated that morphine treatment may in fact exacerbate and prolong neuropathic pain in rats for months following cessation of treatment (Grace et al., 2016). For this reason, along with the propensity for negative side effects, the use of opioids is not recommended as a first-line treatment of neuropathic pain (Dworkin et al., 2010).

The finding that hydrocodone is significantly effective at preventing the development of chronic hyperalgesia following burn is especially surprising in light of the common belief that hydrocodone is a weaker opioid than either morphine or oxycodone, and normally considered insufficient to treat more severe pain (WHO, 1990; Reddy et al., 2014). In contrast, the current results, presented in Chapters V and VI, demonstrate that hydrocodone is capable of providing equianalgesia to conventionally 'strong' opioids, and in addition is actually superior in preventing and/or treating chronic forms of pain which are traditionally considered intractable to opioid treatment (Arner and Meyerson, 1988).

These findings imply that hydrocodone is functionally or mechanistically different from oxycodone and morphine in ways that differentially interact with the mechanisms underlying development of pain following burn injury. Although these drugs provide equivalent levels of antinociception in this model (as confirmed in the previous chapter), repeated treatment with hydrocodone, especially at a relatively high dose, results in significantly better overall outcomes in regards to the trajectory of development of burn-induced hyperalgesia. This is particularly interesting given that despite the high rate of hydrocodone prescriptions relative to other opioids, it is often not

utilized for treating burn pain, as it is considered too weak to be efficacious to treat the more severe pain resulting from burn injuries.

CHAPTER VII

EXPERIMENT 5: ANTINOCICEPTIVE RESPONSE TO OPIOIDS PREDICTS HYPERALGESIA DEVELOPMENT

1. Background

As previously mentioned, burn pain patients often need high doses of opioids to provide adequate analgesia (Patterson et al., 2004; Wiechman Askay et al., 2009), and even so, often report that their pain is not entirely managed (Latarjet, 2002; Wiechman Askay et al., 2009). This implies that burn pain patients are often in a state of pain, even while being treated. Despite this fact, physicians frequently restrict opioid doses, even when the patient reports that pain is not fully controlled, due to concerns of over-prescribing opioids and fostering addiction in their patients. Indeed, the current CDC guidelines for prescribing opioids advises physicians to consider opioids only when nonopioid options fail (or can be reasonably expected to fail) to control pain; to use the lowest possible reasonable dose; to "prescribe no greater quantity than needed for the expected duration of pain severe enough to require opioids", which is suggested to be around 3 days; and the use of opioids beyond 7 days is actively discouraged in most circumstances (Dowell et al., 2016).

Recent evidence suggests that prior experience of inflammatory pain may predispose patients to the development of neuropathic pain (Dieb et al., 2017). In addition, chronic pain itself increases the risk of developing substance use disorders (Blanco et al., 2016) Further, as has already been highlighted, many of the same

molecular mechanisms underlying acute pain in burn injury are also implicated in the development of chronic, neuropathic pain. Thus, the current policy of minimizing opioid use during acute pain may in fact increase the risk of developing later chronic pain, which in turn elevates risk of opioid addiction. Conversely, adequate management of pain during the early phases of injury (even if higher-than-recommended doses of opioids are required to do so) may reduce the incidence of neuropathic pain later on.

Although no differences were observed in antinociceptive potency between opioids (Chapter V), there was considerable individual variation in antinociceptive response, regardless of specific opioid. If the experience of inadequately managed inflammatory pain early in the treatment process does indeed foster the development of chronic pain, then it can be predicted that animals for whom opioids provided more potent early analgesia would ultimately develop less severe hyperalgesia. Conversely, animals for whom opioids were less effective early in treatment should ultimately develop more severe hyperalgesia. To test this hypothesis, I examined whether a correlation exists between opioids' antinociceptive potency early in the course of treatment and subsequent development of burn-induced hyperalgesia in individual animals.

2. Experimental Procedure

Subjects

Adolescent (PND 28-29 at beginning of experiment) C57BL/6 mice were used in this experiment. The animals were housed as described in the general methods.

Approximately 10-15 animals per group were used for this experiment, for a total of 181 animals. Please note that these are the same animals utilized in experiments 1-3.

Burn Injury

Burn or sham injury was induced as described above (Experiment 1).

Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were administered saline, hydrocodone, oxycodone, or morphine. All opioids were given at either 20 or 40 mg/kg doses. Mice received opioids (or saline) twice daily (a.m. and p.m.) for 28 days, beginning the evening of the day of injury.

Behavioral Analysis

All mice were examined for their baseline response to the von Frey mechanical allodynia test the day before receiving the burn injury. The mice were retested on days 4, 7, 11, 14, 21, and 28 after the burn. On each of these testing days, the mice were assessed twice. First, in the morning, their mechanical reactivity in the burn-injured and uninjured feet was examined, to assess their level of pain reactivity in the absence of drug (i.e. ~12 h after their last dose of opioid analgesics). Then the animals received their morning dose of opioids (or saline) and will be re-examined for their pain reactivity 1 h after opioid administration. The effect of opioids to block the development of burn-induced mechanical allodynia was assessed by comparing the daily pre-drug pain

threshold scores of burned animals receiving opioids vs burn animals receiving only saline (i.e. allodynia development in the absence of any pharmacological pain relief). Opioid analgesic potency was defined as the degree of change in reactivity threshold pre-drug to post-drug, daily (i.e. post-drug reactivity threshold minus pre-drug reactivity threshold).

Statistics

The effect of an opioid on the development of burn-induced hyperalgesia was computed as described above. A higher score corresponds to less pain sensitivity, or to a stronger effect of reducing burn-induced hyperalgesia. The antinociceptive potencies of the various drugs were computed as described above. A higher score indicates greater antinociceptive potency. For each mouse and foot, pretreatment pain threshold scores and antinociceptive potency scores were collected. For each mouse, hyperalgesia scores and antinociceptive potency of opioids at Day 4 were correlated with hyperalgesia scores and antinociceptive potency at Day 28. The correlations between the different scores were analyzed using 2-tailed Pearson Correlation coefficient (SPSS Statistics 20, Somers, NY). Differences with p-values of less than .05 were deemed statistically significant.

3. Results

Unsurprisingly, greater degrees of pain shortly after injury (D4) were correlated with greater degrees of hyperalgesia at D28 ($r = 0.286$, $p < .01$) This result is presented graphically in Figure 10.

Animals with injuries were grouped into 4 pain levels based on their individual levels of mechanical allodynia/hyperalgesia on D4. These categories were 'very low' (post-injury pre-test mechanical allodynia threshold 1 gram), 'low' (0.6 gram), 'moderate' (0.4 gram), or 'severe' (0.16-0.04 gram) (Table 1). Given that the 'very low' category had only 4 animals across all drug treatment groups, and that the severity of their burn injury was likely not representative, we did not further analyze this group. The other 3 groups are depicted in Figure 10. Note that no animals were eliminated and no data was manipulated to result in the formation of these groups.

Table 1: *Distribution of animals within each pain group.*

Drug	**Dose**	**Pain Category**			
		Very low	**Low**	**Moderate**	**Severe**
Total		**4**	**24**	**42**	**38**
Saline	NA	0	4	10	4
Morphine	20	1	3	8	5
	40	0	2	5	6
Oxycodone	20	1	2	6	7
	40	0	2	2	9
Hydrocodone	20	1	5	4	5
	40	1	6	6	2

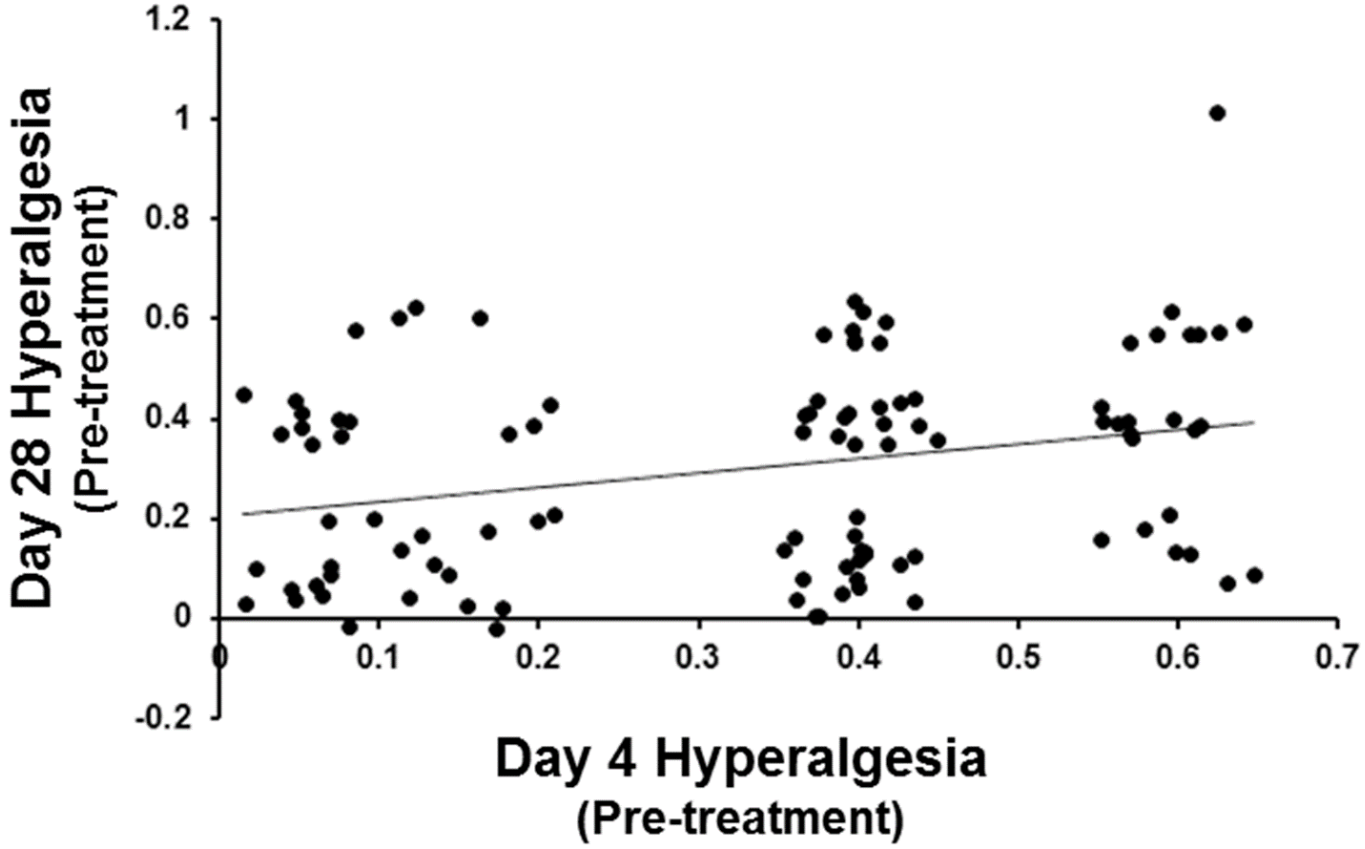

Figure 10. Pain at Day 4 post-burn correlates with pain/hyperalgesia at Day 28. Results are presented as individual data points per experimental subject corresponding to their individual hyperalgesia scores on Days 4 and 28 (in grams). Regression line indicates significant correlation, $r = 0.286$.

Within each pain category, individual response to opioids at D4 (i.e. opioid potency in that individual) was calculated, and this was compared with hyperalgesia development at D28. Across all pain categories, individual opioid response at D4 was significantly correlated with degree of hyperalgesia at D28 ($r = 0.522$, $p < .0001$). Animals for whom opioids demonstrated greater antinociceptive potency at D4 showed less severe hyperalgesia at D28, irrespective of severity of pain at D4. This data is represented in Figure 11.

Low Pain Category

For animals exhibiting a 'low' degree of pain at D4 (0.6 g pre-test threshold), animals were further sub-divided into 'low' or 'high' responders based upon their individual responsiveness to opioids on D4. Mixed model ANOVA indicated a significant main effect of antinociceptive response ($F(1, 22) = 41.16$, $p < .0001$). There was no significant main effect of day ($F(5, 110) = 1.09$, $p > .05$). Additionally, a significant interaction between antinociceptive response and day was present ($F(5, 110) = 2.88$, $p < .05$). Bonferroni post-hoc comparison revealed that the antinociceptive potencies in the high responders were significantly higher than the low responders for the entire duration of the study (Figure 12 A).

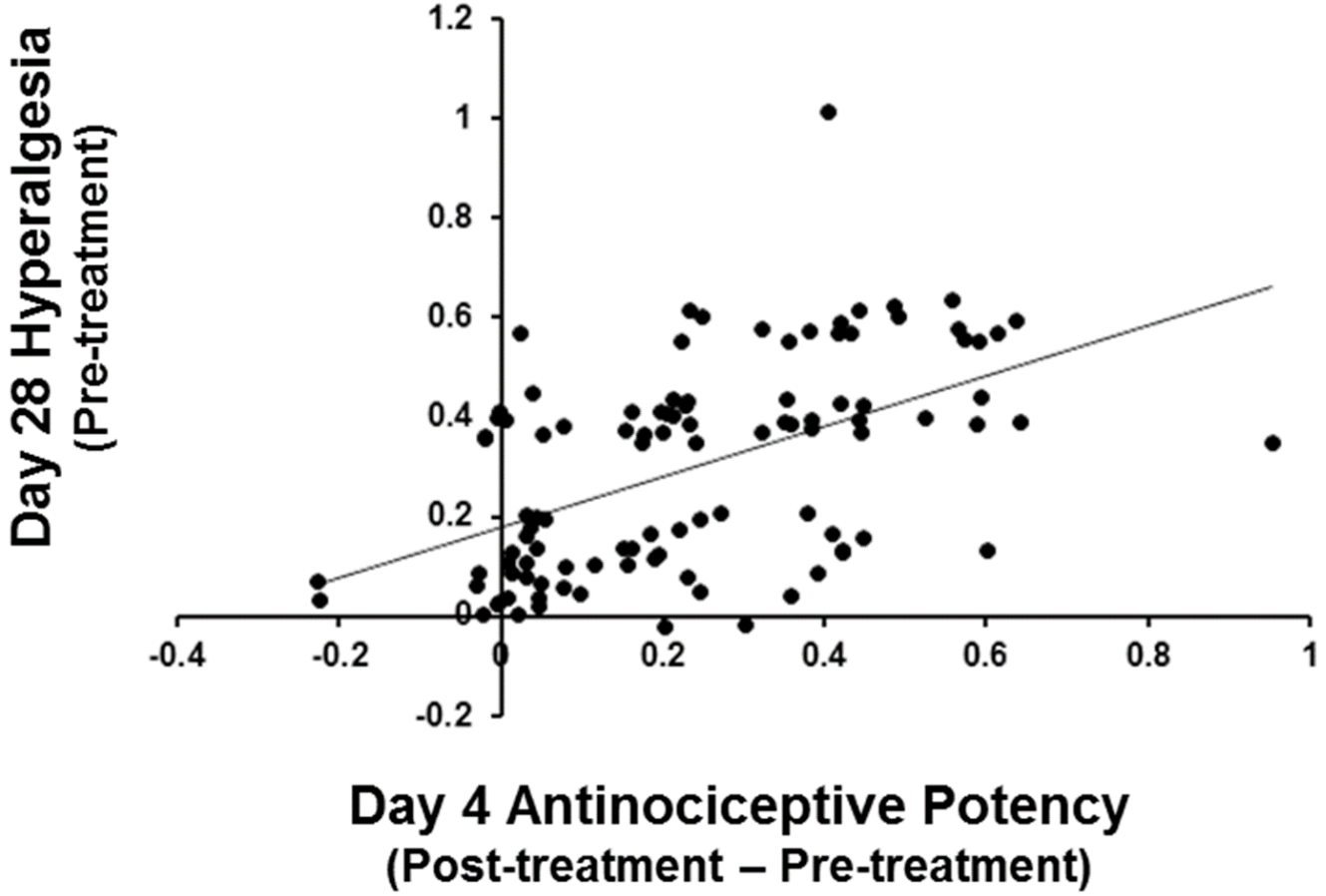

Figure 11. Opioid antinociceptive potency at Day 4 correlates with hyperalgesia at Day 28. Results are presented as individual data points per experimental subject corresponding to their individual antinociceptive potency scores on Day 4 and hyperalgesia scores on Day 28 (in grams). Regression line indicates significant correlation, $r = 0.522$.

Planned comparisons between the antinociceptive responses on D4 and D28 demonstrated the antinociceptive response of the high responders on D28 was significantly lower than their response on D4 ($t(17) = 3.11$, $p < .01$), indicating development of antinociceptive tolerance or OIH in this subgroup. No significant difference between the antinociceptive response on D28 and D4 was observed for the low responders ($t(5) = -1.76$, $p > .05$), indicating that no tolerance or OIH was developed in this subgroup.

Significant hyperalgesia was developed across the 28 days in the low pain category ($F(1, 22) = 6.79$, $p < .05$; Figure 12 B). During the course of the experiment, pain levels increased significantly in both the low and high responders (low responders: $t(5) = 3.54$, $p < .05$; high responders: $t(17) = 3.02$, $p < .01$).

Bonferroni post-hoc comparison revealed that the mechanical sensitivity thresholds were significantly higher in the high responders than the low responders on D11. Despite starting with equal degrees of pain at D4, high responders showed a trend toward improved pain outcomes on D28 (Figure 12 B), though this trend did not achieve significance. However, individual opioid response at D4 was not significantly correlated with hyperalgesia levels at D28 ($r = 0.371$, $p = .75$; Figure 12 C).

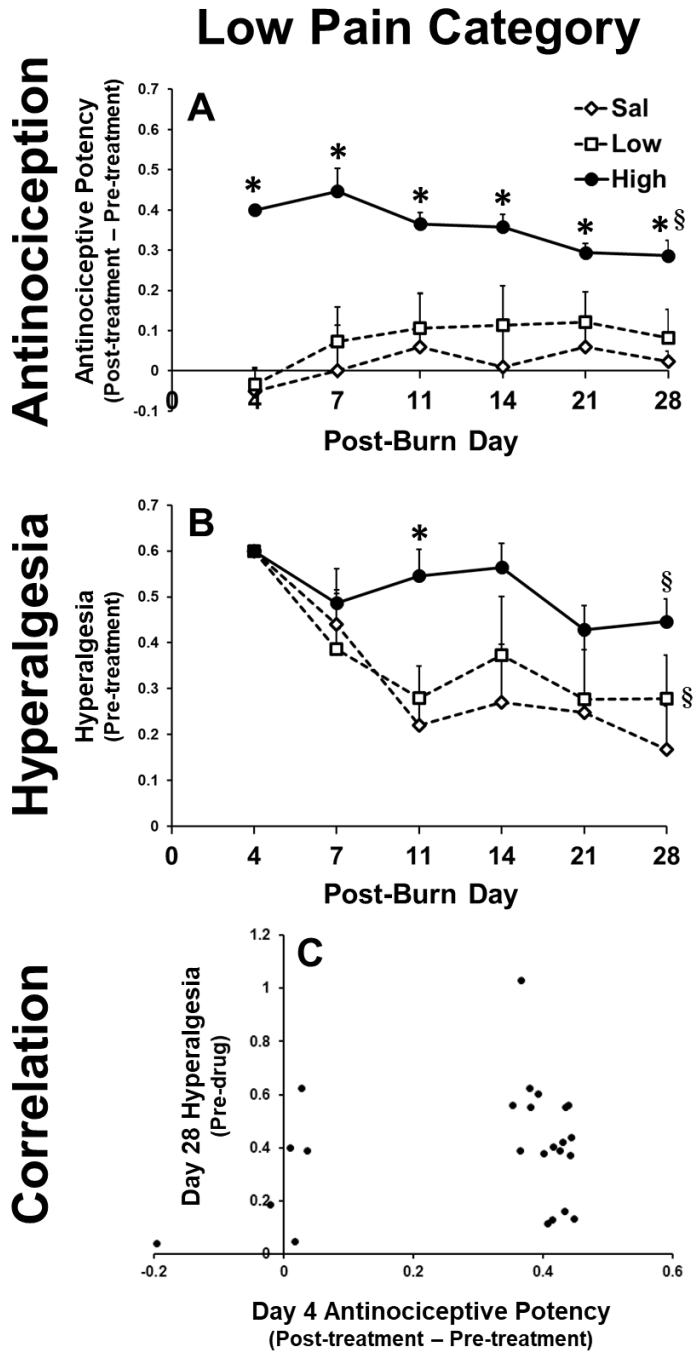

Figure 12. Antinociceptive potency and hyperalgesia development in animals suffering low degrees of burn pain. A. Antinociceptive potency of opioids across days. B. Mechanical sensitivity threshold across days. C. Correlation of D4 antinociceptive potency and D28 hyperalgesia threshold. Opioid potency and hyperalgesia development do not correlate in this subgroup, $r = 0.371$. * Significantly different from low responders ($p < .05$). § Significantly different from D4 within the same experimental group ($p < .05$). Results are presented as mean ± SEM.

Moderate Pain Category

For animals exhibiting a 'moderate' degree of pain at D4 (0.4 g pre-test threshold), animals were further sub-divided into 'low', 'medium', or 'high' responders based upon their individual responsiveness to opioids on D4. Only 1 opioid-injected animal in this pain subcategory was defined a low responder to opioids. Therefore, this animal is graphed with the saline-treated animals for simplicity.

Analysis using mixed model ANOVA indicated a significant main effect of antinociceptive response ($F(2, 39) = 61.50$, $p < .0001$; Figure 13 A). There was no significant main effect of day ($F(5, 195) = 0.79$, $p > .05$) A significant interaction was present between antinociceptive response and day ($F(10, 195) = 4.85$, $p < .0001$). Bonferroni post-hoc comparison revealed that the antinociceptive potencies in the high and medium responders were significantly higher than the low responder/saline group for the entire duration of the study (Figure 13 A).

In the moderate pain category, only the high responders demonstrated reduced antinociceptive response over the course of the study. Planned comparisons demonstrated no significant difference for the low responders between the antinociceptive responses on D4 and D28 ($t(10) = -1.45$, $p > .05$). For the medium responders, the antinociceptive response on D28 was significantly higher than the response on D4 ($t(20) = -2.68$, $p < .05$). In contrast, for the high responders, the antinociceptive response on D28 was significantly lower than the response on D4 ($t(9) = 3.33$, $p < .01$).

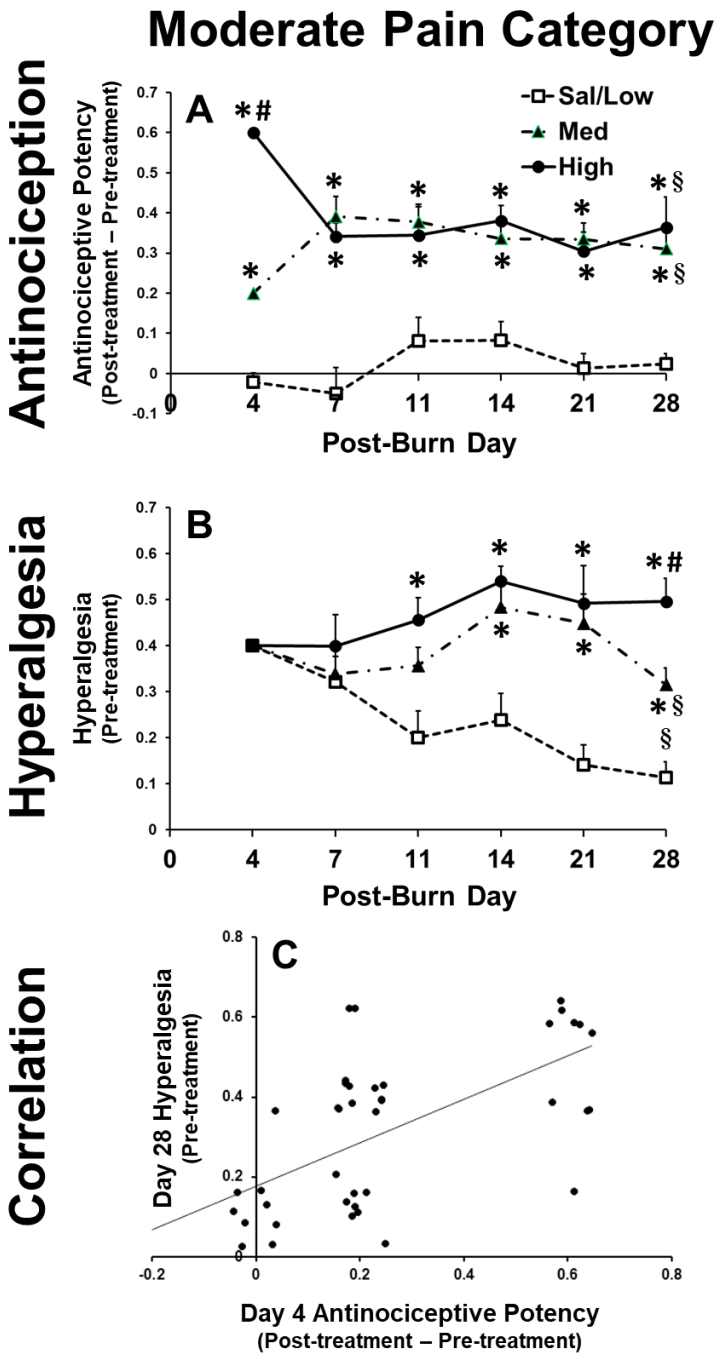

Figure 13. Antinociceptive potency and hyperalgesia development in animals suffering moderate degrees of burn pain. A. Antinociceptive potency of opioids across days. B. Mechanical sensitivity threshold across days. C. Correlation of D4 antinociceptive potency and D28 hyperalgesia threshold. Opioid potency and hyperalgesia development are correlated in this subgroup, $r = 0.671$. * Significantly different from low responders ($p < .05$). # Significantly different between high and medium responders ($p < .05$). § Significantly different from D4 within the same experimental group ($p < .05$). Results are presented as mean ± SEM.

Although all animals in this group experienced equal levels of pain on D4 (by definition), significant differences in hyperalgesia development were present, based upon individual opioid response on D4. Analysis using mixed model ANOVA indicated a significant main effect of hyperalgesia development ($F(2, 39) = 12.29$, $p < .0001$). There was a trend for a main effect of day ($F(4, 156) = 2.32$, $p = .059$), and a significant interaction between hyperalgesia development and day ($F(8, 156) = 2.14$, $p < .05$).

Bonferroni post-hoc comparison revealed that hyperalgesia was significantly less severe (i.e. pretest mechanical sensitivity thresholds were significantly higher) in the high responders than the saline-treated animals from D11 until the end of the experiment (Figure 13 B). Pretest thresholds were also significantly higher in the medium responders than the saline-treated animals from D14 until the end of the experiment. Moreover, pretest thresholds were significantly higher in the high responders than the medium responders on D28 ($p < .05$).

Furthermore, during the course of the experiment, pain levels increased significantly in the saline animals and moderate responders but not in the high responders (Figure 13 B). For the high responders, planned comparisons between the pretest thresholds on D4 and D28 demonstrated a trend for higher pretest thresholds on D28 than on D4, but it did not reach statistical significance ($t(9) = -2.01$, $p = .075$). In contrast, both the moderate responders and saline-treated animals had lower pretest thresholds (i.e. greater pain sensitivity) on D28 than on D4 (medium responders: $t(20) = -2.37$, $p < .05$; saline $t(10) = 8.73$, $p < .0001$).

Additionally, there was a significant positive correlation between D28 pretest pain thresholds and antinociceptive potency on D4 (Figure 13 C). Although all animals had comparable levels of hyperalgesia on D4, animals with higher treatment responses to opioids on D4 were more likely to experience less pain on D28.

Severe Pain Category

Mice categorized as experiencing severe pain levels on D4 (0.16-0.04 gram pretest thresholds) were further divided into three subcategories, 'high', 'medium', and 'low' responders based upon their individual antinociceptive response to opioids on D4. Perhaps due to the high level of pain resulting from the burn, a high number (n = 15) of the opioid-treated animals were categorized as low responders based on their individual responses.

Analysis of opioid antinociceptive response across time using a mixed model ANOVA indicated a significant main effect of antinociceptive response ($F(2, 35) = 22.22$, $p < .0001$; Figure 14 A). There was a trend toward a main effect of day ($F(5, 175) = 2.11$, $p = .066$), and a significant interaction between antinociceptive response and day was present ($F(10, 175) = 3.29$, $p < .001$).

Bonferroni post-hoc comparison revealed that the antinociceptive potencies in the high responders were significantly higher than the low responders for the entire duration of the study, except D14 (Figure 14 A). The antinociceptive potencies in the medium responders were significantly higher than the low responders for the duration of the study, with the exceptions of D7 and D28. On D4, high responders had a

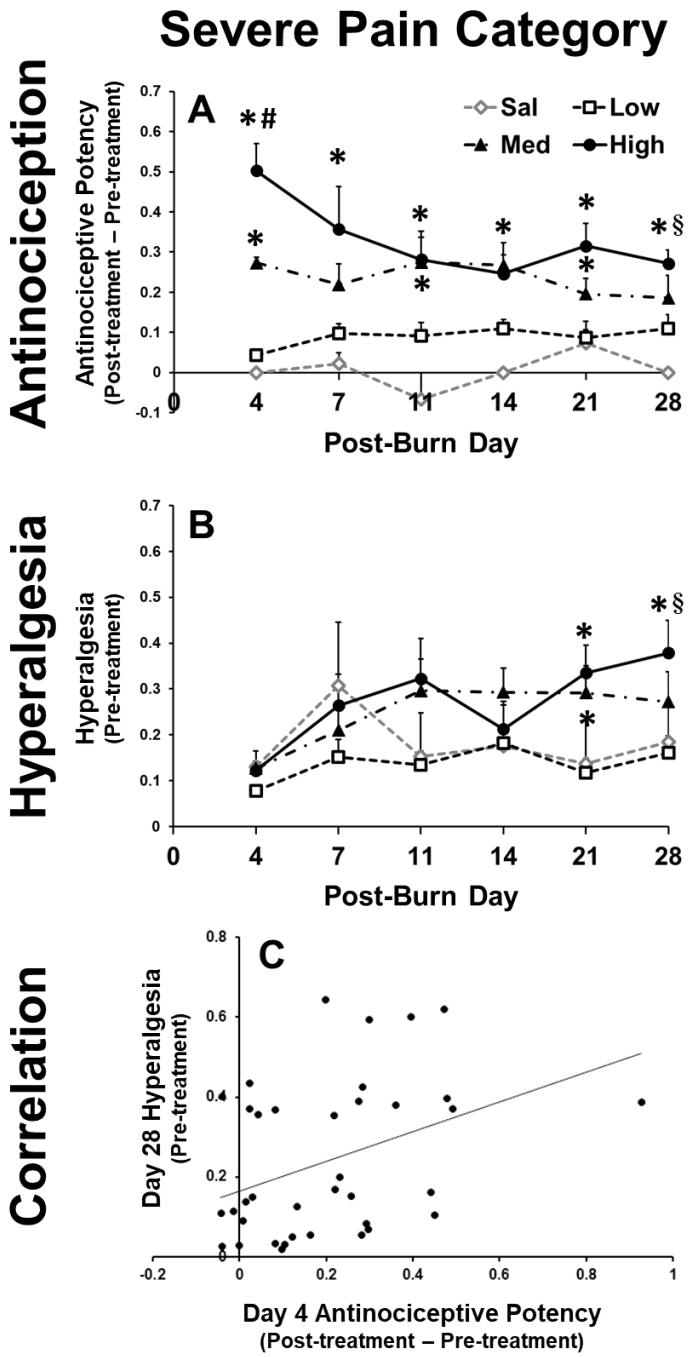

Figure 14. Antinociceptive potency and hyperalgesia development in animals suffering severe degrees of burn pain. A. Antinociceptive potency of opioids across days. B. Mechanical sensitivity threshold across days. C. Correlation of D4 antinociceptive potency and D28 hyperalgesia threshold. Opioid potency and hyperalgesia development are correlated in this subgroup, $r = 0.396$. * Significantly different from low responders ($p < .05$). # Significantly different between high and medium responders ($p < .05$). § Significantly different from D4 within the same experimental group ($p < .05$). Results are presented as mean ± SEM.

significantly higher response than medium responders. However, no significant differences in antinociceptive response were observed between high and medium responders from D7 until the end of the experiment. Additionally, only the high responders demonstrated reductions in antinociceptive response during the duration of the study. Planned comparisons demonstrated no significant difference between the antinociceptive responses on D4 and D28 in the low or medium responders (low responders: $t(18) = -1.99$, $p = .062$; medium responders: $t(10) = 1.51$, $p > .05$). However, in the high responders, the antinociceptive response on D28 was significantly lower than the response on D4 ($t(7) = 2.92$, $p < .05$).

Although all mice in this group experience equal (and severe) pain levels on D4, by definition, levels of hyperalgesia by D28 stayed high in the low opioid responders, while high responders experienced significantly less hyperalgesia (Figure 14 A). Mixed model ANOVA revealed a significant main effect of hyperalgesia development ($F(2, 35) = 5.77$, $p < .01$). There was no significant main effect of day ($F(4, 140) = 1.12$, $p > .05$, n.s.), and no significant interaction between hyperalgesia development and day ($F(8, 140) = 1.47$, $p > .05$).

Bonferroni post-hoc comparison revealed that pretest thresholds were significantly higher (i.e. less pain sensitivity) in the high responders than the low responders on D21 and D28 (Figure 14 B). Pretest thresholds were also significantly higher in the medium responders than the low responders on D21. Furthermore, during the course of the experiment, pain levels decreased significantly only in the high responders but not in the low and medium responders. For the high responders, planned

comparisons between the pretest thresholds on D4 and D28 demonstrated a significant increase in pretest thresholds on D28 from D4 ($t(7) = -3.26$, $p < .05$). A trend for increased pretest thresholds on D28 was seen for the medium and low responders, but it did not reach statistical significance in either subgroup (medium responders: $t(10) = -2.06$, $p = .067$; low responders $t(18) = -2.06$, $p = .054$).

Despite the severity of injury in this group, there was not a significant correlation between the hyperalgesia on D4 and D28. This is to be expected, due to the reduced range of variability in pain experience among animals in this group as compared to the other pain experience categories. However, there was a significant positive correlation between D28 pretest pain thresholds and antinociceptive potency on D4 (Figure 14 C). Animals with higher responses to opioid treatment on D4 were more likely to experience less pain on D28.

4. Discussion

Animals which experienced greater degrees of pain shortly after injury showed more severe hyperalgesia at D28. This is not surprising, given the mediating role that injury severity likely plays in both outcomes; animals with more severe injuries are likely to be in greater pain at D4 and also to develop greater levels of injury-induced hyperalgesia.

Interestingly, animals who demonstrated greater antinociceptive responses to opioids, i.e. animals for whom opioids were more potent, at D4 displayed reduced development of hyperalgesia at D28. This effect cannot be explained simply by the

observed relation of early pain and late pain, as the relationship was observed in animals who demonstrated equal degrees of pain at D4. That is, animals who displayed greater antinociceptive responses to opioids at D4 had significantly less hyperalgesia than animals which displayed equivalent levels of pain at D4 but less response to opioids.

This was true for animals experiencing moderate-to-severe levels of pain, but notably not for animals in low levels of pain. This may be due to the fact that animals in less pain at D4 had overall better outcomes at D28 regardless of opioid response, reducing variability in outcome and therefore reducing the observable impact of opioids in this group, or because animals in low levels of pain had less varied antinociceptive responses to opioids at D4 than animals in moderate-to-severe pain, leading to more consistent outcomes at D28.

In animals experiencing moderate levels of pain, arguably the most clinically relevant of the 3 conditions in this experiment, individual differences in opioid antinociceptive response between medium and high opioid responders present at D4 disappeared by D7 and remained absent for the remainder of the experiment. In the case of the high responders, opioid antinociceptive response significantly decreased from D4 to D7, indicating the emergence of either antinociceptive tolerance or OIH in these animals. On the contrary, opioid antinociceptive response significantly increased in the medium responders from D4 to D7. This could be the result of antinociceptive sensitization.

Regardless of the mechanism of the convergent antinociception levels between these two groups, they displayed significantly different levels of hyperalgesia

development by D28, such that the high responders to opioids on D4 exhibited significantly less hyperalgesia by D28 than medium responders. Because of the antinociceptive convergence in these groups from D7 onward, it can be assumed that whatever mechanism is driving the differences in hyperalgesia and relates early antinociceptive response with later hyperalgesia development is occurring early (within 72 hours) after injury.

It is interesting to note that antinociceptive tolerance and/or OIH developed among all three pain experience categories, but only in the high opioid responders in each category. This can be taken to mean that tolerance development is more likely when pain is completely, or at least more adequately, managed; and that the presence of residual, uncontrolled pain precludes the development of tolerance. This observation parallels earlier reports that pain reduces the rewarding properties of opioids, as measured by both conditioned place preference and self-administration (Lyness et al., 1989; Ozaki et al., 2002). Although reward and tolerance are very different properties, the two seem to share convergent characteristics. The development of tolerance to the rewarding properties of a drug of abuse is often used as one of the two defining criteria for the establishment of addiction, along with physiological withdrawal when the drug is withheld. The current results, interpreted in the light of the prior research regarding the effects of pain experience on reward, indicate that the physiological changes that underlie antinociceptive tolerance may overlap or interact with the physiological changes that accompany addiction. If this is the case, the prevention or minimization of

one will in turn prevent or minimize the other, offering potentially novel avenues for treatment in both the pain and addiction clinics.

Further, this result implies that individuals who, due to their physiology, are more sensitive to one effect of opioids (in this case, analgesic effectiveness of a particular dosage) may also be more sensitive to several other effects of opioids (tolerance development and withdrawal severity, reward, sensitivity of signaling systems, etc.). This finding makes sense, and in fact can be easily predicted, if these disparate effects are all driven by identical or convergent mechanisms, such as a particular opioid receptor (i.e. μ-receptor) or initiation of a particular intracellular signaling cascade (e.g. ERK activation).

However, in light of the findings of wide-ranging drug-specific differences among opioids in this dissertation as well as in the current scientific literature, it seems that these relationships may be far more complex and interdependent with other, so far unidentified factors that direct predictions (e.g. 'an individual who demonstrates high sensitivity to analgesic effects of morphine is at higher-than-average risk to develop addiction') seem premature at best. Indeed, the prediction offered as an example runs directly counter to the results of rodent behavioral studies which show that *decreased* sensitivity to the rewarding and analgesic effects of opioids is predictive of pathological drug-seeking behaviors in operant learning paradigms, not the other way around (Ahmed et al., 2002; Koob, 2013; Cahill et al., 2016). The disparity of these findings indicates that there are moderating factors at work that have not yet been elucidated.

Whatever the relationship between antinociceptive potency and reward, these results imply shared mechanisms between individual differences in opioid antinociceptive potency and the development of hyperalgesia. The mechanisms underlying the correlation between degree of early pain management and subsequent hyperalgesia development remain unknown. However, existing literature implicates several potential mechanisms which may mediate the reported correlation, individually or collectively.

One potential mechanism is the interaction between the immune system, opioids, and pain. This is an especially attractive explanation, as immunological factors are known to be profoundly altered in the wake of burn injury, and are also well established to influence the pharmacology of opioids (Zimmermann, 2001; Sehgal et al., 2011; Grace et al., 2015; Vallejo et al., 2004). For example, the immune receptor TLR4 is known to suppress the potency of opioids (Grace et al., 2015). TLR4 has also been established to be modulated following burn injury, and to play a role in the development of negative outcomes following burn injury (Cho et al., 2004; Maung et al., 2005; Krzyzaniak et al., 2011; Schwacha et al., 2012). Thus, stronger TLR4 signaling and/or higher TLR4 expression in certain individuals following injury could result in decreased opioid potency in these individuals.

Similarly, opioids are often considered immunosuppressive, and have the ability to suppress burn-induced inflammation which would otherwise lead to development of hyperalgesia (Stein and Zöllner, 2009; Sehgal et al., 2011; Rowan et al., 2015). It is easy to see how these two mechanisms might interact, whereby individuals with stronger

TLR4-mediated inflammatory responses might display reduced opioid potency, which in turn reduces the ability of opioids to suppress inflammatory response and prevent hyperalgesia development.

Burn injury has also been demonstrated to alter the expression of pain-related signaling molecules in the dorsal horn of the spinal cord (Wang et al., 2011a), which could in turn impact the function of opioids. The role of NMDA receptors, especially in the spinal cord, must not be overlooked. NMDA receptors are known to have an impact on opioid analgesic tolerance (Trujillo and Akil, 1991, 1994; Ahmadi et al., 2016) as well as the development of chronic pain (Wang et al., 2011a; Zhang et al., 2016; Chen et al., 2016). Pre-existing individual or burn-induced differences in NMDA receptor expression or function could mediate the observed correlation between lower antinociceptive potency of opioids and greater degrees of hyperalgesia.

Another potential explanation includes alterations in Akt/mTOR, p38-MAPK, and JNK signaling which alter both antinociceptive response to opioids and pain hypersensitivity (Alexander et al., 2004; Maung et al., 2005; Zhang et al., 2008; Wang et al., 2011a; Zhang et al., 2013; Sanna et al., 2014; Xu et al., 2014; Marcus et al., 2015) and may themselves be differentially altered by opioids (Emery et al., 2016).

As noted before, these mechanisms are not mutually exclusive. Any or all of them may be contributing to the observations reported here. It is easy to imagine a situation where none of these mechanisms acting alone contributes enough to be independently sufficient to drive the correlation between opioid antinociceptive potency and the development of hyperalgesia, but each factor acting in concert with the others

exerts a multiplicative effect which results in behavioral changes. The contributions of these various potential molecular mechanisms on the behavioral findings reported here should be explored further in future studies.

CHAPTER VIII

EXPERIMENT 6: EFFECTS ON D2 RECEPTOR SENSITIVITY IN A RECREATIONAL USE MODEL*

1. Background

In addition to their legitimate medical uses, prescription opioid painkillers are widely used recreationally, especially among adolescents (SAMSHA, 2011). Opioid painkillers are the second most common 'hard' drug (that is, excluding marijuana and its synthetic substitutes) abused by high school seniors, behind amphetamines (often also in the form of misused prescription medications for ADHD) (Johnston et al., 2014). This is due in part to their widespread availability – as noted before, hydrocodone combinations constitute the most prescribed single medication in the United States (Von Korff et al., 2008).

Opioid misuse has been shown to be associated with the development of multiple psychiatric disorders (Busto et al., 1998; Sullivan et al., 2005; Dowling et al., 2006; Becker et al., 2008), especially mood disorders (Martins et al., 2009). The dopamine D2 receptor (D2DR) has been demonstrated to play a crucial role in many of these same disorders (Glantz et al., 2010; Zou et al., 2012; de Kwaasteniet et al., 2014) and has also been shown to be associated with opioid addiction risk (Clarke et al., 2014), implying a

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potential mechanism whereby opioids interact with the D2DR system to precipitate these disorders.

Additionally, the D2DR, especially in the striatum, has been suggested to play a key role in the negative affective state caused by opioid withdrawal (Funada and Shippenberg, 1996; Georges et al., 1999; Smith et al., 2002; Enoksson et al., 2012). Animals undergoing morphine withdrawal demonstrate hyper-sensitivity of the D2/D3 receptors when challenged with the agonist quinpirole (Lee et al., 1987; Reddy et al., 1993; Piepponen et al., 1996; Druhan et al., 2000).

Our prior work has demonstrated that opioid-mediated hyper-sensitivity of D2DR is both age-dependent, present in adults but highly amplified in adolescents (Hofford et al., 2012), and drug-specific, observed following repeated exposure to some opioids (e.g. morphine, methadone) but not others (e.g. buprenorphine) (Barwatt et al., 2013). However, the drug-specific differences observed in (Barwatt et al., 2013) were between methadone and buprenorphine, which are not widely abused as compared to oxycodone and hydrocodone.

Given the observation of drug-specific alterations of the D2DR system by opioids, and the potential psychological consequences which alteration of the D2DR system may carry, it is important to explore drug-specific influences on D2DR sensitivity by clinically relevant (and therefore widely abused) opioids.

2. Experimental Procedure

Subjects

Adolescent (PND 28 at beginning of experiment) male mice were examined for this experiment. Eight to 19 mice per group, for a total of 129 mice, were used for this experiment.

Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were injected with oxycodone, hydrocodone, or morphine at 10, 20, or 80 mg/kg, once daily for 6 days, via gavage.

Locomotor Testing

On the morning of day 7, mice were taken to the locomotor behavior core room, and allowed to habituate for 30 minutes. Then, they were placed in 8 automated optical beam activity monitors (Model RXYZCM-16; Accuscan Instruments, Columbus, OH, USA). Baseline locomotor behavior was recorded for 30 minutes. The mice were then injected with quinpirole HCl (10mg/kg, 10 ml/kg, i.p.) or vehicle (10 ml/kg) and locomotor activity was recorded for a further 120 minutes post-injection. The apparatus was cleaned thoroughly with ethanol followed by water and completely dried between each run.

Statistics

For each animal, the total distance traveled in the 120 minutes post-quinpirole was normalized to the total distance traveled in the 30-minute baseline, expressed as a percentage. Total distance traveled (normalized) for the entire 120 minutes post-quinpirole was analyzed using a 3 (drug) x 2 (quinpirole/vehicle) between-subjects ANOVA. Additionally, total distance traveled (in cm) was binned in 5-minute increments for each animal. These 5-minute bins were then averaged across animals for each group. Data were normalized using the 5-minute bin immediately prior to quinpirole injection, and expressed as a percentage (i.e. the final 5 minutes of baseline activity was considered 100% of the typical amount of locomotor activity). These data were analyzed using a 2 (quinpirole/vehicle) x 3 (drug) x 24 (time bin) mixed-factor ANOVA. Differences between treatment groups were calculated using Bonferroni post-hoc comparisons. Differences with p-values less than .05 were deemed statistically significant.

3. Results

Significant differences were observed in the response to quinpirole in animals pretreated with the various opioids. Two-way ANOVA revealed a main effect of dose ($F(2, 106) = 4.86$, $p < .01$). Bonferroni post-hoc comparison revealed a significantly larger total distance traveled during the 2 hours post-quinpirole administration in the mice pretreated with 20 mg/kg oxycodone as compared with the mice pretreated with 20 mg/kg morphine ($p < .05$; Figure 15 A). Total distance traveled for mice pretreated with

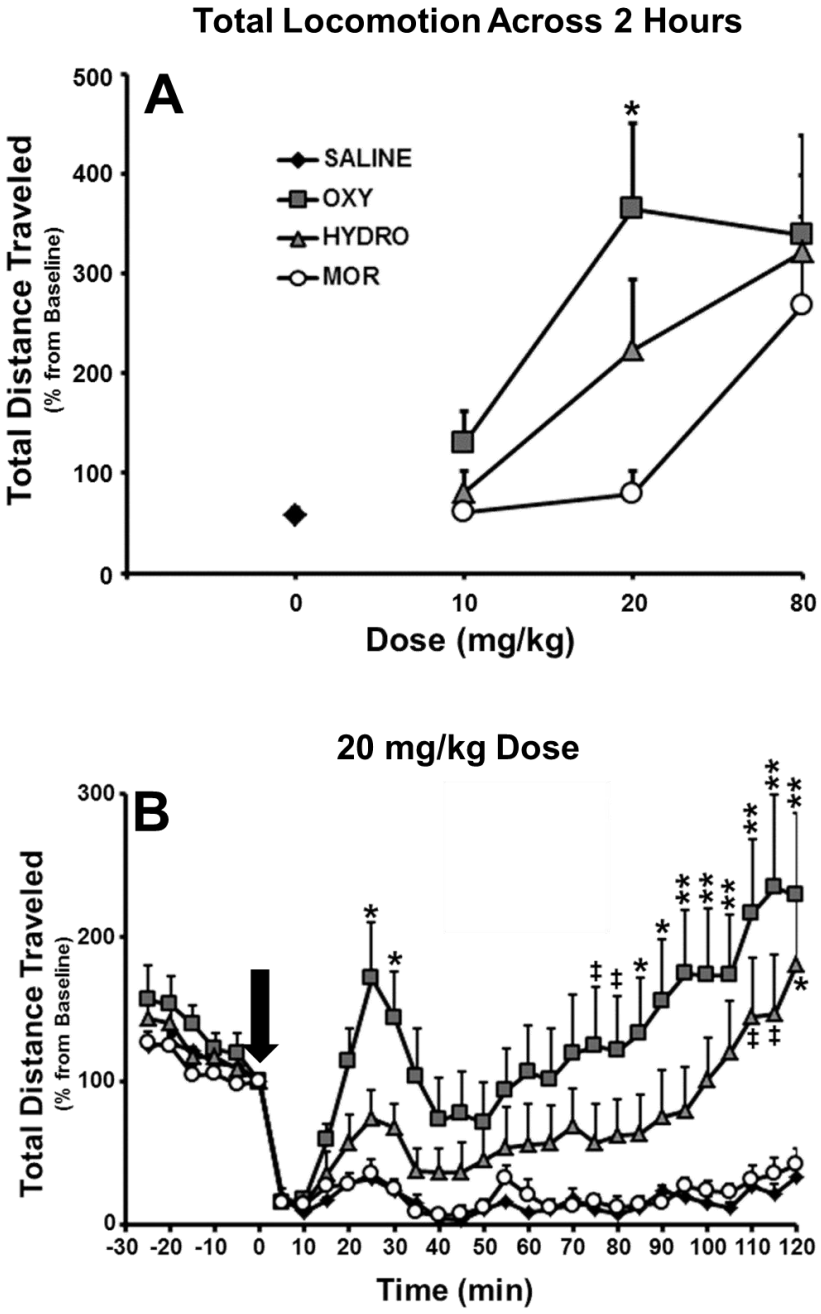

Figure 15. Differential effects of opioids on the hyperlocomotor response to D2/D3 agonist quinpirole. A. Effect on total locomotor activity, by drug and dose. Opioids increase locomotor activity in a dose-dependent manner. There were no locomotor differences between opioids at either 10 mg/kg dose or 80 mg/kg dose. Drug-specific differences were observed at 20 mg/kg dose. B. Temporal analysis of the effects of the 20 mg/kg dose of opioids on locomotor response to quinpirole. Quinpirole was administered at time 0 (indicated by arrow). * Significantly different ($p < .05$) from saline and morphine. ** Significantly different ($p < .001$) from saline and morphine. ‡ Significant difference ($p < .05$) from saline only. Data presented as mean ± SEM.

hydrocodone fell in between oxycodone and morphine and did not significantly differ from either one.

Temporal analyses were also computed for the mice pretreated with saline or pretreated with 20 mg/kg oxycodone, hydrocodone, or morphine using between-group factors of treatment and a within-group factor of time (1-120 minutes post-injection period summed in 5-minute intervals). For this analysis, for each mouse the score of the last 5-minute interval prior to the vehicle or quinpirole injections (i.e. baseline) was used to normalize the data. The results are presented in Figure 15 B. Two-way repeated ANOVA revealed a main effect of time ($F(1, 53) = 14.30$, $p < .0001$), a main effect of pretreatment ($F(3, 53) = 5.22$, $p < .01$) and a significant interaction between time and pretreatment ($F(3, 53) = 4.31$, $p < .01$). Bonferroni post-hoc comparison revealed significant differences in the responses to quinpirole between oxycodone-, hydrocodone- and morphine-pretreated animals. No significant differences were observed between morphine-pretreated and drug-naïve animals.

4. Discussion

In animals treated with either saline or morphine, quinpirole suppressed locomotor activity for the entire 120 min post-quinpirole injection. In contrast, in the oxycodone and hydrocodone-pretreated animals, quinpirole suppressed locomotor activity only during the first 10 min post-injection, followed by a significantly enhanced locomotion response as compared to both drug-naïve and morphine-pretreated animals.

This enhanced locomotor response to quinpirole was significantly larger in the oxycodone-pretreated animals.

The locomotor suppressing effects of quinpirole are well documented in the mouse (Halberda et al., 1997), and differ from the classic biphasic response observed in rats. It is theorized that the locomotor suppressing effects of quinpirole are due largely to action at the presynaptic, D2S isoform of the D2DR, which functions as an autoreceptor. Therefore, when quinpirole binds and activates this presynaptic autoreceptor, dopamine release is suppressed and subsequently so is dopamine-mediated locomotor behavior, in much the same way as dopaminergic antagonists exhibit neuroleptic activity, but via a slightly different mechanism.

Conversely, the locomotor activating effects of quinpirole, which may appear at first glance to be paradoxical, are thought to derive from the action of quinpirole at the post-synaptic D2L isoform, mimicking synaptic dopamine release. Thus, the behavioral switch in the actions of quinpirole, from inducing severe hypo-locomotion in opioid-naïve mice to a hyper-locomotor activation in mice pre-exposed to opioids, implies that exposure to opioids in the 6 days prior to quinpirole testing results in a disturbance of D2DR signaling homeostasis.

This could result from alterations in the protein levels of D2S and D2L receptors, differences in their functional expression levels on the cell surface, alterations in the balance and signature of the milieu of second messenger cascades resulting from activation of these receptors, or, most likely, a combination of all of these mechanisms. Preliminary results from qPCR experiments have indicated that mRNA levels of these

receptor isoforms, at least, appear not to vary following exposure to opioids (unpublished results). However, this does not rule out the possibility of altered rates of translation into functional protein. Experiments presented in the next chapter will explore the possibility of alterations in second messenger responses. Future experiments should examine alterations in protein levels, as well as functional expression of signaling-competent receptors at the cell surface.

CHAPTER IX

EXPERIMENT 7: EFFECTS ON OPIOID-RECEPTOR-MEDIATED AND D2-RECEPTOR-MEDIATED SECOND MESSENGER SYSTEMS*

1. Background

The previous experiments explored drug-specific differences of opioids on behavior, ranging from behavioral antinociception and allodynia to effects on dopaminergic system behavioral sensitivity. However, the question remains as to the mechanism mediating these differences. Drug-specific differences between buprenorphine and methadone on D2DR behavioral sensitivity that have been observed previously in our lab (Barwatt et al., 2013) may be potentially explained as a function of pharmacological differences. Buprenorphine is a partial opioid agonist, while methadone is a full agonist with additional action as an NMDA receptor antagonist. Thus, the differences observed between these drugs may be due simply to these differences in mechanism of action.

However, hydrocodone, oxycodone and morphine are far more pharmacologically similar. These three compounds have incredibly similar chemical structures, and the putative active moiety which binds and activates the μ-receptor is identical for all three. Further, while hydrocodone and oxycodone are more selective for

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the μ-receptor than morphine, they have comparable ratios of affinity to each other for the three opioid receptors. Therefore, drug-specific differences between oxycodone and hydrocodone cannot be explained as due to differential activation patterns of the opioid receptors. Thus, an alternative mechanism, distinct from traditional pharmacodynamic considerations, likely underlies any differences observed between these drugs.

As has already been discussed (Experiments 2 & 3), the opioid receptors, including the μ-receptor, have been demonstrated to exhibit functional selectivity, displaying different intracellular responses to agonism depending on the specific ligand bound, and that this ligand-directed signaling may have profound impacts on the pharmacology of different compounds acting on the same target (Urban et al., 2007; Pradhan et al., 2012). It stands to reason that these drug-specific differences in second messenger cascade activation may be one molecular mechanism which ultimately mediates behavioral differences between otherwise similar opioid drugs.

Multiple second messenger cascades are engaged by the opioid receptors, and are therefore candidates for functionally selective effects. Indeed, many of these pathways have already been demonstrated to exhibit ligand-biased effects in cell culture and other, less directly translatable models (Pradhan et al., 2012). The signaling molecules ERK1/2, members of the MAPK family, have been previously demonstrated to exhibit brain region-specific and event-specific alterations in expression and phosphorylation levels following morphine exposure, development of antinociceptive tolerance, and behavioral sensitization (Eitan et al., 2003). In addition, ERK1/2 have been implicated in the development of antinociceptive tolerance to opioids and hyperalgesia (Wang et al.,

2011b; Merighi et al., 2013; Zhang et al., 2013; Sanna et al., 2015b), and have been shown to be subject to ligand-specific alterations in activity (Kramer and Simon, 2000; Ahn et al., 2004; Gesty-Palmer et al., 2006; Ligeza et al., 2008; Zheng et al., 2008).

The signaling molecule Akt, also known as Protein Kinase B (PKB), is also established to be an important second messenger for the opioid receptors, mediating responses to pain and the analgesic effects of opioids (Polakiewicz et al., 1998; Neary et al., 2005; Sugita et al., 2005; Shahabi et al., 2006; Cunha et al., 2010; Sanchez-Blazquez et al., 2010; Olianas et al., 2011; Merighi et al., 2013). In addition, Akt and several of its downstream targets, such as mTORC1, have been implicated in the development of antinociceptive tolerance development and the emergence of hyperalgesia, and is altered by burn injury (Sugita et al., 2005; Zhang et al., 2008; Xu et al., 2014).

In addition to their association with the opioid receptors, both Akt and ERK1/2 are also well established as effector molecules of the D2DR (Welsh et al., 1998; Beaulieu et al., 2005; Quan et al., 2008; Beaulieu et al., 2011; Huang et al., 2013). It has been demonstrated that different receptor species, when expressed on the same cell, can exhibit cross-talk between shared second messenger systems, where ligand-directed alterations in activation levels of an effector molecule by one receptor can have an impact on the signaling via that effector molecule by the second type of receptor (Tan et al., 2009). Therefore, drug-specific alterations of either ERK or Akt via the opioid receptors in the striatum may influence the signaling capability, and therefore the behavioral responsiveness, of the D2DR system as well.

Even in the absence of direct cross-talk between opioid and D2 receptors via second messenger pools, opioid treatment is known to alter dopamine release in the striatum (Pothos et al., 1991; De Vries and Shippenberg, 2002). Therefore, repeated activation of striatal D2 receptors following opioids could result in alterations of D2 signaling. Either of these potential actions provide a molecular mechanism linking opioid misuse with perturbances in the D2DR system.

Thus, these molecules were attractive targets to look for ligand-directed effects of opioids which may underlie the behaviors that have been previously discussed. These molecules potentially provide a molecular mechanism for differences in antinociception and allodynia development if they are differentially altered by opioids, and they are especially likely to mediate the impact of opioids, directly or indirectly, on the D2DR system.

In this experiment, I examined the hypothesis that opioids differentially alter the expression and/or phosphorylation levels of Akt and/or ERK1/2. Due to the likelihood that exposure to different opioids and their effects on these signaling molecules may 'set the stage' for differential responses to D2 receptor agonism, I also examined the effect of D2 agonism on the expression and/or phosphorylation levels of these molecules following exposure to different opioids.

2. Experimental Procedure

Subjects

Adolescent (PND 28 at beginning of experiment) males were examined for this experiment. Ten to fifteen mice per group were used for this experiment, for a total of 81 mice.

Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were injected with saline, oxycodone, hydrocodone, or morphine once daily for 6 days at 20 mg/kg, the dose found in Experiment 4 to have the greatest impact on D2DR behavioral response.

Tissue Collection

Twenty-four hours following the final opioid/saline administration, mice were injected with quinpirole hydrochloride (10 mg/kg, 10 ml/kg, i.p.) or vehicle (100 µM hydrochloric acid, 10 ml/kg). Thirty minutes following quinpirole/vehicle injection, a timepoint corresponding with the first peak of greatest behavioral response to quinpirole, mice were deeply anesthetized with sodium pentobarbital (120mg/kg, 10 ml/kg, i.p.), their brains were extracted and flash-frozen in a bath of 2-methylbutane (Fisher O3551-4) chilled on dry ice. Dorsal striatum tissue was then bilaterally dissected out of the flash-frozen brains on ice, and stored at -80 °C until processed for Western blot.

Western Blotting

Striatum tissue was allowed to thaw on ice, and then was homogenized in boiling 1% SDS lysis buffer plus 2 µM okadaic acid to inhibit phosphatase activity (~150 µL per sample). Homogenized samples were then boiled for an additional 10 minutes. Homogenized samples were stored on ice from this point forward. Total protein concentration was determined for each sample using DC Protein Assay, a proprietary Lowry assay modified for compatibility with detergents, according to the manufacturer's protocol (Bio-Rad) and using bovine serum albumin (BSA; Bio-Rad Protein Standard II, #500-0007) at known concentrations of 0.18, 0.36, 0.72 and 1.44 mg/mL to generate a standard curve for quantification purposes. Please note that Bio-Rad Protein Standard II concentrations vary by lot, and the numbers reported for the concentration standard curve are specific to the lot used.

Protein samples were analyzed at 50x dilution in PBS. Based on the concentrations calculated by protein quantification, samples were diluted to 200 µg total protein in 3x Laemmli loading buffer with DTT added as a reducing agent to disrupt disulfide bonds and boiled for an additional 10 min. Samples were loaded and resolved on 10% SDS-PAGE ran at constant 150V until the dye front had reached within ~1.5 cm of the bottom of the gel (45 minutes – 1.5 hours), then transferred to PVDF membrane using a wet transfer protocol at constant 200mA for 2 hours on ice.

Blots were blocked using a 5% solution of non-fat powdered milk in PBS and were probed using antibodies to pERK (p-P44/42 MAPK, T202/Y204, 1:1,000, Cell Signaling Technology), total ERK (P44/42 MAPK, 1:1,000, Cell Signaling Technology),

pAkt (S473, 1:1,000, Cell Signaling Technology), and total Akt (1:1,000, Cell Signaling Technology). Membranes were incubated with HRP-conjugated goat-anti-rabbit secondary antibody (1:5,000, Bio-Rad), developed with homemade ECL reagent, and imaged using AlphaView software (Cell Biosciences). Analysis of band signal strength was made via the software. Phospho-protein signals were normalized to total protein signals on the same membrane. Specific details of all reagents are provided in Appendix II.

Statistics

For each animal, the ratio of phospho-protein:total protein was calculated for Akt, ERK 1, and ERK 2, and expressed as a percentage. For each experimental group, this percent change was averaged across individuals for each protein. Data was analyzed using a 4 (drug) x 2 (quinpirole vs vehicle) design, two-way ANOVA. Post hoc contrasts between each treatment group were computed using Bonferroni post hoc procedure. Differences with p-values of less than .05 were deemed statistically significant.

3. Results

Akt

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F(3, 71) = 16.73$, $p < .0001$), a main effect of treatment with quinpirole ($F(1, 71)=24.20$, $p < .0001$), and a significant interaction between pretreatment and treatment ($F(3, 71) = 4.10$, $p < .01$). Bonferroni post-hoc comparison revealed that morphine

pretreatment did not alter Akt activation levels (i.e. phospho-Akt/total Akt) in the dorsal striatum as compared to levels observed in drug-naïve animals ($p > .05$). In contrast, pretreatment with both oxycodone and hydrocodone significantly decreased Akt activation levels in the dorsal striatum ($p < .001$).

Additionally, differential responses to quinpirole were also observed in mice pretreated with various opioids. Quinpirole administration did not significantly alter Akt activation levels in drug naïve animals ($p > .05$). In contrast, quinpirole administration reduced Akt activation levels in morphine pretreated animals ($p < .001$). However, no further reduction in Akt activation levels (from baseline) was observed in mice pretreated with hydrocodone or oxycodone in response to quinpirole ($p > .05$). These results are presented in Figure 16.

ERK 1 (P44 MAPK)

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F(3, 73) = 3.02$, $p < .05$). However, there was no main effect of treatment with quinpirole ($F(1, 73) = 1.24$, $p > .05$), and no significant interaction between pretreatment and treatment ($F(3, 73) = 0.89$, $p > .05$). Bonferroni post-hoc comparison revealed that oxycodone and morphine pretreatment resulted in significantly different levels of ERK 1 activation from one another, driving the observed main effect of pretreatment. Despite this, opioid pretreatment did not significantly alter ERK 1 activation levels (i.e. phospho-ERK 1/total ERK 1) in the dorsal striatum for either drug as compared to levels observed in drug-naïve animals ($p > .05$). Additionally, quinpirole administration did not

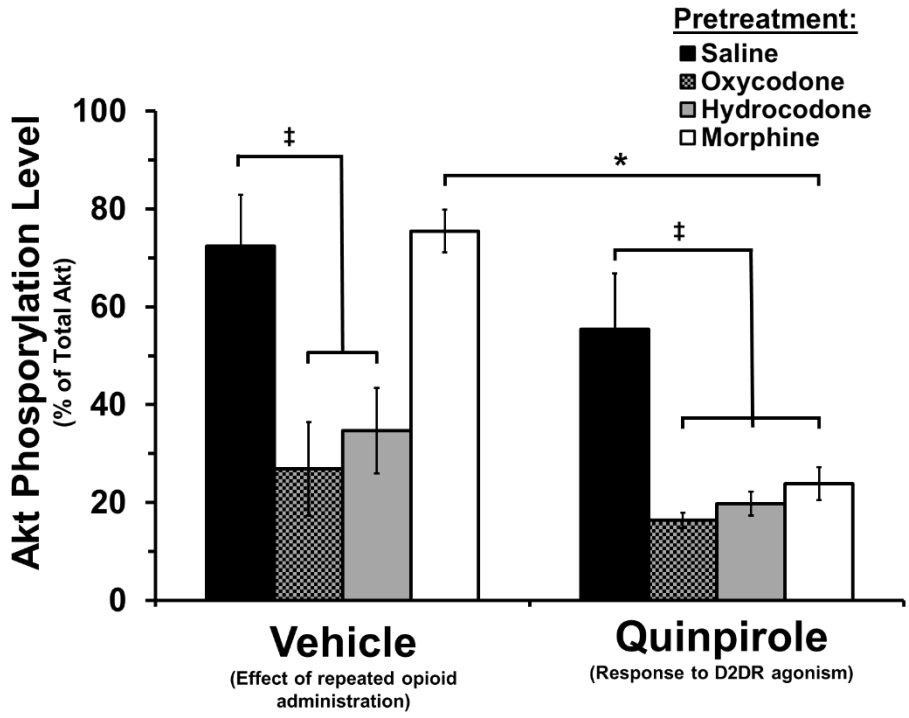

Figure 16. Differential effects of opioids alone and with D2DR agonism on Akt activation in Dorsal Striatum. * Significantly different ($p < .05$) from same pre-treatment vehicle controls. ‡ Significantly different ($p < .05$) from saline pre-treated animals. Data graphed as a percentage of total Akt protein levels and presented as mean ± SEM.

significantly alter ERK 1 activation levels in either the drug-naïve or in the opioid-pretreated animals ($p > .05$). These results are graphed in Figure 17.

ERK 2 (P42 MAPK)

Two-way ANOVA revealed a main effect of pretreatment with the various opioids ($F(3, 73) = 10.86$, $p < .0001$), a main effect of treatment with quinpirole ($F(1, 73) = 27.04$, $p < .0001$), but no significant interaction between pretreatment and treatment ($F(3, 73) = 1.21$, $p > .05$). Bonferroni post-hoc comparison revealed that

opioid pretreatment did not alter ERK 2 activation levels (i.e. phospho-ERK 2/total ERK 2) in the dorsal striatum as compared to levels observed in drug-naïve animals ($p > .05$). Similar to the pattern observed with ERK 1, a trend toward increased ERK 2 activation levels was observed in morphine-pretreated animals, and a trend toward decreased ERK 2 activation levels was observed following oxycodone pretreatment, but neither reached statistical significance.

Quinpirole administration did not significantly alter ERK 2 activation levels in drug naïve animals ($p > .05$). In contrast, increased ERK 2 activation in response to quinpirole was observed in mice pretreated with all of the opioids as compared to their

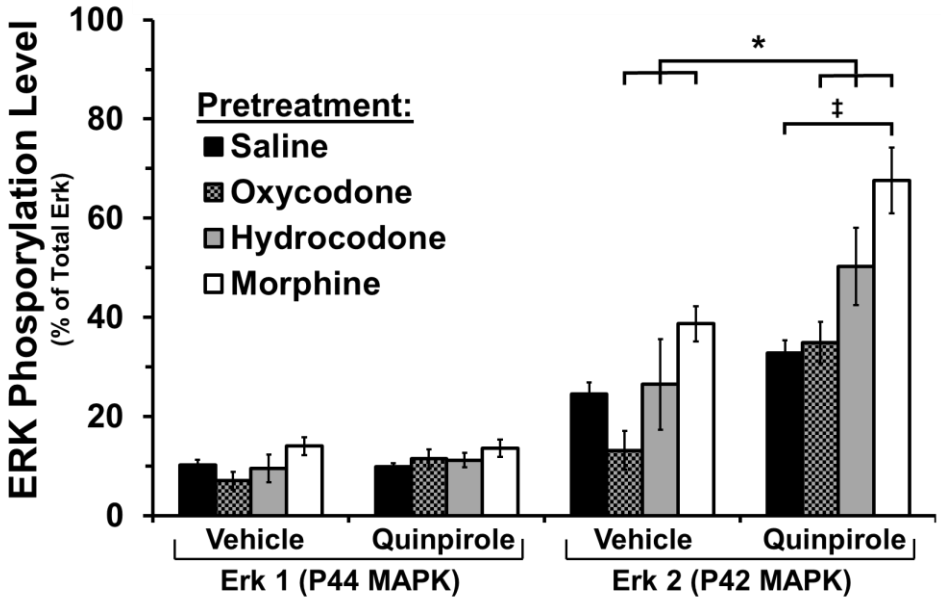

Figure 17. Differential effects of opioids alone and with D2DR agonism on ERK 1/2 activation in Dorsal Striatum. * Significantly different ($p < .05$) from same pre-treatment vehicle controls. ‡ Significantly different ($p < .001$) from saline pre-treated animals. Data graphed as a percentage of total ERK protein levels and presented as mean ± SEM.

own baseline levels ($p < .05$). However, ERK 2 activation level was significantly higher than drug naïve animals following quinpirole only in the morphine-pretreated mice ($p < .001$). These results are graphed in Figure 17.

4. Discussion

Prior exposure to opioids had a significant, and drug-specific, impact on the phosphorylation (activation) state of second messenger molecules in the striatum. In addition, an interaction was observed between opioid exposure and D2DR receptor activation, implying that pre-treatment with opioids can predispose the cellular response to D2DR agonism toward a particular signaling outcome, even when exhibiting no effect in and of itself.

Neither exposure to opioids, nor subsequent activation of the D2DR, had a measurable impact on the phosphorylation state of ERK 1, with this molecule exhibiting uniformly low levels of phosphorylation in all conditions. In the case of the ERK molecules, phosphorylation exerts an activating effect, indicating that ERK 1 has relatively low levels of biological activity in this experiment, and that activity state is not altered significantly by any of the manipulations performed in this experiment.

For the signaling molecule ERK 2, phosphorylation of ERK 2 was significantly different between animals exposed to oxycodone and morphine, indicating a potential drug-specific effect on this molecule. However, neither drug resulted in significant changes in ERK 2 phosphorylation relative to saline control levels, indicating that the effects these drugs have are modest, at least at this dose and treatment paradigm.

Activation of the D2DR signaling pathway via quinpirole resulted in significant increases in ERK 2 phosphorylation, and therefore activity, relative to ERK 2 phosphorylation levels in the absence of D2DR agonism, for all drugs. However, quinpirole also caused a slight (non-significant) increase in ERK 2 phosphorylation in saline-pretreated animals. As a result, only pretreatment with morphine caused a significant increase in ERK 2 activation relative to saline-treated animals exposed to quinpirole.

These results indicate that increased activity through the D2DR signaling pathway results in increased activation of striatal ERK 2 when the animal has a history of opioid exposure, and that the magnitude of this effect differs in a drug-specific manner. ERK 2 is well established to have multiple functions in the cell. As a kinase, active ERK 2 phosphorylates a wide range of downstream targets, activating or inactivating them relative to baseline levels. The pathways targeted by ERK 2 have diverse endpoints, and therefore this widespread alteration in the activity of multiple pathways has the potential to alter multiple outcomes for the cell. In addition to this, ERK 2 also has the potential to have large impacts on gene expression. Multiple targets of ERK 2 are transcription factor pathways, such that alterations in pathway activity by ERK 2 phosphorylation may have impacts on levels of genes whose expression is under the control of these transcription factors. In addition, activated ERK 2 can function as a transcription factor itself if it translocates to the nucleus of the cell, which is a known consequence of exposure to some opioids (e.g. etorphine).

Given the wide range of cellular functions which ERK 2 modulates, this implies that drug-specific alterations in ERK 2 activity may have profound cellular, circuit-level, and behavioral consequences, which have the potential to be persistent even after cessation of opioid use. In addition, the increased ERK 2 activity in the presence of D2DR activation implies the potential of magnified cellular and behavioral responses to dopamine following opioid exposure, which might make the animal more sensitive to dopamine signals of reward or motivation, or might heighten learning about reward-associated cues, exacerbating the addiction state.

The phosphorylation state of the signaling molecule Akt, also known as protein kinase B (PKB), was also significantly altered following exposure to opioids, in a drug-specific manner. In the case of Akt, phosphorylation inactivates the molecule, while de-phosphorylated Akt is functionally active as a kinase. Much like ERK, Akt has a very wide range of target pathways, including but not limited to cell cycle regulation, inhibition of pro-apoptotic pathways, and gene expression via control of several diverse transcription factor pathways.

Treatment with hydrocodone or oxycodone resulted in significant decreases in Akt phosphorylation, even in the absence of D2DR activation. Morphine, however, did not significantly alter Akt phosphorylation. Activation of the D2DR caused no further reductions in Akt phosphorylation in hydrocodone- or oxycodone-pretreated animals. However, D2DR activation caused a significant reduction of Akt phosphorylation in morphine-pretreated mice.

The lack of further decreases in Akt phosphorylation in hydrocodone- or oxycodone-pretreated animals could indicate that the effect of these opioids on Akt activation is independent of D2DR pathways, and mediated via a different mechanism, perhaps through the opioid receptors, or by interactions with other receptors expressed in the striatum (such as NMDA-type glutamatergic receptors). Alternatively, it could be the case that the effect *is* mediated through the D2DR, and that the magnitude of the alteration in the presence of opioids alone is so great that increased D2DR activity is incapable of further reductions (i.e. a floor effect).

The lack of effect of morphine pretreatment alone on Akt phosphorylation, but strong effects of pretreatment on Akt phosphorylation in the context of D2DR activation, imply that morphine is not exerting a direct effect on Akt phosphorylation but is 'setting the stage', or altering conditions within the cell, such that the D2DR signaling pathway is 'primed' for an altered effect. This is of particular note for 2 reasons. First, it implies that drug-specific alterations in cellular responses to paracrine signals may go undetected in the absence of the paracrine signal itself, laying bare the possibility of wide-ranging intracellular consequences of drug exposure that may have gone unseen by prior research. Second, it indicates that drugs of abuse can, at least in some cases, result in a 'response tuning' of reward systems even in the absence of omnipresent dysregulations of cell homeostasis. This could potentially result in a 'signal gain' in dopamine-responsive cells and circuits that is present only when dopamine circuits are active, causing heightened learning, memory, and motivation *specifically* for reward cues. This

could help explain observed peculiarities about the addicted brain, and abnormal learning and motivated behavior in an addicted state.

In any case, the observed drug-specific alterations in intracellular signal pathway activity following exposure to these common opioid analgesic drugs carries important implications. Not least of which, it implies that exposure to different specific opioids, even ones as remarkably similar as these three, can result in significantly different consequences at both the cellular and behavioral level, which have the potential to fundamentally influence the overall outcomes following exposure to these drugs, in perhaps any function that is influenced by any of the altered intracellular pathways.

CHAPTER X

EXPERIMENT 8: BURN INJURY ALTERS OPIOID-SPECIFIC EFFECTS ON D2 RECEPTOR BEHAVIORAL SENSITIVITY

1. Background

As detailed in the previous chapters, alterations in D2DR receptor sensitivity were observed, and differed in a drug-dependent manner based upon which specific opioid the animal had been pre-exposed to. However, this effect may be altered by the use of opioids to treat a burn injury. There is evidence to suggest that the experience of pain reduces the addictive potential of opioids (Ozaki et al., 2002; Niikura et al., 2008), such that the medical use of opioids to treat pain may not carry as much inherent addiction risk as the recreational use of opioids. In light of this, it is also possible that the experience of burn pain may alter the impact opioids have on the D2DR system, which would in turn inform the relative risks for later neuropsychiatric problems associated with medical treatment with opioids.

If the medical use of opioids to treat pain reduces the impact they exert on the D2DR system, this may provide further evidence against the current policy of minimizing medical opioid use in order to reduce negative outcomes such as addiction (Dowell et al., 2016). Further, if a drug-specific difference on the effect of opioids on the D2DR system exists in the context of pain, this would provide more information to physicians regarding the selection of low-risk opioids for the treatment of pain.

However, as noted, opioids are often used at higher and more frequent doses and for longer durations in burn patients than even in patients with other pain phenotypes, and significantly higher than recreational misusers (Patterson et al., 2004; Wiechman Askay et al., 2009). Thus, it is also possible that any reductions in the impact of opioids on D2DR sensitivity in the context of pain may be counteracted by the increased levels of opioid exposure.

Thus, in this experiment, I tested the hypothesis that the experience of burn pain would alter the effect of opioids on the D2DR system, and would do so in a drug-specific manner, even when animals are given greater exposure to opioids.

2. Experimental Procedure

Subjects

Male mice, PND 28 at the beginning of the experiment, were examined for this experiment. There were 9-14 mice per group, for a total 66 mice used for this experiment.

Burn Injury

Burn or sham injury was induced as described above (Experiment 1).

Injection Paradigm

Injection paradigm followed the parameters listed above. Mice were administered hydrocodone, oxycodone, or morphine. All opioids were given at 20

mg/kg, the dose found in Experiment 4 to have the greatest impact on D2DR behavioral response. Mice received receive opioids twice daily (a.m. and p.m.) for 14 days post-injury, beginning the evening of the day of injury.

Locomotor Testing

On the morning of day 15 post-injury, mice were taken to the locomotor behavior core room, and allowed to habituate for 30 minutes. Then, they were placed in 8 automated optical beam activity monitors (Model RXYZCM-16; Accuscan Instruments, Columbus, OH, USA). Baseline locomotor behavior was recorded for 30 minutes. The mice were then injected with quinpirole HCl (10mg/kg, 10 ml/kg, i.p.) and locomotor activity was recorded for a further 120 minutes post-quinpirole. The apparatus was cleaned thoroughly with ethanol followed by water and completely dried between each run.

Statistics

For each animal, the total distance traveled in the 120 minutes post-quinpirole will be normalized to the total distance traveled in the 30-minute baseline, expressed as a percentage. Total distance traveled (normalized) for the entire 120 minutes post-quinpirole were analyzed using a 2 (injury) x 3 (drug) between-subjects ANOVA. Additionally, total distance traveled (in cm) was binned in 5-minute increments for each animal. These 5-minute bins were then averaged across animals for each group. Data was normalized using the 5-minute bin immediately prior to quinpirole injection, and

expressed as a percentage (i.e. the final 5 minutes of baseline activity was considered 100% of the typical amount of locomotor activity). These data were analyzed using a 2 (injury) x 3 (drug) x 24 (time bin) mixed-factor ANOVA. Differences between treatment groups were calculated using Bonferroni post-hoc comparisons. Differences with p-values less than .05 were deemed statistically significant.

3. Results

Opioids Have Similar Impacts on D2DR Response Following Sham Injury

Locomotor activity in the 30-minute baseline period was analyzed using a 5 (time) x 3 (treatment) mixed-model ANOVA. A significant effect of time was observed in the baseline period, indicating the development of locomotor habituation over time ($F(4, 112) = 13.679$, $p < .001$). However, the rate and magnitude of habituation did not differ between opioid treatment conditions. The interaction between time and treatment was non-significant, $F(8, 112) = 0.746$, $p > .05$.

Locomotor activity in the 120-minutes post-quinpirole administration was analyzed with a 24 (time) x 3 (treatment) mixed-model ANOVA. There was a significant effect of time, $F(23, 644) = 37.121$, $p < .001$, but no significant interaction of time and treatment, $F(46, 644) = 0.353$, $p < .05$.

Hydrocodone Treatment Results in Greater Disturbance of D2DR Response Than Oxycodone or Morphine Following Burn Injury

Locomotor activity in the 30-minute baseline period was analyzed using a 5 (time) x 3 (treatment) mixed-model ANOVA. A significant effect of time was observed in the baseline period, indicating the development of locomotor habituation over time ($F(4, 128) = 12.025$, $p < .001$). However, the rate and magnitude of habituation did not differ between opioid treatment conditions. The interaction between time and treatment was non-significant, $F(8, 128) = 1.697$, $p > .05$. These data are presented in Figure 18 A.

Locomotor activity in the 120-minutes post-quinpirole administration was analyzed with a 24 (time) x 3 (treatment) mixed-model ANOVA. There was a significant effect of time, $F(23, 736) = 22.119$, $p < .001$, and a significant interaction of time with treatment, $F(46, 736) = 1.531$, $p < .05$. Post-hoc analysis with Bonferroni technique revealed locomotor activity in hydrocodone-treated animals was significantly higher ($p < .05$) than locomotor activity in morphine-treated animals at 5 and 35 minutes following quinpirole administration. Oxycodone-treated animals did not differ significantly from either hydrocodone- or morphine-treated mice at any timepoint. These data are presented in Figure 18 B.

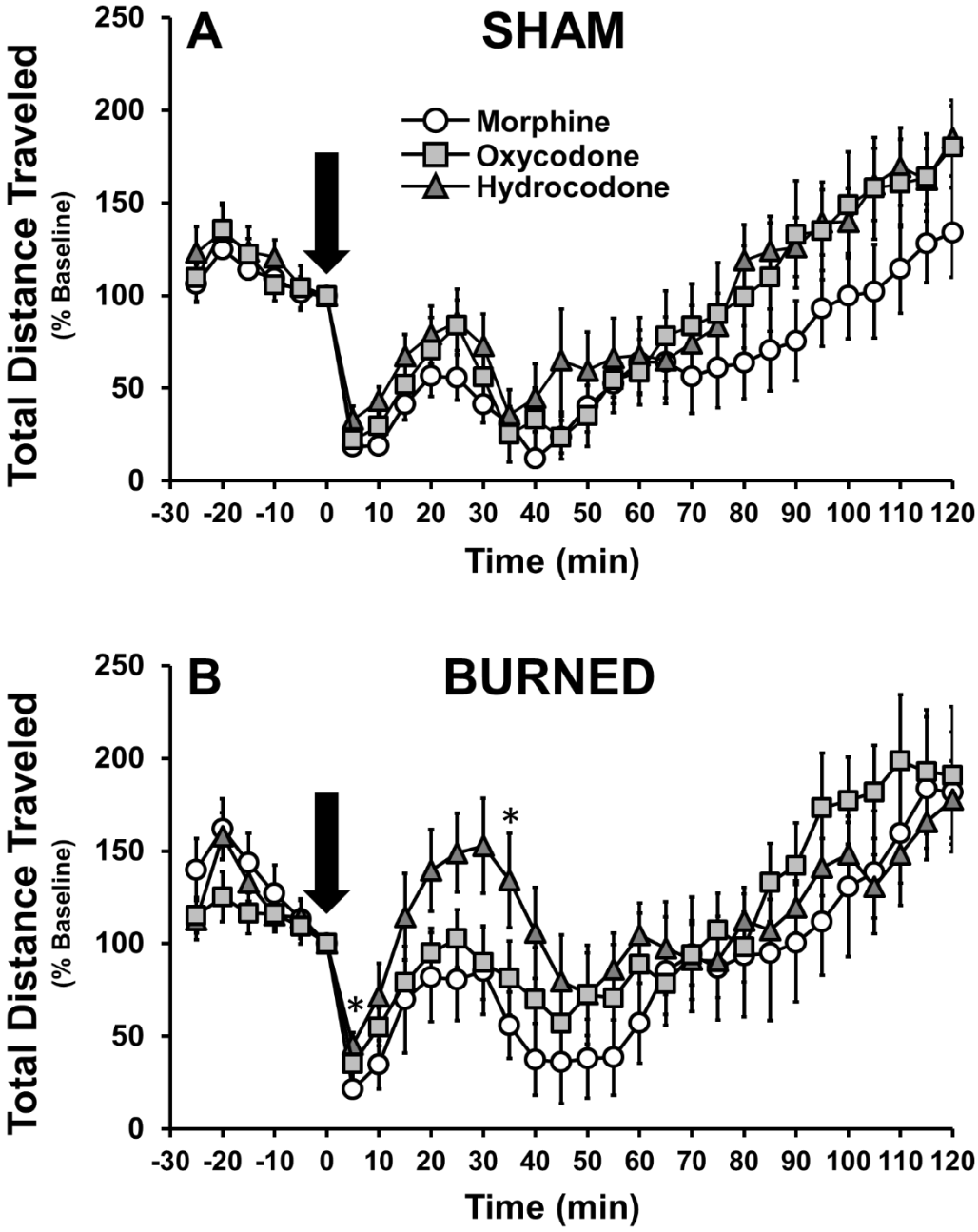

Figure 18. Burn injury causes emergence of drug-specific effects on the hyperlocomotor response to D2/D3 agonist quinpirole. A. Locomotor response to quinpirole in sham animals following repeated opioid administration. No drug-specific differences were observed in sham animals. B. Locomotor response to quinpirole in burn-injured animals following repeated opioid administration. Drug-specific differences in D2DR sensitivity were present among opioids in the presence of burn injury. Quinpirole was administered at time 0 (indicated by arrow). * Significantly different ($p < .05$) from morphine. Data presented as mean ± SEM.

No Effect of Burn Injury on Morphine-induced Hypersensitivity of D2DR Response

The impact of burn-injury on the ability of morphine to disturb D2DR behavioral sensitivity was examined by comparing the locomotor response to quinpirole following 14 days of twice daily treatment with morphine, following either burn or sham injury.

Locomotor activity in the 30-minute baseline period was analyzed using a 5 (time) x 2 (treatment) mixed-model ANOVA. A significant effect of time was observed in the baseline period, indicating the development of locomotor habituation over time ($F(4, 96) = 11.846$, $p < .001$). However, the rate and magnitude of habituation did not differ between injury type. The interaction between time and injury was non-significant, $F(4, 96) = 1.542$, $p > .05$. These data are presented in Figure 19 A.

Locomotor activity in the 120-minutes post-quinpirole administration was analyzed with a 24 (time) x 2 (treatment) mixed-model ANOVA. There was a significant effect of time, $F(23, 552) = 22.413$, $p < .001$, but no significant interaction of time and treatment, $F(23, 552) = 0.547$, $p > .05$. These data are presented in Figure 19 A.

No Effect of Burn Injury on Oxycodone-induced Hypersensitivity of D2DR Response

The impact of burn-injury on the ability of oxycodone to disturb D2DR behavioral sensitivity was examined by comparing the locomotor response to quinpirole following 14 days of twice daily treatment with oxycodone, following either burn or sham injury.

Locomotor activity in the 30-minute baseline period was analyzed using a 5 (time) x 2 (treatment) mixed-model ANOVA. A significant effect of time was observed

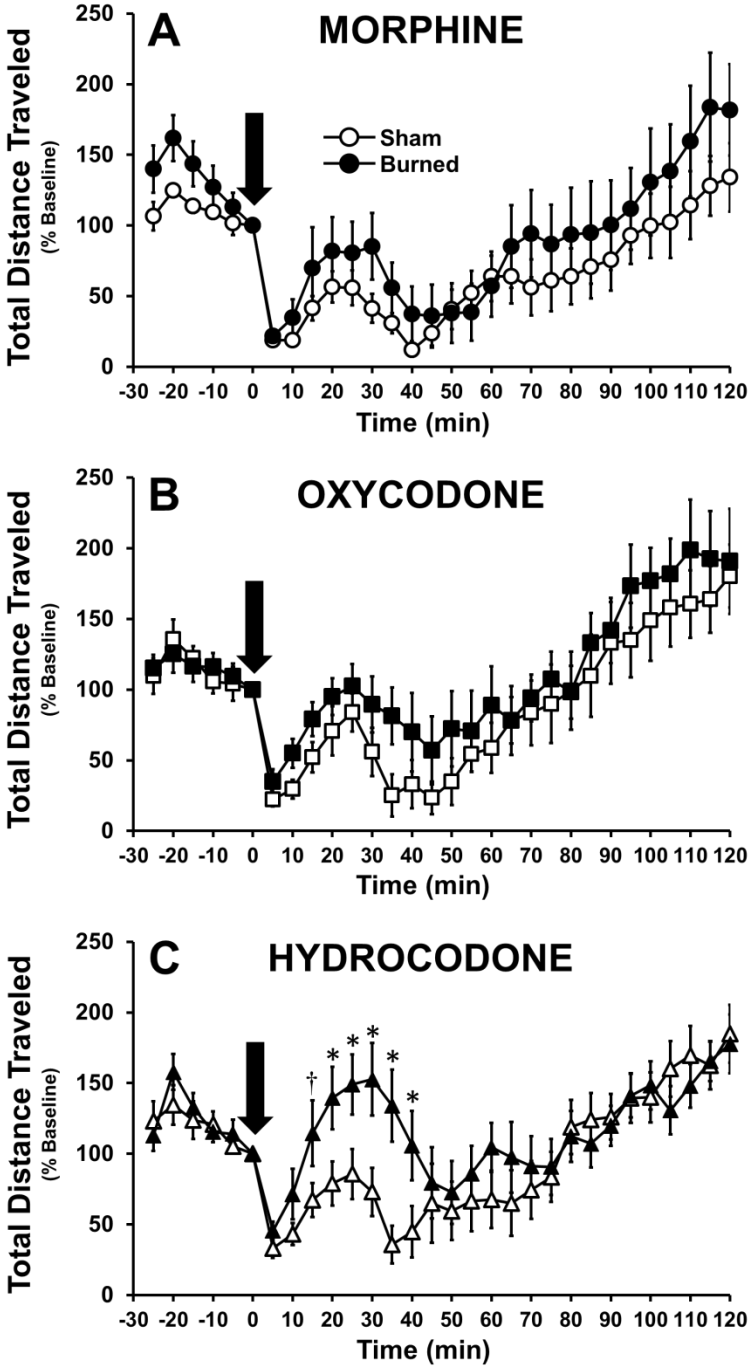

Figure 19. Drug-specific effects of burn injury on the hyperlocomotor response to D2/D3 agonist quinpirole. A. Locomotor response to quinpirole following morphine administration is unaffected by burn injury. B. Locomotor response to quinpirole following oxycodone administration is unaffected by burn injury. C. Locomotor response to quinpirole following hydrocodone administration is significantly increased in the presence of burn injury. Quinpirole was administered at time 0 (indicated by arrow). * Significantly different ($p < .05$) from sham. † Trend toward significantly different ($p = .064$) from sham. Data presented as mean ± SEM.

in the baseline period, indicating the development of locomotor habituation over time ($F(4, 68) = 3.530$, $p < .05$). However, the rate and magnitude of habituation did not differ between injury type. The interaction between time and injury was non-significant, $F(4, 68) = 0.768$, $p > .05$. These data are presented in Figure 19 B.

Locomotor activity in the 120-minutes post-quinpirole administration was analyzed with a 24 (time) x 2 (treatment) mixed-model ANOVA. There was a significant effect of time, $F(23, 391) = 19.574$, $p < .001$, but no significant interaction of time and treatment, $F(23, 391) = 0.354$, $p > .05$. These data are presented in Figure 19 B.

Burn Injury Significantly Increased Hydrocodone-induced Hypersensitivity of D2DR Response

The impact of burn-injury on the ability of hydrocodone to disturb D2DR behavioral sensitivity was examined by comparing the locomotor response to quinpirole following 14 days of twice daily treatment with hydrocodone, following either burn or sham injury.

Locomotor activity in the 30-minute baseline period was analyzed using a 5 (time) x 2 (treatment) mixed-model ANOVA. A significant effect of time was observed in the baseline period, indicating the development of locomotor habituation over time ($F(4, 76) = 11.649$, $p < .001$). However, the rate and magnitude of habituation did not differ between injury type. The interaction between time and injury was non-significant, $F(4, 76) = 2.349$, $p > .05$. These data are presented in Figure 19 C.

Locomotor activity in the 120-minutes post-quinpirole administration was analyzed with a 24 (time) x 2 (treatment) mixed-model ANOVA. There was a significant effect of time, $F(23, 437) = 13.731$, $p < .001$, and a significant interaction of time with treatment, $F(23, 437) = 2.636$, $p < .001$. Post-hoc analysis with Bonferroni technique revealed locomotor activity in burn-injured animals was significantly higher ($p < .05$) than locomotor activity in sham-injured animals at 20-40 minutes following quinpirole administration, and a trend toward significantly higher locomotor activity in the burn-injured animals ($p = .064$) was seen at 15 minutes post-quinpirole. These data are presented in Figure 19 C.

4. Discussion

In sham animals, no drug-specific differences were seen in the effect of opioid exposure on the behavioral sensitivity of the D2DR response to agonism. The 'natural' response to quinpirole in mice has been well established in the literature to be locomotor-suppressive (Halberda et al., 1997). This effect persists across the developmental age of the mouse, and is well replicated in our hands ((Emery et al., 2015), Chapter VIII). Twice daily treatment with opioids for two weeks resulted in a behavioral hypersensitivity to quinpirole as compared to the well-established locomotor suppression seen following quinpirole in drug-naïve mice. However, no drug-specific differences in the degree of this hypersensitivity were present in this experiment. Importantly, these results appear to differ from my previous experimental result (Chapter VIII) which showed significant drug-specific differences in the magnitude of D2DR

hypersensitivity following opioid pre-treatment. In the previous experiment, mice were injected with one of the 3 opioids once daily for 6 days, and tested 24 hours after the final opioid administration. In the current design, mice were administered opioids twice daily for 14 days, resulting in approximately 4x the degree of exposure to opioids (twice as many daily exposures, for twice as long); and were tested 12 hours following the final opioid dose. Please note that also in my previous studies, the differential effects of pretreatment with various opioids on quinpirole responses were observed at moderate opioid doses, and was not significant at very low or very high doses of opioids. Thus, the lack of significant differences among the opioids in this experiment might be explained by dose and duration. The difference in the timing of the quinpirole administration following the final dose of opioids likely has little impact, if any. Prior experiments have demonstrated that the opioid-induced impact on D2DR response is observable while opioids are still on-board, and persists for at least 3 days following the last exposure to opioids (Barwatt et al., 2013). Thus, a 12-hour difference within the first 72 hours is likely negligible.

In the previous study, drug-specific differences were present such that morphine-pretreated mice showed no behavioral activation after quinpirole, and oxycodone-pretreated mice showed behavioral hypersensitivity such that the early spike in locomotor activity, ~25-35 minutes following quinpirole, had a peak magnitude of ~175% of baseline activity, rising to ~200% baseline at the late peak (around 120 minutes post-quinpirole). Hydrocodone-pretreated animals showed a midrange effect

between these two extremes, with an early locomotor spike peaking at ~75% baseline, and ~150% baseline by 120 minutes.

In the current experiment, behavioral activity in oxycodone-pretreated mice decreased, while activity in morphine-pretreated mice increased such that all three groups demonstrated similar activity levels, with an early locomotor spike peaking at ~75% baseline, and ~150% baseline by 120 minutes, which represented 'moderate' locomotor hyperactivation in the previous study. This can be interpreted to mean that increased exposure to oxycodone resulted in the development of tolerance to the behavioral hypersensitivity of the D2DR, while increased exposure to morphine resulted in a sensitization of the effect.

While this finding is interesting in itself, a potentially more important general interpretation arises after further consideration. The presence of profound drug-specific differences between opioids which disappear following the passage of more time/greater exposure to drugs implies a 'window of sensitivity' for drug-specific impacts of opioid exposure. That is, drug-specific effects may only be present for a short period of time, which resolve and after which, the drugs do not appear to be different from one another. However, the impacts and consequences which resulted from the differences could be much more tenacious, long outlasting the differences which gave rise to them.

In animals which received a burn injury, small but significant differences were present in the effect of opioids on D2DR behavioral sensitivity. Specifically, animals in which the burn injury pain was treated using hydrocodone demonstrated significantly greater quinpirole-induced locomotor activity than burned animals treated with

morphine. This indicates that the presence of burn injury and/or pain interacts with opioids in a drug-specific manner to drive differential impacts on the D2DR system.

To further explore this interaction, the impact of each opioid on the locomotor-activating effects of quinpirole in the burn-injured animals was compared with the impact of that opioid on the effects of quinpirole in the sham animals. Neither burn-injured animals treated with morphine or oxycodone showed any difference in the locomotor response to quinpirole, compared to sham animals treated with each respective opioid. This indicates that the experience of burn injury and/or associated pain plays no role in the impact of morphine or oxycodone on D2DR system sensitivity. While exposure to oxycodone and morphine both increase D2DR sensitivity to agonism, the experience of burn injury does not modulate this increase in any way.

However, burn-injured animals which were treated with hydrocodone showed significant increases in locomotor hyperactivity following quinpirole administration compared to sham animals who received comparable treatment with hydrocodone. This indicates that the mechanism by which hydrocodone affects the D2DR system is uniquely impacted (and increased) by burn injury. The role of the D2DR system in addiction and mental health has been discussed in some depth in previous chapters, and I will not labor the point to repeat those roles here. The significant increase in the impact of hydrocodone on D2DR sensitivity in the presence of burn injury may imply that treatment of burn pain with hydrocodone carries unique risks for the precipitation of D2-mediated negative outcomes, including addiction and depression. This is particularly unfortunate considering the fact that my prior experiments demonstrated hydrocodone to

be the most effective of the 3 opioids to prevent the development of chronic hyperalgesia symptoms following burn (Chapter VI).

However, it is potentially of significance that of the three opioids examined, hydrocodone is uniquely different from both oxycodone and morphine in its ability to prevent the development of burn-induced hyperalgesia, and that it is also the only drug examined which displays an interaction between burn injury and D2DR sensitivity. These findings, taken together, indicate that the mechanism of action of hydrocodone on burn injury pain, chronic pain/hyperalgesia, and striatal D2DR system signaling may share a common mechanistic feature, which differs from that of oxycodone and morphine.

Research specifically focused on hydrocodone is currently distressingly sparse. This research has been further hampered by the fact that in humans, hydrocodone is very rarely given alone; instead it is typically prescribed in combination with acetaminophen/paracetamol (as Vicodin) or ibuprofen (as Vicoprofen). The first pure hydrocodone preparation was approved by the FDA for prescription use only last year (2016). However, one of the few studies available on hydrocodone reveals that, while chronic treatment with Vicodin results in the development of thermal hyperalgesia in much the same way as most other opioids, treatment with pure hydrocodone does not (O'Connell et al., 2014). Thus, it appears that combining hydrocodone with acetaminophen may result in it behaving more conventionally. It could be the case that the mechanistic feature which makes hydrocodone unique from other opioids may be antagonized by acetaminophen. Unfortunately, at the time of writing, the exact

mechanism of acetaminophen's action remains unknown (McKay and Walters, 2013), leaving this avenue tantalizing but not immediately pursuable. It may be that discovery of acetaminophen's pharmacology will elucidate the peculiarities of hydrocodone's action; or conversely that research into the nonconformity of hydrocodone amongst the opioids will bear fruit which will guide research in the molecular mechanisms of acetaminophen. Only time will tell.

Notably, this significant increase in locomotor hyperactivity is present only in the early activity spike (~25-35 minutes post-quinpirole). The late behavioral activity rise (occurring in the second hour following quinpirole administration) is the same between the sham and burn-injured animals. This observation lends additional support to the hypothesis that the early and late locomotor peaks are driven by distinct mechanisms, one (mediating the early phase) which is affected by burn injury and another (driving the late rise) which is not affected by burn.

CHAPTER XI

EXPERIMENT 9: EFFECTS ON GENE EXPRESSION AND IDENTIFICATION OF NOVEL MOLECULAR TARGETS ALTERED BY OPIOID EXPOSURE

1. Background

Although alterations in cell signaling cascades by opioids may have profound behavioral consequences, activation levels of signaling molecules downstream of opioid receptors often return to baseline levels in the order of minutes to hours following dissociation of the ligand from the receptor (Ahn et al., 2004). Indeed, even longer-lasting effects on second messenger systems associated with withdrawal tend to resolve in temporal parallel with the abatement of withdrawal symptoms. However, the use and abuse of opioids increases the risk of negative consequences, such as increased addiction/relapse susceptibility and increased risk for mood disorders, for months (in animal models) or years (in human clinical populations) following cessation of drug use, with some of the consequences appearing to be life-long. These long-lasting consequences of opioid exposure implies a potential genomic component.

In addition to their short-acting role as second messenger molecules, ERK, Akt, JNK, and other signal transducers whose activity are altered by opioids are also known to translocate to the nucleus, where they act as transcription factors either directly or indirectly (such as JNK which phosphorylates/activates the promiscuous transcription factor c-jun). Thus, drug-specific alterations in the activation states of these molecules has the potential to alter the expression levels of any gene regulated by them or their

downstream targets. Some potential target genes are obvious, such as β-arrestin 2, D2 dopamine receptor, arginine vasopressin, and oxytocin, which have all been suggested to mediate addiction behaviors such as drug seeking and reinstatement, and withdrawal effects (van Ree and de Wied, 1977a, b; Kovacs et al., 1985; Blum et al., 1991; Reddy et al., 1993; Kovacs et al., 1998; Wang et al., 2000; You et al., 2000; Smith et al., 2002; Zhang et al., 2004; Raehal et al., 2005; Raehal and Bohn, 2011; Baracz and Cornish, 2013; Zanos et al., 2014; Georgiou et al., 2015; Zhou et al., 2015).

In addition, the signaling molecules affected by opioids activate multiple transcription factors, including multiple members of both the fos and jun families, as well as many acting directly as transcription factors themselves (i.e. ERK-ELK complex). Thus, the number of genes potentially affected by opioid exposure but not already implicated in the literature is far too large to screen blindly. Therefore, in collaboration with the Texas A&M AgriLife Genomics and Bioinformatics service, we conducted a full transcriptome, next generation RNA-Seq experiment to identify genes whose expression were altered by hydrocodone, oxycodone or morphine compared to saline controls, as well as genes that were differentially altered by the different opioids. The results of this RNA-Seq experiment identified genes previously not associated with opioid addiction or pain, which appeared to be altered by exposure to opioids, as well as genes which were altered in drug-specific ways.

In this experiment, I, along with collaborators in the Texas A&M Agrilife Genomics and Bioinformatics Service, utilized high-throughput, next generation RNA sequencing to identify novel candidate genes altered by opioid exposure, and

differentially altered by different opioids. Further, I utilized quantitative PCR to explore differential alteration of mRNA levels of proteins previously implicated in the effects of opioids, and to confirm and expand upon the findings of the RNA-Seq experiment identifying drug-specific alterations in genes previously not identified to be associated with opioid exposure.

2. Experimental Procedure

Subjects

Adolescent (PND 28 at beginning of experiment) males were examined for this experiment. Ten mice per group for a total of 40 mice were used for this experiment.

Injection Paradigm

Injection paradigm followed the parameters described in the General Methods. Mice were injected with oxycodone, hydrocodone, or morphine at 20 mg/kg once daily for 6 days. This dose was chosen based on its efficacy to elicit behavioral and molecular alterations with drug-specific differences, as seen in Experiments 4 & 5.

Tissue Collection

Twenty-four hours following the final opioid/saline administration, mice were deeply anesthetized with sodium pentobarbital (120 mg/kg, 10 ml/kg), their brains were extracted and flash-frozen in a bath of 2-methylbutane (isopentane) chilled on dry ice.

Dorsal striatum tissue was bilaterally dissected out of the flash-frozen brains on ice and stored at -80 °C until processed to extract RNA.

RNA Extraction

Total cellular RNA was extracted from striatal tissue using Qiagen RNeasy® Lipid Tissue Mini kit, according to the manufacturer's protocol (Qiagen), with the modifications of an additional chloroform/phenol extraction step prior to column isolation of RNA and the inclusion of manufacturer-recommended RNase-free DNase treatment of the final RNA, both of which served to increase purity of the RNA. Total RNA was quantified and checked for quality/purity using UV absorption readings at 230, 260, and 280 nm as determined by NanoDrop™. Extracted RNA was stored at -80 °C at all times except when being processed for downstream applications.

RNA-Seq

Samples of extracted RNA were analyzed using high-throughput next generation sequencing (RNA-Seq). For each biological replicate, 2 µg total RNA was submitted to the Texas A&M AgriLife Genomics and Bioinformatics Service. Libraries were prepared by them for each sample by using the Illumina TruSeq RNA Sample Preparation Kit following the manufacturer's instructions. Data was sequenced on ten lanes of Illumina HiSeq 2500 sequencer. The study sequenced the full transcriptome of all 40 subjects – 10 samples per group. Barcoding was used to multiplex biological

replicates. Base calling and data preparations were completed using Illumina CASAVA software.

The data analysis began with quality control of the generated FASTQ files, using FastQC software (Andrews, 2015). Reads were aligned to the reference genome to locate the origin of each RNA fragment on the reference genome. The reference genome of *Mus musculus* (mm10) and the gene annotation file were downloaded from UCSC and the Tuxedo protocol proposed by Trapnell et al. (2012) was employed.

Quantitative PCR

In addition to RNA sent for RNA-Seq, extracted total RNA was reverse transcribed into cDNA using New England Biolabs M-MuLV RT protocol (NEB) and random hexamers. Primers for various genes of interest, including those identified by RNA-Seq, were designed using NCBI primer-BLAST software and NCBI *Mus musculus* mRNA reference sequences for the genes of interest, and ordered from Eurofins MWG Operon, LLC (Huntsville, AL). In cases where multiple reference sequences or splice variants were present, selections were made in order to favor the most canonical sequence, or the longest sequence. Primers were then chosen to favor the most general expression (amplicons present in all known splice variants) to avoid unintentionally biasing the results by favoring a particularly enriched or poorly expressed variant. Gene accession numbers for the genes of interest and the primer sequences designed for each are provided in Appendix III. Primer pair specificity was confirmed by melt curve

analysis and ethidium bromide gel analysis. Any primer pair which produced multiple bands in EtBr gel *or* multiple melt curve peaks were redesigned.

Quantitative PCR was run on each sample, using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich, St. Louis, MO) on a StepOnePlus™ 96-well Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Each reaction took place in a volume of 25 μL, with final concentrations of 200 nM of each primer (400 nM primer pair) and 0.8 nM cDNA. Cycle conditions were an initial 10 minutes holding step at 94 °C to activate the HotStart *Taq* polymerase, followed by 40, 2-stage cycles of melt temp (94 °C) for 15 seconds, annealing and extension temp (61 °C) for 1 minute with a fluorescence reading, followed by a melt curve determination with a resolution of 0.3 °C. Baseline fluorescence was defined between cycle 3 and 15. Threshold values were set manually based on the logarithmic amplification curve. Genes of interest were normalized to levels of the housekeeping gene β-actin.

Statistics

For RNA-Seq, the differential gene expression analysis for each group was performed between all the pairs using Cuffdiff 2, software included as part of Cufflinks (Trapnell et al., 2012). Cuffdiff 2 provides corrected p-values for multiple hypothesis testing, usually called q-values. Genes were sorted based on the q-values and the False Discovery Rate (FDR) by the Agrilife Genomics and Bioinformatics Service.

For the qPCR, Ct values for each sample were determined automatically by the software based on the manually set threshold. Differences between groups were

calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and based on fold change relative to the housekeeping gene β-actin. Fold change values were transformed into their multiplicative inverse (x^{-1}) and were then analyzed for each gene using a one-way, between-subjects ANOVA using drug treatment group as the factor. Post hoc contrasts between each treatment group were computed using Bonferroni post hoc procedure, and planned comparisons using Dunnett's test were used to make individual paired comparisons between each opioid treatment versus saline controls. Differences with p-values of less than .05 were deemed statistically significant.

3. Results

RNA-Seq

Analysis of the RNA-Seq data revealed several genes whose expression levels were significantly dysregulated (either up- or down-regulated) following opioid treatment in comparison to RNA expression levels of the same gene in saline-exposed control animals. Out of approximately 15,000 genes, comparatively few were dysregulated following opioid exposure. A total of 88 genes were significantly up-regulated by opioid exposure, while a total of 189 were down-regulated. Interestingly, and somewhat unexpectedly, the majority of these dysregulated genes were specific to particular opioid treatment conditions, rather than being dysregulated by exposure to opioids non-specifically. The numbers of genes found to be dysregulated by exposure to opioids, and the opioid treatment conditions resulting in their significant dysregulation, are presented in Figure 20.

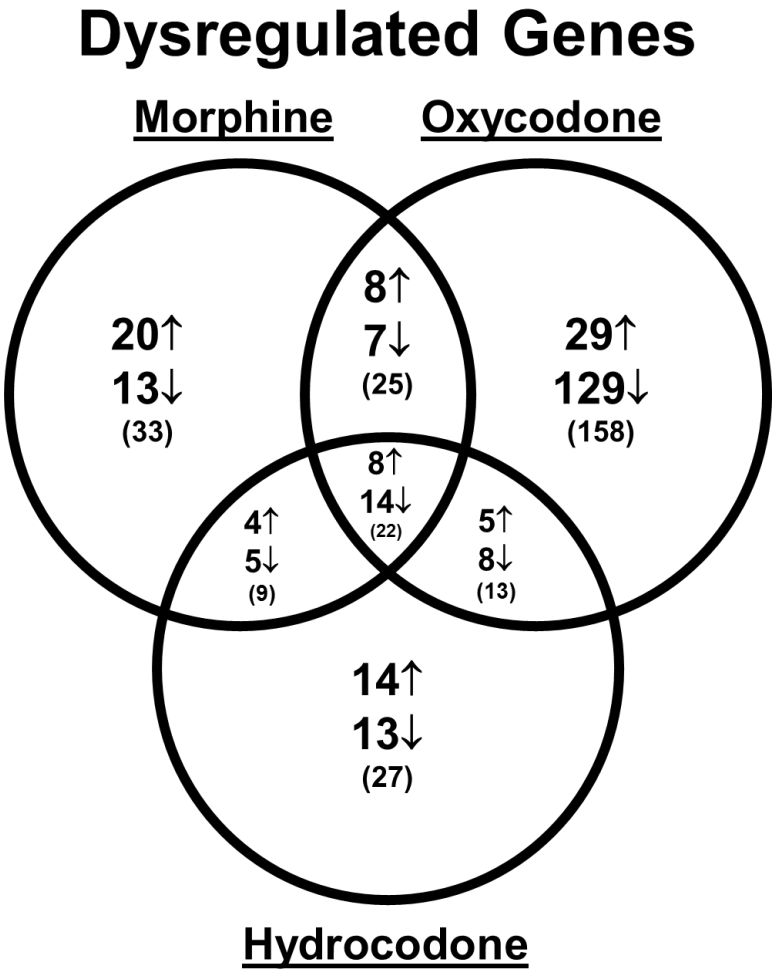

Figure 20. Venn diagram of genes dysregulated by opioids. Number indicates genes whose expression levels were significantly altered in that condition relative to expression levels following saline administration. ↑ Genes up-regulated following treatment with indicated opioid. ↓ Genes down-regulated following treatment with indicated opioid. Number in parentheses indicates total number of genes dysregulated in that condition. Relatively few genes (22 total) were dysregulated by exposure to opioids in general. Significantly more genes were dysregulated by treatment with oxycodone than either other opioid.

In addition to analyzing the numbers of genes dysregulated by the various opioid treatment conditions, I also analyzed the dysregulated genes for their functional classification, as presented in Table 2. Some genes naturally fall under multiple categories, and other (often relatively newly discovered genes) have only putative functional classification. Despite this, all genes in the current analysis were

Table 2. *Functional classification of genes altered by exposure to opioids. Genes were not sorted into multiple categories; in the case where a gene product has multiple cellular functions, the canonical or most ubiquitous function was used for classification purposes. Particular attention is called to the large number of signaling, transcription, cell structure, and metabolic/mitochondrial genes whose expression is altered by exposure to oxycodone.*

FUNCTION	Oxycodone		Hydrocodone		Morphine	
	Up	Down	Up	Down	Up	Down
Cell Death	-	4	-	1	-	1
Development	-	7	2	2	3	3
DNA binding protein	-	3	-	-	-	1
Hemoglobin	-		-	2	2	-
Hormone	3	2	4	1	1	-
Immune	-	6	3	2	1	1
Metabolism	1	5	1	2	1	2
MicroRNA	5	5	3	5	3	8
Mitochondrial	9	7	2	1	4	1
non-coding RNA	3	1	-	2	2	1
Protein translation	5	5	2	2	8	-
Receptor	-	5	-	2	-	-
Regulatory protein	-	3	-	-	1	-
RNA Regulation	-	4	-	-	-	-
Signaling	6	27	3	2	4	6
Structural	4	24	2	3	2	3
Transcription	9	29	8	13	4	9
Unknown or unclassified function	5	21	1	-	4	3
Total	**50**	**158**	**31**	**40**	**40**	**39**

placed in only one category. In the case where multiple functions are known for that gene, it was classified according to its canonical function, or the function it is most commonly associated with. In the case of newly discovered genes that lack clear classification in bioinformatics resources, they were classified according to structural homology analysis.

Quantitative PCR

Following RNA-Seq analysis, approximately 30 genes were chosen for qPCR analysis. Some were genes identified by the RNA-Seq to be differentially altered following exposure to differential opioids; while others were chosen based on the preponderance of literature indicating that they are genes with crucial roles in drug addiction, opioid system function, or pain. In the latter case, genes were chosen regardless of whether they appeared dysregulated in the RNA-Seq results. The genes chosen for qPCR analysis are summarized in Table 3 below, and the specific primers utilized are given in Appendix III.

Analysis of inverse-transformed fold change values using one-way, between subjects ANOVA revealed 3 genes which demonstrated significant differences among treatment conditions. Expression levels of oxytocin (*Oxt*) were significantly different based on treatment condition, $F(3, 34) = 3.671$, $p = .022$. Expression levels of myosin heavy chain 6 (*Myh6*) were significantly different based on treatment condition, $F(3, 36) = 3.886$, $p = .017$. Expression levels of *Darpp-32* were significantly different based on treatment condition, $F(3, 36) = 2.884$, $p = .05$.

Table 3. *Genes analyzed by qPCR.*

GENE	SYMBOL
Arginine Vasopressin	*Avp*
β−Actin	*Actb*
β−Arrestin 2	*Arrb2*
D1 Dopamine Receptor	*D1dr*
D2 Dopamine Receptor	*D2dr*
Dopamine and cAMP-Regulated Neuronal Phosphoprotein	*Darpp-32*
Dopamine Transporter	*Dat*
Forkhead Box Protein J2	*Foxj2*
Interleukin-1 Receptor-associated Kinase	*Irak1*
c-Jun n-Terminus Kinase 2	*Jnk2*
c-Jun n-Terminus Kinase 3	*Jnk3*
JunD	*Jund*
Potassium Voltage-gated Channel, Shaw-related subfamily, 3	*Kcnc3*
Mouse Double Minute 2 homolog	*Mdm2*
Myosin Heavy Chain 6	*Myh6*
Oxytocin	*Oxt*
Oxytocin Receptor	*Oxtr*
Tumor Protein p53	*P53*
Platelet-Derived Growth Factor Receptor Alpha	*Pdgfra*
Rotatin	*Rttn*
Superoxide Dismutase	*Sod1*
Vasopressin 1a Receptor	*Avpr1a*

Bonferroni post hoc analysis revealed no statistically different conditions amongst any pairwise comparisons for any of the 3 genes demonstrated to be significantly different by ANOVA. However, this is likely due to the small differences in means, and comparatively large variances, coupled with the highly conservative nature of the Bonferroni test.

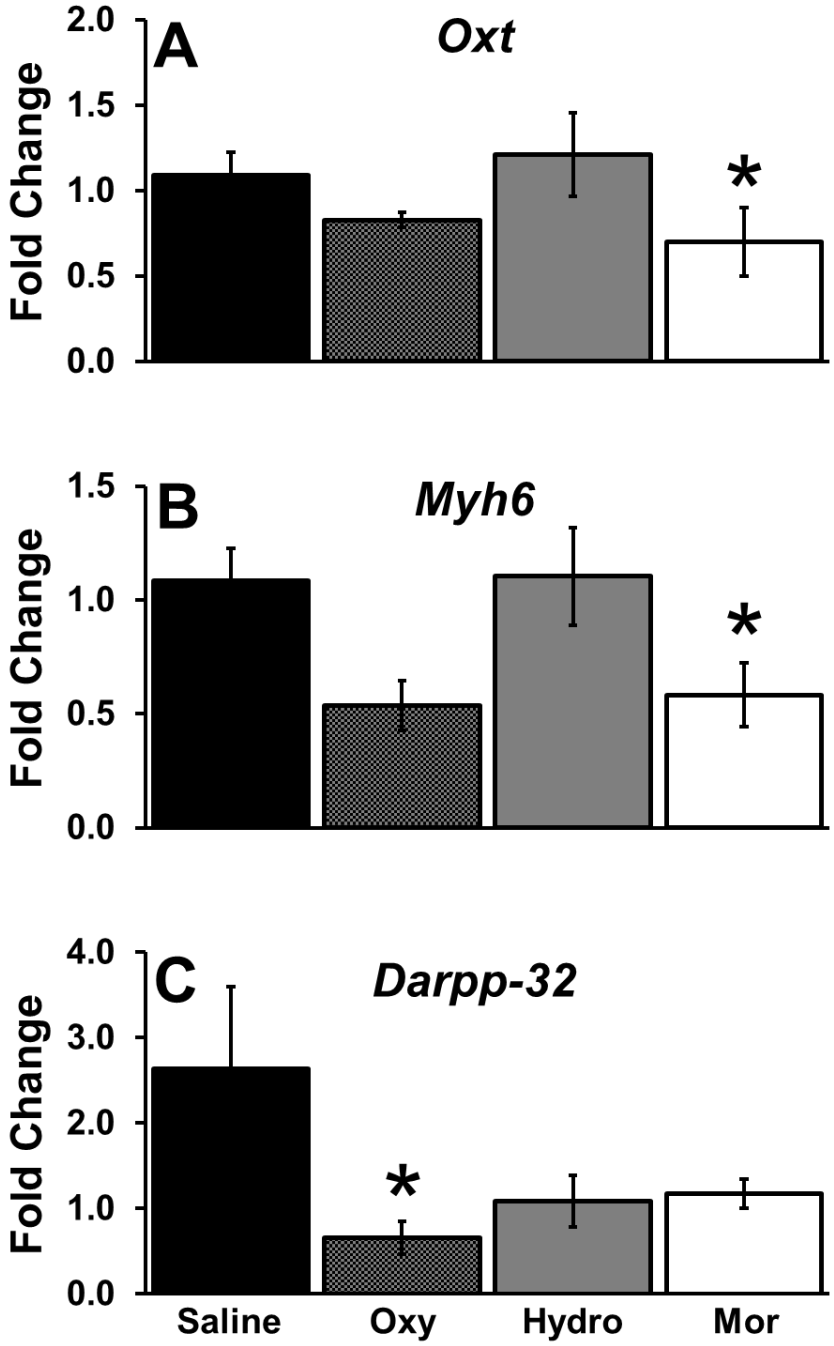

Figure 21. Results of qPCR analysis. Expression levels of selected gene products in the striatum following 6-day exposure to 1 of 3 opioids, compared to expression observed in animals exposed to saline. A. Oxytocin mRNA expression levels. B. Levels of Myosin heavy chain 6 mRNA. C. Levels of DARPP-32 mRNA. All data is graphed as fold change normalized to expression levels in saline-treated animals and presented as mean ± SEM. * Significantly different ($p < .05$) from expression levels in saline-treated animals.

Dunnett's comparisons of each opioid treatment versus saline control levels of gene expression revealed that morphine significantly reduced levels of *Myh6* mRNA, relative to saline levels ($p = .038$). Neither oxycodone nor hydrocodone resulted in any alteration of *Myh6* transcript levels ($p > .05$). Dunnett's test revealed that morphine also significantly decreased the expression levels of *Oxt* mRNA relative to levels observed in saline-treated animals, $p = .020$. Again, neither oxycodone nor hydrocodone altered oxytocin expression relative to saline treatment, $p > .05$. In contrast, oxycodone significantly decreased expression of *Darpp-32* transcript in the striatum as compared to treatment with saline, $p = .035$, while neither morphine nor hydrocodone significantly altered *Darpp-32* expression relative to saline control levels, $p > .05$. These results are presented graphically in Figure 21.

In addition to the 3 genes found to be significantly altered by opioid exposure, 7 additional genes were found to trend toward significance ($p = .051$ - $.094$). These genes are presented in Table 4 below.

Due to the relatively high variance as compared to sample size in this experiment, it is possible that the genes found to trend toward statistical significance represent true differences that did not have adequate power to meet statistical thresholds of detection. For this reason, despite the failure to demonstrate global statistically significant differences in the ANOVA test, planned comparisons using Dunnett's test were executed on these 7 genes to compare individual opioid treatment conditions specifically to saline control levels.

Table 4. *Genes approaching statistical significance in ANOVA analysis.*

GENE	SIGNIFICANCE (P VALUE)
Kcnc3	.051
Avpr1a	.068
Irak1	.069
Jnk2	.071
Jund	.074
Avp	.08
Dat	.094

As compared to levels of each gene observed in saline-treated control samples: oxycodone significantly reduced *Kcnc3* transcript levels, (p = .025); and *Jnk2* levels (p = .045). No other drug treatment conditions significantly altered gene expression levels as compared to levels observed in saline-treated animals, for these genes or for any of the 5 remaining genes found to have near-significant differences via ANOVA test.

4. Discussion

The results from the RNA-Seq experiment were very promising, yielding findings which justify the exploration of several future research questions. Perhaps most importantly for the purposes of the initial hypothesis was the finding of highly drug-specific effects on gene expression dysregulation. It was expected that the vast majority of dysregulated genes would be altered by exposure to opioids in general, and would be genes involved with neuroadaptations related to drug addiction generally. In fact, what

we saw was that a relatively small number of genes (22) were dysregulated by opioid treatment generally (i.e. those genes dysregulated by all 3 drugs). Additionally, in at least some cases, genes altered by all 3 drugs were not always altered in the same direction by all 3 drugs, indicating drug-specific differences even in effects on these genes.

Somewhat surprisingly, relatively high numbers of genes were uniquely dysregulated by exposure to morphine (33 genes), hydrocodone (27 genes) or oxycodone (158 genes). The much higher number of genes dysregulated by oxycodone, relative to the other two opioids, is particularly intriguing. Of those genes uniquely dysregulated by oxycodone, most (~82%) were downregulated. This is in contrast to genes dysregulated by morphine or hydrocodone, which showed relative balance between upregulated and downregulated gene expression.

Of the genes downregulated by exposure to oxycodone (though not necessarily *uniquely* by oxycodone), over half (55%) code for gene products exhibiting a role in intracellular or paracrine signaling, gene transcription, or cellular structure. The full implications of this finding have yet to be discovered, but it is possible that it represents a narrowing of neuroplasticity, such that exposure to oxycodone 'cements' neural physiology by decreasing novel neurite growth and shaping. This would support prior research findings that chronic drug taking facilitates the development of inflexible, habit-like behavior and a decrease in neurocognitive flexibility. If this is the case, it indicates that oxycodone may have a particularly high likelihood to provoke the

progression from typical use (either recreational or medicinal) to problematic use and addiction.

An alternative, and more troubling, explanation is that oxycodone is uniquely neurotoxic compared to the other opioids, and the downregulation in signaling, structure, and transcription related genes reveal an overall degradation in the health of striatal cells, and by extension, likely other brain cells as well. Future experiments should explore the health of striatal cells by examining apoptotic, pyroptotic, and necrotic markers. Further, experiments should examine measures of neuroplasticity such as neurite diameter, synaptic spine density and alterations to these variables following exposure to novelty. This will help determine whether exposure to oxycodone is in fact having a detrimental effect on plasticity.

In addition to pathways in which large numbers of genes are dysregulated, indicating broad influences of opioids on these functions, there are also a number of categories where small numbers of genes are differentially dysregulated by exposure to opioids, but the specific genes in these categories are critical. One interesting category in which relatively critical genes are dysregulated is metabolic/mitochondrial related genes. Although relatively few genes in this class are dysregulated, the ones which are dysregulated point to fundamental alterations in cellular respiration and energy utilization. This again may imply a deterioration in overall cell health following opioids.

Another very promising result of the RNA-Seq experiment is the drug-specific dysregulation of small non-coding RNAs and microRNAs. These molecules, formerly considered to be ‘junk’ genomic content, have recently captured attention in molecular

biology as critical regulators of mRNA translation. Despite relatively low expression levels in the cell (which makes them less amenable to qPCR analysis and helped contribute to the overlooking of their importance by the scientific community), ncRNAs and miRNAs are incredibly powerful regulators, both of each other and of mRNA translation and post-translational modification and degradation of proteins. As such, ncRNA and miRNA networks add a high degree of nuance to the intracellular environment, and alterations in expression levels and balances of these molecules have the potential for rather profound consequences.

Indeed, recent work suggests there may be as many as 5,600 unique miRNA sequences in the human genome, half of which are shown to associate with Argonaute 2 and therefore be functional in the RNAi pathway responsible for alterations in post-transcriptional gene expression (Londin et al., 2015). This does not include sncRNAs which act as miRNA sponges, siRNAs crucial to RNAi pathways, lncRNAs, or circRNAs, all of which may play key regulatory roles in cell processes.

Recent work in the field of alcohol abuse and alcoholism has revealed that a primary mechanism by which alcohol exerts its effects at the cellular and molecular level to foster the neuroadaptations leading to an addictive state, is by altering miRNA and sncRNA expression, thereby altering the regulation of downstream molecules (Balaraman et al., 2013; Most et al., 2014; Most et al., 2016). It stands to reason that this mechanism is not specific to alcohol addiction, but rather is general to the neuroadaptations underlying all addictive states (Most et al., 2014).

This theory, coupled with the observation of miRNAs dysregulated by opioids in this experiment, is quite intriguing. Future research should examine the regulatory targets of all miRNAs and functional ncRNAs found to be dysregulated by opioid exposure. Because of the mechanism of action of miRNA regulation of mRNA expression, it is possible that several key systems may be functionally influenced by altered expression levels of only a single miRNA. Further, because of the mechanism of miRNA action, it is possible that these alterations would not be observed in the RNA-Seq results, as miRNA regulation in some cases only prevents translation of mRNA into protein, leaving detected levels of mRNA unaltered but nevertheless affecting functional expression.

Among the qPCR findings, the fact that oxytocin transcript is significantly impacted by opioid exposure is not altogether surprising, given prior knowledge that opioids interact with this system. However, the fact that the impact of opioids on the oxytocin system is drug-specific is interesting. While oxycodone and morphine result in decreased oxytocin transcript in the striatum, hydrocodone exerted no similar effect. It should be noted that oxytocin is not a transcript normally expressed at high levels in the striatum. The alterations observed here may indicate genomic impacts of opioids that occur in all neuronal tissue.

Thus, it is worth considering that this may be accompanied by a similar, and amplified, reduction in oxytocin expression in cells which *do*, in their normal physiological function, express oxytocin protein. This would imply drug-specific differences in addiction risk, given the observed role of the oxytocin system in drug

addiction. Thus, further investigation into the drug-specific impacts of opioids on oxytocin system function in the hypothalamus is warranted by the current findings.

It is interesting, and curious, that DARPP-32 is significantly altered by exposure to different opioids. This is curious because, of all dopamine-system-related genes investigated, this is the only one which demonstrated an effect. While the possibility exists and must be acknowledged that this is a statistical anomaly, an alternative explanation presents itself as well. Although it is well established, both in the literature and in our prior findings, some of which are presented in this dissertation, that opioids have wide-ranging and drug-specific impacts on the function of the dopamine signaling system, it often appears to be the case that these functional impacts are not due to expression changes of membrane-bound proteins such as receptors or transporters. Instead, the alterations seem to be mediated by downstream effectors. Thus, the current finding that *Darpp-32,* a downstream signal regulator which inhibits protein phosphatase activity, is altered while *D1dr, D2dr,* and *Dat* transcript levels are unaltered, is congruent with this pattern. This finding adds further evidence that opioids exert drug-specific effects on the dopaminergic signaling pathways at multiple levels including at the transcriptome (*Darpp-32*), as well as expression and activity of downstream signaling molecules (ERK, Akt; Chapter VIII).

The alteration of *Myh6* transcript levels by exposure to morphine may at first seem strange. *Myh6* represents the α isoform of the myosin protein, most commonly expressed in cardiac atrial muscle. However, myosin proteins are very common in neurons, and are thought to play structural and transport roles in cellular compartments

lacking microtubules but still exhibiting organelle transport activity (Bridgman, 2004). The protein encoded by *Myh6* represents one of the heavy-chain components of Myosin II protein. Although the role of Myosin II in the neuron is less well understood as compared to other Myosin proteins (e.g. I or V), its presence in non-muscle cells is well established and is often found in association with the Golgi apparatus (Musch et al., 1997). In addition, myosin II is known to be involved in neurite outgrowth (Wylie et al., 1998; Wylie and Chantler, 2001). The highly specific alteration in gene expression of *Myh6* subunit in the absence of alterations by other myosin subunit isoforms may be in itself inconsequential due to functional compensation by alternate isoforms. However, in light of the rather large number of signaling and structural genes demonstrated to be dysregulated by opioids in the RNA-Seq results, it is possible that this represents an important alteration of structural and/or cell trafficking mechanisms, or alternatively it may represent a global decrease in the number or size of neurites in the striatum, which implies a commensurate loss of synaptic connections. Further investigation into both alterations of other myosin components and structure/intracellular transport mechanisms more broadly in the context of opioid exposure is warranted.

In addition to these three genes that were significantly altered, seven additional genes demonstrated near-threshold levels of difference. Planned comparisons conducted on these genes revealed only modest differences in 2 of the genes, *Kcnc3* and *Jnk2* following oxycodone administration. The modest, but significant, reduction in transcript levels of these genes following oxycodone treatment relative to saline levels is of potential interest. *Kcnc3* encodes a voltage-gated potassium channel subunit. While its

alteration by oxycodone was modest, it could imply slight alterations in electrophysiological function of the neuron following oxycodone exposure. This possibility is made more interesting in light of the numerous structural and signaling genes downregulated by oxycodone in the RNA-Seq results, as it adds an additional piece of support to the hypothesis that oxycodone exposure is uniquely altering the structure, function, and synaptic integrity of neurons.

Down-regulation of *Jnk2* is perhaps even more interesting, considering the established role of JNK signaling in opioid analgesia, tolerance, and hyperalgesia (Chen et al., 2008; Guo et al., 2009; Manassero et al., 2012; Sanna et al., 2014; Marcus et al., 2015; Sanna et al., 2015a; Sanna et al., 2015b). This down-regulation of *Jnk2* mRNA by oxycodone may imply the development of a cellular state which is primed for altered analgesic response to opioids. Typically, the development of antinociceptive tolerance or opioid-induced hyperalgesia is accompanied by increases in JNK levels, and these behavioral developments can often be rescued by suppressing JNK expression/activity. Thus, the down-regulation of *Jnk2* by oxycodone may partially account for its greater success in treating long-term, chronic pain relative to morphine, or it may represent a compensatory mechanism by the cell in order to adjust for another expression change. Additional research should be done on drug-specific differences in the effects on the JNK pathway. This is especially true in the case of drug-specific differences in antinociception, as spinal JNK levels have been shown to be associated with this effect (*unpublished results*).

It is interesting to compare the current qPCR results to those obtained by Bates in parallel work from our lab (Bates, 2017). Although the work done by Bates was investigating a different question (i.e. the effect of social housing conditions on morphine-induced gene expression alterations), he utilized an experimental design that included saline-treated and morphine-treated mice, and then analyzed gene expression changes in the dorsal striatum as well, and investigated many of the same gene targets as the present work. Interestingly, he found that morphine treatment significantly increased the expression of 5 genes also analyzed in the present study (i.e. *Foxj2, Cdk12, Pde12, D1dr,* and *Rttn*). However, none of these genes were significantly altered by morphine exposure, or indeed exposure to any opioid, in the present study.

A likely explanation for the discrepancy is that Bates employed a different route of administration, specifically subcutaneous injection, which results in drastically different pharmacokinetic profiles as compared to gavage administration employed here. Beyond the altered kinetics, subcutaneous administration avoids first-pass metabolism while p.o. administration is subject to first-pass metabolism, resulting in the same administered dose (i.e. 20 mg/kg) to have very different peak plasma concentrations. Additionally, Bates administered opioids for 14 days, rather than the current study's 6-day regimen. This indicates that exposure to opioids for a longer period of time, and/or with different kinetics of absorption and elimination or plasma concentration, may significantly alter the gene expression consequences which result.

Taken in this context, the genes which trended toward statistically significant alteration but failed to reach threshold differences are more interesting. It may be that

these are genes whose expression was ‘captured’ in the midst of opioid-induced alteration, and the drug-specific differences in expression of these genes may become statistically significant if the experiment were to be repeated with either higher opioid doses or longer durations of administration.

Regardless, it is of note that the expression levels of multiple mRNAs were significantly altered by exposure to different opioid analgesic drugs. While it has been established previously that different opioids may lead to different levels of activation among signaling molecules (Pradhan et al., 2012), the current finding demonstrates a drug-specific impact at the level of the genome. The implications of this are profound.

As demonstrated previously (Chapters IV and V), the doses and administration routes employed in the present set of studies yield a state of equianalgesia, indicating that they are here equivalent and comparable with regards to their analgesic function, which is often the only factor considered in the clinical setting. Despite this analgesic equivalence, exposure to these supposedly comparable compounds results in remarkably large differences in gene expression perturbances, as measured by RNA-Seq, with exposure to oxycodone resulting in an order of magnitude more gene dysregulation than an equianalgesic dose of either hydrocodone or morphine.

These differences in gene expression levels following exposure to these different compounds persists at the level of qPCR. This finding also further demonstrates that the observed behavioral and signaling differences among the opioids are unlikely due to pharmacokinetic differences as it is unlikely that the relatively minor pharmacokinetic

differences between these compounds could result in such large differences at the level of the transcriptome.

CHAPTER XII

GENERAL DISCUSSION, FUTURE DIRECTIONS, & CONCLUSIONS

The findings presented in this dissertation provide additional evidence that there are wide-ranging drug-specific differences among opioids at multiple levels including behavioral, intracellular signaling, and genetic. In addition, it provides a direct comparison among 3 commonly prescribed and commonly abused opioid analgesic medications, oxycodone, hydrocodone, and morphine. This direct cross-drug analysis is novel and fills a crucial gap in the current research literature.

Multiple interdependent molecular mechanisms are hypothesized to be mediating the genetic and signaling differences observed in this series of experiments, which have the advantage of accounting for the behavioral findings herein observed as well. These various hypothetical molecular mechanisms mediating opioid-specific differences are illustrated in Figure 22. Many of the hypothesized mechanisms have empirical support presented within this dissertation, which are referenced in the figure legend. However, more support is needed before the model presented can be considered comprehensive and exhaustive. In addition, please note that the model presented here focuses on intracellular mechanisms of opioid-specific differences. These mechanisms likely underlie and mediate drug-specific differences which emerge at higher levels (i.e. synaptic changes, neuronal circuits, etc.).

Several important conclusions can be drawn from the present work. The first is that it provides compelling evidence against the *status quo* of opioid research and

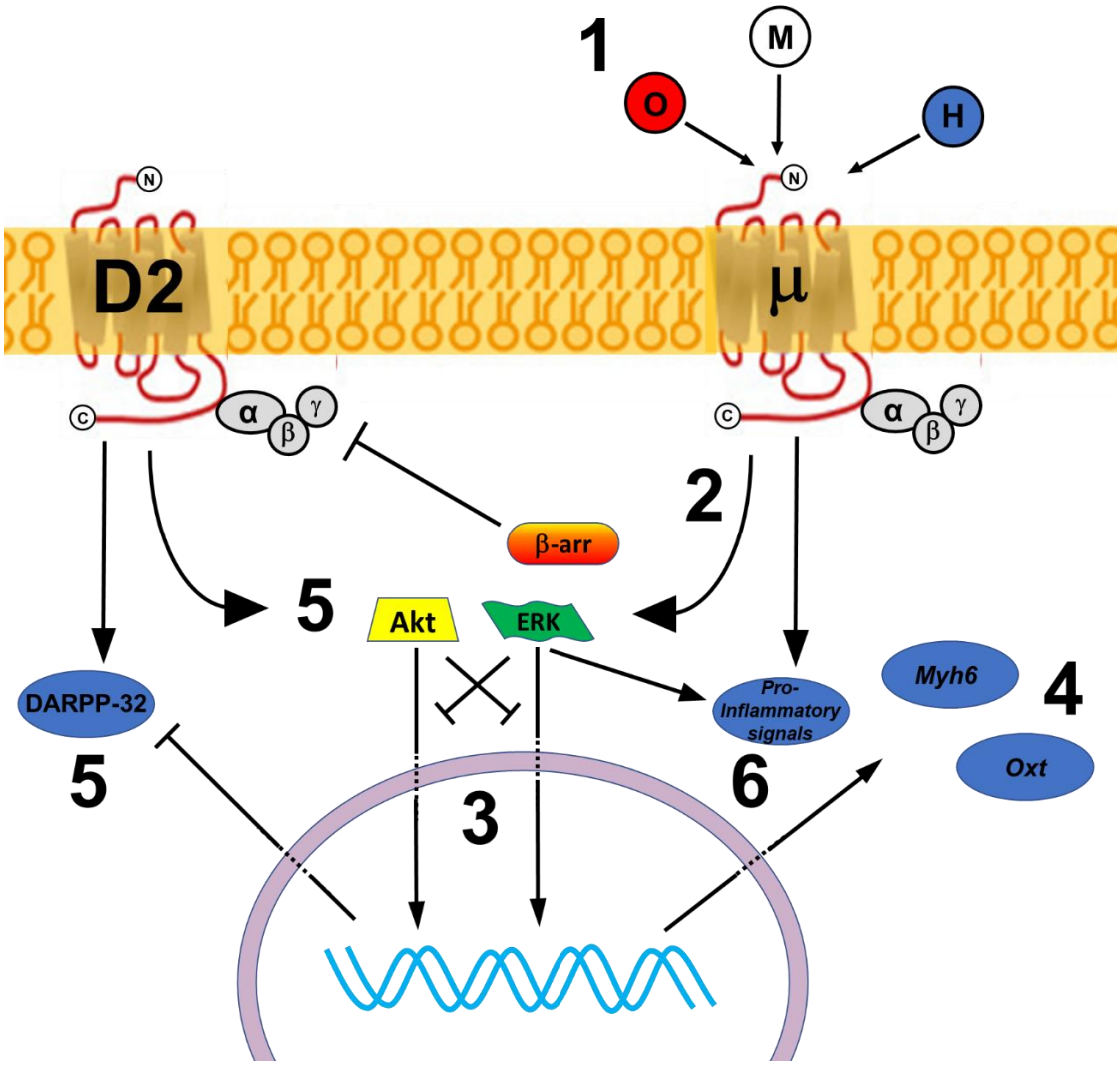

Figure 22. Molecular mechanisms of opioid-specific differences: A hypothesis. Different opioids are subject to different degrees of pharmacokinetic and pharmacodynamic actions prior to and while interacting with their receptors (1). Even when accounting for these differences, drug-specific differences exist in both molecular and behavioral outcomes of exposure to different opioids. This difference can occur via several different mechanisms. These include differential degrees of activation of non-canonical signaling pathways (2, Ch. IX). These various second messenger pathways are known to bi-directionally interact, as well as function as transcription regulators (3). Therefore, differential activation of these signaling molecules can result in profound, drug-specific differences in gene expression profiles (4, Ch. XI). These drug-specific alterations can influence the signaling response of non-opioid receptors as well, either by directly influencing the availability of shared second messenger molecules or indirectly by altering expression of downstream pathway components (5, Ch. IX & XI). Lastly, ligand-specific signaling by opioids differentially influence interactions with the immune system, either directly or indirectly (6). Please note that these various mechanisms are not mutually exclusive, and are likely active concomitantly.

treatment. This *status quo* has for years entailed exhaustive research and analysis on a particular archetypal opioid, most often morphine, followed by extrapolation of those findings to other classical opioids with adjustments for known differences between the compounds. While this research model has been particularly effective for deducing the basic workings of opioid pharmacology, receptor structure and function, mechanisms of action, etc., it does not allow for the prediction of the types of drug-specific differences observed herein.

Further, as a clinical parallel of the 'model drug' research paradigm, pain physicians have long made opioid prescribing and dosing decisions based on the notion of opioid equivalence and interconversion; that is, that different classical opioids can be considered functionally interchangeable when pharmacological differences such as metabolic differences (i.e. absorption and elimination rates), peak plasma concentrations, receptor affinities, etc. are considered and accounted for. Because of the (true, if incomplete) understanding that both beneficial (analgesia) and harmful effects (e.g. respiratory depression, reward/addiction, opioid-induced hyperalgesia, etc.) associated with opioids were mediated largely by action at the μ receptor, it was believed that these negative effects were by and large inseparable from the analgesic functions.

This led to the assumption that opioids acting with equivalent degrees of antinociceptive potency (i.e. equivalent occupancy and activation of the μ receptor) would likewise have equivalent levels of risk for the negative effects associated with opioid use. This in turn guided a treatment philosophy centered around using the

minimal adequate dose of opioid to manage patients' pain, while simultaneously largely ignoring the specific identity of the opioid used.

Contrary to this model, the results presented here indicate that the specific identity of the opioid used for pain management may have vastly differential ramifications for the results of treatment, even in spite of functional equianalgesia. This implies that research should be performed upon individual opioid compounds with regards to not only their analgesic profile but also wide-ranging and diverse functional characteristics which may differ from one another in ways that are unpredictable based solely on chemical features and/or pharmacological characteristics.

This research demonstrates that opioid compounds with functionally equivalent abilities to relieve pain can differ in key ways including, paradoxically, their ability to control pain. This is to say, despite both acute and chronic equianalgesia observed between these three compounds given at the doses and administration routes utilized in the present study, a fundamental difference which was revealed was the differential ability to prevent/control the development of long-term injury-induced pain. To begin with, this result provides compelling, if indirect, evidence that the pain mechanisms mediating acute pain response and those responsible for chronic pain development are dissociable processes.

This result also reveals that different opioid compounds can control the former type of pain equally well but have very different efficacies for the latter type of pain. Considering the prevalence of chronic pain and its resistance to treatment with many

opioid drugs, this finding hints at potential opioid-based pain management strategies for chronic pain which may be significantly more effective than current solutions.

This result also indicates that there are some opioid compounds which should be preferentially used over others for pain treatment despite apparently similar analgesic potencies. This conclusion somewhat directly contradicts the current research and clinical *status quo* which assumes that analgesic potency and risk are intrinsically linked. Future research in this area should examine and characterize additional opioids, with a particular focus on opioids which have normally not been favored because of perceptions that they are pharmacologically 'weak' compounds. The current results indicate that not only can pharmacologically 'weak' compounds such as hydrocodone provide equivalent analgesic potency to 'strong' opioids, they may do so with greater benefits and fewer adverse effects. Additionally, future research should address discovering the mechanisms in which these compounds differ from one another. Elucidation of these differences would greatly enhance our ability to predict which opioids would result in preferable outcomes, as well as greatly aiding in the development of novel opioid compounds which are biased toward beneficial mechanisms and pathways, and away from harmful ones.

It can also be concluded from the current work that different common opioid drugs can have markedly different impacts on the functional sensitivity of the D2DR system. The D2DR system plays an important role in many common psychiatric issues and disorders, including but not limited to depression, bipolar disorder, schizophrenia, alcoholism, addiction, anxiety, and others. Notably, many of these disorders are

comorbid with opioid use and abuse. It is intriguing that different opioids have differential effects on the sensitivity of this system, as it implies that different opioids will carry differential degrees of risk for precipitating or worsening disorders comorbid with opioid use, including other drug and alcohol use and depression. This again calls into question the assumption that opioids with equivalent analgesic potencies have equivalent levels of risk. Rather, some opioids may carry significantly less risk for the precipitation or worsening of comorbid psychiatric disorders both when used medically and recreationally.

Of course, recreational users of opioids are often not tailoring their use patterns based on specific preferred drugs, but rather are using whatever drugs are readily available to them. However, given the current information, it is perhaps advantageous for physicians to prescribe opioids which are safer and less risky to the clinical population. Considering many of the prescription opioids that are abused are diverted from the clinical prescription supply, altering prescription patterns would in turn also impact which opioids are misused by recreational users as well.

The data presented here also indicates that the impact of opioids on the D2DR system is altered by the experience of inflammatory (burn) pain. This lends some empirical evidence to support the common but largely anecdotal understanding that the experience of pain alters, and it is assumed reduces, the rewarding and addictive nature of opioids. It also indicates a drug-specific nature of this pain-dependent alteration of opioid properties. While the evidence here presented supports the belief that the experience of pain alters the rewarding properties of opioids, at least insofar as their

ability to alter the signaling sensitivity of the D2DR receptor system, this does not necessarily imply a *reduction* in reward. Indeed, in the current work, the ability of hydrocodone to alter D2DR behavioral sensitivity may be enhanced by the presence of burn injury pain.

This finding calls for several additional studies which promise to be revealing. An examination of drug-specific differences in reward, using CPP, will be particularly revealing. In addition to drug-specific differences in opioid reward acquisition, an examination of the impact of burn pain on reward will also be interesting, and will give a more direct indication as compared to D2DR behavioral sensitivity. An analysis of additional opioid drugs, especially focusing on 'weak' opioids, is also warranted.

Finally, the drug-specific impact upon gene expression, as observed via 2 parallel techniques, indicates that the drug-specific differences among opioid analgesics are fare more profound than simple pharmacokinetic differences or functional variations. The three compounds investigated here, despite equal degrees of painkilling potency, result in large differences in gene expression. These differences exist both in the number and identity of the genes so altered, as well as the magnitude and sometimes direction of dysregulation in genes which are altered by multiple opioids. Because of the potential for widespread downstream consequences of gene expression dysregulation, this opioid-specific alteration in gene expression is perhaps quite significant.

This finding implies that apparently similar and comparable opioid compounds, with equivalent functional utility for the treatment of pain, may have remarkably different long-term consequences resulting from their use. These long-term

consequences may be difficult to predict, given the complex nature of gene expression changes, plus the extensive interrelations between gene expression, translation, and post-translational functionality which serve as cellular checks against large disruptions in cellular function.

It should be noted that some of the gene transcripts that were observed to be altered by exposure to opioids in this study serve as this type of regulator, indicating that not only is gene expression itself affected by exposure to opioids, but that the regulatory mechanisms intended to prevent or minimize the adverse consequences of such alteration may themselves be disrupted by treatment with opioids. The long-term consequences of this remain to be explored by future work, but may be significant for behavioral and clinical outcomes following opioid use. Future research should explore the significance and impact of each gene and group of genes disrupted by opioid exposure, as well as the potential to mitigate behavioral consequences of opioid exposure by preventing or rescuing gene expression level alterations.

In conclusion, the current work described in this doctoral dissertation provides compelling evidence that there exist wide-ranging and clinically relevant differences among pharmacologically comparable and commonly used opioid analgesic medications. These differences carry implications of differential functional outcomes following exposure to these seemingly comparable compounds. Although the common clinical assumption that these compounds can be rendered equipotent for antinociceptive effects is supported by the evidence presented here, the current results do not support the related assumption that equivalent pain relief capabilities is correlated with similar

negative side effect risks. Indeed, the current work demonstrates that some opioids may have significantly reduced adverse side effects and long-term outcomes, despite equal analgesic potency.

These findings underscore the need for thorough and extensive biomedical and preclinical investigation of specific opioid compounds, as opposed to the conventional research model utilizing a model drug and extrapolating to all chemically similar opioids (e.g., researching morphine and extrapolating the findings to all phenanthrene opioids; investigating fentanyl and extending the findings to all fentanyl-based synthetic derivatives; etc.).

At the time of this writing, opioid use, misuse, and overdose death are rising exponentially in the United States, reaching levels characterized by public health officials as epidemic proportions. This rise in opioid-related adverse outcomes has been driven, in part, by the increasingly common use of opioids to treat pain disorders in the clinical population, as well as illicit synthesis and use of increasingly potent opioids such as fentanyl and its derivatives. However, the choice of which specific opioids to be prescribed has largely not been guided by empirical, scientific, evidence-based research, because of the assumed inseparability of analgesic function from adverse outcomes. In light of the current research, as well as concurrent research being conducted in other labs indicating similar findings, it may be the case that a more careful selection in favor of specific opioid drugs over others can provide similar pain relief outcomes while reducing opioid-associated risks. Much more research is needed to parse out the pros and cons of specific common opioids across a variety of pain modalities, but the current

work indicates that this research investment of time, energy and money is one worth making, as it is likely to bear fruit.

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APPENDIX I

ABBREVIATIONS

Akt	Ak mouse strain thyoma-related factor; also known as PKB
β-Arr2	Beta Arrestin 2
cDNA	Complimentary DNA
CPP	Conditioned Place Preference
D2DR	D2-Type dopamine receptor
DOR	Delta (δ) Opioid Receptor
ERK	Extracellular signal related kinase; a member of the MAPK family
GPCR	G-Protein Coupled Receptor
GRK	G-Protein Coupled Receptor Related Kinase
IL	Interleukin
i.p.	Intraperitoneal
JNK	c-Jun N-terminal kinase; a member of the MAPK family
MAPK	Mitogen Activated Protein Kinase
MOR	Mu (μ) Opioid Receptor
NF-κB	Nuclear Factor Kappa light chain enhancer of activated B cells
NMDA	N-Methyl-D-Aspartate Activated Glutamate Receptors
PKB	Protein Kinase B, also known as Akt
TLR4	Toll-Like Receptor 4
TRPV1	Transient Receptor Potential Vanilloid Type 1

APPENDIX II

PROTOCOLS AND REAGENTS

Homemade ECL (Enhanced Chemiluminescence) Reagent

Reagents:

Luminol
p-Coumaric acid
Tris HCl
H_2O_2
DMSO

Stock Solutions:

0.1 M Tris HCl, pH 8.5
250 mM Luminol in DMSO (light sensitive) 0.443 g in 10 mL.
90 mM p-Coumaric acid in DMSO (light sensitive) 0.1478 g in 10 mL.

*Store all at room temperature, wrap the light sensitive solutions with foil.

Procedure:

Immediately before assay, prepare the reagent.

- To 20 mL Tris HCl, add 44 µL p-coumaric acid, mix by inversion.
- Add 100 µL Luminol, mix by inversion.
- Add 5.5 µL H2O2, mix by inversion.
- Immediately add to blot, let blot develop for 1 minute (may need longer, depending on the strength of the signal.)
- Remove excess, image blot.

3x Laemmli Sample Buffer (Western blot loading buffer)

1.75 mL DDW
3.75 mL 4x Upper Buffer
0.3g SDS
Bromophenol Blue just until visible
3 mL Glycerol

-Freeze in 850 mL aliquots. Immediately before use, add 150 µL DTT or β-

Mercaptoethanol (BME) to denature proteins in sample.

Antibodies for Western Blotting

All primary antibodies were diluted 1:1000 in 5% milk solution. Secondary antibody was diluted 1:5000 in 5% milk solution.

p-ERK (T202/Y204) ..... Cell Signaling Technology, #9101, Lot 23

Total ERK ..... Cell Signaling Technology, #9102, Lot 19

p-Akt (S473) ..... Cell Signaling Technology, #9271, Lot 10

Total Akt ..... Cell Signaling Technology, #9272, Lot 24

Goat anti-rabbit HRP-conjugate ..... Bio-Rad, , #170-5046 Control NB167917

Reagents for PCR & qPCR

Maloney Murine Leukemia Virus Reverse Transcriptase
(M-MuLV RT) ..... New England Biolabs M0253S

RNase inhibitor, murine ..... New England Biolabs, M0314S

Random Hexamers ..... New England Biolabs, S1230S

SYBR® Green JumpStart™ *Taq* ReadyMix™ ..... Millipore Sigma, S4438

APPENDIX III

GENE ACCESSION NUMBERS & PRIMER SEQUENCES

GENE	ACCESSION NUMBER	STRAND	SEQUENCE
AVP	NM_009732.2	Forward (Sense)	TCCGTGGATTCTGCCAAGC
		Reverse (Antisense)	AAGTTTATTTTCCATGCTGTAGGG
β−Actin	NM_007393.5	Forward (Sense)	TCAAGATCATTGCTCCTCCTG
		Reverse (Antisense)	TGTAAAACGCAGCTCAGTAAC
β−Arrestin	NM_001271358.1	Forward (Sense)	TACACACTGGACCCATCAC
		Reverse (Antisense)	ATTCACTCCTTGCGTTCAC
D1DR	NM_010076.3	Forward (Sense)	CCTGTTTTCTGTCCCTGCTTA
		Reverse (Antisense)	GACACAGCTAAAGAGATGACAAAGA
D2DR	NM_010077.2	Forward (Sense)	ACCACTCAAGGGCAACTGTA
		Reverse (Antisense)	ATCCATTCTCCGCCTGTTCA
DARPP-32	NM_144828.2	Forward (Sense)	AGAGGTTAAAGCCAGAGTCC
		Reverse (Antisense)	TTAATGAAGTCCAGCGGTGA
DAT	NM_010020.3	Forward (Sense)	CATTGCCACATCCTCCAT
		Reverse (Antisense)	TAGGCCAGTTTCTCTCGGAA
FoxJ2	NM_021899.3	Forward (Sense)	CCCAAGACTCAGCAGGATAC
		Reverse (Antisense)	AAGTCCCAGTCGAAGTCATC
IRAK1	NM_001177973.1	Forward (Sense)	TGACCCAGAGGCAAAACTCC
		Reverse (Antisense)	CTTAGTTCCACAGAGCACCTCC
JNK2 (MAPK9)	NM_207692.2	Forward (Sense)	ATATCTGGTCAGTGGGTTGC
		Reverse (Antisense)	TGGTCAGTACCTTGGAATATCAC
JNK3 (MAPK10)	NM_001081567.2	Forward (Sense)	CGCCACAAAATCCTCTTTCCC
		Reverse (Antisense)	CTGGACACGGAGTTCCTAGC
JunD	NM_010592.5	Forward (Sense)	GTTGGTTGCGTGTTGAGTG
		Reverse (Antisense)	GGAACAGGAATGTGGACTCG
KCNC3	NM_008422.2	Forward (Sense)	GTCCTCATCTTTGCCACCAT
		Reverse (Antisense)	ATGTCTCCATAGCCCAGTGT
MDM2	NM_010786.4	Forward (Sense)	CTGCAAGCACCTCACAGATT
		Reverse (Antisense)	CCAACGGACTTTAACAACTTCA
Myh6	NM_001164171.1	Forward (Sense)	GACATTGGTGCCAAGAAGATG
		Reverse (Antisense)	GGCAGAGTCGAACGTTTATG
OXT	NM_011025.4	Forward (Sense)	CTTCGCCCTGCCAGTCT
		Reverse (Antisense)	GCTAAAGGTATTCCCAGAAAGTG
OXTR	NM_001081147.1	Forward (Sense)	CTGTTCTCAACCATCCTCGG
		Reverse (Antisense)	AGGAGGGATGCAAACCAATC

GENE	ACCESSION NUMBER	STRAND	SEQUENCE
P53	NM_011640.3	Forward (Sense)	TACCCCACACCCTGTAAGATTC
		Reverse (Antisense)	GGCTTTGCAGAATGGAAGGAA
PDGFRa	NM_011058.2	Forward (Sense)	TCCGGGTATCGGATTTTCTTTG
		Reverse (Antisense)	ATAAGAGCTGGCAGGAGATGAG
Rotatin	NM_175542.3	Forward (Sense)	TCTACTCAAGGGGTGGATAGC
		Reverse (Antisense)	CCATCTCTCCGCCACAATC
SOD1	NM_011434.1	Forward (Sense)	CTCAGGAGAGCATTCCATCATT
		Reverse (Antisense)	ACTTTCTTCATTTCCACCTTTGC
V1aR	NM_016847.2	Forward (Sense)	CGTTCTGAGCATACCACAGTA
		Reverse (Antisense)	GATGAAGGTAGCCCAGCAAT