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Abuse-related Behavioral Effects of Oxycodone in the Mouse and their Modulation by HIV-1 Tat Expression

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASR	acoustic startle response
βArr2	beta-arrestin-2
B6	C57BL/6J, strain of mice
CPP	conditioned place preference test
CNS	central nervous system
DOX	doxycycline
FR	fixed ratio
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
HAND	HIV-associated neurocognitive disorders
HIV	human immunodeficiency virus
i.p.	intraperitoneal
i.v.	intravenous
LA	locomotor activity

MOR	mu-opioid receptor
NLX	naloxone
NMDA	N-methyl-D-aspartate
OXY	oxycodone
pp	prepulse intensity level
PPI	prepulse inhibition of the startle response
REG	regular
RTTA	reverse tetracycline transactivator
SAL	saline
s.c.	subcutaneous
S.E.M.	standard error of the mean
STIM	119 dB startle stimulus
Tat	HIV-1 transactivator of transcription
VTA	ventral tegmental area

Abstract

ABUSE-RELATED BEHAVIORAL EFFECTS OF OXYCODONE IN THE MOUSE AND THEIR MODULATION BY HIV-1 TAT EXPRESSION

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Abuse of prescription opioids has become epidemic and oxycodone is among the most frequently abused of these drugs. Opioid misuse is a risk factor for HIV infection and its chronic use by HIV-infected individuals can be accompanied by worsened progression to AIDS, cellular damage, and behavioral deficits collectively termed “neuroAIDS”. This toxicity is likely attributable, in part, to the interaction of opioids with the neurotoxic HIV-1 Tat protein. The ultimate objective of this dissertation was to characterize the interaction of HIV-1 Tat expression with the abuse-related effects of oxycodone.

Physical dependence, drug self-administration, and sensitization are three classes of phenomena observed in laboratory animals suggested to have relevance to opioid dependency. There have been few reports of oxycodone's physical dependence, self-administration, or its sensitization effects in mice; therefore, the initial objective of

the present studies was to establish methodologies in the mouse to characterize these effects. Subsequently, these methodologies would be applied to examine the effects of HIV-1 Tat expression on these abuse-related phenomena.

A novel escalating dosing regimen (9-33 mg/kg, s.c.) of oxycodone was developed to induce physical dependence in which naloxone dose-dependently (0.1-10 mg/kg, s.c.) increased somatic signs of withdrawal. In other mice administered a similar regimen, precipitated withdrawal effects were observed using the acoustic startle response and its related measure, habituation. These oxycodone regimens also produced evidence of locomotor sensitization. Using a novel oral operant self-administration procedure, C57BL/6J mice volitionally consumed oxycodone solutions (0.056-1.0 mg/ml) under post-prandial conditions to behaviorally-active levels (i.e., produced hyperlocomotion and Straub tail). Subsequently, HIV-1 Tat-expressing mice were examined under these behavioral conditions. HIV-1 Tat-expressing mice showed altered oxycodone abuse-related effects relative to non-expressing mice in that they: (i) increased oral oxycodone self-administration, (ii) had attenuated oxycodone physical dependence-related effects as measured by acoustic startle and habituation, and (iii) had blunted expression of oxycodone locomotor sensitization. Together, these effects are consistent with previous findings of reduced morphine efficacy and dependence in Tat-expressing mice, and suggest that opioid sensitivity is reduced by HIV-1 Tat. Further studies are needed to determine the rate at which opioid sensitivity is altered by HIV-1 Tat expression.

Chapter I: Introduction

1. Prescription Opioid Abuse

The use of opium extends over thousands of years but it was not until the early 1800s when Friedrich Sertürner isolated the poppy plant's active ingredient, morphine, which has since been largely used for the treatment of pain (Brownstein, 1993). Through technological developments and refinement of methodologies for chemical synthesis and purification, more potent and efficacious opioids have been synthesized or semi-synthesized. The growing number of opioids, such as oxycodone, hydrocodone, and fentanyl, has provided alternatives for the treatment of pain but not without possible consequences, such as respiratory depression, constipation, antinociceptive tolerance, and dependence. In more recent decades, the non-medical use and abuse of prescription opioids has dramatically increased, where between the years 1999-2008, the Centers for Disease Control and Prevention found the overdose deaths due to prescription opioids surpassed those due to cocaine and heroin combined (Centers for Disease Control and Prevention, 2011). In 2014, it was estimated that approximately 1.9 million Americans met criteria for prescription painkiller use disorder (Center for Behavioral Health Statistics and Quality, 2015). Another study found the availability as well as the rate of abuse of prescription opioids increased between the years 2002 and

2010 but plateaued from 2010 to 2013, possibly due to the introduction of abuse deterrent reformulations and more stringent rules to limit the number of prescriptions (Dart et al., 2015). These increased opioid abuse patterns undoubtedly poses an increased risk of Human Immunodeficiency Virus (HIV) infection. Despite stronger regulations and physician monitoring of prescription opioid use, national surveillance programs have reported an increase in the rates of heroin abuse and heroin overdose-related deaths which, although correlative, also coincided with the release of extended-release oxycodone in August 2010 (Compton et al., 2016; Dart et al., 2015).

Several demographic features, including geographic regional differences and sex, regarding prescription opioid abuse have been characterized. In the United States, the non-medical use of prescription opioids has been heavily reported in rural areas as opposed to urban areas (Havens et al., 2007; Keyes et al., 2014; Wang et al., 2013; Wunsch et al., 2009). Clinical studies and meta-analyses have reported sex differences in both the opioid analgesic response and the subjective effects of opioids in which females exhibit greater efficacy for treating pain but also experience greater adverse side effects than males (Niesters et al., 2010; Zacny and Drum, 2010). The Nationwide Emergency Department Sample showed that from 2006 through 2010 the majority of emergency room visits due to prescription opioid overdoses were females, further suggesting an important sex difference in opioid response which may in part be attributable to the influence of hormonal variability (Tadros et al., 2015). Mechanisms behind these sex differences, such as the influence of hormones as well as pharmacokinetic and pharmacodynamic differences are being further examined in preclinical research.

2. Oxycodone

2.1. General Pharmacology

Prescription opioids, such as oxycodone, primarily act on the mu opioid receptor to exert antinociceptive and subjective effects (Beardsley et al., 2004; Zacny and Gutierrez, 2003). Mu opioid receptors are located in both the central and peripheral nervous systems and on various cell types, including neurons and glial cells. Mu opioid receptors are one of the three classical opioid receptors (mu, delta, and kappa) and are distributed throughout the brain, including the mesolimbic dopaminergic system which is implicated in food and drug reinforcement, with higher density than other subtypes in certain regions such as the amygdala and thalamus (for review, Le Merrer et al., 2009). Due to their wide distribution, these receptors actively play a role in many physiological processes including stress and immune responses. Mu-opioid receptors (MORs) are seven-transmembrane G protein-coupled receptors (GPCRs) of the Gi/o subtype which, once activated by either endogenous peptides, such as beta-endorphin, or by exogenous MOR agonists, lead to downstream signaling effects including inhibition of adenylyl cyclase and decreased neuronal excitability and neurotransmitter release. However, MORs can be present on interneurons that release gamma-aminobutyric acid (GABA), therefore, by disinhibiting interneurons through MOR activation a downstream increase in dopaminergic activity (Johnson and North, 1992).

MOR agonists, such as morphine and oxycodone, can differ in their pharmacological profile. For example, in *in vivo* studies, oxycodone is reportedly two to three times more potent than morphine for antinociceptive effects and is equipotent to heroin (Beardsley et al., 2004; Curtis et al., 1999; Zacny and Lichtor, 2008; Zhukovsky

et al., 1999). This potency difference is also conserved between controlled-release formulations of oxycodone and morphine (Curtis et al., 1999). In *in vitro* studies, however, oxycodone has a lower binding affinity to the MOR than morphine as well as lower efficacy as determined by the [^{35}S]GTP-γS assay (Peckham and Traynor, 2006; Thompson et al., 2004). These various pharmacological effects support the claim that no two opioids are functionally alike, and may have a large difference in their abuse liability thereby warranting further investigation of their individual abuse-related effects.

First synthesized from thebaine for clinical use in 1917, oxycodone is primarily metabolized via N- and O-demethylation by the cytochrome P450 enzymes, CYP3A and CYP2D6, into noroxycodone and oxymorphone, respectively (Kalso, 2005; Lalovic et al., 2004). The active metabolite oxymorphone has a greater affinity for the MOR than oxycodone; however, the antinociceptive effects of oxycodone are largely mediated by the parent compound rather than its metabolites which may be advantageous for patients with renal impairment (Cleary et al., 1994; Thompson et al., 2004). A greater abundance of the parent compound rather than its metabolites in the brain further supports that oxycodone is responsible for the centrally-mediated effects (Lalovic et al., 2006). The therapeutic actions of the parent compound in oxycodone formulations along with oxycodone's higher oral bioavailability (~60-87%) than morphine (~20%) may, in part, play into the popularity and diversion of oxycodone (Hoskin et al., 1989; Leow et al., 1992; Poyhia et al., 1992). The terminal elimination half-life of oral oxycodone is reported to be approximately 3.5-h in humans as well as in rats, although a lower oral bioavailability is reported in the rat possibly due to a greater first-pass metabolism in the rat as compared to humans (Chan et al., 2008; Lalovic et al., 2006).

2.2. Abuse Liability

As seen with other opioids, in addition to antinociceptive effects, oxycodone use produces positive subjective effects and may result in the development of antinociceptive tolerance, physical dependence, and abuse. As measured by clinical assessments, positive subjective effects of this drug include dose-dependent increases in drug-liking, “feeling high”, and drug-wanting (Comer et al., 2008; Zacny and Gutierrez, 2003). The oral route of administration is the most widely used and favored among prescription opioid abusers, although other common routes include intravenous as well as insufflation (Gasior et al., 2016; Kirsh et al., 2012). Oxycodone is similar to other opioids, including morphine and hydrocodone, in producing positive subjective effects and there is reportedly little difference in their ability to do so (Stoops et al., 2010; Zacny and Gutierrez, 2009). Moreover, oxycodone can also similarly produce unpleasant side effects including constipation, dysphoria, and respiratory depression. However, oxycodone may differ from morphine in its effect on expression of various genes in hippocampal and striatal regions that may facilitate synaptic plasticity and abuse-related behaviors (Mayer-Blackwell et al., 2014; Zhang et al., 2009). It has also been suggested that oxycodone differs from morphine in rodents by differentially altering D2-like dopamine receptor responses as well as dopamine transmission as measured by fast-scan cyclic voltammetry in the nucleus accumbens (Emery et al., 2015a; b; Vander Weele et al., 2014). Additionally, unlike morphine, naloxone has been shown to precipitate similar degrees of withdrawal in both wildtype and beta-arrestin-2 knockout mice made dependent on oxycodone via osmotic minipumps (Raehal and

Bohn, 2011). Together, these observations suggest that MOR agonists can differ and these differences may influence their abuse-related effects.

Chronic opioid exposure and withdrawal from opioids affects the individual across multiple biological levels. Upon precipitating withdrawal in morphine-dependent rats via intracerebroventricular administration of methylnaloxonium, a derivative of the mu-opioid antagonist naloxone, symptoms of physical withdrawal were most robust when the antagonist was administered to the locus coeruleus and the periaqueductal gray region (Maldonado et al., 1992). The ventral tegmental area (VTA) and the nucleus accumbens, which are part of the mesolimbic dopaminergic pathway, have also been strongly implicated in drug reinforcement (Koob, 1992). After chronic morphine exposure to the VTA in rats, a reduction in neuronal area was found in only dopaminergic neurons despite a lack of change in total number of dopaminergic neurons in the VTA (Sklair-Tavron et al., 1996). In prescription opioid-dependent humans, volumetric loss in the amygdala, decreased anisotropy in axonal pathways of the amygdala as measured by diffusion tensor imaging, and decreases in functional connectivity in the amygdala as well as the nucleus accumbens have all been found (Upadhyay et al., 2010; Younger et al., 2011).

In the dorsal striatum of adult C57BL/6J mice, extended access (4-h) to intravenous (i.v.) oxycodone self-administration resulted in a significant decrease in mRNA levels of $GABA_A$, subunits beta 2 and alpha 1, but a significant increase in GABA, subunits rho 1 and 2 mRNA levels (Zhang et al., 2014). These effects were found in conjunction with escalated oxycodone consumption levels across 14 days of extended access to i.v. self-administration. The dopaminergic system in the VTA and

striatal regions has been found to be altered in mice by chronic oxycodone exposure (specifically for dopamine release, D1 and dopamine transporter mRNA expression) and varied depending on if exposure occurred during adolescence or adulthood (Sanchez et al., 2016; Zhang et al., 2009). Together, these biological changes after chronic opioid administration and withdrawal may be important biological factors that influence further misuse.

3. Preclinical Assessment of Abuse-related Effects

To assess the potential abuse-related effects of a drug, preclinical researchers can use certain procedures that have been shown to have high reliability and face validity: (i) self-administration, (ii) physical dependence, and (iii) locomotor sensitization. Together, these assays provide a profile for a drug's abuse-related effects and the environmental and biological determinants of these reinforcing effects can then be evaluated. Preclinical assessment of oxycodone's abuse-related effects using these procedures is limited in the mouse, but the current literature is reviewed below.

3.1. Self-administration

Self-administration can be defined as the volitional intake or consumption of a drug, and can be measured via different routes of administration such as the intravenous or oral routes. Oxycodone has been demonstrated to serve as a positive reinforcer in intravenous self-administration studies in rats and mice (Beardsley et al., 2004; Mavrikaki et al., 2017; Neelakantan et al., 2017; Zhang et al., 2015b; Zhang et al., 2014; Zhang et al., 2009) in which an operant response was required for its delivery, although the oral route has not yet been investigated. In fact, the oral self-administration

of any opioid in the mouse in which an operant contingency was required to obtain drug delivery has been confined to the potent benzimidazole opioid, etonitazene (Elmer et al., 1995). Previous preclinical studies using the oral route have examined oxycodone's antinociceptive properties or other behaviors, such as in learning and memory tasks, in which oxycodone was administered to laboratory mice or rats via oral gavage (Davis et al., 2010; Nozaki et al., 2006). Gavage techniques to study oral oxycodone's effects on behavior, pharmacokinetics, or dopamine receptor responses (e.g., Chan et al., 2008; Emery et al., 2015b) have been useful as they precisely control the level of oxycodone exposure across subjects. However, the use of volitional oral consumption entailing an operant response (i.e., oral self-administration) is imperative to assess oxycodone's effects related to its abuse liability because, unlike experimenter-administered oxycodone, the response requirement to obtain oxycodone access can be manipulated to examine motivational levels, the neuropharmacological and genetic effects produced by self-administered versus experimenter-administered opioid can markedly differ (Jacobs et al., 2003), and self-administered oxycodone would likely be better predictive of actual oxycodone abuse.

3.1.1. The use of post-prandial conditions in oral self-administration

In preclinical studies of oral self-administration, one technique to induce consumption of the liquid delivery uses food-induced (i.e., post-prandial) conditions in typically food-restricted subjects, which amounts to the feeding of a daily allotment of chow to the subject prior to the operant self-administration session (Campbell and Carroll, 2000). This method is used to induce thirst which consequentially assists in shaping behavior of the animal to allocate behavior (i.e., responding on an "active"

operant lever) to obtain a liquid delivery. After acquisition of self-administration behavior, the drug is thought to be reinforcing if it: (i) maintains self-administration after removal of post-prandial conditions (i.e., chow is given after operant sessions) and (ii) achieves greater levels of deliveries than those of water. The oral route is particularly unique, however, insofar as the consumption of a drug can be influenced by other factors such as the taste and palatability of the drug-containing drinking solution as well as the thirst and hunger state of the subject prior to operant session. This makes for interpreting the reinforcing efficacy of a drug in oral self-administration studies particularly challenging. Other tests, such as progressive ratio tests, can be used in conjunction to examine the oral reinforcing efficacy of a drug. Progressive ratio tests measure the degree to which a laboratory animal or human will “work” for a delivery or dose of a drug (Richardson and Roberts, 1996) and has been used previously for measuring abuse liability of oral oxycodone in humans (Babalonis et al., 2013). In preclinical studies, this is done by progressively increasing the ratio requirement (e.g., the number of active lever presses) needed to receive a single liquid delivery. The “breakpoint” is referred to as the final ratio requirement completed by the subject to receive at least one liquid delivery, prior to the subsequent ratio step in which no deliveries were obtained. By comparing the breakpoint averages for drug versus water, the drug is thought to serve as a positive reinforcer if it maintains a higher breakpoint average than water.

3.2. Physical Dependence

In addition to self-administration, physical dependence is another abuse-related phenomenon that can be measured preclinically. Here again few preclinical reports

have characterized the physical dependence effects of oxycodone, especially in the mouse. Oxycodone has been reported to dose-dependently suppress somatic signs of withdrawal in morphine-dependent rhesus monkeys suggesting cross-dependency to morphine (Beardsley et al., 2004). In addition, characteristic somatic signs of opioid withdrawal (e.g., jumping, body shakes, and diarrhea) have been reported in ICR mice administered a subcutaneous slow-release emulsion mixture of oxycodone to a similar degree as those produced by morphine (Mori et al., 2013). Oxycodone delivered to rats via osmotic minipumps resulted in substantial weight loss upon termination of drug administration after pump removal indicative of physical dependence (Hutchinson et al., 2009). The authors reported that they did not analyze the somatic signs of oxycodone withdrawal because of the "severity of … withdrawal" it produced (Hutchinson et al., 2009). Disruption of operant behavior can also be used to infer a type of “behavioral dependence” even when not accompanied by somatic signs of withdrawal (Schuster and Thompson, 1969). Naloxone-precipitated withdrawal disruption of lever pressing maintained by intracranial self-stimulation in rats indicative of behavioral dependence has also been reported (Wiebelhaus et al., 2016). Disruption of another behavior, the acoustic startle response, has been characterized to be an additional measure of opioid dependence, specifically morphine, as discussed below.

3.2.1. Acoustic Startle as Measure of Dependence

The acoustic startle response (ASR) is the whole-body reflexive response to a loud acoustic stimulus, typically in preclinical studies will be a brief (millisecond) exposure to a 120 dB sound. A special advantage of this non-invasive procedure is its translational value across species in addition to not requiring pre-training and can be

tested repeatedly, although conflicting reports suggest the possibility of some habituation with repeated testing (Abel et al., 1998; Braff et al., 2001; Cadenhead et al., 1999; Geyer and Dulawa, 2003; Plappert et al., 2006). Moreover, the ASR and its related measures, habituation and prepulse inhibition, is a useful laboratory preclinical assay as it is fairly predictive of clinical startle effects to drugs (for review, Braff et al., 2001).

The ASR has been shown to be altered by opioids, specifically morphine. Chronic morphine administration to rats resulted in increases in ASR, and naloxone-precipitated withdrawal resulted in significant decreases in ASR (Mansbach et al., 1992). Other studies have found naloxone-precipitated withdrawal from morphine in rats resulted in significant increases in ASR (Harris and Gewirtz, 2004). Interestingly, in rats trained to self-administer morphine, ASR measured one week after withdrawal was inversely correlated with the amount of morphine self-administered in that low intake rats showed increased ASR, and high intake rats showed reduced ASR (Le et al., 2014). These differences in withdrawal effects on startle have also been noted in preclinical ethanol studies (Chester and Barrenha, 2007; Rassnick et al., 1992; Slawecki et al., 2006). In rats, cessation of cocaine self-administration or withdrawal from nicotine did not affect ASR (Mansbach et al., 1994; Wilmouth and Spear, 2006), although the related measure prepulse inhibition has shown complex nicotine withdrawal effects in mice (Semenova et al., 2003; Stoker et al., 2008), suggesting ASR is not sensitive to the dependence-related effects of all drugs of abuse. Together, these results converge to the conclusion that the acoustic startle procedure may be

advantageous to use for measuring opioid dependence-related effects that bypass the observer subjective effects found in measures of somatic signs of withdrawal.

In addition to startle, sensorimotor gating, defined as the process of filtering out excessive stimuli, can be evaluated using the startle apparatus. Prepulse inhibition of the startle reflex (PPI) is an operational measure of sensorimotor gating that is mediated by cortico-striato-pallido-pontine circuitry and can be defined as the inhibition of a whole-body motor startle reflex when a small acoustic stimulus (prepulse) is presented before (usually in milliseconds) a loud acoustic stimulus (STIM/pulse). PPI has been widely used for preclinical models of schizophrenia and Alzheimer's disease; moreover, drugs of abuse such as amphetamine and ketamine which alter dopamine neurotransmission decrease PPI, suggesting a role of the dopaminergic system in information processing, however not all drugs of abuse have been fully characterized (Geyer et al., 2001). Opioids are reported to have negligible acute effects on PPI; however, pretreatment of naloxone, an opioid antagonist, blocks amphetamine's PPI-impairing effects and, depending on the length of exposure, morphine has diverse effects on both startle and PPI, suggesting the complexity the role the opioid system has in sensorimotor gating that warrants further characterization (Harris and Gewirtz, 2004; Meng et al., 2010; Swerdlow et al., 1991).

3.3. Locomotor Activity

In addition to self-administration and physical dependence, locomotor activity and its sensitization is another procedure to assess a drug's preclinical abuse-related effects. Locomotor sensitization has been shown to correspond to the reinforcing properties of multiple drugs of abuse (Robinson and Berridge, 1993). The endogenous

opioid system has been implicated in the expression of locomotor sensitization of various drugs of abuse, such as cocaine, nicotine, and methamphetamine (Chiu et al., 2006; Hummel et al., 2004; Shen et al., 2010; Yoo et al., 2004). Administration of MOR agonists results in increased locomotor activity and repeated exposure results in further increases in locomotion, or locomotor sensitization. Oxycodone has previously been shown to increase locomotor activity in rats and mice and its repeated exposure results in locomotor sensitization (Collins et al., 2016; Leri and Burns, 2005; Liu et al., 2005; Niikura et al., 2013; Zhang et al., 2016). The active component of the herb *Corydolis yanhusuo*, l-tetrahydropalmatine, was reported to block both the development and expression of oxycodone locomotor sensitization in mice (Liu et al., 2005). In conjunction with unpublished findings of l-tetrahydropalmatine reducing oxycodone-induced increases of striatal extracellular dopamine in the rat, the authors suggested that l-tetrahydropalmatine was blocking oxycodone's effects via a D1- or D2-linked mechanism although this compound has also shown to inhibit pro-inflammatory cytokine expression (Oh et al., 2010; Zhang et al., 2015a). Thus, further characterization of MOR agonist-induced locomotor sensitization can assist determining its underlying determinants.

4. Mouse Models to Investigate Opioid Interactions

The use of mouse models in preclinical research allows for the investigation of a plethora of opioid interactions to characterize mechanisms of opioid dependence and tolerance. Genetic manipulations in mice have been carried out to examine the role of beta-arrestin-2, alpha3beta4* neuronal nicotinic receptors, and AMPA-type glutamate receptors in opioid tolerance and dependence (Bohn et al., 2003; Muldoon et al., 2014;

Raehal and Bohn, 2011; Vekovischeva et al., 2001). Typically for these studies, the C57BL/6J mouse strain is utilized as a background strain for its large breadth of genomic data and its popularity in knockout studies. This strain of mice is particularly sensitive to opioids as compared to DBA counterparts as shown by greater potentiation by morphine in intracranial self-stimulation, greater intravenous morphine self-administration, and a higher preference for morphine in saccharin solution over tap water (Elmer et al., 2010; Horowitz et al., 1977). Additionally, this strain has been observed to consistently display characteristic effects of opioid dependence (e.g., naloxone-precipitated jumping) across various methods of morphine physical dependence, although at times with less intensity than some other strains such as Swiss-Webster mice (Kest et al., 2002).

One laboratory technique of genetic manipulation in the mouse is the use of the tetracycline ("Tet")-On system of expression, in which a gene is activated to produce gene products of interest via exposure to a tetracycline or its derivatives such as doxycycline. The Tet-On system is advantageous over murine knockout techniques as it is a reversible, conditional expression system thereby allowing the normal development of the mouse without potential genetic compensation as well as having the ability to parametrically control gene expression. The use of this system allows the investigation of neurological diseases and the interaction with potential pharmacotherapies or drugs of abuse. One mouse model of interest is a mouse model of neuroAIDS through doxycycline-inducible expression of the neurotoxic HIV-1 transactivator of transcription, "Tat", protein. In this mouse model, there is overwhelming evidence to show opioids

have a worsening effect of Tat-induced consequences at multiple biological levels, as discussed below.

4.1. NeuroAIDS

NeuroAIDS is the culmination of symptoms that affect the CNS after systemic HIV-1 infection. The result in a subset of patients is the development of HIV-associated neurocognitive disorders, or HAND. HAND can range in severity, with the most severe form called HIV-associated dementia, and is characterized by motor and/or behavioral impairments leading to difficulties in daily functioning which may result in impairments in: attention-concentration, information processing, as well as learning and memory (Antinori et al., 2007). The advent of highly active antiretroviral therapy in the 1990s led to a reduction of the incidence of HIV-associated dementia from 16% to 5%; however, the prevalence of the development to HAND persists (Harezlak et al., 2011; Heaton et al., 2010; Nath and Sacktor, 2006). The challenge of treating HAND persists as co-morbidities with drug abuse and other mental disorders may exist, as well as a high incidence rate of progression to more severe subtypes of HAND in previously asymptomatic individuals (Alfahad and Nath, 2013; Robertson et al., 2007; Sacktor and Robertson, 2014).

It has been suggested that the expression of neurotoxic viral proteins are the underlying cause of the impairments observed. The two proteins that have garnered the most evidence for these effects are the envelope glycoprotein gp120 and the transactivator of transcription, Tat, protein. These viral proteins have received attention as their expression or circulating levels in the bloodstream are not affected by antiretroviral treatment. Moreover, the ability for these proteins to form reservoirs in the

brain, where antiretrovirals are mostly unable to penetrate, may allow for further damage to be caused. The focus of certain studies within this dissertation surrounds HIV-1 Tat protein expression.

4.1.1. HIV-1 Tat

The HIV-1 transactivator of transcription, or Tat, protein is a viral neurotoxic protein that regulates HIV-1 transcription and replication. Tat can be secreted by intact, infected cells, circulate in the bloodstream at nanomolar concentrations, bind to various target cells, cross the plasma membrane to affect host gene expression and trigger various responses (for review, Debaisieux et al., 2012). HIV-1 Tat protein exposure causes damage to dopaminergic neurons, increases pro-inflammatory cytokine production potentially through an NF-κB-mediated pathway, and disrupts the integrity of the blood-brain barrier (Andras et al., 2003; Buonaguro et al., 1992; El-Hage et al., 2008; Kim et al., 2003; Nath et al., 2000; Nookala and Kumar, 2014). Additionally, Tat can induce: apoptosis, gray matter density reductions in various brain regions in mice, and increases in excitability of enteric neurons (Carey et al., 2013; New et al., 1997; Ngwainmbi et al., 2014).

HIV-1 Tat expression in rodents has demonstrated effects on behavioral measurements as well. HIV-1 Tat exposure in rodents has been shown to impair learning, memory, and motor ability, as well as increase anxiety and alter drug effects, such as from cocaine and morphine (Carey et al., 2012; Fitting et al., 2012; Hahn et al., 2013; Harrod et al., 2008; Li et al., 2004; Paris et al., 2013). HIV-1 Tat expression in female and male mice has shown to decrease locomotor activity, albeit to a greater degree in males, as well as disrupt cocaine-induced sensitization in ovariectomized rats

receiving intra-accumbal microinjections of Tat (Hahn et al., 2015; Harrod et al., 2008). Additionally, deficits in PPI have been reported in HAND patients (Minassian et al., 2013). HIV-1 proteins have been studied in sensorimotor gating procedures using preclinical rodent models. For example, PPI deficits have been observed in female HIV-1 transgenic rats generated to express seven of the nine viral proteins, in neonatal or adult rats that received intra-hippocampal Tat injections, and in Tat-expressing mice (Fitting et al., 2006a; Fitting et al., 2006b; Moran et al., 2013; Paris et al., 2015). Moreover, neonatal gp120 intra-hippocampal injections in rats altered other sensorimotor gating-related measurements such as reduced latency to the peak of the acoustic startle response (Fitting et al., 2007). As mentioned previously, HIV-1 Tat has been shown to directly alter behavioral effects of drugs of abuse and the focus of this dissertation is Tat's interactions with opioids.

4.1.2. HIV-1 Tat interacts with opioids

There is evidence linking HIV-1 infection and the modulation of the opioid system to translate to changes in behavioral outcomes, such as antinociception and abuse-related behaviors. It is known that opioid abuse can increase the risk of HIV-1 infection through needle-sharing, but opioids have also been reported to accelerate HIV-1 infection to AIDS and worsen associated impairments (Arora et al., 1990; Donahoe and Vlahov, 1998). In non-human primates infected with simian immunodeficiency virus (SIV), chronic morphine was found to worsen performance on behavioral measures such as motor skill (Marcario et al., 2016). Despite this, opioids are still considered as a major line of therapy for treating HIV-associated neuropathic pain. This is concerning considering in healthy human volunteers who received administration of antiretroviral

medication, ritonavir or the combination of ritonavir and lopinavir, increased oxycodone plasma concentrations and an increased elimination half-life of oral oxycodone was observed, which may lead to accidental overdose or undertreatment of pain in HIV-infected individuals receiving both opioids and antiretroviral therapy (Nieminen et al., 2010). Moreover, through the synthesis and characterization of bivalent ligands, there is evidence to suggest the existence of dimerization of MORs with others such as the chemokine receptor 5 (CCR5), an important co-receptor for HIV-1 infection, which may subsequently affect pain modulation, opioid sensitivity, as well as HIV-1 neuropathology (Akgun et al., 2015; Arnatt et al., 2016; Yuan et al., 2013).

The worsened impairments by opioids are suggested to be due to exacerbation of neurotoxic effects of the viral proteins gp120 or Tat leading to microglial activation, neuroinflammation, and neuronal damage which may accelerate neurocognitive deficits (El-Hage et al., 2005; Fitting et al., 2010; for review, Hauser et al., 2012; Hauser et al., 2009; Hu et al., 2005; Zou et al., 2011). Tat expression and morphine co-exposure worsens oligodendrocyte survival, increases cellular expression of opioid receptors, and prevents down regulation of cell surface opioid receptors in microglia (Hauser et al., 2009; Turchan-Cholewo et al., 2008). Moreover, morphine exposure significantly worsens Tat-mediated reduction of spine density in striatal medium spiny neurons, increases glial activation, increases neuronal loss, and increases pro-inflammatory chemokine/cytokine production (Bruce-Keller et al., 2008; El-Hage et al., 2008; El-Hage et al., 2005; Fitting et al., 2010; Gurwell et al., 2001). Similar effects have been observed with the MOR agonists methadone and buprenorphine, but to varying degrees to where morphine showed the most robust effects (Fitting et al., 2014). Finally, HIV-1

Tat has been shown to interact with cocaine to potentiate its abuse-related behavioral effects and alter morphine efficacy as measured by antinociception and rotarod performance assays (Fitting et al., 2012; Harrod et al., 2008; Paris et al., 2014a). Despite this, there are no studies examining the abuse-related behavioral effects of HIV-1 Tat expression with the clinically relevant MOR agonist, oxycodone. Moreover, given oxycodone's different pharmacological profile than that of morphine, characterization of Tat's effects on oxycodone's abuse-related effects is needed to better clarify its interactions with opioids.

5. Rationale

Drug self-administration, physical dependence, and locomotor sensitization are effects pertinent to the abuse-related properties of drugs that can be studied in laboratory animals. Oxycodone's preclinical abuse-related effects have been minimally characterized, and in order to investigate potential interactions of HIV-1 Tat protein with this opioid's effects, development of new methodologies and further characterization of oxycodone's effects are needed. New methodologies would also facilitate future evaluation of potential pharmacotherapies and mechanisms of oxycodone dependence.

The establishment of oxycodone oral self-administration methodology is critical for evaluating determinants of oxycodone's abuse through the oral route especially considering the use of this route clinically. Self-administration requires volitional drug consumption. HIV-1 Tat-expressing mice have not been examined under conditions involving the volitional consumption of any opioid. Therefore, developing oxycodone oral self-administration methodologies would enable examinations of the interactions of oxycodone and mouse models of diseases, such as neuroAIDS.

There have been few published reports describing regimens to examine oxycodone physical dependence in the mouse and most have used invasive techniques, such as implantation of osmotic mini-pumps. Moreover, in previous studies physical dependence upon oxycodone has been inferred by subjective measurements such as by observer counts of somatic signs of withdrawal. Disruptions of the acoustic startle response has been used previously to measure withdrawal effects indicative of morphine dependence in rodents, but has not been explored with other opioids, including oxycodone. Use of this objective, quantifiable dependent measure to infer oxycodone dependence-related effects would eliminate potential observer variability and bias, and facilitate analysis. Moreover, this would provide a rapid procedure to examine the interactions of HIV-1 Tat on oxycodone dependence-related effects as well as for other applications such as to screen potential pharmacotherapies.

Locomotor activity is typically increased in rodents after mu-opioid agonist administration and opioids can generate locomotor sensitization. Locomotor sensitization has been associated with motivational sensitization and the abuse liability of drugs. Further investigation of oxycodone's effects on locomotor activity and its sensitization is needed to better understand determinants of its use. Importantly, characterization of the effects of HIV-1 Tat expression on oxycodone-induced locomotor sensitization is important to determine how they interact. This might then assist in determining the underlying mechanisms behind opioid's deleterious effects in HAND-related impairments and serve as guidance for physicians to express further caution when prescribing opioids as a line of therapy for pain in HIV-infected individuals.

6. Hypothesis

There are two major objectives of this dissertation. One objective is to establish methodologies in order to further evaluate oxycodone's abuse-related effects in the mouse. A second major objective is to use these methodologies to investigate oxycodone's interactions with HIV-1 Tat expression in the doxycycline-inducible, HIV-1 Tat-expressing mouse. Opioid misuse is a risk factor for HIV infection and its chronic use by HIV-infected individuals is accompanied by worsened progression to AIDS, cellular damage and behavioral deficits. HIV-1 Tat has been shown to alter the abuse-related effects of other drugs, such as cocaine, and morphine has shown to interact with HIV-1 Tat to worsen neuronal pathology. Therefore, the central hypothesis of this dissertation is that long-term co-exposure to oxycodone and HIV-1 Tat in mice will augment the abuse-related behavioral effects of oxycodone.

7. Research Approach

1) Establish oxycodone's oral self-administration in C57BL/6J mice
2) Identify regimens of oxycodone administration that will induce physical dependence in C57BL/6J mice
3) Evaluate the acoustic startle response as a measure of oxycodone dependence in C57BL/6J mice
4) Evaluate the effects of HIV-1 Tat expression on oral oxycodone self-administration and dependence as measured by acoustic startle
5) Determine the effect of HIV-1 Tat expression on acute and chronic oxycodone-induced hyperactivity and opioid locomotor sensitization

Chapter II: Oxycodone physical dependence and its oral self-administration in C57BL/6J mice[1]

1. Introduction

The oral route of administration is the most commonly used route for the use and misuse of the highly abused prescription opioid oxycodone (Kirsh et al., 2012). Oxycodone differs in its pharmacological profile from morphine in several manners, including its high oral bioavailability, and therefore the mechanisms behind oxycodone dependence may differ. At present, the only known report of an oral opioid self-administration procedure in mice lies with the highly potent opioid etonitazene (Elmer et al., 1995). Therefore, the purpose of the present study was to establish an oral oxycodone operant self-administration procedure in C57BL/6J mice to enable further investigations of oxycodone's abuse-related effects and their treatment using a route of administration that coincides with a popular medical and nonmedical route of its administration. Another objective of this study was to establish a regimen for reliably and efficiently inducing physical dependence upon oxycodone in C57BL/6J mice, and to determine the sensitivity of dependence to precipitated withdrawal. Together, these novel procedures and regimens would define critical methodology in which to later lead

[1] Some content in Chapter II is adapted from *Eur J Pharmacol*, 2016,**789**:75-80

to the investigation of abuse-related behavioral interactions of HIV-1 Tat expression and oxycodone.

2. Methods

2.1. Subjects

Male C57BL/6J mice were obtained at approximately 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and were allowed to acclimate to the vivarium for at least one week prior to commencement of training and testing. Mice were housed in an AALAC-accredited animal facility, kept on a 12-h/12-h light/dark cycle (lights on from 06:00 to 18:00 hours), and given water *ad libitum*. Mice in oral self-administration studies were provided daily allotments of chow (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories Inc., Indianapolis, IN), sufficient to maintain them at 85% of their free-feeding weight, whereas mice in physical dependence procedures were given *ad libitum* access throughout the study. All procedures were conducted during the light phase and were in accordance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press, 2011), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Oral operant self-administration

2.2.1. Apparatus

Sixteen mouse operant chambers (MED Associates, Inc., St. Albans, VT) enclosed in sound- and light-attenuating cubicles equipped with a viewing peep hole

were used for this study. Each operant chamber contained a house light mounted on the rear wall, a Sonalert® tone-generating device, and panels of cue lights mounted above two response levers between which was positioned a well into which a drinking cup was positioned. Vendor-supplied drinking cups were replaced with fabricated dipper cups into which were soldered stainless steel liquid delivery tubes. Attached to each stainless steel delivery tube was silicone tubing (0.79 mm ID/ 3.99 mm OD; Helix Medical, Carpinteria, CA) that was routed behind the operant chamber and attached to a syringe that when compressed by a Razel Model R-ES syringe infusion pump (Razel Scientific Instruments, St. Albans, VT), delivered 20 ul of liquid. Recording of lever presses, activation of house and cue lights, sonalerts, and syringe pumps were accomplished via computer-controlled circuitry and software (MED-PC IV, MED Associates, Inc., St. Albans, VT).

2.2.2. Procedure

The overall procedure was adapted and modified as previously described by Meisch and collaborators that had been used to establish the highly potent opioid, etonitazene, as an oral reinforcer in rats and mice (Beardsley and Meisch, 1981; Elmer et al., 1995; Meisch and Kliner, 1979). Training and testing proceeded according to the following phases: 1) Post-prandial induction of water reinforcement; 2) Post-prandial induction of increasing concentrations of oxycodone; 3) Maintenance of OXY consumption without prandial induction.

2.2.3. General training and testing conditions

Mice were trained and tested daily during 3-h experimental sessions that began each day between 11:00 and 11:45 hours. Mice were transported from the vivarium to their testing rooms in their home cages, and weighed. During the 1.5-h period immediately prior to the experimental session, daily portions of chow or access to water bottles were provided or not depending upon the phase of the study (see below).

2.2.4. Phase I: Post-prandial induction of water reinforcement

Daily chow allotments were provided in the home cages during the 1.5-h pre-session period to induce thirst. Any uneaten chow was placed on the floor of the operant chambers during experimental sessions, and any uneaten chow at the end of the session was returned to the mice when returned to their vivarium home cages. Mice were initially trained to press the right lever reinforced with deliveries of water according to a fixed-ratio 1 (FR1) reinforcement schedule, with the ratio requirement progressively increasing to a FR4 as individual performance permitted. Presses of the left lever prior to completion of the fixed ratio contingency reset the ratio requirement, but otherwise were without scheduled consequences. At the initiation of each liquid delivery, the Sonalert® sounded and the cue lights above the right-side lever were illuminated for 6 s. During the 6-s reinforcement period, lever presses were not counted toward completing the FR4 contingency, but were recorded.

2.2.5. Phase II: Post-prandial induction of increasing concentrations of oxycodone

After performances of the mice had stabilized in which there were no increasing or decreasing trends in the number of water deliveries across three consecutive

experimental sessions, water was replaced with increasing concentrations of oxycodone aqueous solutions (0.056, 0.1, 0.3, 0.56, and 1.0 mg/ml) under FR4 reinforcement contingencies and post-prandial conditions. Five experimental sessions were conducted at each concentration before advancing to the next phase. Testing was completed in an uninterrupted order up to 0.56 mg/ml that was interrupted by a brief winter holiday (1 day) after which the mice were put through a re-training period and tested at the highest concentration, 1 mg/ml, before moving onto the next phase.

2.2.6. Phase III: Maintenance of OXY consumption without prandial induction

Deliveries of 1 mg/ml oxycodone solutions continued to be available according to FR4 reinforcement contingencies. Uneaten pre-session chow was no longer provided during experimental sessions, but instead was given post session in the home cages. After five experimental sessions had occurred, pre-session feedings were reduced to 50, 25, and finally 0% of the total daily food allotment with each reduction in effect for five consecutive experimental sessions. Any uneaten chow, and the complement to provide 100% of their total daily food allotment, was provided after experimental sessions in the home cages. Mice were then maintained on 1 mg/ml OXY during test sessions without prior prandial induction.

2.3. Physical dependence

In separate groups of male C57BL/6J adult mice, oxycodone was administered subcutaneously for eight days with increasing doses of oxycodone of 9, 17.8, 23.7, and 33 mg/kg b.i.d. (~7-h separating injections) on days 1–2, 3–4, 5–6, and 7–8, respectively, and then on the morning of the 9th day was administered 33 mg/kg

oxycodone followed 2-h later with an injection of either 0.1, 1, 3 or 10 mg/kg s.c. naloxone. A separate group of mice was administered saline instead of oxycodone for eight days, and were challenged with 10 mg/kg s.c. naloxone on Day 9. Immediately following naloxone injections, mice were individually placed in Plexiglas cages and were observed and scored for manifestation of somatic signs of withdrawal including the total number of jumps, wet dog shakes, paw tremors, backing, ptosis and diarrhea for 30-min using methods previously reported in testing morphine-dependent mice (Muldoon et al., 2014). Changes in body weight (g) immediately before and 30-min after naloxone injections were also recorded. All testing was conducted in a blind manner.

2.4. Drugs

Oxycodone HCl (Mallinckrodt Inc., St. Louis, MO) was initially prepared in an aqueous sterile stock solution of 10 mg/ml for self-administration studies, which was then diluted with deionized water to make working solutions for oral self-administration tests of 0.056, 0.1, 0.3, 0.56, and 1.0 mg/ml. For physical dependence studies, oxycodone was prepared in a sterile stock solution of 10 mg/ml in non-heparinized 0.9% saline before diluting in sterile saline to make working solutions in the following concentrations: 0.09, 1.78, 2.37, and 3.3 mg/ml. Naloxone HCl (Sigma-Aldrich, St. Louis, MO) was prepared in sterile 0.9% saline to make working solutions in the following concentrations: 0.01, 0.1, 0.3, and 1 mg/ml. All injections were given at a 10 ml/kg injection volume.

2.5. Data analysis

For self-administration tests, numbers of liquid deliveries as well as active and inactive lever (i.e., non-reinforced) presses were recorded. A two-tailed, paired t-test was used to compare number of liquid deliveries before and after complete removal of pre-session feeding. For physical dependence tests, somatic signs and body weight changes indicative of withdrawal were scored quantitatively by a blinded research assistant and used as dependent measures. Individual one-way ANOVA tests were conducted to analyze somatic signs of withdrawal. All data were analyzed and graphed using microcomputer software (Prism 6 for Windows, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if $p<0.05$.

3. Results

3.1. Oral operant self-administration

3.1.1. Post-prandial induction of oxycodone self-administration

Mice learned to lever press reinforced with water delivery under post-prandial conditions before introduction of oxycodone availability (Fig. 1, empty symbols). Little change in the number of lever presses (Fig. 1A) or in the number of liquid deliveries (Fig 1B) occurred when 0.56 and 0.1 mg/ml of oxycodone were available. With further increases beyond 0.1 mg/ml in oxycodone concentration, numbers of reinforced lever presses and the number of deliveries obtained decreased. Numbers of presses of the left-side (unreinforced) lever were low, and unsystematically related to oxycodone concentration, and never overlapped with the numbers of right-side (reinforced) lever

presses (Fig. 1A). Despite decreases in liquid deliveries with increases in oxycodone concentration, estimated OXY consumption (mg/kg body weight) increased (Fig. 2). Behavioral signs characteristic of opioid-like effects in mice, such as hyperlocomotion and presence of Straub tail, were observed at the two highest oxycodone concentrations in routine observations during and after test sessions. In addition, during hyperlocomotion events, mice were occasionally observed pressing the reinforced lever without liquid consumption before re-commencing rapid, circular movements within the test chamber.

3.1.2. Oxycodone self-administration is maintained after withdrawal of prandial induction

After completing concentration-response tests during post-prandial oxycodone induction, test solutions were maintained at 1 mg/ml OXY for the remainder of the study. Pre-session feedings were then gradually decreased until all daily food allotments were given following test sessions in the home cages. As pre-session feeding was reduced, self-administration of OXY was maintained, and increased once all pre-session feedings were withdrawn (Fig. 3), although this increase was not statistically significant once pre-session feedings were completely withdrawn ($t=2.575$, $df=4$, $p=0.0616$).

3.2. Physical dependence

The ability of naloxone (0.1, 1, 3 and 10 mg/kg) to precipitate signs of dependence was examined in groups of mice that were either treated with vehicle or oxycodone. As seen in Figure 4, naloxone precipitated oxycodone somatic withdrawals signs such as jumps, paw tremors, and loss of body weight. Near-zero levels of total number of withdrawal signs (Fig. 4A), paw tremors (Fig. 4B), and jumps (Fig. 4C) were

elicited by the 10 mg/kg naloxone dose in control mice administered the saline dosage regimen (empty circles in Fig. 4) and they experienced little change in body weight (Fig. 4D). In contrast, two or more of the tested naloxone doses significantly elevated these measures in oxycodone-treated mice (total number of signs, [$F(4,44)=37.15$; $p<0.0001$] (Fig. 4A); paw tremors, [$F(4, 44)=5.857$; $p=0.0007$] (Fig. 4B); jumps, [$F(4,44)=27.37$; $p<0.0001$] (Fig. 4C); and loss of body weight, [$F(4, 44)=6.066$; $p=0.0006$] (Figure 4D). Post-hoc analysis revealed that all four doses of naloxone produced significant increases in total number of signs and jumps, whereas 1 and 10 mg/kg naloxone produced significant increases in paw tremors, and only the two highest doses tested, 3 and 10 mg/kg naloxone, significantly decreased body weight.

4. Summary

In oral oxycodone self-administration, as the concentration of oxycodone increased, the number of deliveries obtained increased before decreasing with higher concentrations, whereas the estimated consumption increased reaching an average maximum of approximately 40 mg/kg. These levels of oxycodone were sufficient to reach behaviorally active levels inducing mu-opioid receptor-like mediated effects in the mice including hyperlocomotion and Straub tail (Aceto et al., 1969; Hecht and Schiorring, 1979). Oxycodone was later orally self-administered to similar levels without the use of post-prandial conditions, suggesting, although does not definitively confirm, that oral oxycodone was serving as a positive reinforcer. Limitations of this study, including hardships endured in the investigation of reinforcement effects, as well as stereotypic behaviors observed in some subjects after reaching behaviorally active levels are discussed in depth in Chapter VI.

In the oxycodone physical dependence study, physical dependence upon oxycodone was induced in C57BL/6J mice after nine days of its b.i.d. subcutaneous administration as inferred by naloxone-precipitated somatic signs of opioid-like withdrawal syndrome. Using a wide range of naloxone doses (0.1 to 10 mg/kg), a naloxone dose-dependent increase in withdrawal severity was observed in number of paw tremors, jumps, bodyweight loss, and total number of signs. The regimen used in the present study therefore provides an easy novel method to accurately measure dependence-related effects on future behavioral endpoints.

In summary, experimental conditions have been identified in which oxycodone was orally self-administered by mice in which an operant response contingency was required. This procedure enables future studies examining determinants of its self-administration heretofore restricted to intravenous self-administration procedures (Wade et al., 2015; Zhang et al., 2009). In addition, an oxycodone administration protocol (9-day) was identified for inducing physical dependence in mice and thus enables future studies examining mechanisms of the induction of dependence and the modulation of the expression of signs of withdrawal such as in the search for pharmacotherapeutics ameliorating the malaise of withdrawal. Both procedures define foundational methodology to be used to investigate the unique interactions of HIV-1 Tat expression on opioid abuse liability, specifically with the prescription opioid oxycodone.

5. Figures

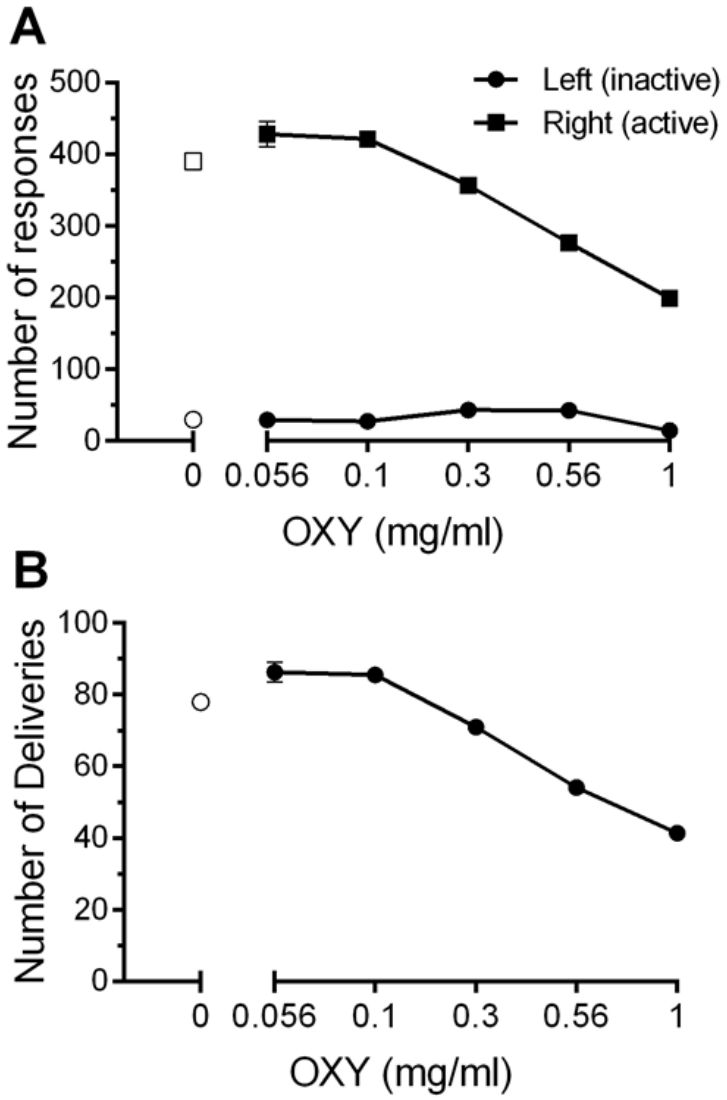

Figure 1. Effects of oxycodone concentration on the number of active and inactive lever presses emitted (A) and liquid deliveries obtained (B) by mice under post-prandial conditions during 3-h test sessions. N=15-16, with 2-5 test sessions per concentration. Data are expressed as mean (± S.E.M.).

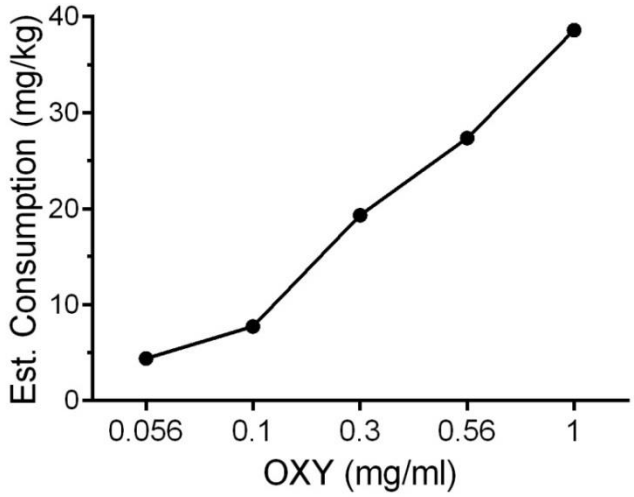

Figure 2. Estimated consumption of oxycodone (mg/kg body weight) as a function of available concentration (mg/ml) under post-prandial conditions. Consumption was estimated based on individual subject's daily body weight and the total deliveries of oxycodone obtained. N=15-16, with 2-5 test sessions per concentration. Each symbol represents the mean oxycodone consumption (± S.E.M.).

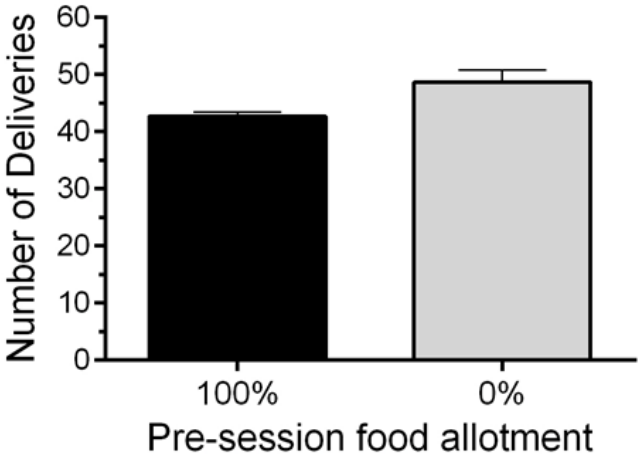

Figure 3. Effects of removing pre-session chow on obtained 1 mg/ml oral oxycodone deliveries during 3-h test sessions. N=14. Data represent the mean number of deliveries (± S.E.M.) obtained.

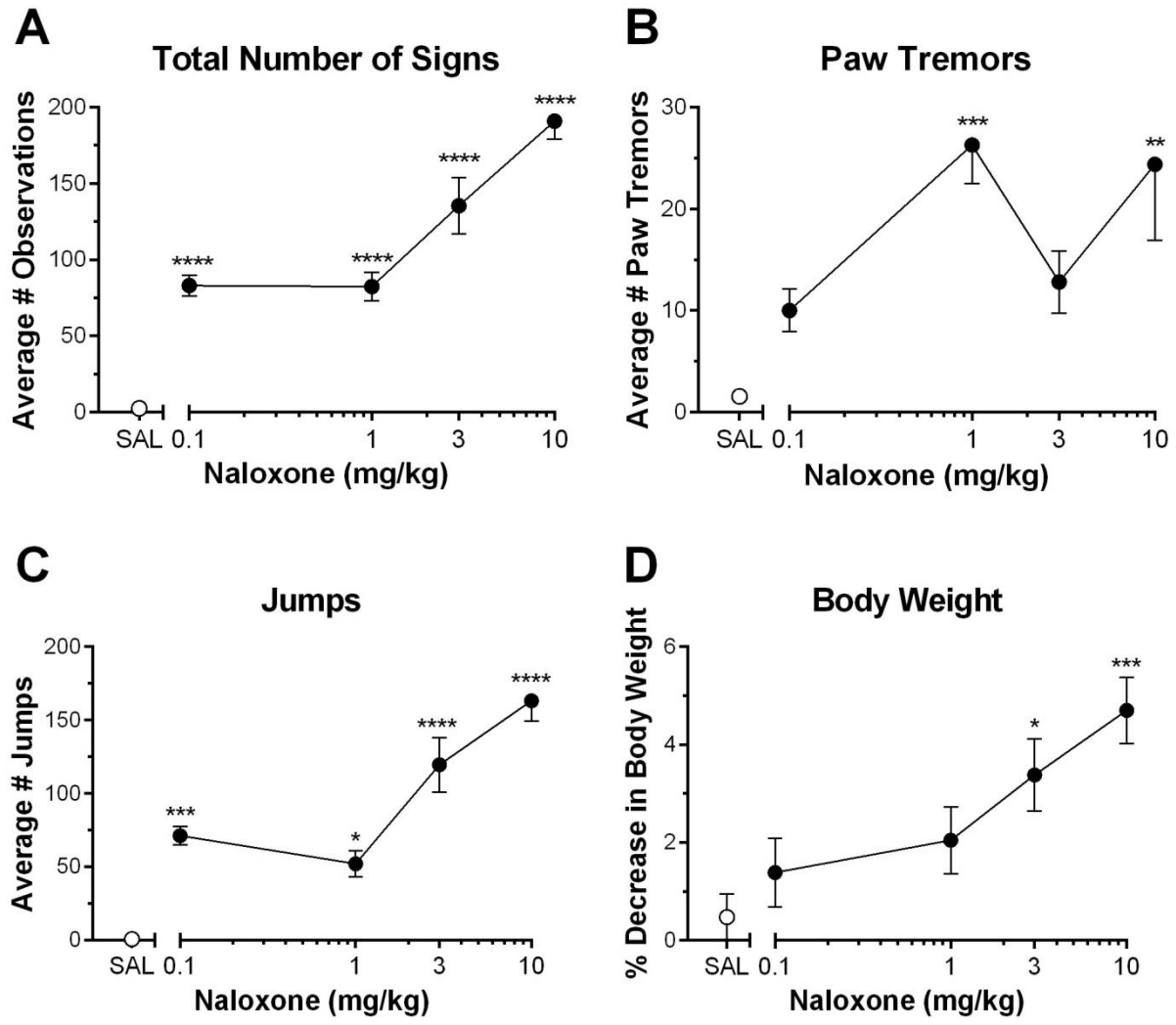

Figure 4. Naloxone precipitated somatic withdrawal signs in oxycodone chronically injected mice. Behaviors scored were (A) total somatic signs (B) paw tremors (C) total numbers of jumps (D) body weight decrease. N=9-10/group. Data are expressed as mean (± S.E.M.). *P < 0.05, **P < 0.01, ***P< 0.001, ****P< 0.0001 to SAL-treated control mice.

Chapter III: The acoustic startle response and habituation, but not prepulse inhibition, serves as a measure of oxycodone dependence in C57BL/6J mice

1. Introduction

The acoustic startle response (ASR) is an immediate, reflexive response to a loud acoustic stimulus and is often used in the initial behavioral phenotypic characterization of rodents with genetic manipulations. It has also been used to evaluate how drugs of abuse alter sensory excitability and habituation, and has previously been found to be sensitive to precipitated withdrawal from morphine (Davis, 1980; Harris and Gewirtz, 2004; Mansbach et al., 1992). The use of acoustic startle to measure dependence-related effects has advantages relative to other measures due to its sensitive, quantifiable nature that is not compromised by subjective bias as is possible in traditional measurements of somatic signs of withdrawal. At the present time, to my knowledge, there are no published reports describing the effects of oxycodone on the ASR or its related measures in either the preclinical or clinical literature. Therefore, this study was conducted to determine the ability for the ASR to reliably measure oxycodone dependence in C57BL/6J mice.

Additionally, the nonspecific effects of the tetracycline derivative doxycycline were investigated for reasons that are two-fold. First, there is widespread use of doxycycline (via injection or infused chow administration) in animal models employing the use of the “tet on” system of genetic expression for a protein of interest and its nonspecific behavioral effects must be evaluated and reported to better understand more sophisticated behavioral effects in these animal models. This method of expression, for example, is the basis for the HIV-1 Tat mouse model of neuroAIDS discussed in subsequent chapters. Secondly, and also as important, there is growing evidence of antibiotics altering characteristics of classical opioids, such as morphine, and other drugs of abuse in both preclinical and clinical assessments (Hutchinson et al., 2008; McIver et al., 2012; Syapin et al., 2016). For example, oral gavage of various antibiotics to mice significantly prevented morphine antinociceptive tolerance as assessed by warm water tail-immersion and acetic acid stretch assays (Kang et al., 2017). Moreover, morphine dependence, as inferred by naloxone-precipitated jumping, was attenuated by administration of the beta-lactam antibiotic ceftriaxone (Habibi-Asl et al., 2014). Therefore, doxycycline’s interactions with the expression of oxycodone dependence, as well as its nonspecific effects, were assessed in C57BL/6J mice. These tests would provide the critical control measures for subsequently investigating the role of HIV-1 Tat expression on oxycodone abuse-related behavioral effects.

2. Methods

2.1. Subjects

Male C57BL/6J (“B6”) mice were obtained at approximately 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and were allowed to acclimate to the vivarium for

at least one week prior to commencement of training and testing. Mice were housed in an AALAC-accredited animal facility, kept on a 12-h/12-h light/dark cycle (lights on from 06:00 to 18:00 hours), and given *ad libitum* access to water and standard rodent chow (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories Inc., Indianapolis, IN). The investigation of doxycycline's effects were evaluated in separate groups of mice fed an *ad libitum* diet consisting of 6 g of doxycycline ("DOX") per kg of chow (Harlan Industries Inc., Indianapolis, IN) beginning immediately after preliminary baseline tests and for the duration of the studies. All procedures were conducted during the light phase and were in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press, 2011), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Overall procedure

The overall procedure for these studies followed our previously established methodologies for oxycodone ("OXY") physical dependence induction as well as acoustic startle and locomotor activity testing with minor modifications (Enga et al., 2016; Enga et al., 2017). Mice were first tested in a preliminary ASR and locomotor activity baseline test (i.e., "Baseline I"). Two weeks later, mice were re-tested during which an acute saline ("SAL") injection was administered immediately prior to both startle and locomotor tests to establish a second baseline (i.e., "Day 0/SAL"). Chronic SAL or OXY b.i.d. subcutaneous injections then began as outlined in Figure 5. Two hours after the Day 9 morning ("AM") injection (33 mg/kg), mice received an acute subcutaneous injection of SAL immediately prior to both startle and locomotor tests to

determine its effects on behavior that would enable subsequent comparisons to naloxone challenge, and which consequentially also captured the effects of chronic OXY administration up to that point. The next day on Day 10, 2 h after receiving an AM injection of OXY (33 mg/kg) an acute subcutaneous injection of naloxone ("NLX", 1 mg/kg) was administered immediately prior to startle and locomotor tests to determine if this opioid antagonist would precipitate effects indicative of dependence. Within this chapter, the term "naloxone-challenge" on Day 10 is used interchangeably with "precipitated withdrawal", as this regimen demonstrated naloxone dose-dependent increases in somatic signs of withdrawal, as discussed in Chapter II. Bodyweights were recorded at the beginning of each day, and immediately following behavioral testing on Days 9 and 10, as changes in bodyweight has been used to infer dependence upon opioids (Aceto et al., 1985; Martin et al., 1963). This chapter discusses results from acoustic startle tests; results from locomotor activity tests will be discussed in Chapter V.

2.2.1. Investigation of nonspecific effects of doxycycline and its effects on the expression of oxycodone dependence

To assess the effects of DOX, groups of B6 mice (n=8/group) were tested under baseline conditions with REG chow, and then either maintained on REG chow or switched to DOX chow and re-tested after 2, 9, 16, 30, and 58 days. To test interactions of DOX and oxycodone dependence, the oxycodone dependence and testing regimen described earlier in this chapter (shown in Figure 5) was used in DOX-maintained B6 mice. Here, after baseline I tests, mice were switched to and maintained on a DOX diet

to permit the characterization of DOX's effects on oxycodone dependence expression, its interactions with NLX , and its nonspecific effects on acoustic startle.

2.3. Acoustic startle apparatus

Startle responses were measured using eight commercially-supplied startle chambers (San Diego Instruments, San Diego, CA). Chambers were ventilated and illuminated, and contained a clear, nonrestrictive Plexiglas® cylindrical animal enclosure secured on a platform. Acoustic stimuli were presented through a loud speaker that was located directly above the animal enclosure. Mouse startle movements were transduced by a piezoelectric sensor attached to the bottom of the animal enclosure platform, which were digitized and recorded by a computer. Beginning with the onset of the startle pulse (STIM) for each trial, 1000 readings were taken at 1-ms intervals. To ensure accuracy of sound levels and stabilimeter sensitivity, routine (at least monthly) calibrations were conducted throughout the study.

2.4. Procedure

A test session consisted of 75, 200-ms trials, with five trial types: STIM alone, 73pp (i.e., "73dB **p**re**p**ulse")+STIM, 77pp+STIM, 85pp+STIM, and NO STIM. Both prepulse and pulse stimuli were 20-ms in duration, with an interstimulus interval of 100-ms between the onset of their presentations, and an intertrial interval average of ~15-s across the session (range: 10-20-s). The STIM intensity was set at 119 dB, while prepulses were set at 4, 8, and 16 dB above the background level of 69 dB (i.e., 73, 77, and 85 dB). During NO STIM trials, mice were subjected to only the background level of noise. A test session was initiated with a 5-min acclimation period to the background

level of noise before the presentation of five STIM alone trials. Following this, 13 replicates of all trial types were presented in a mixed sequence to prevent consecutive presentation of identical trial types and possible habituation to trial types. Test sessions ended with five STIM alone trials. Total session time was approximately 30 min.

2.5. Drugs

Oxycodone HCl (Mallinckrodt Inc., St. Louis, MO) was initially prepared in a sterile stock solution of 10 mg/ml in non-heparinized 0.9% saline before diluting in sterile saline to make working solutions in the following concentrations: 0.09, 1.78, 2.37, and 3.3 mg/ml. Naloxone HCl (Sigma-Aldrich, St. Louis, MO) was prepared in sterile 0.9% saline to make working solution at 0.1 mg/ml. All injections were given at a 10 ml/kg injection volume and there was a seven hour period between any AM and PM injections. AM injections occurred between 08:00 and10:00 hours and PM injections occurred between 15:00 and 17:00 hours.

2.6. Data analysis

For analyses of startle response, the startle magnitude for the “STIM alone” trial type was averaged across replicates. For PPI, startle magnitude for each trial type was averaged across replicates and these averaged magnitudes were used to calculate %PPI with the following formula: 100x[(STIM alone-prepulse)/(STIM alone)]. Habituation was inferred by measuring the %decrease in startle reactivity where, using the average startle magnitudes of the first and last five STIM alone trials of the session, %decrease in startle reactivity was calculated using the following formula: 100x[(First STIM alone - Last STIM alone)/First STIM alone)]. Data were analyzed using a two-way ANOVA, with

a within-subject factor of "day" and between-subject factor of "group". Within- and between-group comparisons were made using Bonferroni post-hoc tests where appropriate. All statistical tests were conducted using microcomputer software (Prism 6 for Windows, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if $p<0.05$.

3. Results

3.1. Acoustic startle response serves as a measure of oxycodone dependence

Baseline I and baseline II (i.e., “Day 0/SAL”) startle responses did not differ between SAL and OXY groups (not shown). There was a significant main effect of day [$F(2, 36)=25.03$; $p<0.0001$] as well as a significant interaction between day and group [$F(2, 36)=13.95$; $p<0.0001$]. Post-hoc analysis revealed chronic OXY significantly increased the ASR as compared to Day 0/SAL ($p=0.0004$) and NLX-precipitated withdrawal significantly decreased startle ($p=0.0003$ vs Day 0/SAL; $p<0.0001$ vs Day 9/SAL) as shown in Figure 6A. There were, however, no significant differences between SAL- and OXY-treated groups at any timepoint. In habituation analyses, there was a significant main effect of group [$F(1, 18)=11.02$; $p=0.0038$] as well as a significant interaction of group and day [$F(2, 36)=4.197$; $p=0.0230$]. In SAL-treated mice, there was no difference across days in habituation (Fig. 6B). In OXY-treated mice, chronic OXY did not affect habituation, however its precipitated withdrawal significantly reduced habituation ($p<0.05$ vs Day 0/SAL and Day 9/SAL; $p=0.0003$ vs SAL-treated mice).

In PPI analyses, there was a significant main effect of prepulse intensity on all days tested [Day 0/SAL: $F(2, 36)=56.37$; $p<0.0001$; Day 9/SAL: $F(2, 36)=75.29$;

p<0.0001; Day 10/NLX: F (2, 36)=32.40; p<0.0001]. In SAL-treated mice, PPI increased over days tested and was significantly different from Day 0/SAL on Days 9 and 10, but only at the 77dB prepulse intensity (Day 9: p=0.0132 vs Day 0/SAL; Day 10: p=0.0044 vs Day 0/SAL). In OXY-treated mice, PPI did not change as a result of chronically administered OXY nor its precipitated withdrawal. There were significant between-group differences at Days 9 and 10 at all prepulse intensities (see Table 1 for individual p-values).

3.2. Effects of doxycycline on acoustic startle

At baseline, REG-fed mice had non-significantly higher (p=0.06) startle magnitudes relative to DOX-fed mice (Fig. 7A). Over the course of 58 days, only on day 2 was there a significant difference in startle magnitude between the two groups with the REG-fed mice having a greater startle magnitude than the DOX-fed mice. The ASR of the mice did not significantly change as a result of time in either the DOX-fed or REG-fed mice although there was a non-significant decreasing trend in startle magnitude in the REG-fed mice that converged towards levels exhibited by the DOX-fed mice. Habituation was unaffected over time in either group (Figure 7B). As shown in Figure 8, PPI did, however, significantly increase over time in both REG- and DOX-fed groups at all prepulses and to similar degrees [73pp: F(5, 70)=11.88, p<0.0001; 77pp: F (5,70)=12.27, p<0.0001; 85pp: F (5, 70)=8.997, p<0.0001].

3.3. Effects of doxycycline on the expression of oxycodone dependence

Similar to the previously discussed REG-fed groups of C57BL/6J mice, there were no statistical differences between DOX-fed SAL- or OXY-designated groups in the

baseline I or Day 0/SAL tests on acoustic startle (not shown). After chronic OXY, there was a significant main effect of day [F (2, 36)=6.134; p=0.0051], as well as a significant interaction of day and group [F (2, 36)=8.232; p=0.0011]. There was a lack of effect of DOX alone on startle, as there were no changes across tests in SAL-treated mice. Unlike REG-fed mice, post hoc analysis did not reveal a significant difference in acoustic startle after chronic OXY administration. Naloxone-precipitated withdrawal, however, did result in a significant decrease in startle as shown in Figure 9A (p=0.0006 vs Day 0/SAL; p<0.0001 vs Day 9/SAL). This decrease was also lower than SAL-treated mice, although nonsignificantly so (p=0.057). In habituation, there was a lack of a significant main effect of both day (p=0.11) and group (p=0.072). Moreover, there was no significant change in habituation in the SAL-treated group. Similar to REG-fed mice, chronic OXY did not affect habituation; however, its precipitated withdrawal did significantly decrease habituation as shown in Figure 9B (p=0.027 vs Day 0/SAL; p=0.023 vs SAL-treated mice).

There was a significant main effect of prepulse intensity on all days tested [Day 0/SAL: F (2, 36)=37.18; p<0.0001; Day 9/SAL: F (2, 36)=35.21; p<0.0001; Day 10/NLX: F (2, 36)=26.79; p<0.0001]. In SAL-treated mice, there was a lack of change in %PPI across days as shown in Table 2, suggesting a lack of an effect of doxycycline alone on PPI. Similar to REG-fed mice, there was a lack of an effect of chronic OXY and its precipitated withdrawal on PPI in DOX-fed mice.

In regards to body weight loss measurements as a separate index of physical dependence upon oxycodone, body weight was measured at the beginning of Day 9 and Day 10 as well as at the conclusion of behavioral testing on those days (i.e., a 3.5-h

span between body weight measurements). For both REG- and DOX-fed mice, bodyweight loss was decreased after testing on both Days 9 and 10, in both SAL- and OXY-treated groups. This decrease was to a greater degree in OXY-treated groups after naloxone challenge (not shown). However, there was only a significant difference between Days 9 and 10 or between SAL and OXY groups in DOX-fed mice ($p<0.05$). That is, body weight loss did not serve as a reliable, additional indicator of physical dependence upon OXY in this study, most likely due to the large timespan and behavioral testing between measurements.

4. Summary

Nine days of oxycodone administration to C57BL/6J mice fed a REG-chow diet produced a significant increase in the acoustic startle response, whereas naloxone-precipitated withdrawal resulted in a significant decrease in startle. Habituation, on the other hand, was only affected by naloxone-challenge as shown by its significant decrease shown on Day 10/NLX. PPI was unaltered by either chronic oxycodone administration as well as precipitated withdrawal.

Doxycycline alone did not affect acoustic startle, habituation, or PPI up to 58 days of exposure in C57BL/6J mice. Unlike REG-fed mice, feeding doxycycline appeared to prevent OXY-induced increases in startle, although naloxone-challenge significantly decreased startle as well as habituation in both groups. Also similar in both groups, PPI was unaffected after both chronic oxycodone administration as well as precipitated withdrawal.

In summary, this study demonstrated the ability of the acoustic startle response to serve as a sensitive index of oxycodone dependence. Habituation, but not PPI, also

was responsive to apparent precipitated withdrawal conditions. These findings support the use of the startle response as an objective and quantifiable procedure to evaluate oxycodone dependence-related effects. Moreover, the results with doxycycline tests support the use of this tetracycline derivative for transgenic mouse models without important concerns of its nonspecific effects on abuse-related behavioral measures. Therefore, a novel and reliable procedure for the future evaluation of oxycodone dependence-related effects in mice expressing the neurotoxic HIV-1 Tat protein was established.

Table 1. Mean %PPI for all prepulse intensities at each test day in REG-fed C57BL/6J mice.

	Prepulse Intensity(dB)	Day 0/SAL	Day 9/SAL	Day 10/NLX
		AVG (± S.E.M)	AVG (± S.E.M)	AVG (± S.E.M)
SAL	73	45.2 (5.03)	55.88 (4.60)	58.27 (4.01)
	77	52.39 (4.88)	65.91 (3.05)[a]	67.72 (2.59)[a]
	85	67.05 (3.14)	76.32 (1.76)	75.35 (2.28)
	Prepulse Intensity(dB)	Day 0/SAL	Day 9/SAL	Day 10/NLX
		AVG (± S.E.M)	AVG (± S.E.M)	AVG (± S.E.M)
OXY	73	31.57 (5.17)	28.0 (5.12)[b(p=0.0008)]	38.1 (6.02)[b(p=0.0193)]
	77	45.83 (4.16)	39.74 (6.06)[b(p=0.0004)]	47.65 (5.20)[b(p=0.0077)]
	85	58.40 (3.63)	58.28 (4.20)[b(p=0.0011)]	59.47 (4.33)[b(p=0.0046)]

[a] P<0.05 to Day 0/SAL
[b] Significant difference was found between SAL- and OXY-treated mice

Table 2. Mean %PPI for all prepulse intensities at each test day in DOX-fed C57BL/6J mice.

	Prepulse Intensity(dB)	Day 0/SAL	Day 9/SAL	Day 10/NLX
		AVG (± S.E.M)	AVG (± S.E.M)	AVG (± S.E.M)
SAL	73	54.44 (6.71)	57.32 (6.25)	64.70 (5.11)
	77	62.02 (6.30)	66.40 (6.22)	70.90 (4.02)
	85	70.52 (4.43)	74.98 (3.16)	78.75 (1.92)
	Prepulse Intensity(dB)	Day 0/SAL	Day 9/SAL	Day 10/NLX
		AVG (± S.E.M)	AVG (± S.E.M)	AVG (± S.E.M)
OXY	73	59.24 (2.01)	45.75 (4.42)	53.82 (3.87)
	77	66.57 (2.77)	61.23 (4.17)	63.01 (3.04)
	85	76.33 (1.85)	68.45 (4.05)	69.29 (3.88)

5. Figures

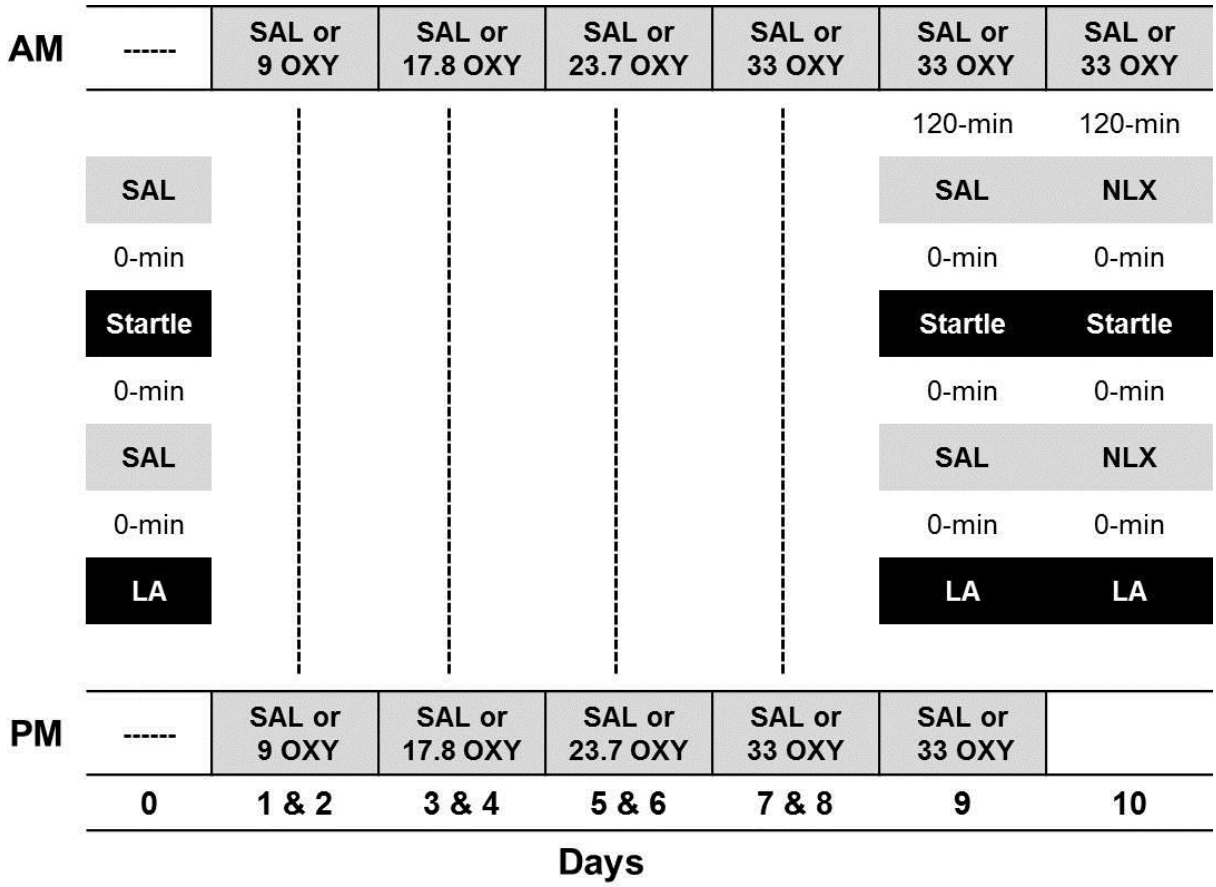

Figure 5. Conditions of the oxycodone physical dependence and testing regimen. Mice (n=10/group) were first tested in a preliminary behavioral baseline acoustic startle and locomotor activity ("LA") test and then re-tested under similar conditions two weeks later before receiving chronic (10-day) administration of saline (SAL) or oxycodone (OXY). Bodyweights (g) were measured at the beginning of each day, and after testing on Days 9 and 10.

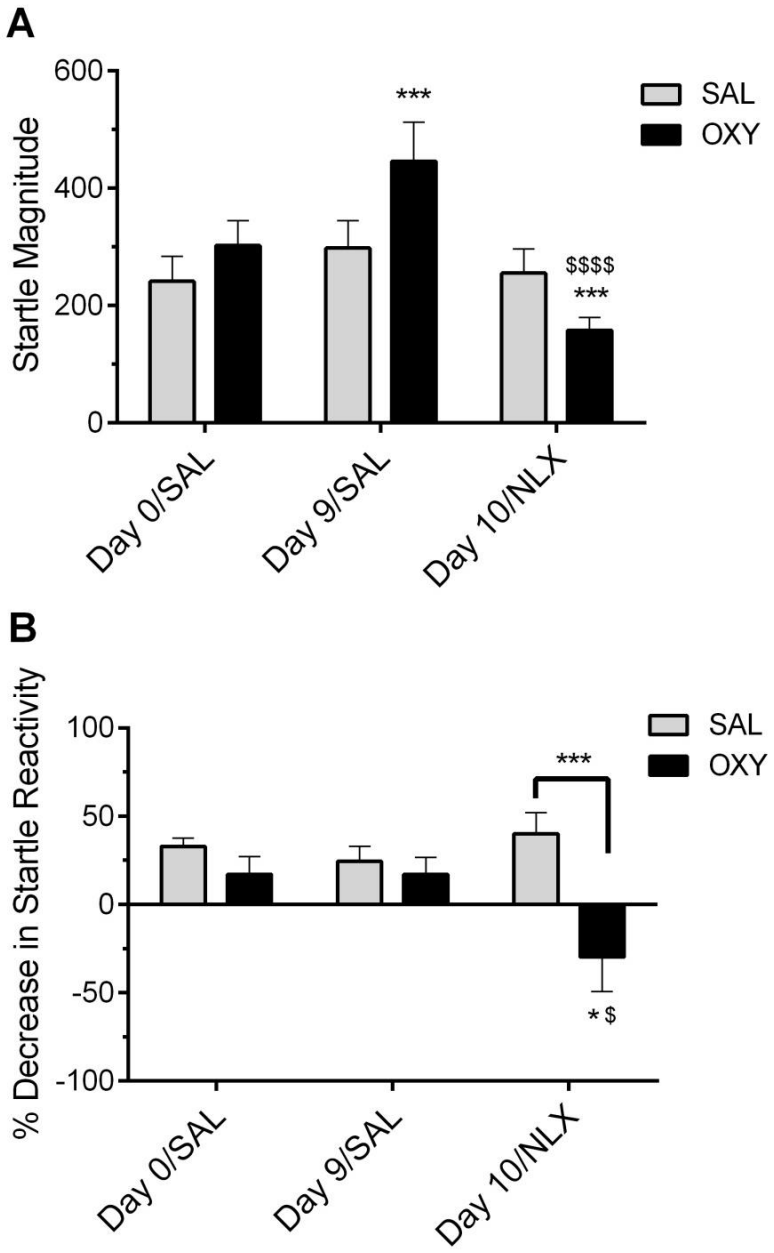

Figure 6. Acoustic startle response (A) and habituation (B) after chronic OXY and during naloxone challenge (precipitated withdrawal) in adult, male C57BL/6J mice. N=10/group. Data represent the mean startle amplitude (± S.E.M.) or the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05, ***P<0.001 to Day 0/SAL and $P<0.05, $$$$P<0.0001 to Day 9/SAL.

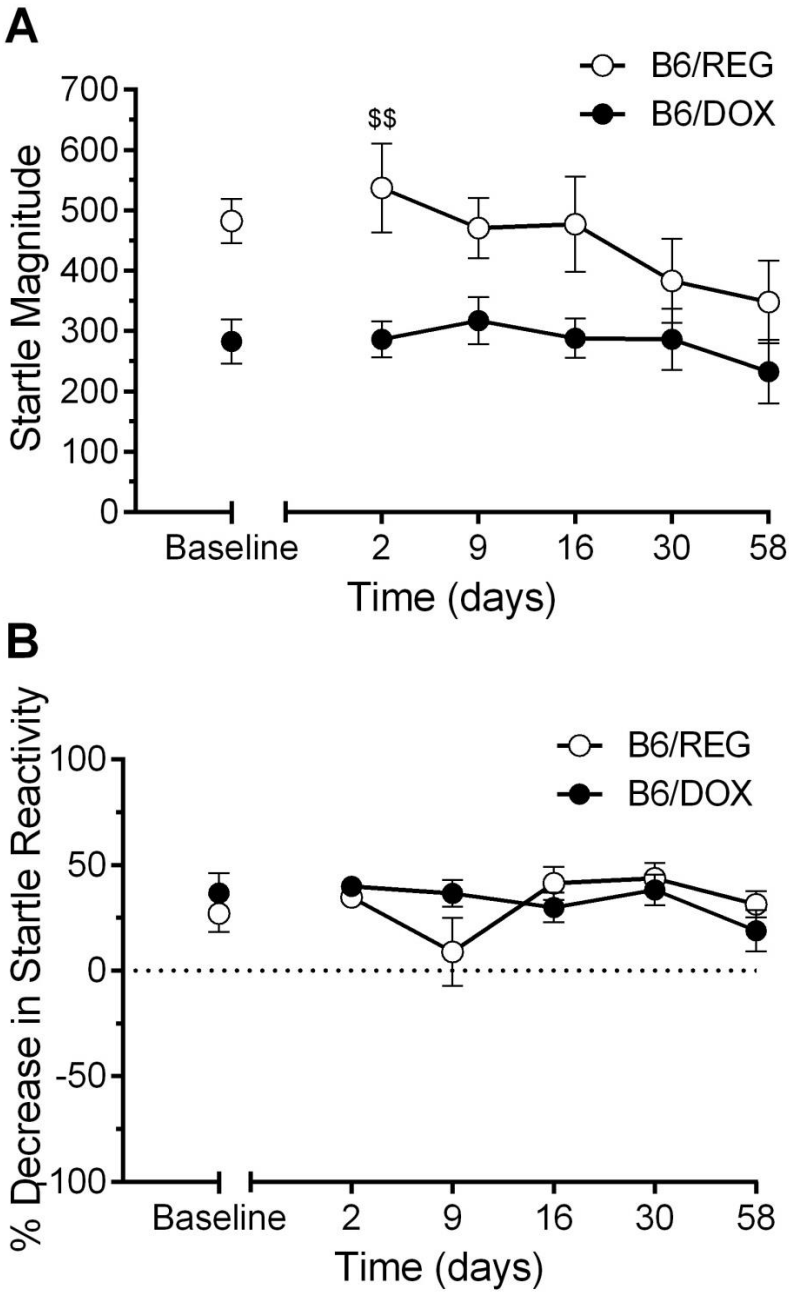

Figure 7. Acoustic startle response (A) and habituation (B) across feeding days for REG- or DOX-fed C57BL/6J mice. N=8/group. Data represent the mean startle amplitude (± S.E.M.) or the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. $$P<0.01 to DOX-fed mice.

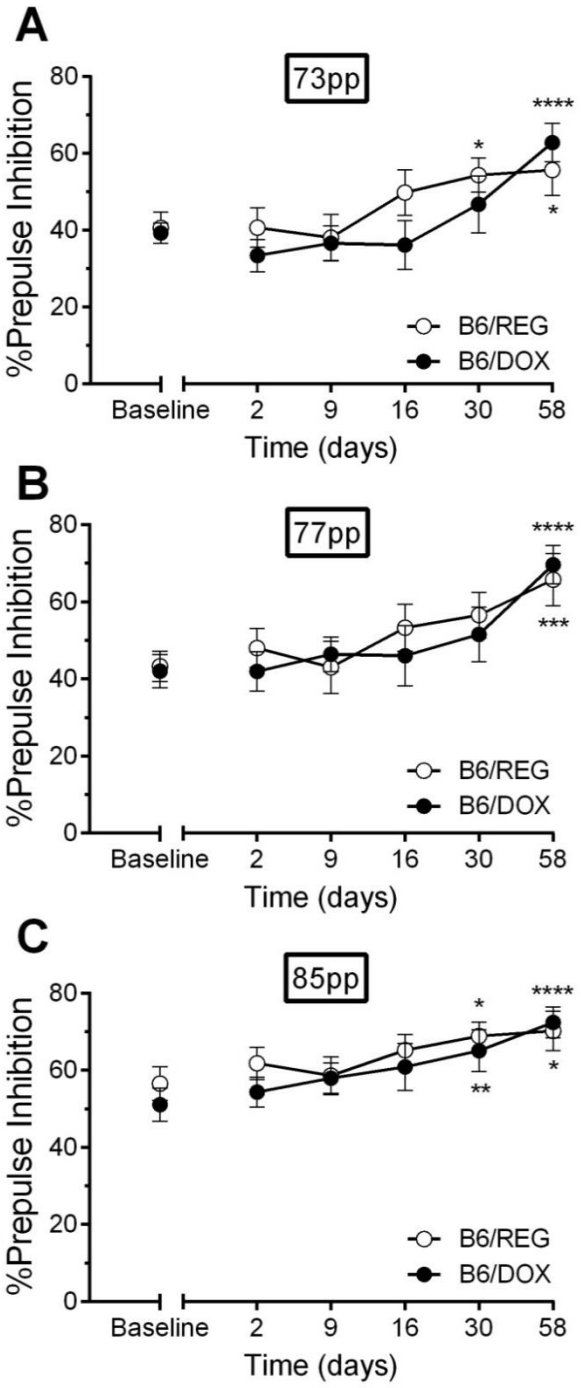

Figure 8. Percent prepulse inhibition over time with 73 dB (A), 77 dB (B), and 85 dB (C) prepulse level intensities for REG- or DOX-fed C57BL/6J mice. N=8/group. Data are represented as the mean %PPI (± S.E.M.). *P< 0.05, **P<0.01, and ****P<0.0001 to Day 0/SAL.

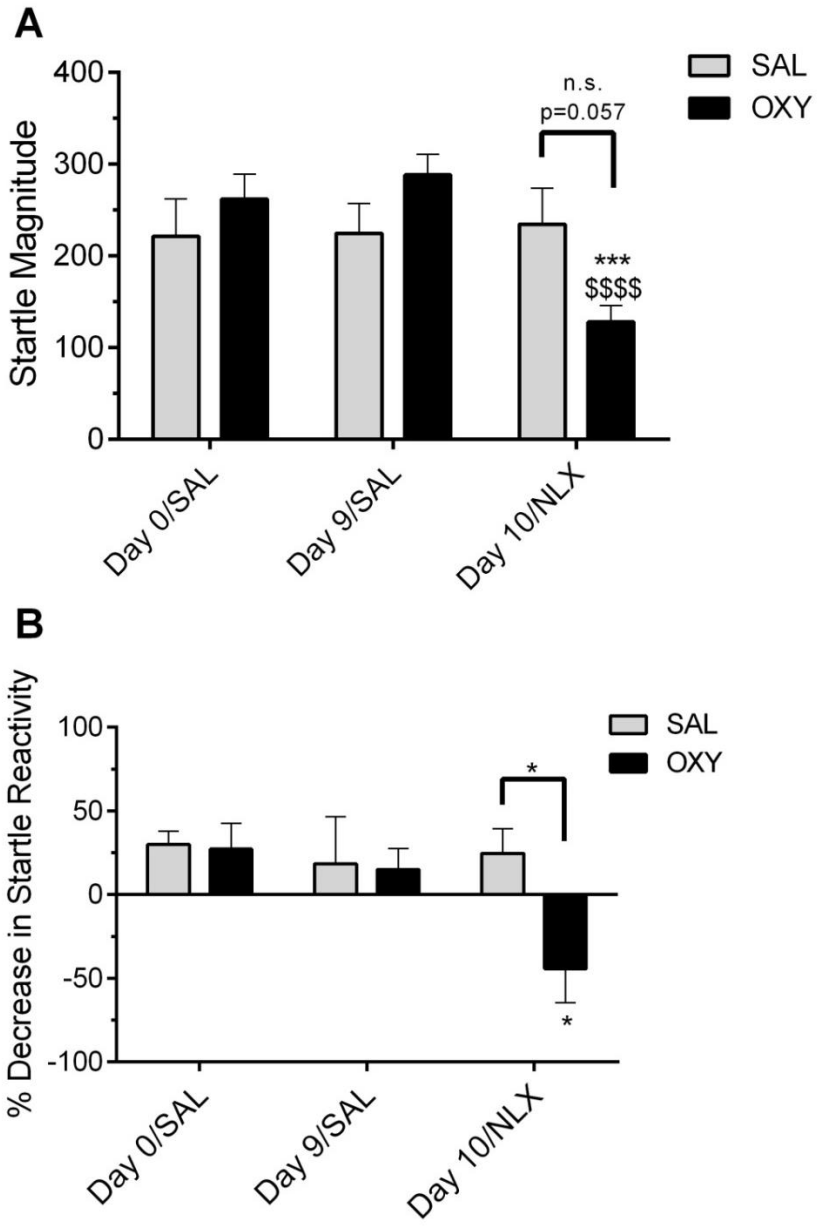

Figure 9. Acoustic startle response (A) and habituation (B) after chronic OXY and its precipitated withdrawal in DOX-fed adult, male C57BL/6J mice. N=10/group. Data represent the mean startle amplitude (± S.E.M.) or the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05 to Day 0/SAL or SAL-treated mice, ***P< 0.001 to Day 0/SAL and $$$$P<0.0001 to Day 9/SAL.

Chapter IV: HIV-1 Tat transgenic mice readily self-administer oral oxycodone but are resistant to physical dependence as measured by acoustic startle and habituation

1. Introduction

HIV-1 Tat expression has previously been shown to interact with opioids, primarily morphine, to worsen neuronal damage, increase glial activation, as well as dampen morphine antinociceptive responsiveness (Bruce-Keller et al., 2008; Fitting et al., 2010). Moreover, Tat has been shown to potentiate the rewarding properties of drugs of abuse, such as cocaine and ethanol (McLaughlin et al., 2014; Paris et al., 2014a). Thus, it was hypothesized that HIV-1 Tat expression in the mouse would also facilitate oxycodone abuse-related behavioral effects. At present, the preclinical literature surrounding HIV-1 viral protein expression and operant self-administration of a drug is limited to two reports using intravenous cocaine or heroin self-administration and HIV-1 transgenic rats which express seven of the nine HIV-1 viral proteins (see Chapter VI for further discussion). These reports however do not address other routes of administration, such as the oral route. While the HIV-1 transgenic rat model may possess a greater clinical relevance to the neuropathophysiology of HIV-associated neurocognitive disorders (HAND) as a whole, it does not address the individual actions

of the various HIV-1 viral proteins. Isolating the individual actions of viral proteins, such as Tat, are essential for characterizing and understanding their contribution to the larger system, as well as to better understand their interactions with mechanisms of drug dependence. Therefore, one objective of the present study was to evaluate the effects of HIV-1 Tat expression on the volitional consumption (via oral self-administration) of oxycodone.

Acoustic startle and prepulse inhibition of the startle response (PPI) are reported to be compromised in HAND and have been found to be directly affected by HIV-1 Tat (Fitting et al., 2006a; Fitting et al., 2006b; Minassian et al., 2013; Moran et al., 2013). It is hypothesized that PPI deficits induced in HAND are attributable to attenuation of critical inhibitory systems, which may consequentially promote impulsive and risky behavior, such as drug abuse, in some patients (Minassian et al., 2013). Similar to the paucity of published self-administration reports, the scientific literature evaluating interactions of HIV-1 viral protein expression and drugs of abuse on acoustic startle and its related measures is limited. The only published reports involve methamphetamine administration and HIV-1 transgenic rats or mice expressing the envelope glycoprotein gp120. Although these studies utilized different rodent models, opposing PPI effects were found in these studies in which acute methamphetamine administration attenuated PPI, and withdrawal from chronic methamphetamine administration increased it (Henry et al., 2014; Moran et al., 2012). Using the newly developed oxycodone physical dependence regimen and acoustic startle testing protocol described in previous chapters, the second objective of the present study was to investigate the effects of HIV-1 Tat expression on acoustic startle, its related measures, and the interactions of

oxycodone dependence on these measurements. It was hypothesized that acoustic startle would be decreased in Tat-expressing, oxycodone-dependent mice after precipitating withdrawal. Moreover, it was hypothesized Tat-expressing mice would incur PPI deficits and to a greater degree after oxycodone administration.

2. General Methods

2.1. Subjects

Male, doxycycline-inducible Tat_{1-86} transgenic mice were generated on a C57BL/6J and C3H background as described previously (Bruce-Keller et al., 2008; Hauser et al., 2009), and backcrossed to the C57BL/6J strain which further serves as rationale for the use of this strain as a useful control group for the studies within this dissertation. The tat transgene activity was under control of a glial fibrillary acidic protein (GFAP) promoter, therefore limiting expression to astroglia in the CNS. Tat protein expression was induced in mice positive for the tat transgene, referred to as “Tat(+)”, and the reverse tetracycline transactivator (RTTA) gene once placed on a diet consisting of 6 g doxycycline (“DOX”) per kg of chow (Harlan Laboratories, Inc., Indianapolis, IN). Tat(-) mice were not positive for the tat transgene, but did possess the RTTA gene. In oral oxycodone (OXY) self-administration studies, Tat(+) and Tat(-) mice (n=8/group) were maintained at an 85% free-feeding weight with daily allotments of standard (“REG”) rodent chow (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories Inc., Indianapolis, IN) or DOX chow, depending on the phase of the study as described below. During acute OXY and OXY dependence studies, Tat(+) and Tat(-) mice (n=10/group) were allowed *ad libitum* access to water and either REG

or DOX chow. All mice were kept under a 12-h/12-h light/dark cycle (lights on from 06:00 to 18:00 hours) in an AAALAC-accredited animal facility. All procedures were conducted during the light phase and were in accordance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press, 2011), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Oral operant self-administration

2.2.1. Apparatus

Sixteen mouse operant chambers (MED Associates, Inc., St. Albans, VT) enclosed in sound- and light-attenuating cubicles equipped with a viewing peep hole were used for this study. Each operant chamber contained a house light mounted on the rear wall, a Sonalert® tone-generating device, and panels of cue lights mounted above two response levers between which was positioned a well into which a drinking cup was positioned. Vendor-supplied drinking cups were replaced with fabricated dipper cups into which were soldered stainless steel liquid delivery tubes. A separate stainless steel liquid drainage tube was soldered to capture any liquid spillage and was funneled to a 1 ml microcentrifuge conical tube. Attached to each stainless steel delivery tube was silicone tubing (0.79 mm ID/ 3.99 mm OD; Helix Medical, Carpinteria, CA) that was routed behind the operant chamber and attached to a syringe that when compressed by a Razel Model R-ES syringe infusion pump (Razel Scientific Instruments, St. Albans, VT), delivered 20 ul of liquid. Recording of lever presses, activation of house and cue lights, sonalerts, and syringe pumps were accomplished via computer-controlled circuitry and software (MED-PC IV, MED Associates, Inc., St. Albans, VT).

2.2.2. Procedure

The overall procedure was as we had described (Enga et al., 2016) with minor modifications. Training and testing proceeded according to the following phases: 1) Pre-DOX post-prandial induction of water and OXY consumption; 2) DOX post-prandial induction of water and OXY consumption; 3) Post-DOX post-prandial induction of water and OXY consumption. Within each phase, mice were exposed to increasing concentrations of OXY with the final concentration of each phase being 1 mg/ml.

2.2.3. General training and testing conditions

Mice were trained and tested daily during 2-h experimental sessions, which were later shortened to 1-h, which began each day between 09:00 and 11:45 hours. Mice were transported from the vivarium to their testing rooms in their home cages, and weighed. After, began the "pre-session" period as described below. During the pre-session period, home-cage water bottles were absent and returned immediately following the test session.

2.2.4. Phase I: Pre-DOX post-prandial induction of water and OXY consumption

During the training component of the study, the pre-session and operant test session periods had durations of 1.5 and 2 h, respectively. Daily REG-chow food allotments were provided in the home cages during the 1.5-h pre-session period to induce thirst. Uneaten chow remained in the home cage during the 2-h test session. Mice were trained to press the active lever (lever side counterbalanced across mice), under a fixed-ratio 1 (FR1) that was progressively increased to a FR10 schedule of reinforcement reinforced with deliveries of water. Presses of the inactive lever prior to

completion of the fixed ratio contingency reset the ratio requirement, but otherwise were without scheduled consequences. At the initiation of each delivery, the Sonalert® sounded and the cue lights above the active lever were illuminated for 6 s. During the 6-s reinforcement period, lever presses were not counted toward completing the FR10 contingency, but were recorded.

Once mice obtained selective pressing of the lever reinforced with water delivery (emitted ≥80% of total presses on the active lever during the entire session) under FR10 conditions for three consecutive sessions, and during which there were no increasing or decreasing trends in the numbers of deliveries obtained and deliveries during each session were ±20% the average of those sessions, the pre-session and operant test session periods were both shortened to 1-h to efficiently capture active drinking behavior (for rationale, see Chapter VI for discussion of stereotypic behaviors seen in C57BL/6J mice during 3-h test sessions). After satisfying the above criteria, increasing concentrations of OXY at 0.1, 0.3, 0.56, and 1 mg/ml were made available for three consecutive sessions each.

2.2.5. Phase II: DOX post-prandial induction of water and OXY consumption

After completing the concentration-effect curve in Phase I (i.e., “Pre-DOX”), mice were removed from daily testing and placed on a DOX-containing diet for approximately two weeks before resuming testing under DOX feeding conditions. As in the Pre-DOX phase, testing began with water as the delivered liquid for presses at the active lever according to FR10 reinforcement schedules. Once mice satisfied performance criteria as described for the Pre-DOX phase, the oxycodone concentration-effect curve was re-determined.

2.2.6. Phase III: Post-DOX, post-prandial induction of water and OXY consumption

After completing Phase II (i.e. “DOX phase”), mice were removed from daily tests and were returned to REG chow feedings for one day before resuming testing under REG chow feeding conditions. One day was chosen for the resumption of testing as the half-life of doxycycline in the mouse is estimated to be approximately 2.8-h, thus providing an estimate of less than 0.5% of doxycycline for its body availability after 24-h (Bocker et al., 1981). This phase allowed the characterization of Tat expression alone on oral OXY self-administration, as Tat is still expressed in the mouse three weeks after removal of DOX chow (Ngwainmbi et al., 2014). As in the Pre-DOX and DOX phases, pressing the active lever according to FR10 reinforcement schedules resulted in deliveries of water. Once mice satisfied the previously described criteria, the oxycodone concentration-effect curve was re-determined.

2.3. Acoustic startle response

As mentioned previously, the acoustic startle response (ASR) may serve as an additional measure of opioid dependence. Moreover, HIV-1 Tat has been found to modulate acoustic startle and its prepulse inhibition. Therefore, the effects of HIV-1 Tat expression on acoustic startle and its prepulse inhibition was evaluated in three parts: (i) effects of Tat alone, (ii) effects of Tat and acute OXY administration, and (iii) effects of Tat and chronic OXY and its precipitated withdrawal.

2.3.1. Acoustic startle apparatus

Startle responses were measured using eight commercially-supplied startle chambers (San Diego Instruments, San Diego, CA). Chambers were ventilated and

illuminated, and contained a clear, nonrestrictive Plexiglas® cylindrical animal enclosure secured on a platform. Acoustic stimuli were presented through a loud speaker that was located directly above the animal enclosure. Mouse startle movements were transduced by a piezoelectric sensor attached to the bottom of the animal enclosure platform, which were digitized and recorded by a computer. Beginning with the onset of the startle pulse (STIM) for each trial, 1000 readings were taken at 1-ms intervals. To ensure accuracy of sound levels and stabilimeter sensitivity, routine (at least monthly) calibrations were conducted throughout the study.

2.3.2. Procedure

Test session conditions

A test session consisted of 75, 200-ms trials, with five trial types: STIM alone, 73pp (i.e., "73dB prepulse")+STIM, 77pp+STIM, 85pp+STIM, and NO STIM. Both prepulse and pulse stimuli were 20-ms in duration, with an interstimulus interval of 100-ms between the onset of their presentations, and an intertrial interval average of ~15-s across the session (range: 10-20-s). The STIM intensity was set at 119 dB, while prepulses were set at 4, 8, and 16 dB above the background level of 69 dB (i.e., 73, 77, and 85 dB). During NO STIM trials, mice were subjected to only the background level of noise. A test session was initiated with a 5-min acclimation period to the background level of noise before the presentation of five STIM alone trials. Following this, 13 replicates of all trial types were presented in a mixed sequence to prevent consecutive presentation of identical trial types and possible habituation to trial types. Test sessions ended with five STIM alone trials. Total session time was approximately 30 min.

Effects of HIV-1Tat expression alone

Tat(-) and Tat(+) mice (n=8/group) were initially tested in a 30-min baseline acoustic startle session under REG feeding conditions. Subsequently, mice were either maintained on REG chow or switched to DOX chow and re-tested for startle after 2, 9, 16, 30, and 58 days.

Effects of HIV-1 Tat expression and acute OXY administration

On Day 1, Tat(-) and Tat(+) mice (n=8/group) maintained on a REG chow diet were administered saline ("SAL") approximately 25-min before being tested in a 30-min acoustic startle test session followed by a 1-h locomotor activity test session. Mice were then switched to a DOX diet for a week (to induce Tat expression in Tat(+) mice). On Day 8, mice were again administered SAL 25-min prior to the initiation of the acoustic startle tests. Three days later on day 11, mice were assigned to one of four OXY treatment groups (9, 17.8, 23.7, or 33 mg/kg) using a within-subject Latin square design. Mice were then again randomized into treatment groups to test remaining OXY doses on days 14, 17, and 20 (i.e., a two-day interval between subsequent tests). For all OXY tests, mice were administered OXY 25-min prior to startle sessions.

Effects of HIV-1 Tat expression and chronic OXY and its precipitated withdrawal

Tat(-) and Tat(+) mice (n=10/group) were assigned to groups that received either chronic SAL or chronic OXY and were maintained on a REG or DOX-containing diet as shown in Table 4. Mice were first tested in a preliminary ASR and locomotor activity baseline test (i.e., "baseline I") under REG-chow conditions. Following baseline I, mice were maintained on either REG or DOX chow for two weeks. Two weeks later, mice

were re-tested during which an acute saline (“SAL”) injection was administered immediately prior to both startle and locomotor tests to re-establish baseline effects (i.e., “Day 0/SAL). Following this, chronic SAL or OXY was administered subcutaneously for ten days, as described in Chapter III and Fig. 5, with slight changes (i.e. additional naloxone doses). Importantly, 2 h after the Day 9 AM injection (33 mg/kg), mice received an acute subcutaneous injection of SAL immediately prior to both acoustic startle and locomotor activity tests to assess the effects of chronic OXY on behavior. On Day 10, 2 h after receiving an AM injection of OXY (33 mg/kg), mice received an acute subcutaneous injection of naloxone (“NLX”, 0.1-10 mg/kg) to assess the ability of naloxone to precipitate perturbations of the dependent measures. Within this chapter, the term “naloxone-challenge” on Day 10 is used interchangeably with “precipitated withdrawal”, as this regimen demonstrated naloxone dose-dependent increases in somatic signs of withdrawal, as discussed in Chapter II. Bodyweights of mice were recorded at the beginning of each day, and immediately following behavioral testing on Days 9 and 10. This chapter discusses results from acoustic startle tests; results from locomotor activity tests will be discussed in Chapter V.

2.4. Drugs

Oxycodone HCl (Mallinckrodt Inc., St. Louis, MO) was initially prepared in an aqueous sterile stock solution of 10 mg/ml for self-administration studies, which was then diluted with deionized water to make working solutions for oral self-administration tests of 0.1, 0.3, 0.56, and 1.0 mg/ml. For acute OXY and OXY dependence studies, oxycodone was prepared in a sterile stock solution of 10 mg/ml in non-heparinized 0.9% saline before diluting in sterile saline to make working solutions in the following

concentrations: 0.09, 1.78, 2.37, and 3.3 mg/ml. Naloxone HCl (Sigma-Aldrich, St. Louis, MO) was prepared in sterile 0.9% saline to make working solutions in the following concentrations: 0.01, 0.1, and 1 mg/ml. All injections were given at a 10 ml/kg injection volume and there was a seven hour period between any AM and PM injections. AM injections occurred between 08:00 and10:00 hours and PM injections occurred between 15:00 and 17:00 hours.

2.5. Data analysis

For self-administration studies, numbers of liquid deliveries and active and inactive lever (i.e., non-reinforced) presses were recorded. A Grubbs' test with an alpha level set at 0.05 was conducted to eliminate outliers (see Chapter VI for further discussion). Data were then analyzed using a one-way or two-way ANOVA, with a within-subject factor of "concentration" and a between-subject factor of "phase" or "group" as appropriate.

For acoustic startle studies, the startle magnitude for the "STIM alone" trial type was averaged across replicates and was used to infer the startle response. For PPI, startle magnitude for each trial type was averaged across replicates and these averaged magnitudes were used to calculate %PPI with the following formula: 100x[(STIM alone-prepulse)/(STIM alone)]. Habituation was inferred by measuring the %decrease in startle reactivity where, using the average startle magnitudes of the first and last five STIM alone trials of the session, %decrease in startle reactivity was calculated using the following formula: 100x[(First STIM alone -Last STIM alone)/First STIM alone)]. Data were analyzed using a two-way ANOVA, with a within-subject factor of "day" and between-subject factor of "group". Within- and between-group comparisons

were made using Bonferroni post-hoc tests where appropriate. For DOX-fed, OXY-treated Tat transgenic groups, data were analyzed in separate two-way ANOVAs based on genotype to analyze naloxone dose effects; a separate two-way ANOVA was used to detect between-genotype effects. All data were analyzed and graphed using microcomputer software (Prism 6 for Windows, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if $p<0.05$.

3. Results

3.1. Oral oxycodone self-administration

Both Tat(-) and Tat(+) mice learned to press for water deliveries under post-prandial conditions with REG or DOX chow in all phases before being introduced to increasing concentrations of OXY. As shown in Figure 10, lever discrimination between active and inactive levers was maintained for both Tat(-) and Tat(+) mice across all phases (i.e., Pre-DOX, DOX and Post-DOX phases). There was a significant main effect of concentration on active lever presses for both genotypes [Tat(-): $F (4, 87)=45.55$, $p<0.0001$; Tat(+): $F (4, 92)=33.33$, $p<0.0001$], as concentration increased the number of active lever presses decreased. There was a significant main effect of phase for Tat(+) mice [$F (2, 92)=5.389$, $p=0.0061$] with generally the greatest number of lever presses emitted during the Post-DOX phase at similar concentrations. There was a lack of significant differences in the number of active lever presses between genotypes at all concentrations during all phases of the study. Numbers of inactive lever presses did not differ across OXY concentration or phase for both genotypes at any concentration or phase of the study.

During all phases and both genotypes, as OXY concentrations increased, the number of deliveries obtained decreased (Fig. 11A and 11B). Separate two-way ANOVA analyses revealed a significant main effect of concentration for both genotypes [Tat(-): $F(4, 87)=50.33$, $p<0.0001$; Tat(+): $F(4, 92)=34.58$, $p<0.0001$], and a significant main effect of phase for Tat(+) mice [$F(2, 92)=3.218$, $p=0.0446$]. Both genotypes showed a concentration-dependent increase in estimated "mg/kg" consumed of OXY during all phases of the study except for the Tat(+) mice during the DOX phase (Fig. 11C and 11D) in which there was a decrease in consumption from 0.56 mg/ml to 1 mg/ml. Consumption estimates were analyzed and both genotype groups had a significant main effect of concentration across phases [Tat(-): $F(3, 69)=9.539$, $p<0.0001$; Tat(+): $F(3, 72)=11.79$, $p<0.0001$]; however, there was no main effect of phase on consumption.

Feeding conditions were identical during the Pre-DOX and Post-DOX phases, and comparisons were consequentially conducted at 1 mg/ml OXY concentration when consumption was generally the highest and behavioral activation was mostly observed. Within genotypes, more liquid deliveries and consumption of OXY (mg/kg) occurred during the Post-DOX phase (Figure 12A and B). A separate one-way ANOVA analysis of OXY deliveries at the 1 mg/ml OXY revealed a significant difference between Tat(-)/Pre-DOX and Tat(+)/Post-DOX, as shown in Figure 12A [$F(3, 23)=3.032$, $p=0.0498$]. Although both Tat(-) and Tat(+) groups increased their numbers of deliveries in the Post-DOX phase relative to their Pre-DOX phase, there were no significant within-group effects. Unlike numbers of OXY deliveries obtained, one-way ANOVA analysis of consumption estimates at 1 mg/ml did not reveal significant differences within or

between genotype groups (Fig. 12B). Despite similar feeding conditions during the Pre- and Post-DOX phases, more 1 mg/ml OXY deliveries occurred, and more consumption of OXY (mg/kg) occurred during the Post-DOX phase within each genotype, although these differences only obtained statistical significance for deliveries for the Tat (+) mice.

3.2. Acoustic startle

3.2.1. Effects of HIV-1 Tat expression alone

The effects of HIV-1 Tat expression on acoustic startle and its related measures, PPI and habituation are shown in Figures 13-15. Tat(-) and Tat(+) mice did not significantly differ in acoustic startle responses under baseline conditions, regardless of the provided chow type. As shown in Figure 13A and 13B, DOX chow did not affect startle in Tat(-) or Tat(+) mice up to 58 days of exposure. There was, however, a significant increase in startle response relative to baseline for Tat(-)/REG mice after 16, 30, and 58 days ($p<0.01$). Habituation was mostly unaffected by feeding days regardless of chow type; however, in Tat(+)/DOX group there was a significant ($p=0.0268$) increase relative to baseline after 58 days of DOX exposure as seen in Figure 13C and 13D. In PPI, the three prepulse intensities tested (73pp, 77pp, and 85pp) were analyzed in separate two-way ANOVA tests and results are summarized in Table 3. Within each prepulse intensity, lower %PPI measures occurred on all DOX feeding days relative to baseline in the Tat(+) but not Tat(-) mice, and beginning 16 days after DOX exposure, these decreases were statistically significant at several prepulse intensities (Table 3).

3.2.2. Effects of HIV-1 Tat expression and acute OXY administration

The interactions of HIV-1 Tat expression and acute OXY on acoustic startle and its related measures, PPI and habituation, were examined in Tat(-) and Tat(+) mice, either maintained on *ad libitum* REG or DOX chow. For acute studies, there was no difference in ASR between Tat(-) and Tat(+) mice under baseline REG-chow conditions (not shown). After being maintained on DOX for a week, mice were tested under acute doses of OXY. There was a significant main effect of dose on acoustic startle [$F(4, 56)=10.43$, $p<0.0001$]. As shown in Figure 14A, acute administration of all OXY doses (9-33 mg/kg) significantly decreased acoustic startle in Tat(-) mice, and at most doses (17.8-33 mg/kg) in Tat(+) mice. Acute OXY administration in neither genotype affected habituation (Fig. 14B). Similarly, PPI at all prepulse intensities was unaffected by acute OXY administration in all mice as shown in Figure 15.

3.2.3. Effects of HIV-1 Tat expression and chronic OXY and its precipitated withdrawal

The effects of HIV-1 Tat expression and chronic OXY as well as naloxone-precipitated withdrawal on acoustic startle and its related measures are shown in Figures 16-18. Chronic SAL administration followed by NLX (1 mg/kg) challenge on Day 10 did not significantly affect startle in either REG- or DOX-maintained Tat(-) or Tat(+) mice (Fig. 16A and 16B), despite a significant main effect of time [$F(2, 70)=5.067$, $p=0.0088$]. Importantly, these results essentially replicated the earlier described timecourse study with REG and DOX chow in Tat transgenic mice. Habituation was also unaffected after chronic SAL and acute NLX in REG- or DOX-fed Tat transgenic mice (Fig. 16C and 16D). Chronic SAL and acute NLX challenge did not affect PPI in most groups insofar as only mice in the Tat(-)/DOX/SAL group significantly increased PPI

over time at the two lower prepulse intensities (Table 5). In REG-fed Tat(-) and Tat(+) mice, chronic OXY did not significantly affect startle, PPI, or habituation (see Fig. 17A and 17B, and Table 6). Precipitated withdrawal with 1 mg/kg NLX, however, significantly decreased startle and nonsignificantly decreased habituation in both REG-fed Tat(-) and Tat(+) mice. Similar to results in B6 mice, PPI was unaffected after precipitated withdrawal from oxycodone in REG-fed Tat transgenic mice.

Finally, in DOX-fed Tat transgenic mice, separate groups of mice received naloxone (0.1, 1.0 or 10 mg/kg) on Day 10 to characterize a dose-effect curve with naloxone. There was a significant main effect of day [$F(2, 54)=15.09$, $p<0.0001$] as well as a significant interaction of day and group [$F(4, 54)=4.792$, $p=0.0022$] on startle magnitude in Tat(-) mice. For Tat(+) mice, there was only a significant main effect of day [$F(2, 54)=8.897$, $p=0.0005$]. As shown in Figure 18A and 18B, chronic OXY decreased the ASR relative to Day 0/SAL for most groups and significantly so for mice in the Tat(-)/OXY/0.1 NLX group ($p=0.0002$) and the Tat(+)/OXY/1 NLX ($p=0.011$). Precipitated withdrawal with the lowest dose of naloxone tested (0.1 mg/kg) increased startle relative to chronic OXY conditions, and significantly so in Tat(-) mice ($p=0.0044$); however, higher doses of naloxone significantly decreased startle in both Tat(-) and Tat(+) mice to similar levels.

There was a significant main effect of day in both genotypes [Tat(-): $F(2, 54)=6.554$, $p=0.0028$; Tat(+): $F(2, 54)=6.343$, $p=0.0034$] on habituation of the startle response. As shown in Figure 18C and 18D, chronic OXY administration did not affect habituation in Tat(-) mice or Tat-expressing mice. Precipitated withdrawal, however, decreased habituation in both groups but was only significantly decreased in Tat(-) mice

at 1 mg/kg NLX (p=0.0419 vs Day 9/SAL) and 10 mg/kg (p=0.0435 vs Day 0/SAL; p=0.0446 vs Day 9/SAL). In Tat-expressing mice, precipitated withdrawal significantly decreased habituation at the highest dose of naloxone (10 mg/kg: p=0.0370 vs Day 9/SAL). In Tat(-) mice, there was a significant main effect of day at all prepulse intensities [73pp: F (2, 54)=5.974, p=0.0045; 77pp: F (2, 54)=4.414, p=0.0168; 85pp: F (2, 54)=5.381, p=0.0074] for PPI. For Tat(+) mice, there was a significant main effect of day only at the 77 dB prepulse [F (2, 54)=3.881, p=0.0266]. In general, there was a lack of change in PPI at any prepulse intensity for either Tat(-) or Tat(+) mice after chronic OXY administration, although post-hoc analyses revealed several significant differences from baseline for the Tat(+)/OXY/0.1 NLX group as shown in Table 7. Moreover, there was a general lack of effect by precipitated withdrawal from OXY on PPI in most groups, although post-hoc analyses revealed a significant shift from baseline for the Tat(+)/OXY/1 NLX group at the 77 dB prepulse intensity. There were no between-genotype differences for any condition within each naloxone dose tested.

In regards to body weight loss measurements as a separate index of physical dependence upon oxycodone, bodyweight was measured at the beginning of Day 9 and Day 10 as well as at the conclusion of behavioral testing on those days (i.e., a 3.5-h span between body weight measurements). For both REG- and DOX-fed Tat transgenic mice, bodyweight loss was decreased after testing on both Days 9 and 10, in SAL- and OXY-treated groups. This decrease was to a significantly greater degree in all OXY-treated groups after naloxone challenge ($p<0.05$). These results did not differ between REG- or DOX-fed Tat(-) or Tat(+) mice, suggesting a lack of nonspecific effects of DOX as well as Tat expression. Moreover, there was a lack of naloxone dose-dependent

effects on bodyweight loss, which is contrary to the naloxone dose-dependent effects on bodyweight loss observed in Chapter II and Figure 4. Therefore, bodyweight loss did serve as an additional indicator of physical dependence upon OXY in this study, but its reliable inference is limited due to a lack of naloxone dose-dependent effects. Moreover, as mentioned in Chapter III, the timespan between bodyweight measurements and behavioral testing may have had lingering effects to alter this output.

4. Summary

During OXY self-administration tests, the number of active lever responses decreased as a function of increasing oxycodone concentration while maintaining a separation between active and inactive lever presses for both genotypes, as was seen in C57BL/6J mice in Chapter II. Moreover, for both Tat(-) and Tat(+) mice, as oxycodone concentration increased, the number of deliveries decreased while consumption increased. This pattern repeated under all phases, and Tat(+) mice had higher levels of liquid deliveries obtained than Tat(-) mice under DOX and Post-DOX phases. Further analysis showed a significant difference in numbers of obtained liquid deliveries of 1 mg/ml oxycodone between the Pre-DOX phase in Tat(-) mice and the Post-DOX phase in Tat(+) mice, which suggests, although does not definitively confirm, that Tat expression facilitates oral oxycodone self-administration. Limitations of this study and its observations are discussed in depth in Chapter VI.

Tat expression alone did not affect the acoustic startle response up to 58-d of DOX exposure. Tat expression alone significantly increased habituation only after 58-d of DOX, but deficits in PPI were observed after 16-d. Acute oxycodone administration significantly decreased startle in both Tat(-) and Tat(+) mice fed a DOX diet, while

habituation and PPI were left unaffected, suggesting Tat expression does not alter the acute behavioral effects of oxycodone. Similar to findings reported in Chapter III with C57BL/6J mice, chronic oxycodone, in general, did not affect startle or its related measures in Tat(-) or Tat(+) mice, however, naloxone dose-dependently decreased startle and habituation but not PPI in both groups. Moreover, there was a lack of nonspecific effects of DOX on any measurement. It is important to note that although there were no significant differences between Tat(-) and Tat(+) mice under the dependence conditions tested, there was an attenuation of severity of the withdrawal effects observed in Tat-expressing mice. Limitations of this study and its observations are discussed in Chapter VI.

In summary, the results from this chapter indicate that Tat expression can alter some of oxycodone's abuse-related behavioral effects as measured by oral self-administration, acoustic startle, and habituation. These results and the limitations of these studies are further discussed in Chapter VI. The procedures described herein further demonstrate that oxycodone is volitionally orally consumed via operant self-administration and its dependence-related effects may be assessed by the acoustic startle response. These procedures allow for further characterization of the underlying biological determinants of opioid-Tat interactions as well as screening of potential pharmacotherapies to block the behavioral effects observed.

Table 3. Mean %PPI over time in REG- or DOX-fed Tat transgenic mice.

Group	Days	Prepulse Intensity (dB)		
		73	77	85
Tat(-)/REG	Baseline	41.33 (5.81)	50.08 (3.51)	63.23 (3.53)
	2	38.73 (4.68)	46.82 (5.26)	69.57 (3.69)
	9	36.52 (8.92)	47.47 (7.01)	61.22 (6.06)
	16	42.54 (5.94)	51.85 (4.39)	67.97 (3.13)
	30	51.57 (4.95)	58.32 (4.24)	73.04 (2.75)
	58	54.09 (4.11)	60.17 (4.84)	60.17 (4.84)
Tat(-)/DOX	Baseline	40.50 (3.51)	50.92 (4.87)	59.88 (4.94)
	2	38.06 (5.05)	50.84 (4.51)	61.45 (5.37)
	9	42.16 (5.87)	45.73 (6.48)	52.84 (7.26)
	16	38.57 (2.85)	46.36 (3.18)	62.52 (3.12)
	30	38.25 (8.47)	49.18 (7.08)	63.07 (4.54)
	58	39.23 (3.77)	44.63 (6.03)	61.61 (4.98)
Tat(+)/REG	Baseline	51.58 (4.73)	58.78 (3.07)	71.04 (4.12)
	2	49.98 (4.69)	61.64 (3.94)	71.69 (4.03)
	9	55.25 (2.26)	62.58 (2.13)	73.54 (2.70)[b (p=0.024)]
	16	50.46 (6.04)	58.80 (5.36)	71.74 (5.74)
	30	44.07 (8.30)	51.07 (8.06)	61.04 (6.99)
	58	43.61 (7.14)	54.15 (5.91)	67.52 (6.57)
Tat(+)/DOX	Baseline	57.15 (4.36)	62.47 (4.10)	70.60 (3.26)
	2	42.58 (5.84)	51.32 (5.17)	59.91 (5.60)
	9	45.94 (3.76)	50.20 (4.68)	60.38 (3.56)
	16	40.11 (6.01)[a]	50.30 (5.33)	56.48 (7.39)[a]
	30	43.97 (6.17)	54.27 (5.54)	66.29 (5.23)
	58	41.46 (6.08)	43.49 (7.42)[a]	59.34 (6.17)

[a] P<0.05 to baseline
[b] Significant difference was found between Tat(+)/REG and Tat(-)/DOX at one timepoint

Table 4. Summary of test conditions for chronic OXY and dependence-related effects on acoustic startle, PPI, and habituation.

Genotype (Tat +/-)	Chow (REG/DOX)	Chronic treatment (SAL/OXY)	Naloxone dose (mg/kg)
Tat(-)	REG	SAL	1
Tat(+)	REG	SAL	1
Tat(-)	DOX	SAL	1
Tat(+)	DOX	SAL	1
Tat(-)	REG	OXY	1
Tat(+)	REG	OXY	1
Tat(-)	DOX	OXY	0.1
Tat(+)	DOX	OXY	0.1
Tat(-)	DOX	OXY	1
Tat(+)	DOX	OXY	1
Tat(-)	DOX	OXY	10
Tat(+)	DOX	OXY	10

Table 5. Mean %PPI after chronic SAL treatment in REG- or DOX-fed Tat transgenic mice.

Group	Prepulse Intensity(dB)	Day 0/SAL AVG (± S.E.M)	Day 9/SAL AVG (± S.E.M)	Day 10/NLX AVG (± S.E.M)
Tat(-)/REG/SAL	73	43.80 (3.58)	39.62 (4.97)	38.15 (6.10)
	77	49.70 (4.33)	47.98 (5.19)	50.91 (5.48)
	85	65.25 (4.71)	62.09 (5.41)	62.11 (6.13)
Tat(-)/DOX/SAL	73	34.85 (7.72)	49.97 (3.39)[a]	51.74 (2.82)[a]
	77	51.73 (4.89)	57.79 (2.89)	61.91 (3.71)[a]
	85	73.52 (5.03)	75.62 (3.18)	77.28 (3.35)
Tat(+)/REG/SAL	73	47.72 (3.29)	52.57 (3.84)	52.47 (4.86)
	77	61.85 (3.04)	59.96 (3.82)	60.60 (4.58)
	85	69.36 (4.81)	71.86 (3.51)	72.52 (4.03)
Tat(+)/DOX/SAL	73	52.60 (3.64)	52.66 (4.80)	54.58 (4.69)
	77	61.25 (3.81)	66.36 (3.10)[b (p=0.01)]	65.47 (3.32)
	85	75.85 (3.42)	74.25 (3.10)	78.03 (1.74)[b (p=0.048)]

[a] P<0.05 to Day 0/SAL
[b] Significant difference was found between Tat(+)/DOX/SAL and Tat(-)/REG/SAL

Table 6. Mean %PPI after chronic OXY treatment in REG-fed Tat transgenic mice.

Group	Prepulse Intensity(dB)	Day 0/SAL AVG (± S.E.M)	Day 9/SAL AVG (± S.E.M)	Day 10/NLX AVG (± S.E.M)
Tat(-)/REG/OXY	73	42.62 (5.93)	40.37 (4.66)	47.91 (4.68)
	77	53.11 (6.03)	49.24 (5.60)	53.26 (4.82)
	85	68.91 (3.29)	63.83 (3.50)	66.32 (3.22)
Tat(+)/REG/OXY	73	51.77 (5.14)	48.07 (6.33)	56.49 (3.88)
	77	61.04 (5.61)	60.39 (4.68)	57.85 (4.71)
	85	74.95 (4.77)	73.30 (3.81)	76.89 (3.88)

Table 7. Mean %PPI after chronic OXY treatment in DOX-fed Tat transgenic mice.

Group	Prepulse Intensity(dB)	Day 0/SAL AVG (± S.E.M)	Day 9/SAL AVG (± S.E.M)	Day 10/NLX AVG (± S.E.M)
Tat(-)/OXY/0.1 NLX	73	45.15 (6.48)	29.04 (4.77)	50.05 (6.16)[b(p=0.019)]
	77	52.99 (7.42)	40.22 (4.48)	61.03 (5.70)[b(p=0.0078)]
	85	70.56 (5.16)	59.99 (4.13)	74.93 (3.27)[b(p=0.05)]
Tat(-)/OXY/1 NLX	73	45.16 (2.73)	49.96 (4.18)	53.67 (4.02)
	77	53.32 (1.86)	56.98 (3.64)	59.83 (5.10)
	85	70.91 (2.33)	68.04 (3.96)	73.17 (3.30)
Tat(-)/OXY/10 NLX	73	47.78 (4.69)	41.21 (5.80)	51.49 (4.67)
	77	54.67 (4.24)	54.57 (4.33)	62.28 (2.15)
	85	73.06 (2.81)	70.51 (3.85)	75.05 (1.80)
Tat(+)/OXY/0.1 NLX	73	50.41 (4.74)	28.24 (8.91)[a(p=0.012)]	38.13 (10.39)
	77	61.26 (3.42)	44.00 (6.26)[a(p=0.0358)]	51.35 (7.57)
	85	66.16 (2.89)	56.12 (6.63)	60.09 (8.31)
Tat(+)/OXY/1 NLX	73	57.22 (5.34)	50.47 (4.83)	40.90 (6.42)
	77	66.96 (4.52)	60.90 (3.07)	46.27 (5.45)[a(p=0.0082)]
	85	77.51 (2.21)	69.42 (1.98)	68.27 (5.34)
Tat(+)/OXY/10 NLX	73	44.77 (3.77)	36.08 (4.42)	47.74 (6.91)
	77	51.49 (4.09)	47.09 (3.79)	50.09 (6.27)
	85	67.12 (4.25)	64.06 (4.17)	58.56 (8.16)

[a] Significant difference as compared to Day 0/SAL
[b] Significant difference as compared to Day 9/SAL

5. Figures

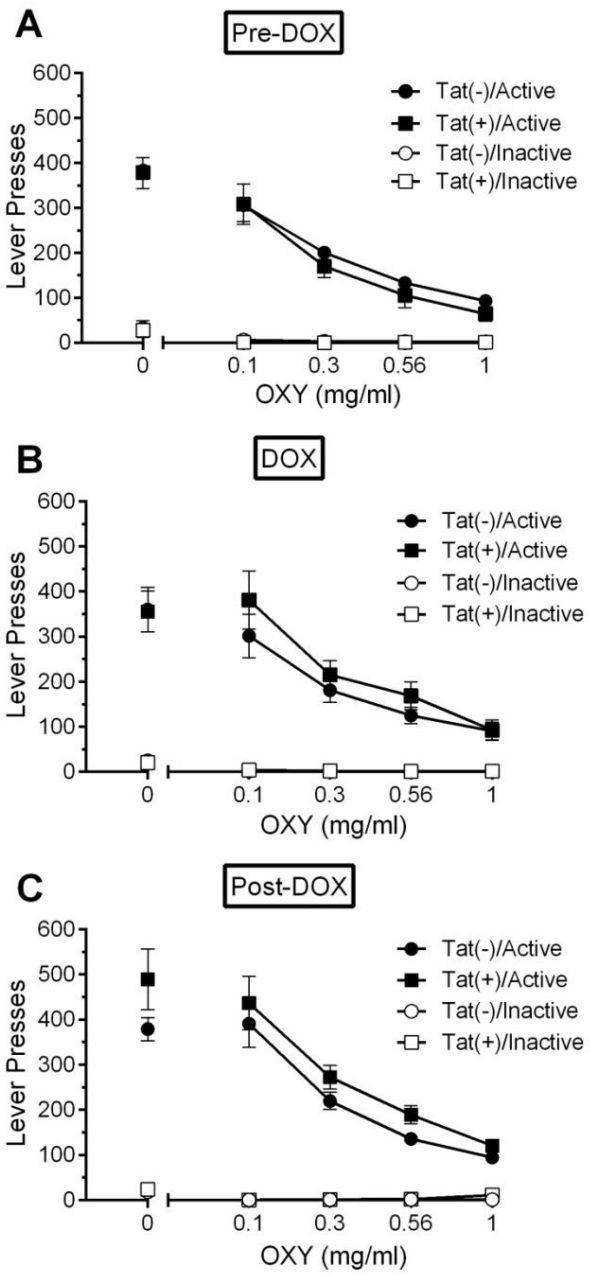

Figure 10. Effects of available oxycodone concentration on the number of active and inactive lever presses during 1-h test sessions emitted by Tat transgenic mice during the pre-DOX (A), DOX (B), and post-DOX (C) phases. Each symbol represents the mean of three test sessions per concentration (N=6-8/group). Brackets through symbols represent ± S.E.M. Filled symbols represent active lever presses, whereas unfilled symbols represent inactive lever presses.

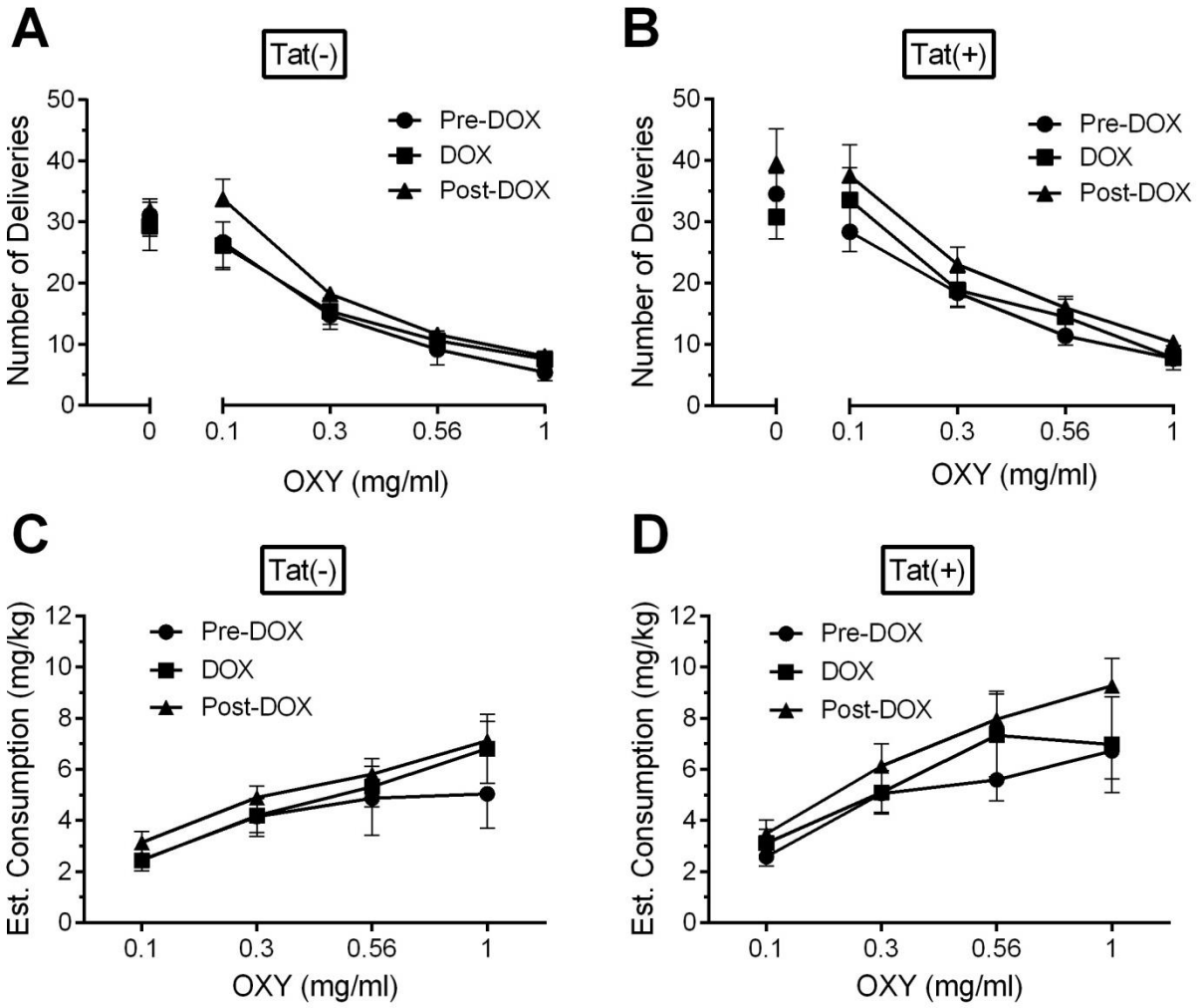

Figure 11. Number of liquid deliveries (A and B) obtained and the estimated consumption (C and D) of oxycodone (mg/kg body weight) as a function of the available concentration (mg/ml) in Tat transgenic mice at all phases. Consumption was estimated based on individual subject's daily body weight and the total deliveries of oxycodone obtained. Each symbol represents the mean number of liquid deliveries or consumption across three test sessions per concentration (N=6-8/group). Brackets through symbols represent ± S.E.M.

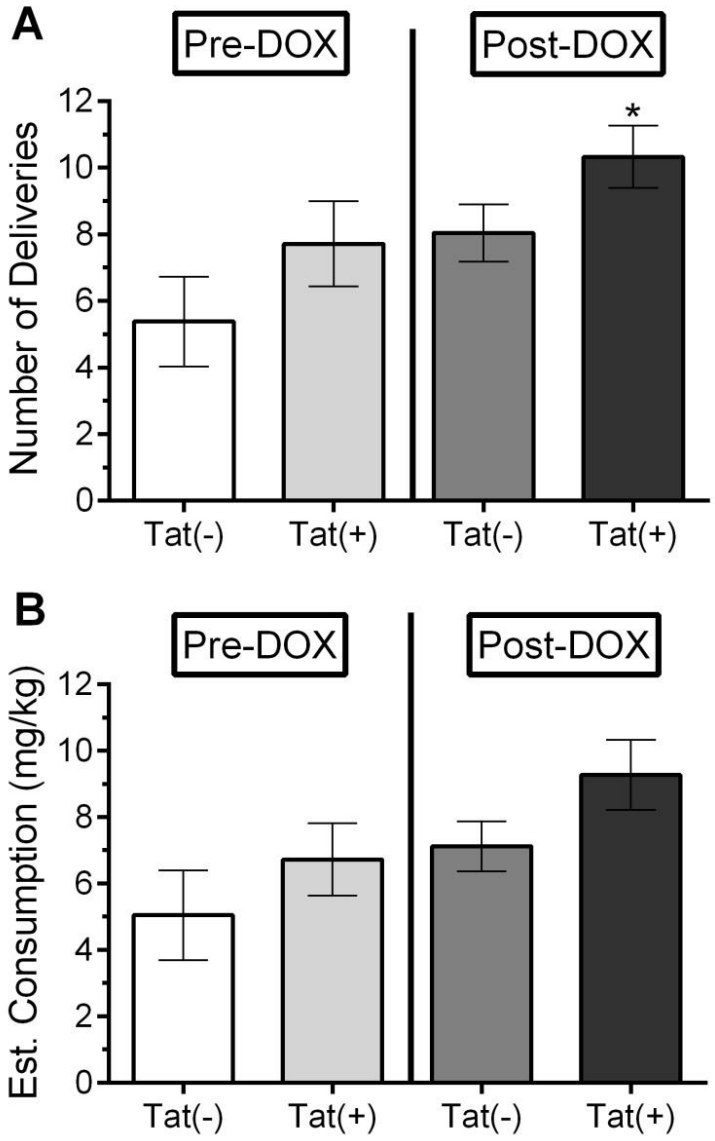

Figure 12. Comparison between Pre-DOX and Post-DOX phases for Tat transgenic mice of number of liquid deliveries (A) obtained and estimated consumption (B) of oxycodone at the highest concentration available, 1 mg/ml. Consumption was estimated based on individual subject's daily body weight and the total deliveries of oxycodone obtained. Each symbol represents the mean oxycodone consumption (± S.E.M.) of 1 mg/ml OXY across three test sessions. *P<0.05 compared to the Tat(-)/Pre-DOX group.

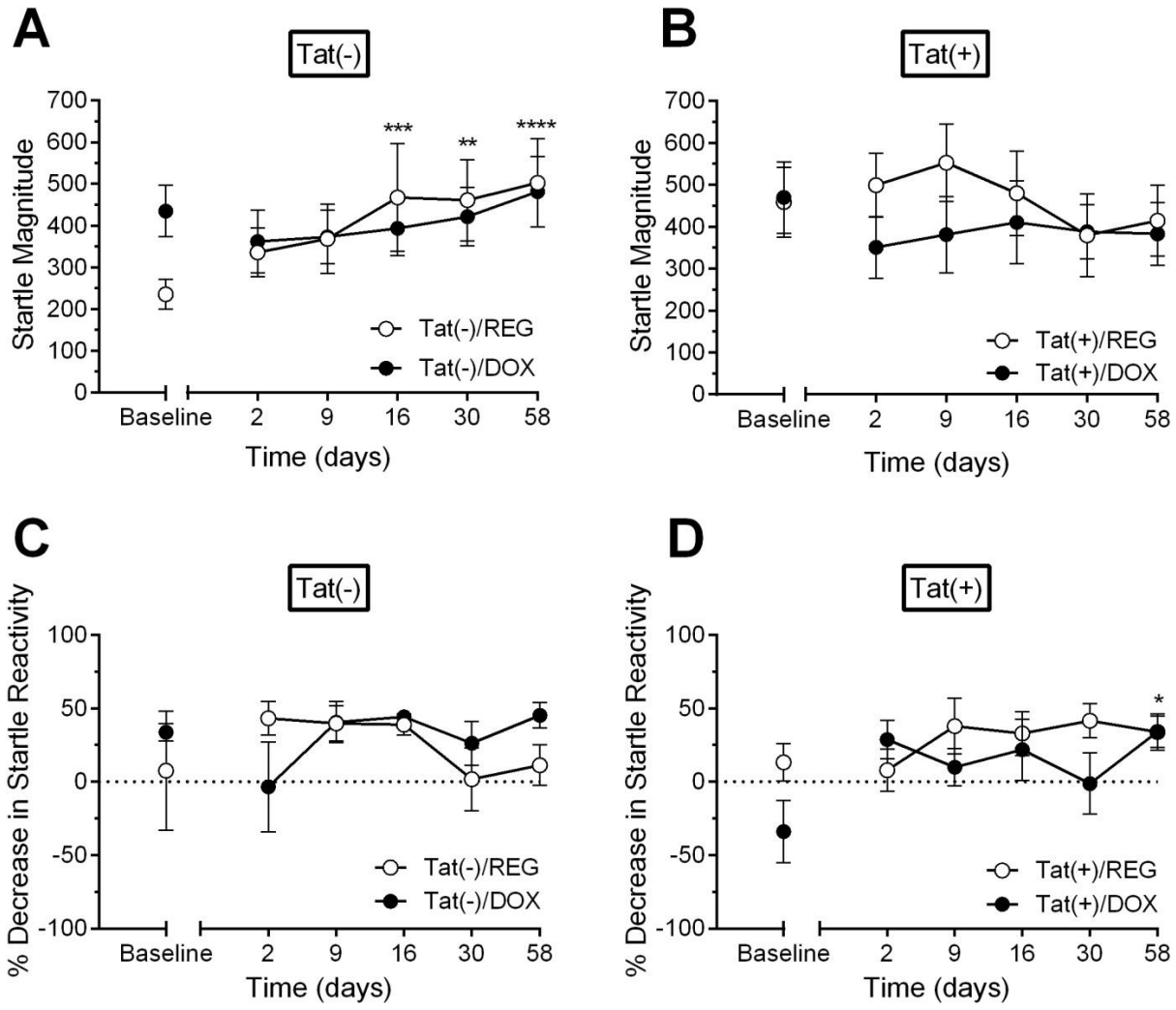

Figure 13. Effects of days fed REG or DOX chow on acoustic startle (A and B) and habituation (C and D) in Tat(-) and Tat(+) mice. N=8/group. Data are represented as the mean (± S.E.M.) startle amplitude or as the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to baseline.

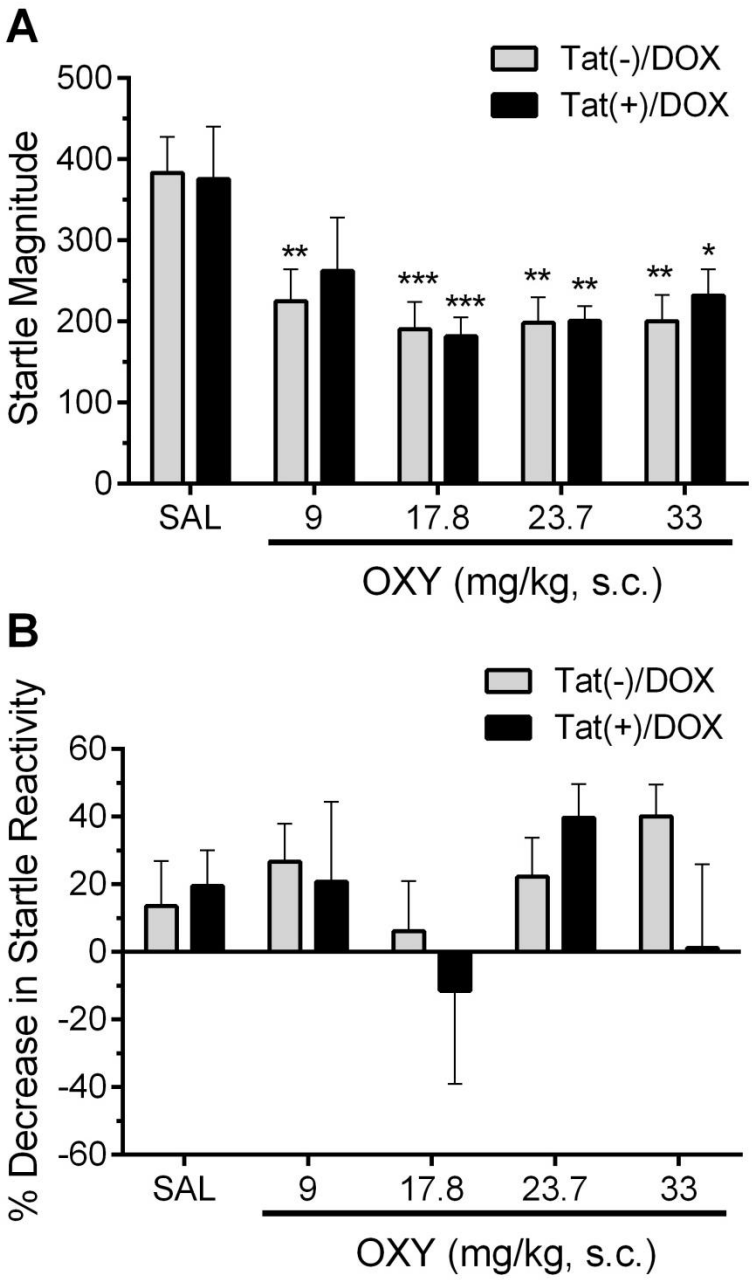

Figure 14. Effects of acute OXY and Tat expression on acoustic startle (A) and habituation (B). N=8/group. Data are represented as the mean (± S.E.M.) startle amplitude or as the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05, **P<0.01, ***P<0.001 compared to SAL treatment.

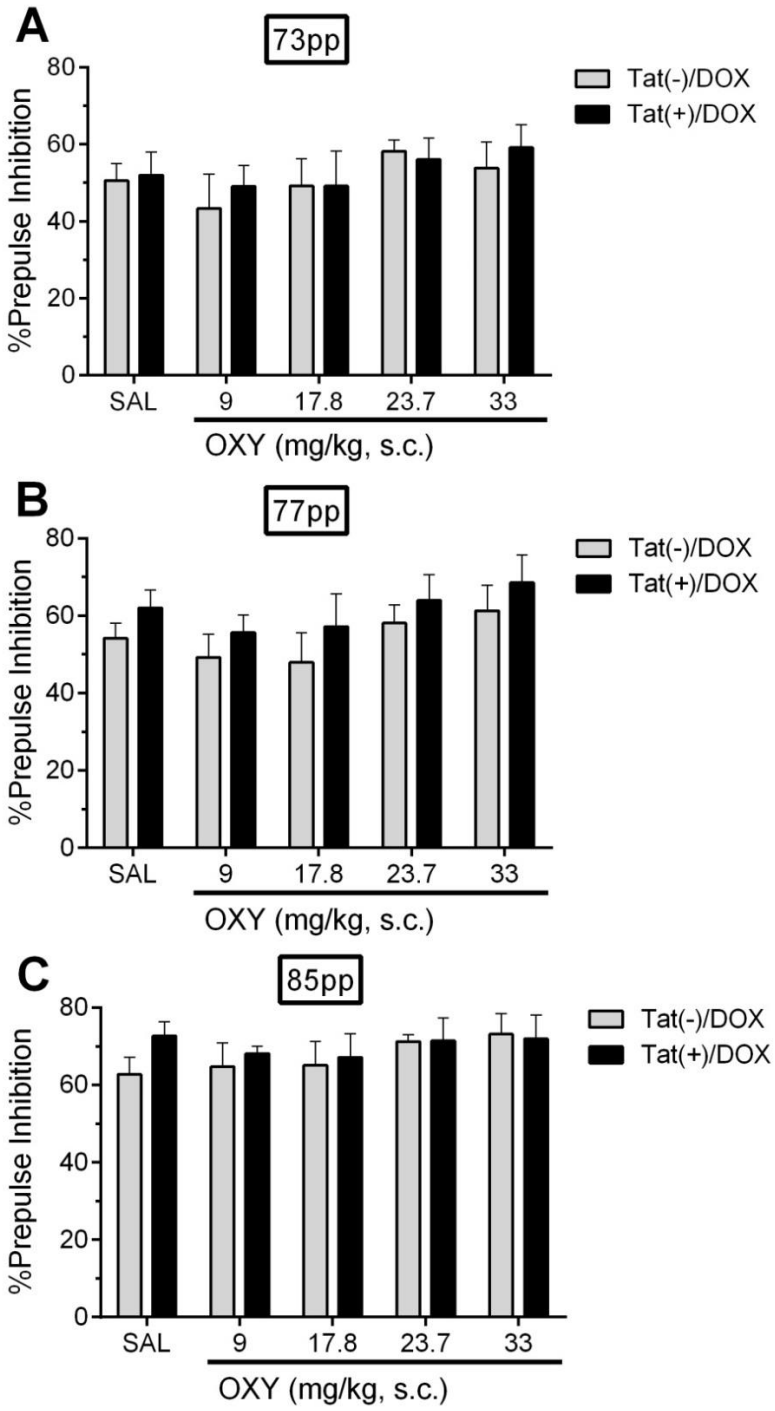

Figure 15. Effects of acute OXY and Tat expression on prepulse inhibition.
N=8/group. Data are expressed as mean percent PPI (± S.E.M.) for each prepulse intensity level (73, 77, and 85 dB).

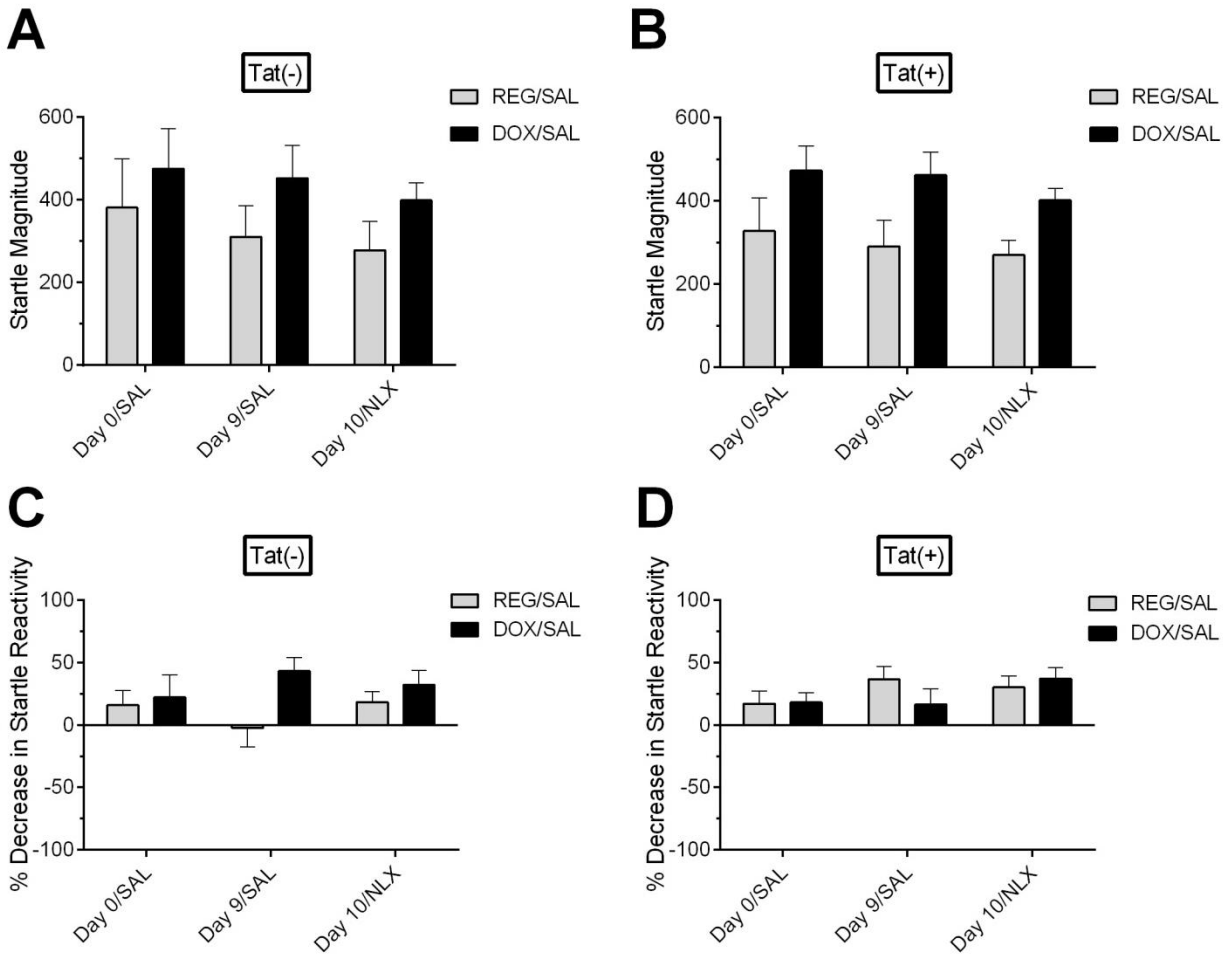

Figure 16. Effects of chronic SAL, acute NLX, and DOX on acoustic startle (A and B) and habituation (C and D) in Tat(-) or Tat(+) mice. N=9-10/group. Data are represented as the mean (± S.E.M.) startle amplitude or as the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05 compared to Tat(-)/REG mice.

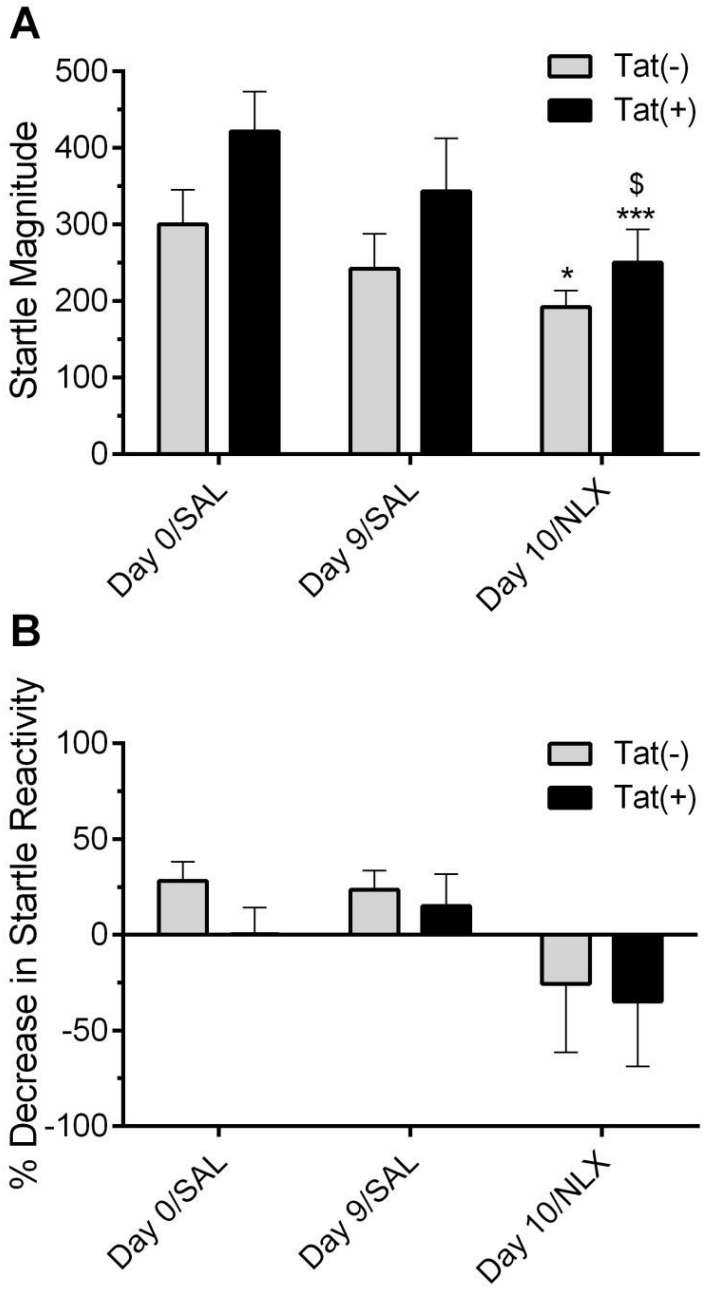

Figure 17. Effects of chronic OXY and NLX-precipitated withdrawal on startle (A) and habituation (B) in REG-fed Tat(-) and Tat(+) mice. N=10/group. Data are expressed as mean (± S.E.M.) startle amplitude or as the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. *P<0.05, ***P<0.001 compared to Day 0/SAL; $P<0.05 compared to Day 9/SAL.

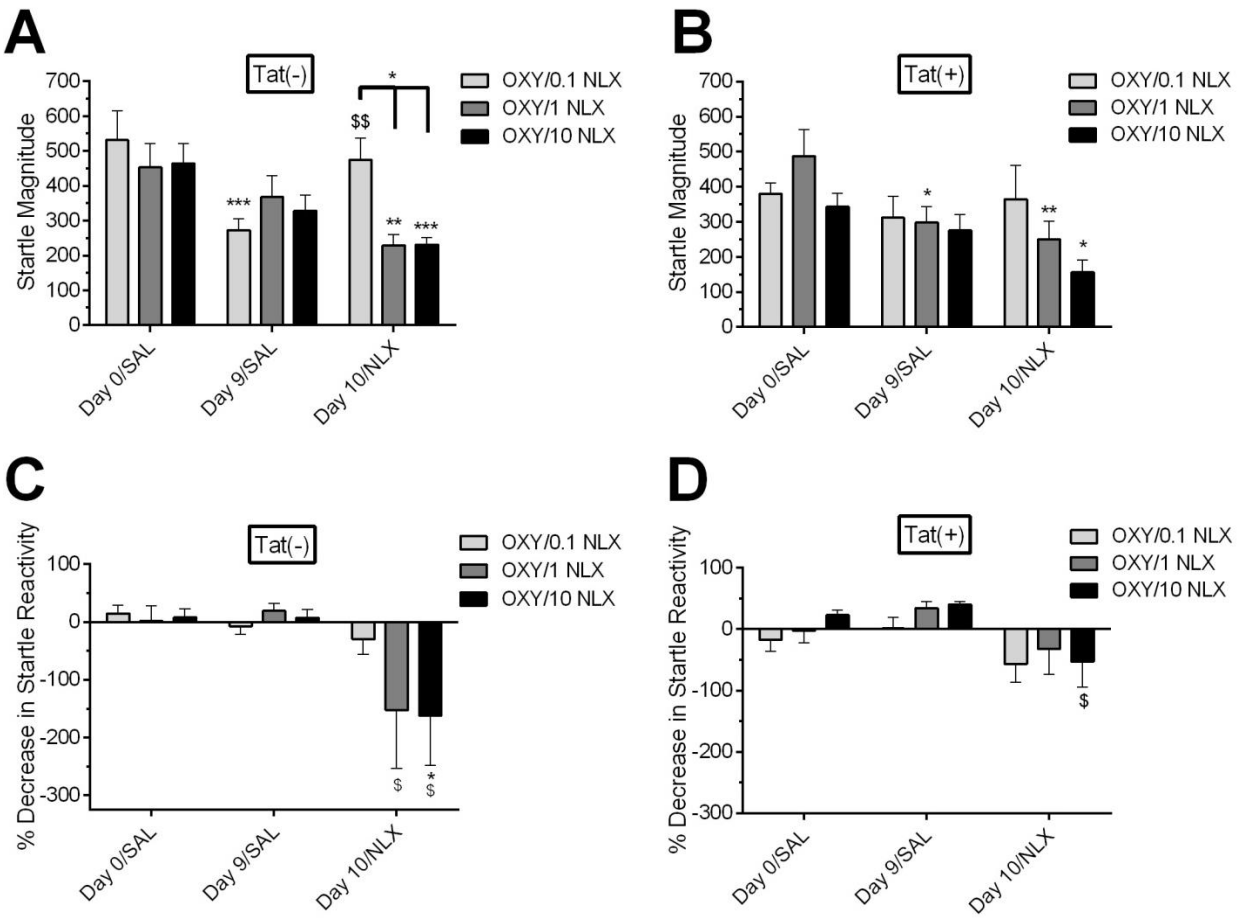

Figure 18. Effects of chronic OXY and NLX-precipitated withdrawal on acoustic startle (A and B) and habituation (C and D) in DOX-fed Tat(-) and Tat(+) mice.
N=10/group. Data are represented as the mean (± S.E.M.) startle amplitude or as the mean (± S.E.M.) percent decrease in startle reactivity within a 30-min test session. Note: mice in the Tat(-)/OXY/0.1 NLX group had a two-day interruption (i.e., received no injections) between Day 3 and 4 of the OXY dosing regimen.*P<0.05, **P<0.01, ***P<0.001 compared to Day 0/SAL; $P<0.05, $$P<0.01 compared to Day 9/SAL.

Chapter V: HIV-1 Tat expression does not alter acute or chronic oxycodone-induced hyperactivity, but does alter opioid locomotor sensitization

1. Introduction

Locomotor activity and its sensitization has been hypothesized to correspond to the reinforcing properties of drugs of abuse, and typically involves the repeated administration of a locomotor activating drug (Robinson and Berridge, 1993). Oxycodone has previously been shown to increase locomotor activity in mice after acute subcutaneous administration and can induce locomotor sensitization (Liu et al., 2005). Because the previously described dependence regimen in Chapters II-IV involves the repeated administration of oxycodone, it was hypothesized that sensitization would extend to it. The previously described oxycodone physical dependence regimen was thus used to evaluate its effects on locomotor activity, its sensitization, and stereotypy in C57BL/6J mice. HIV-1 Tat expression in female and male mice has shown to decrease locomotor activity as well as disrupt cocaine-induced sensitization in ovariectomized rats receiving intra-accumbal microinjections of Tat (Hahn et al., 2015; Harrod et al., 2008). Therefore, it was hypothesized that HIV-1 Tat would inhibit oxycodone's effects on locomotor activity and its sensitization. The effects

of HIV-1 Tat expression on oxycodone-induced locomotor hyperactivity, as well as morphine and oxycodone locomotor sensitization, were thus additionally evaluated.

2. Methods

2.1. Subjects

Male C57BL/6J ("B6") mice were obtained at approximately 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and were allowed to acclimate to the vivarium for at least one week prior to commencement of training and testing. Male, doxycycline ("DOX")-inducible Tat_{1-86} transgenic mice were generated on a C57BL/6J and C3H background as described in Chapter IV and in published reports by others, and Tat expression was only induced in mice positive for the Tat transgene ["Tat(+)"] via a diet consisting of 6 g DOX per kg of chow (Harlan Laboratories Inc., Indianapolis, IN) whereas those lacking the Tat transgene ["Tat(-)"] did not express Tat (Bruce-Keller et al., 2008; Hauser et al., 2009).

In all studies, mice were allowed *ad libitum* access to water and either standard ("REG") rodent chow (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories Inc., Indianapolis, IN) or DOX chow Mice were housed in an AALAC-accredited animal facility, kept on a 12-h/12-h light/dark cycle (lights on from 06:00 to 18:00 hours). All procedures were conducted during the light phase and were in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press, 2011), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Overall procedure

2.2.1. Effects in C57BL/6J mice

As described in Chapter III and Fig. 5, adult male B6 mice underwent chronic subcutaneous saline (SAL) or oxycodone (OXY) administration under REG or DOX feeding conditions. On day 9, under chronic OXY conditions, OXY was administered approximately 2-h prior to a 30-min acoustic startle test session, and consequentially 2.5-h prior to locomotor test sessions. Immediately prior to locomotor tests, animals received an acute SAL injection. On day 10, OXY was again administered 2.5-h prior to locomotor testing and, immediately before testing began, animals received an acute naloxone (NLX) injection (1 mg/kg).

In a separate cohort of adult, male B6 mice, locomotor sensitization was evaluated following the regimen outlined in Figure 20. On day 1, mice received a subcutaneous AM injection of SAL or 9 mg/kg OXY and were immediately placed in locomotor chambers for a 1-h session. Twice-daily injections of SAL or OXY were then given over the next seven days. On day 8, mice received a subcutaneous AM injection of SAL or 9 mg/kg OXY and immediately tested in a 1-h locomotor activity session.

2.2.2. Effects in Tat transgenic mice

To assess the effects of Tat expression alone on locomotor activity, Tat(-) and Tat(+) mice (n=8/group) were first tested in a 2-h baseline locomotor session under REG feeding conditions. Subsequently, mice were switched to DOX chow and re-tested in 2-h locomotor sessions after 2, 9, 16, 30, and 58 days. After 60 days on DOX, these mice were used to test morphine locomotor sensitization as described below.

In separate Tat transgenic mice, acute OXY effects on locomotor activity were assessed following the regimen described in Chapter IV. Briefly, Tat(-) and Tat(+) mice (n=8/group) on REG chow were tested in a 1-h baseline locomotor session following 30-min acoustic startle tests as described in Chapter IV. Mice were then switched to DOX for one week and re-tested on day 8 following subcutaneous SAL administration. Using a within-subject Latin square design, on days 11, 14, 17, and 20, mice were re-tested after acute administration of 9, 17.8, 23.7, or 33 mg/kg OXY (~55-min pre-treatment time prior to locomotor tests; s.c.).

For chronic OXY and dependence effects in Tat(-) and Tat(+) mice, the subcutaneous b.i.d. injection and behavioral testing regimen outlined in Chapter III and Fig. 5 was utilized, but followed the modifications as described in Chapter IV (i.e., NLX dose-response curve added). Here, Tat(-) and Tat(+) mice (n=10/group) were maintained on REG or DOX chow and administered chronic SAL or OXY. On day 9, OXY and SAL were administered approximately 2.5-h and 0-min, respectively, prior to locomotor sessions to assess the effects of chronic OXY on the described behavioral measures. On day 10, OXY and NLX (0.1-10 mg/kg) were administered 2.5-h and 0-min, respectively, prior to locomotor tests activity tests to assess the effects of precipitated withdrawal on the described behavioral measures. Within this chapter, the term “naloxone-challenge” on Day 10 is used interchangeably with “precipitated withdrawal”, as this regimen demonstrated naloxone dose-dependent increases in somatic signs of withdrawal, as discussed in Chapter II.

Finally, locomotor sensitization to morphine and oxycodone were assessed. For morphine tests, Tat(-) and Tat(+) mice (n=8/group) that had been on DOX for 12-weeks

were first assessed in a 2-h baseline locomotor activity session in which SAL was administered (0-min pre-treatment time; i.p.). Over the next four days (i.e., Days 1-4), mice were given 32 mg/kg morphine (i.p.) and immediately placed in a 2-h locomotor activity session. On Days 5-7, no testing occurred but on Day 8, mice were administered 32 mg/kg morphine and re-tested. On Day 9 and 10 no testing occurred, but on Day 11, 32 mg/kg morphine was administered and mice were re-tested. For oxycodone tests, Tat(-) and Tat(+) mice (n=5-6/group; at least 13 weeks of age) were first tested in a cumulative oxycodone dose-response curve under REG-chow conditions. After a 30-min habituation period to the locomotor chambers, mice were removed and given an acute administration of SAL and placed back in the chamber for a 10-min recording period. Subsequently, the mice were removed and administered 0.1 mg/kg OXY (s.c.) and placed back in the chamber for the 10-min period. This process repeated three more times to obtain a complete dose-response curve. Subjects were administered acute injections of 0, 0.1, 0.9, 2, and 30 mg/kg OXY to generate cumulative doses of 0.1, 1, 3, and 33 mg/kg. After this, mice were switched to DOX chow for two weeks, before re-testing the cumulative OXY dose-response curve under DOX-chow conditions.

2.3. Apparatus

For locomotor activity tests, mice were placed in eight commercially obtained, automated activity monitoring devices each enclosed in sound- and light-attenuating chambers that recorded distance traveled in centimeters via computer-controlled circuitry (AccuScan Instruments, Columbus OH). The interior of each device was divided into separate 20×20×30 cm arenas permitting the independent and

simultaneous measurement of two mice in diagonally opposite compartments. Sixteen photobeam sensors per axis were spaced 2.5 cm apart along the walls of the chamber and were used to detect movement.

2.4. Procedure

For baseline measurements, mice were placed in locomotor chambers following acoustic startle testing for a 1-h or 2-h test session with distance traveled recorded in 10-min bins, as described below. For stereotypy, the “stereotypy count” variable was defined by the software that records the number of times the mouse breaks the same beam in succession without breaking an adjacent beam (Tilley and Gu, 2008). Additionally, the time a rodent spends in the periphery versus the center area of the chamber is regarded as an indirect measure of anxiety-like behavior as it has been hypothesized that the center area poses as a threatening environment to the rodent. Decreased time spent in the center is reported in rodent models of anxiety-like disorders and can be reversed by anxiolytic drugs such as benzodiazepines (Prut and Belzung, 2003). Therefore, time spent in the center of locomotor chambers was measured for acute and chronic OXY effects in Tat transgenic mice as an indirect test for anxiety-like behavior. Center time was measured in seconds for each recorded bin.

For oxycodone locomotor sensitization tests in B6 mice, a 1-h test session was conducted with distance traveled recorded in 2-min bins. For morphine locomotor sensitization tests in Tat transgenic mice, a 2-h session was conducted with distance traveled recorded in 10-min bins. Test session durations for sensitization of oxycodone and morphine differed due to reported differences in time to peak effect and possibly consequently duration of action of these two MOR agonists (Nielsen et al., 2000;

Ordonez Gallego et al., 2007; Ross and Smith, 1997). Finally, for oxycodone locomotor sensitization tests in Tat transgenic mice, after a 30-min habituation period an oxycodone cumulative dose-response curve was generated over 5, 10-min recording periods recorded in 2-min bins. Stereotypy counts were also recorded and analyzed in all tests.

2.5. Drugs

Oxycodone HCl (Mallinckrodt Inc., St. Louis, MO) was initially prepared in a sterile stock solution of 10 mg/ml in non-heparinized 0.9% saline before diluting in sterile saline to make working solutions in the following concentrations: 0.09, 1.78, 2.37, and 3.3 mg/ml. Naloxone HCl (Sigma-Aldrich, St. Louis, MO) was prepared in sterile 0.9% saline to make working solution at 0.01, 0.1, or 1.0 mg/ml. Morphine sulfate (Mallinckrodt Inc., St. Louis, MO) was prepared in non-heparinized 0.9% saline at a working solution of 3.2 mg/ml. All injections were given at a 10 ml/kg injection volume and there was a seven hour period between AM and PM injections in chronic OXY and OXY dependence tests.

2.6. Data analysis

Total distance traveled (in cm), stereotypy counts, and time in center were recorded. Data were analyzed using a two-way ANOVA, with a within-subject factor of "day" and between-subject factor of "group". Within- and between-group comparisons were made using Bonferroni post-hoc tests where appropriate. For time in center analyses, raw data were analyzed but graphically the data are expressed as percentages of saline or baseline control tests to better illustrate results. All statistical

tests were conducted using microcomputer software (Prism 6 for Windows, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if $p<0.05$.

3. Results

3.1. Effects in C57BL/6J mice

Figure 19 shows locomotor activity results of REG- and DOX-fed mice. In REG-fed SAL-treated B6 mice total distance traveled was significantly greater during the initial baseline ($p<0.0001$; not shown) than their second baseline (i.e., Day 0/SAL), possibly attributable to adaptation to locomotor activity chambers. This effect was not seen in the OXY-treated group. Two-way ANOVA analysis revealed a significant main effect of day and group [Day: $F(2, 32)=8.20$; $p=0.0013$; Group: $F(1, 16)=6.53$; $p=0.0212$] as well as a significant interaction [$F(2, 32)=7.18$; $p=0.0026$]. Total distance traveled did not change significantly in SAL-treated mice on Days 9 or 10 (Fig. 19A). In OXY-treated mice, chronic OXY significantly increased locomotor activity ($p<0.0001$ vs Day 0/SAL and vs SAL-treated mice), and was significantly decreased after its precipitated withdrawal ($p<0.0001$ vs Day 9/SAL). Analysis of stereotypy counts revealed a significant main effect of day [$F(2, 32)=18.95$; $p<0.0001$] as well as a significant interaction of day and group [$F(2, 32)=13.30$; $p<0.0001$]. Stereotypy was unaffected across days in SAL-treated mice (Fig. 19C). In OXY-treated mice, chronic OXY significantly increased the number of stereotypy counts ($p=0.0002$ vs Day 0/SAL; $p=0.0002$ vs SAL-treated mice) whereas precipitated withdrawal significantly decreased

stereotypy counts (p=0.0015 vs Day 0/SAL; p<0.0001 vs Day 9/SAL; p=0.0379 vs SAL-treated mice).

In DOX-fed B6 mice, there was a significant difference in initial baseline locomotor activity between the SAL- and OXY-designated groups (p<0.0001; not shown), but not at Day 0/SAL. Moreover, at Day 0/SAL, there was a significant within-group significant decrease in total distance traveled in the OXY-designated group as compared to their preliminary baseline (p<0.0001; not shown). There was a significant main effect of both day and group [Day: F (2, 36)=19.56; p<0.0001; Group: F (1, 18)=28.61; p<0.0001] as well as a significant interaction between the two [F (2, 36)=15.08; p<0.0001]. There were no significant differences across days in SAL-treated mice. Chronic OXY significantly increased locomotor activity (Fig. 19B; p<0.0001 vs Day 0/SAL and SAL-treated mice) and naloxone-precipitated withdrawal significantly decreased locomotor activity (p<0.0001 vs Day 9/SAL). Finally, there was a significant main effect of both day and group in stereotypy counts [Day: F (2, 36)=60.87; p<0.0001; Group: F (1, 18)=29.09; p<0.0001] as well as a significant interaction between day and group [F (2, 36)=22.45; p<0.0001]. As shown in Figure 19D, in SAL-treated mice there was no difference in the number of stereotypy counts from Day 0/SAL on Day 9/SAL, however, there was a significant decrease from Day 0/SAL on Day 10/NLX (p=0.012). In OXY-treated mice, chronic OXY significantly increased the number of stereotypy counts as compared to both Day 0/SAL and SAL-treated mice (p<0.0001 vs Day 0/SAL and SAL-treated mice). Naloxone-precipitated withdrawal significantly decreased stereotypy in OXY-treated mice as compared to Day 0/SAL and to chronic OXY conditions (p<0.0001 vs Day 0/SAL and Day 9/SAL).

3.1.1. Oxycodone locomotor sensitization in C57BL/6J mice

Locomotor sensitization after chronic oxycodone administration was also evaluated in B6 mice as depicted in Figure 20 and whose results are shown in Figure 21. Two-way ANOVA analysis conducted on total distance traveled revealed a significant main effect of day and group [Day: $F(1, 18)=23.89$; $p<0.0001$; Group: $F(1, 18)=167.2$; $p<0.0001$] as well as a significant interaction of day and group [$F(1, 18)=29.38$; $p<0.0001$]. Acute OXY (9 mg/kg) on Day 1 significantly increased locomotor activity as shown in Figure 21B ($p<0.0001$ vs SAL). OXY-treated mice had significantly greater distance traveled again on Day 8 as compared to SAL-treated mice ($p<0.0001$). Locomotor activity sensitization occurred in OXY-treated mice as there was a significantly greater distance traveled on Day 8 as compared to Day 1 ($p<0.0001$)., There was a significant main effect of group on stereotypy counts [$F(1, 18)=14.20$; $p=0.0014$]. As shown in Figure 21C, OXY significantly increased stereotypy counts to similar levels at Day 1 and Day 8 (Day 1: $p=0.0093$ vs SAL; Day 8: $p=0.0012$ vs SAL).

3.2. Effects in Tat transgenic mice

Locomotor activity and stereotypy counts decreased over time in both Tat(+) and Tat(-) mice, where Tat(+) mice traveled significantly less than Tat(-) mice and to their baseline and these effects lasted over time up to 58 days of DOX exposure (Figure 22). There was a significant main effect of time [$F(5, 70)=13.04$, $p<0.0001$] and group [$F(1, 14)=16.73$, $p=0.0011$] on locomotor activity. For stereotypy counts, there was also a significant main effect of time [$F(5, 70)=8.442$, $p<0.0001$] and group [$F(1, 14)=5.902$, $p=0.0292$].

Acute OXY administration (9-33 mg/kg) to Tat transgenic mice resulted in an opioid prototypic increase in locomotor activity as well as stereotypy, regardless of genotype (Fig. 23A and 23B). There was a significant main effect of dose on total distance traveled [$F (4, 56)=22.56$, $p<0.0001$] as well as on stereotypy counts [$F (4, 56)=9.136$, $p<0.0001$]. The time subjects spent in the center of the locomotor chamber was also measured as an indirect test of anxiety-like behavior. Acute OXY administration dose-dependently decreased center time in both Tat(-) and Tat(+) mice [Main effect of dose: $F (4, 56)=15.01$, $p<0.0001$], but was not significantly different from SAL at the lowest dose of OXY (9 mg/kg) for Tat(+) mice (Fig. 23C).

Chronic OXY administration to REG-fed Tat transgenic mice resulted in a significant increase in locomotor activity as well as stereotypy, whereas precipitated withdrawal decreased locomotor activity and stereotypy (Fig. 24A and 24B). There was a significant main effect of day on total distance traveled [$F (2, 36)=23.93$, $p<0.0001$] as well as on stereotypy counts [$F (2, 36)=67.16$, $p<0.0001$]. There was a significant main effect of day as well as group on time spent in the center of the chamber [Day: $F (2, 36)=4.929$, $p=0.0128$; Group: $F (1, 18)=8.023$, $p=0.011$]. In both Tat(-) and Tat(+) mice, center time was unaffected by chronic OXY administration or during naloxone challenge relative to Day 0/SAL, although there a nonsignificant increase in center time occurred during the latter condition (Fig. 24C).

Chronic OXY administration to DOX-fed Tat transgenic mice also resulted in a significant increase in locomotor activity and stereotypy, whereas precipitated withdrawal decreased locomotor activity and stereotypy in a naloxone dose-dependent manner (Fig. 25)., There was a significant main effect of day in Tat(-) mice [F (2,

54)=44.97, p<0.0001] and a significant main effect of day and NLX dose group in Tat(+) mice [Day: F (2, 54)=67.67, p<0.0001; Group: F (2, 27)=8.119, p=0.0017] in total distance traveled. Moreover, there was a significant interaction of day and group in Tat(+) mice [F (4, 54)=5.281, p=0.0012]. There was a significant main effect of day in Tat(-) mice [F (2, 54)=119.4, p<0.0001] and a significant main effect of day and NLX dose group in Tat(+) mice [Day: F (2, 54)=90.31, p<0.0001; Group: F (2, 27)=9.742, p=0.0007] in stereotypy counts. There was also a significant interaction of day and NLX dose group in Tat(+) mice [F (4, 54)=4.548, p=0.0031]. In general, there was a lack of between-group differences under chronic OXY or precipitated withdrawal conditions. In center time, chronic OXY administration to DOX-fed Tat transgenic mice did not affect center time; however, naloxone dose-dependently increased center time (Fig. 26). Importantly, Tat(-) mice were affected by precipitated withdrawal on center time at the two highest doses of naloxone (1 and 10 mg/kg), whereas Tat(+) mice were only affected at the highest dose of naloxone. As a control to evaluate DOX effects, preliminary baseline and Day 0/SAL tests were compared for both genotypes and no significant differences were found (not shown). Moreover, SAL-treated Tat transgenic mice fed a REG or DOX diet did not show significant changes in total distance traveled over time (not shown).Both SAL-treated, REG-fed Tat(-) and Tat(+) mice did have significant decreases in stereotypy counts relative to Day 0/SAL at Day 10/NLX (not shown). In center time, there was a significant decrease from Day 0/SAL at Day 10/NLX for REG-fed, SAL-treated Tat(+) mice (not shown).

3.2.1. Interactions of Tat expression and opioid locomotor sensitization

Locomotor sensitization in Tat-expressing mice was evaluated using the “gold standard” mu-opioid, morphine. Tat transgenic mice had been supplied DOX chow for approximately 12-weeks before baseline locomotor testing occurred with SAL pre-treatment. Therefore, there was a significant ($p<0.05$) difference in total distance traveled during a 2-h baseline test session (i.e., see Figure 22A for Tat expression timecourse study). In general, there was a significant main effect of morphine day on total distance traveled [$F(6, 84)=17.78$, $p<0.0001$]. On Day 1 of morphine (32 mg/kg, i.p.) administration, total distance traveled was significantly increased for both Tat(-) and Tat(+) mice to similar levels (Fig. 27) during a 2-h test session. This testing repeated over the next three days, and total distance traveled further increased dramatically in Tat(-) mice but to a slower degree in Tat(+) mice, where there was a significant between-group difference at Day 3 ($p=0.0453$). However, by Day 8, Tat(+) mice surpassed Tat(-) mice in total distance traveled before finally performing to similar levels at Day 11.

Oxycodone locomotor sensitization was also probed in Tat transgenic mice using a cumulative dosing regimen. Mice were first evaluated under REG-chow conditions during which between-group differences were not found in total distance traveled during the 30-min habituation period. OXY dose-dependently increased total distance traveled for both genotypes under REG-chow conditions as shown in Figure 28. After two weeks on DOX chow, mice were re-tested and both Tat(-) and Tat(+) mice showed decreased distance traveled during the 30-min habituation period and significantly so for Tat(-) mice as compared to their REG-chow baseline (not shown). Under DOX-chow

conditions, OXY again dose-dependently increased total distance traveled for both genotypes, and locomotor sensitization relative to the REG-chow baseline occurred at the two highest doses for Tat(-) mice but only at the highest dose for Tat(+) mice. For both Tat(-) and Tat(+) mice, there was a significant main effect of OXY dose on total distance traveled [Tat(-): $F(4, 32)=303.4$, $p<0.0001$; Tat(+): $F(4, 40)=43.95$, $p<0.0001$]. Moreover, for Tat(-) only, there was a significant main effect of test day (i.e., under REG/baseline or DOX testing) [$F(1, 8)=11.77$, $p=0.0090$] as well as a significant interaction of OXY dose and test day [$F(4, 32)=6.672$, $p=0.0005$].

4. Summary

In REG- or DOX-fed C57BL/6J mice, chronic oxycodone administration increased locomotor activity and its stereotypy. Naloxone-precipitated withdrawal decreased these measures. These results suggest doxycycline does not possess nonspecific effects on locomotor activity nor interfere with oxycodone's effects on locomotor activity and its related measures. Moreover, locomotor sensitization was observed after chronic oxycodone administration. Interestingly, the time to peak locomotor activity did not change after chronic oxycodone administration, but there was a leftward shift in the descending limb of oxycodone's effects on locomotor activity. This effect is discussed further in Chapter VI. Together, these results support other preclinical reports of oxycodone's mu-opioid prototypic effects on locomotor activity and its sensitization.

Tat expression in mice attenuated total distance traveled, supporting previous findings of reduced locomotion in Tat-expressing mice (Hahn et al., 2015). Tat expression did not alter oxycodone's ability to increase locomotor activity or stereotypy,

however the time spent in the center was significantly reduced in Tat-expressing mice after acute oxycodone administration to a lesser degree than Tat(-) control mice. That is, oxycodone's effects on center time were not as robust in Tat-expressing mice. Interpretation of these results relative to an anxiety-like index is discussed in Chapter VI. Chronic oxycodone administration increased locomotor activity and stereotypy in both Tat(-) and Tat(+) to similar levels, regardless of chow type provided. Moreover, naloxone-precipitated withdrawal dose-dependently decreased both locomotor activity and stereotypy in both genotypes, but again to similar levels. Unlike acute oxycodone, chronic oxycodone administration did not affect center time. Precipitated withdrawal increased center time in both Tat(-) and Tat(+) mice, but only significantly so at the highest dose of naloxone for Tat-expressing mice. Interestingly, in morphine and oxycodone locomotor sensitization tests, Tat expression attenuated, but did not abolish, locomotor sensitization. This is discussed in depth in Chapter VI. Overall, these results demonstrate that Tat expression affects locomotor activity and its related measures, modulates mu-opioid induced locomotor sensitization, and provides further evidence that Tat expression alters abuse-related behavioral effects of oxycodone.

5. Figures

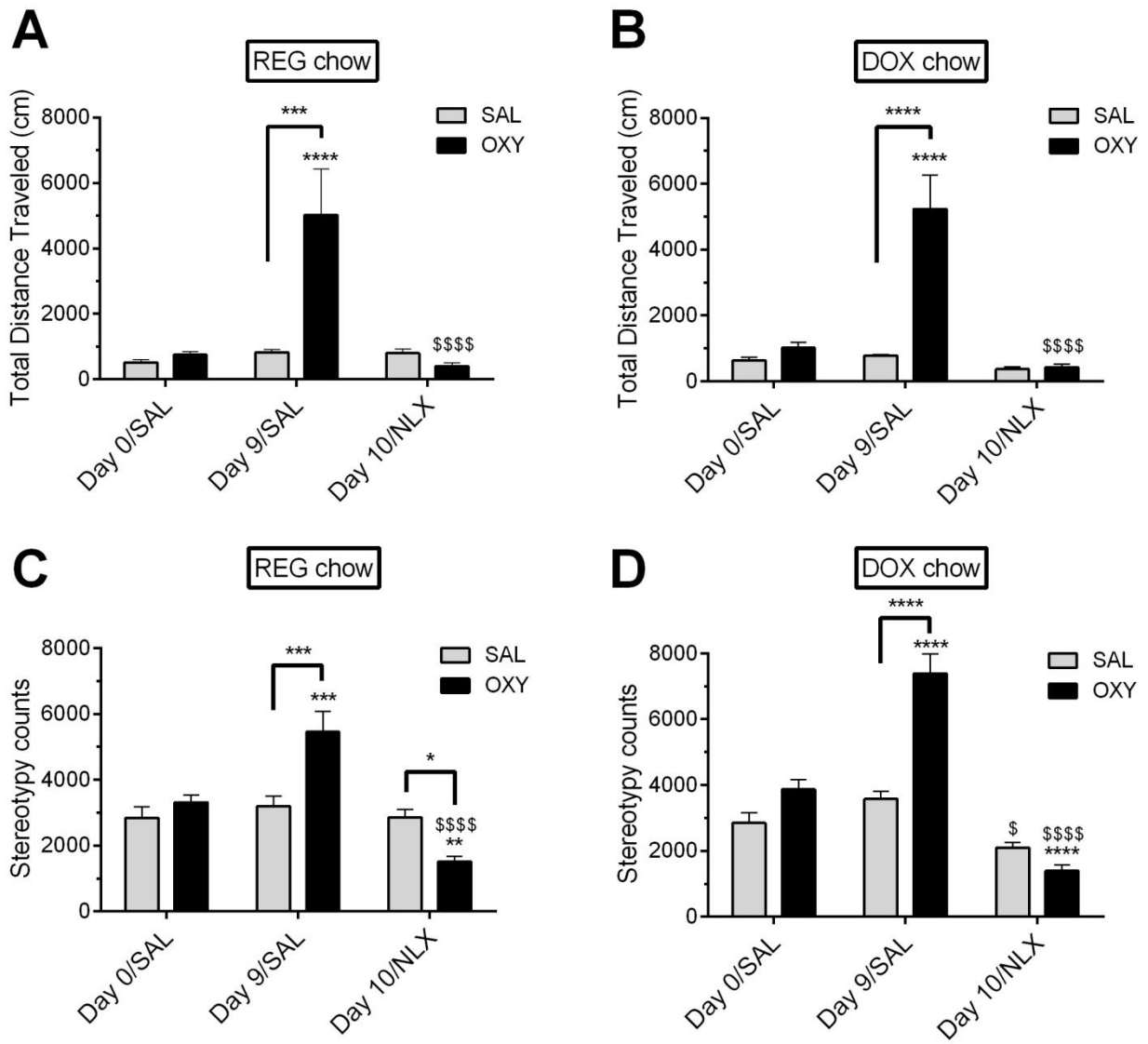

Figure 19. Total distance traveled (A and B) and stereotypy counts (C and D) after chronic OXY and its precipitated withdrawal in REG- and DOX-fed C57BL/6J mice.
N=8-10/group. Data are expressed as the mean (± S.E.M.) total distance traveled or the mean stereotypy counts (± S.E.M.) during a 1-h test session. *P<0.05, ***P<0.0001, ****P<0.0001 to Day 0/SAL or SAL-treated mice; $$$$P<0.0001 to Day 9/SAL.

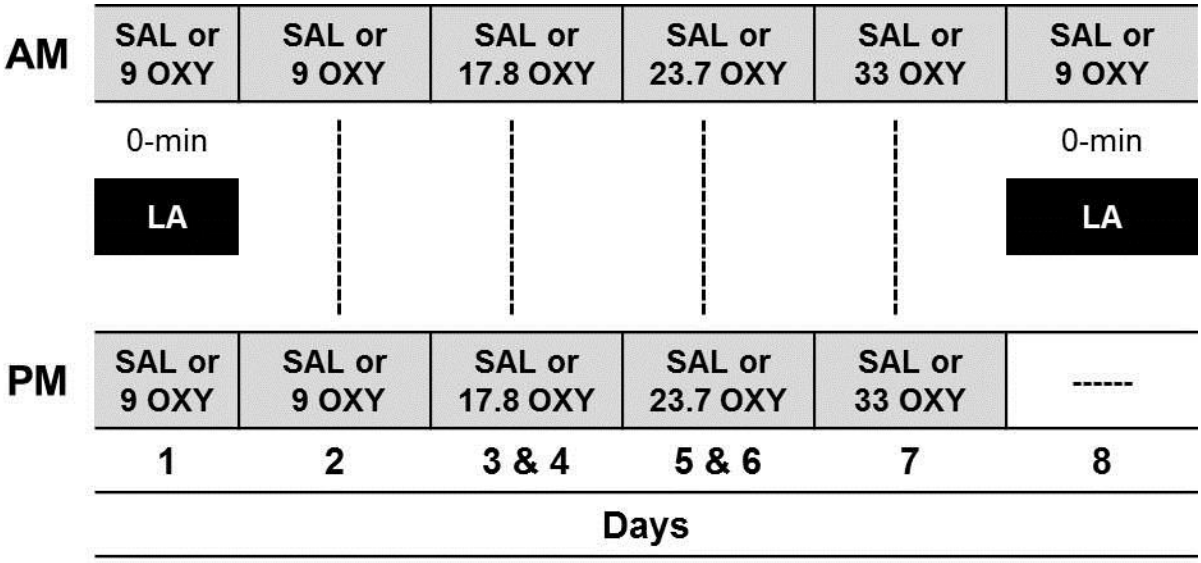

Figure 20. Timecourse of oxycodone locomotor sensitization regimen in C57BL/6J mice. Mice (n=10/group) were administered either SAL or 9 mg/kg OXY and immediately tested in a 1-h locomotor activity session on Day 1. Mice were then chronically administered SAL or OXY (9-33 mg/kg, s.c.) the following seven days. On Day 8, mice were once again administered SAL or 9 mg/kg OXY and immediately tested during a 1-h locomotor activity session.

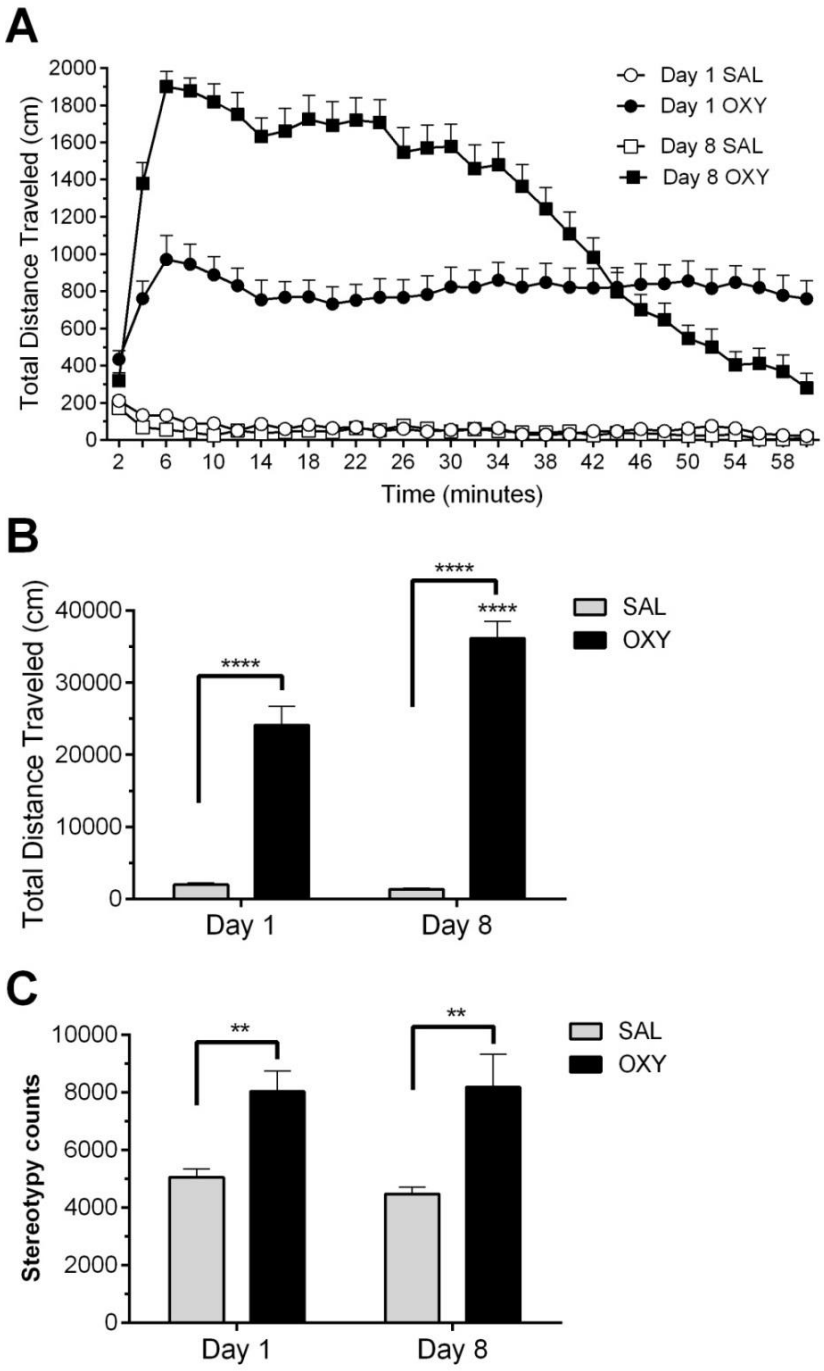

Figure 21. Total distance traveled (A and B) and stereotypy (C) was evaluated at Days 1 and 8 of a chronic OXY administration regimen using C57BL/6J mice. N=10/group. Data are represented as the mean total distance traveled (± S.E.M.) or the mean stereotypy counts (± S.E.M.) during 1-h test sessions. **P<0.01, ****P<0.0001 to Day 1 or SAL-treated mice.

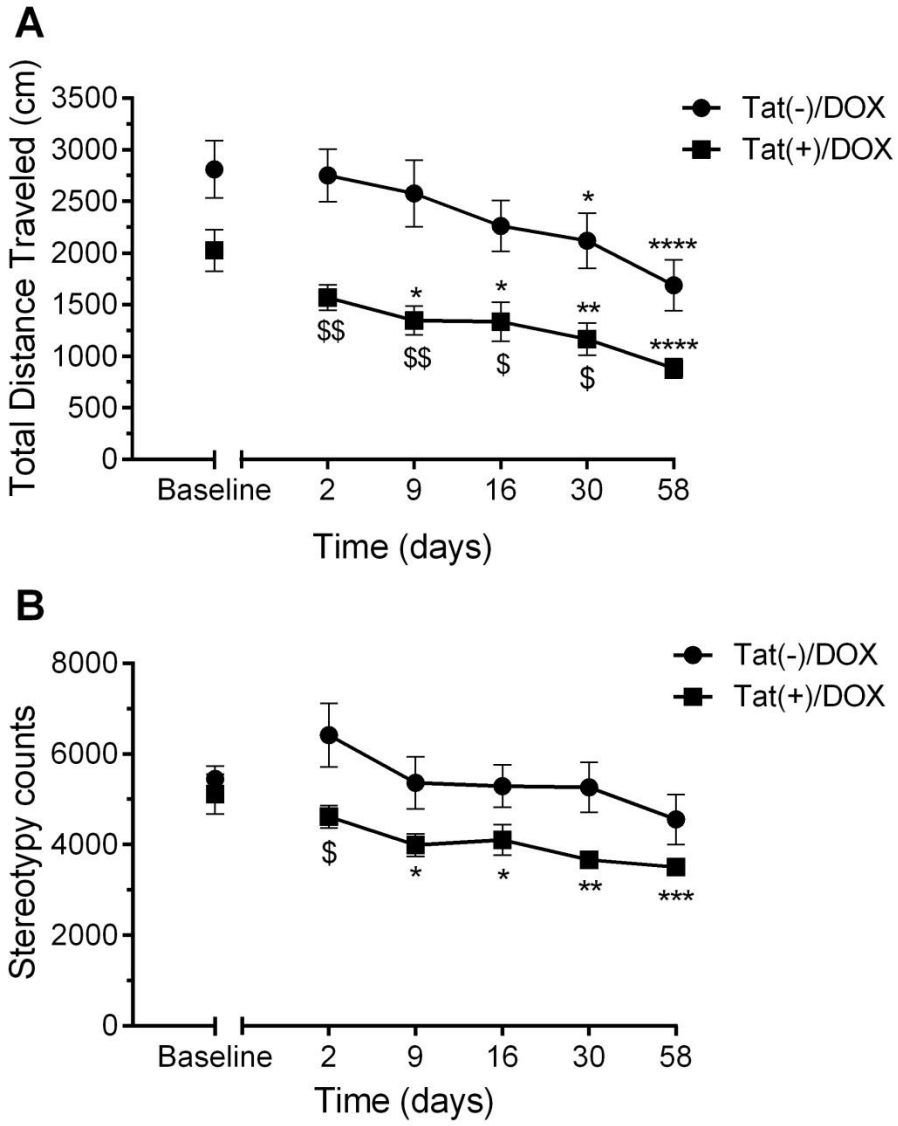

Figure 22. Effects of Tat expression on total distance traveled (A) and stereotypy counts (B) during 2-h test sessions across days of DOX exposure. N=8/group. Each symbol represents the mean (± S.E.M.) total distance traveled or the mean (± S.E.M.) stereotypy counts during 2-h test sessions. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to baseline; $P<0.05 and $$P<0.01 to Tat(-)/DOX mice.

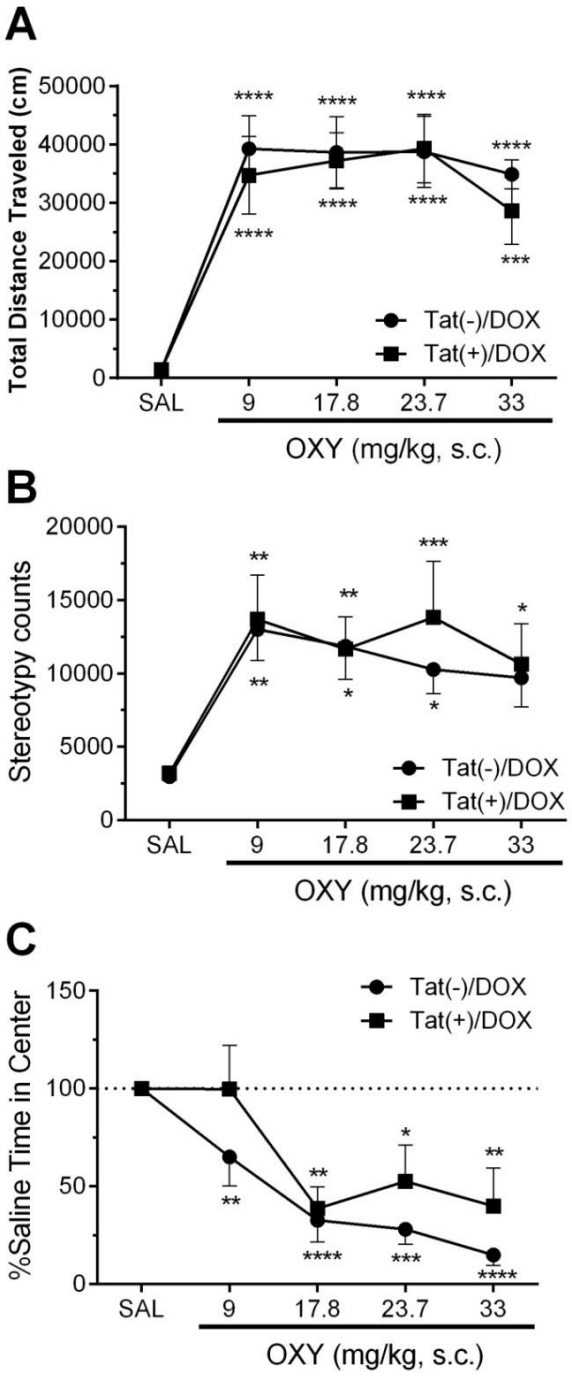

Figure 23. Effects of acute OXY and Tat expression on total distance traveled (A), stereotypy counts (B), and center time (C) during 1-h test sessions. N=8/group. Each symbol represents the mean (± S.E.M.) total distance traveled, stereotypy counts, or center time as a percentage of SAL treatment during 1-h test sessions. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to SAL.

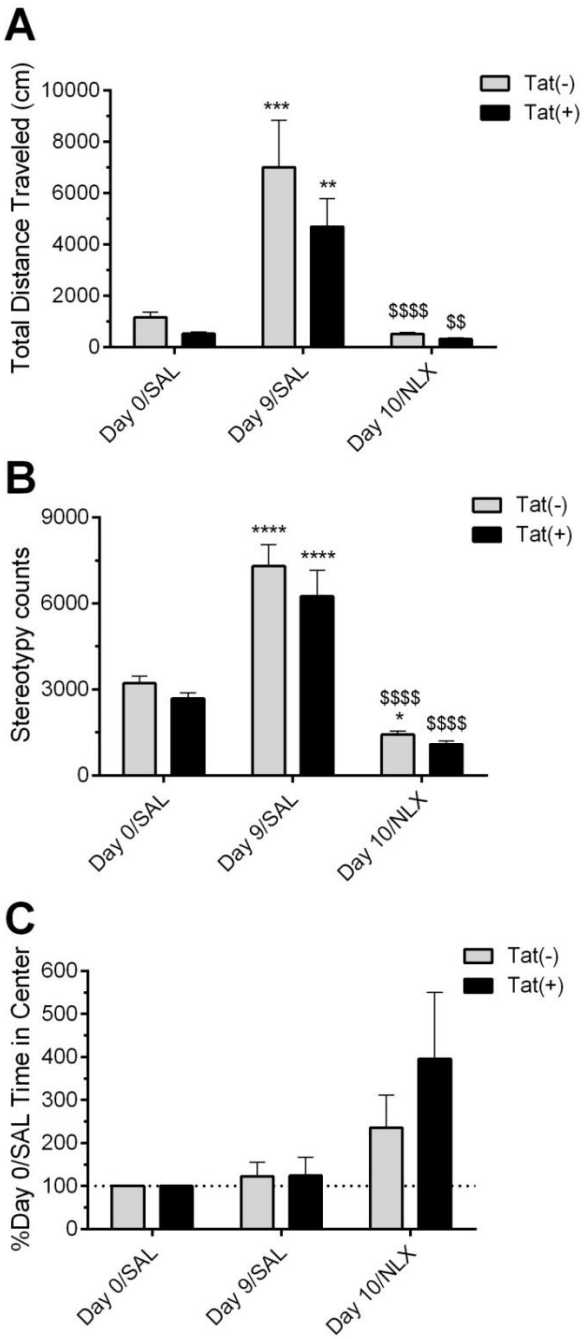

Figure 24. Total distance traveled (A), stereotypy counts (B), and center time (C) after chronic OXY and its precipitated withdrawal in REG-fed Tat transgenic mice. N=10/group. Data are expressed as mean (± S.E.M.) total distance traveled, stereotypy counts, or center time as a percentage of Day 0/SAL during a 1-h test session. *P<0.05, **P<0.01, ***P<0.0001, ****P<0.0001 to Day 0/SAL; $$$$P<0.0001 to Day 9/SAL.

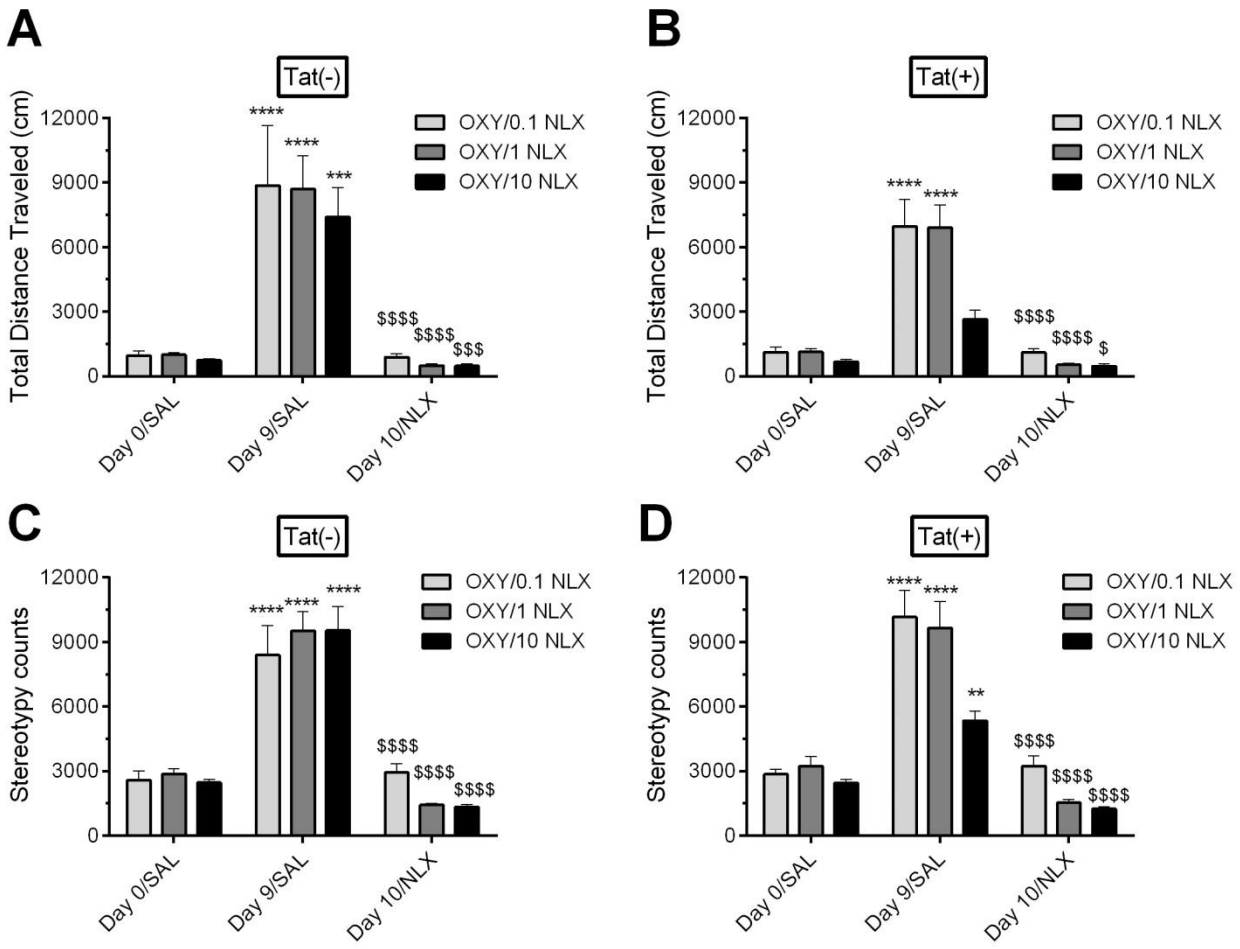

Figure 25. Total distance traveled (A and B) and stereotypy counts (C and D) after chronic OXY and its precipitated withdrawal in DOX-fed Tat transgenic mice. N=10/group. Data are expressed as mean (± S.E.M.) total distance traveled or the mean (± S.E.M.) stereotypy counts during a 1-h test session. **P<0.01, ***P<0.0001, ****P<0.0001 to Day 0/SAL; $P<0.05, $$$P<0.001, $$$$P<0.0001 to Day 9/SAL.

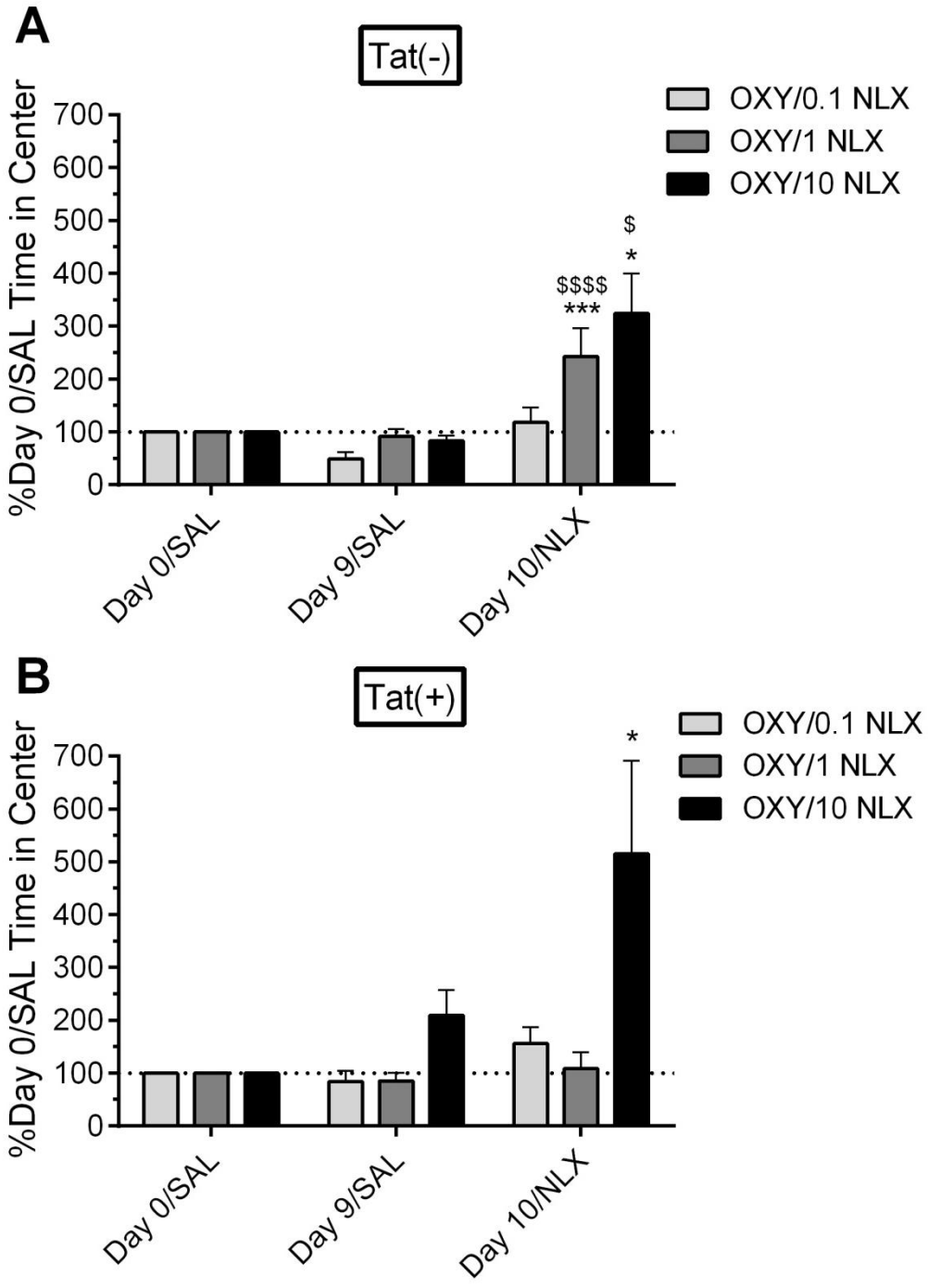

Figure 26. Center time spent after chronic OXY and its precipitated withdrawal in DOX-fed Tat(-) (A) and Tat(+) (B) mice. N=10/group. Data are expressed as mean (± S.E.M.) center time as a percentage of Day 0/SAL during a 1-h test session. *P<0.05, ***P<0.0001 to Day 0/SAL; $P<0.05, $$$$P<0.0001 to Day 9/SAL.

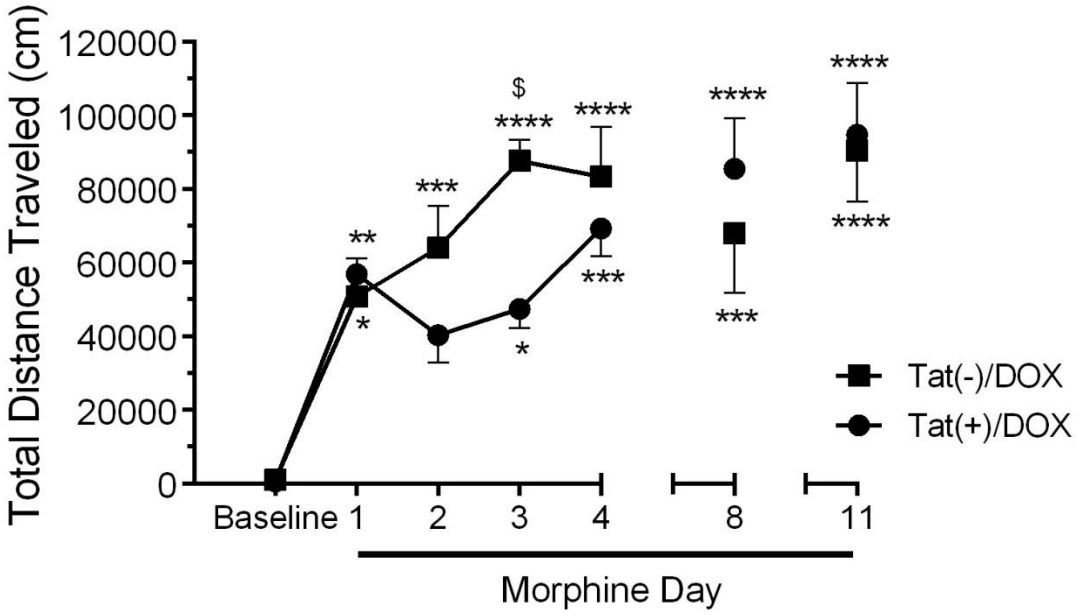

Figure 27. Effects of Tat expression on morphine locomotor sensitization. Mice had been exposed to 12-weeks of DOX chow as well as locomotor tests as shown in Figure 24A prior to SAL baseline measurements in a 2-h locomotor test. The next four days, morphine (32 mg/kg, i.p.) was administered immediately prior to locomotor tests. This was again re-tested on Days 7 and 10. N=8/group. Each symbol represents the mean (± S.E.M.) total distance traveled during a 2-h test session. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to baseline/SAL test; $P<0.05 to Tat(+) mice.

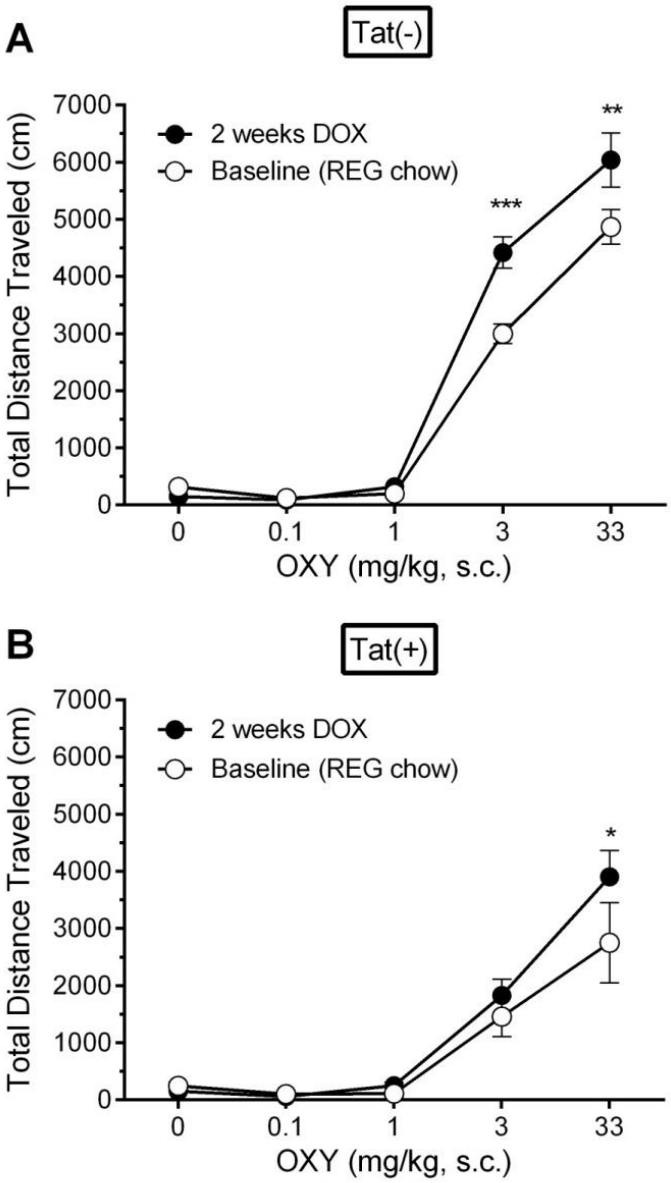

Figure 28. Effects of Tat expression on oxycodone locomotor sensitization. REG-fed Tat(-) and Tat(+) mice (A and B, respectively) were first habituated to locomotor chambers for 30-min before receiving a SAL injection (s.c.) and tested during a 10-min test session. Following this, mice were administered 0.1 mg/kg OXY and re-tested. This process continued to create a cumulative OXY dose-effect curve (0.1- 33 mg/kg). Mice were then switched to DOX chow for two weeks and, after which, were re-tested. N=5-6/group. Each symbol represents the mean (± S.E.M.) total distance traveled during a 10-min bin for each dose of OXY. *P<0.05, **P<0.01, ***P<0.001 to REG-chow baseline test.

Chapter VI: Discussion and Conclusions

1. Introduction

The evidence from this dissertation is of importance from both methodology development and scientific standpoints. The first demonstration of volitional, oral operant self-administration of oxycodone in C57BL/6J mice was observed and refinement of this procedure led to similar observations in HIV-1 Tat transgenic mice. Moreover, the acoustic startle response demonstrated its ability to serve as a measure of oxycodone dependence in a quantifiable, objective manner. These two procedures will provide tools to illuminate oxycodone's abuse liability, its mechanisms of dependence, and for developing pharmacotherapies for attenuating its abuse-related effects. Additionally, these procedures will concurrently facilitate investigating mechanisms behind comorbid disorders, such as that of opioid abuse and neuroAIDS.

Oxycodone self-administration and physical dependence as measured by acoustic startle were partially altered by expression of the neurotoxic HIV-1 Tat protein. Tat-induced PPI deficits were not worsened by oxycodone (see below). Tat expression did not shift the acute effects of oxycodone on locomotor activity, but did shift its chronic effects. Tat expression also attenuated, but did not abolish, the development to and

expression of locomotor sensitization to morphine and oxycodone. Together, these results suggest chronic HIV-1 Tat expression and chronic oxycodone administration alters the abuse-related behavioral effects of oxycodone in a manner opposite of originally predicted. Importantly, chronic expression of HIV-1 Tat may reduce overall sensitivity to chronically administered opioids and their abuse-related effects, resulting in attenuated oxycodone or morphine locomotor sensitization, resistance to dependence-related effects, and possibly explain increased self-administration responding as discussed in detail below.

2. Chapter II

The results from Chapter II demonstrated that oxycodone can be volitionally, orally self-administered in mice with and without post-prandial conditions and oxycodone induces physical dependence as inferred by naloxone dose-dependent increases in the number of observed somatic signs of withdrawal. These results were found using novel methodologies and regimens developed in our lab, and laid an essential foundation for investigating the interactions of HIV-1 Tat expression on oxycodone abuse-related behaviors.

2.1. Oral oxycodone self-administration in C57BL/6J mice

It was observed that as the concentration of oxycodone increased, the number of deliveries obtained increased before decreasing with higher concentrations, whereas the estimated consumption increased reaching an average maximum of approximately 40 mg/kg. In the only other oral opioid, operant self-administration report using mice, consumption of the potent benzimidazole opioid, etonitazene, also increased with

increases in concentration in four different strains of mice (Elmer et al., 1995). In regards to the inverted U-shaped relationship between oxycodone infusions and dose, a similar relationship was observed in a rat oxycodone intravenous self-administration study (Beardsley et al., 2004), and which is characteristic of other reports of self-administered drugs under limited access conditions (e.g., Moreton et al., 1977; Suzuki et al., 1988). In an intravenous oxycodone self-administration procedure with adolescent and adult C57BL/6J mice, however, it was reported that the number of infusions dose-dependently decreased with increases in dose in both age groups, while intake increased resulting in a maximum intake of 8.25 mg/kg i.v. in adult mice (Zhang et al., 2009). In rats, a similar decrease in number of oxycodone infusions and an increase in total intake was found in an intravenous self-administration procedure examining duration of drug access (Wade et al., 2015). Differences in these reported patterns of self-administered oxycodone might be a function of the range of doses and concentrations tested, in that the ascending limb of the dose-effect curve may have been missed if doses not low enough were untested.

The observed levels of oxycodone intake reached behaviorally active levels inducing mu-opioid receptor-like mediated effects in the mice including hyperlocomotion and Straub tail (Aceto et al., 1969; Hecht and Schiorring, 1979). Likely, in part, attributable to these observed effects, it is important to note that the present self-administration procedure is not without its limitations, specifically regarding the precise measurement of oxycodone consumption. For example, one subject was observed engaging in stereotypic biting/chewing upon the active lever that resulted in oxycodone deliveries that were not consumed. These observations were noted after ~30 min into

the test session (see Fig. 29), and after a bout of oxycodone consumption had been observed, suggesting that the drug elicited these stereotypic effects. These stereotypic effects have been previously observed in rats that had orally self-administered the highly potent opiate, etonitazene (Beardsley and Meisch, 1981; Carroll and Meisch, 1981; Meisch and Kliner, 1979).

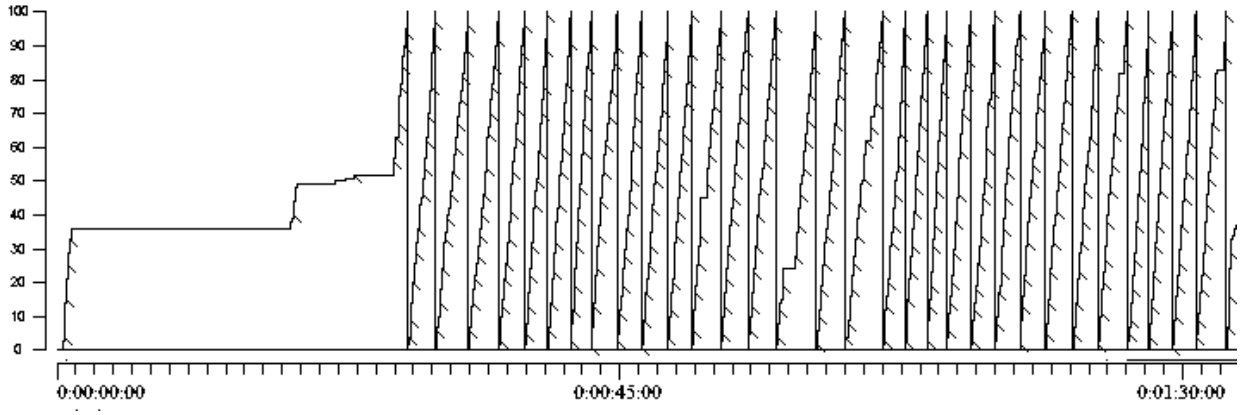

Figure 29. Cumulative record of oxycodone obtained liquid deliveries during a 3-h test session in a C57BL/6J mouse subject. Note: this record shows the first 1.5-h of the test session to demonstrate the likely stereotypic effect. Each diagonal tick mark represents one 20ul liquid delivery.

Pre-session feedings were incrementally reduced from 100% to 50, 25, and finally 0% of the total daily food allotment with each reduction in effect for five consecutive experimental sessions in which 1 mg/ml oxycodone was the available oxycodone concentration. Interestingly, this reduction resulted in the transitory decrease in deliveries and consumption as shown in Figure 30A. Moreover, this coincided with an increase in inactive-lever responding, as shown in Figure 30B.

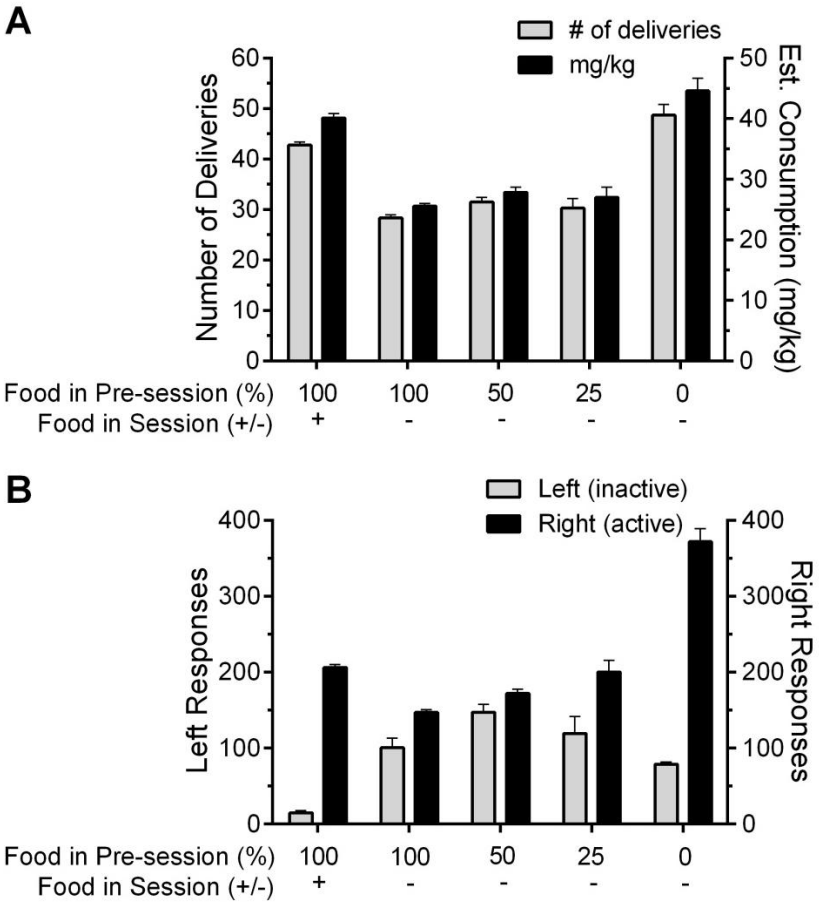

Figure 30. Panel A, Number of deliveries (left ordinate) and consumption estimates (right ordinate); Panel B, Number of inactive lever responses (left ordinate) and active lever responses (right ordinate) as a function of food availability during 3-h test sessions. N=14. Food allotments were supplied in the pre-session and subsequently present or absent in the operant chamber during the test session. Data represents the mean (± S.E.M.) of five consecutive test sessions in which 1 mg/ml oxycodone was available.

This pattern of behavior is similar to previously described effects of food disruption or deprivation in oral self-administration studies of opioids and other drugs of abuse. In oral etonitazene self-administration studies with rats, an increase in liquid deliveries occurred after food restriction although this effect was later shown to differ in Sprague-Dawley rats in which a decrease in etonitazene deliveries was observed (Carroll and Meisch, 1979; Carroll et al., 1986). Most self-administration studies utilizing food restriction manipulated bodyweight as a percentage of the free-feeding weight,

whereas in the present study bodyweight was kept at 85% of the free-feeding weight throughout. Therefore, in the present study, the schedule of feedings themselves may have had an important role on the transitory effects observed more than just the satiation of the animal prior to the test session. Sharpe et al. found methamphetamine-induced locomotor sensitization was altered in food-restricted mice depending on if their daily allotment of chow was provided as one meal or separated into three equal-sized meals (Sharpe et al., 2012). Consequently, the presence, frequency, and timing of delivery of a natural reinforcer in the context of drug self-administration may in turn alter the sensitivity to, or reinforcing efficacy, of the drug itself.

Conversely, food is a natural reinforcer and may therefore affect general operant responding rather than possess interactions of biological importance with non-natural reinforcers, such as drugs of abuse. In the present study, as pre-session food percentage is reduced from 100% to 25% the number of deliveries is maintained at similar levels; however, the number of inactive lever presses changed as a function of pre-session food percentage. This allocation of behavior to the inactive lever, despite maintaining similar total session deliveries is an interesting consequence of an external manipulation (pre-session feeding) that resolves once pre-session feeding is completely removed. Because this pattern resolves itself after complete withdrawal of pre-session feeding, it is difficult to suggest this increased inactive lever responding is simply due to rate-altering effects of oxycodone itself in a potentially hungry-state mouse but more so to do with ancillary operant learning cues in the context of pre-session feeding. Alternatively, it is important to note that the endogenous opioid system and dopaminergic systems have both been implicated in the hedonic value of food as well

as the rewarding properties of opioids. The extensive overlap of these systems in the context of the experiment itself (using postprandial conditions to induce oxycodone oral self-administration) may play a key role in the observed behaviors. This complicates distinguishing the biological determinants of these observations where pre-session feeding differentially affected oxycodone self-administration which, in addition, utilized the oral route of administration making it a multilayered challenge. Therefore, the relationships between feeding behavior and oral oxycodone self-administration are complex to interpret within the confines of this dissertation and additional investigation is necessary to resolve them.

Once pre-session feedings were withdrawn, the mice progressively and once again proportioned most presses to the active lever as well as increased the numbers of oxycodone deliveries to previous levels indicating that pre-session feedings were no longer needed to induce self-administration of oxycodone solutions. This observation is consistent with the inference, but does not definitively confirm, that oral oxycodone was serving as a positive reinforcer. Similar maintenance of behavior has been found previously in an oral operant self-administration study in mice with the highly potent opioid, etonitazene, once pre-session feeding was withdrawn (Elmer et al., 1995). Attempts to further confirm oral oxycodone was serving as a positive reinforcer met with challenges, and because they amounted to "probes" in now what were aging mice with complex behavioral histories, were not included in Chapter II. For example, the maximal amounts of behavior that are maintained by different reinforcer deliveries using progressive ratio schedules has been one way to disentangle the strengths of different reinforcers (Richardson and Roberts, 1996). In probe studies, progressive ratio tests

were performed for both water and 1 mg/ml oxycodone. Briefly, after complete removal of pre-session feedings, mice were maintained on 1 mg/ml oxycodone before being switched to water (minimum of five days) to wash out oxycodone and stabilize performance. During this time, as an example, one mouse required 29 days of lever pressing reinforced with water deliveries to finally reach stability criteria (Fig. 31A). After this period of water reinforcement, mice were returned to 1 mg/ml oxycodone availability and tested until reaching stability criteria again. Then, progressive ratio tests for 1 mg/ml oxycodone began in which the ratio to receive a liquid delivery increased between-sessions using the formula 2^x, where x began at 2 (i.e., to receive one liquid delivery, mice pressed 4, 8, 16, 32, 64, etc. times) until a breakpoint was hit. The breakpoint was defined as the final ratio step completed before the subsequent ratio step in which the animal failed to receive at least one liquid delivery. After oxycodone tests, mice were switched back to water, stabilized, and then tested in progressive ratio for water deliveries. Shown in Figure 31B are the breakpoint averages for seven subjects that underwent progressive ratio tests for oxycodone and water.

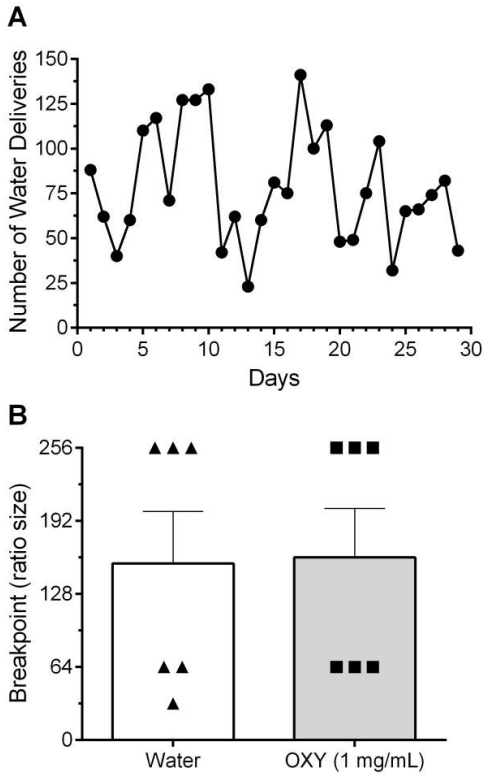

Figure 31. Panel A, Number of deliveries for a mouse subject undergoing water extinction; Panel B, Breakpoints of mice for water and 1 mg/ml oxycodone.
Symbols in A represent the mean (± S.E.M.) number of deliveries during a 3-h session; bars in B represent the mean (± S.E.M.) breakpoint ratio size (N=6; one outlier removed) to obtain at least one liquid delivery during a 3-h session.

As seen in Figure 31B, the breakpoints for water and oxycodone were similar, with high responding subjects allocating high responses for both water and oxycodone and the same pattern with low responding subjects. The definitive inference for a drug serving as a positive reinforcer in an operant task is for its ability to maintain greater levels of responding than in its absence such as when it is replaced by vehicle (such as water). In the literature, there are very few preclinical studies that examine oxycodone in progressive ratio tests and neither compare to a nondrug reinforcer or placebo. In those reports, oxycodone breakpoints are compared either by duration of access, or to a combination of oxycodone and ultra-low dose of the opioid antagonist naltrexone, in an

intravenous self-administration procedure using rats (Leri and Burns, 2005; Wade et al., 2015). Therefore, it is difficult to conclude that the oxycodone breakpoint observed in the present study suggests oxycodone is truly serving as a positive reinforcer. In the clinical literature, oxycodone has been used in progressive ratio tests and compared to either placebo or a nondrug reinforcer, which is often money. In one report, oxycodone obtained higher breakpoints than placebo as well as money as a function of magnitude of dose (Babalonis et al., 2013). In another study, oxycodone served as a positive reinforcer to prescription opioid abusers and non-drug abusers but only in the presence of experimentally-induced pain (i.e., hand immersed in cold water via cold pressor test) and breakpoints for money again shifted as a function of oxycodone dose (Comer et al., 2010). In summary, exceptionally high breakpoints often exceeding FR200 were maintained by both water and oxycodone deliveries in non-liquid deprived mice, suggesting other factors beyond those of intrinsically reinforcing effects were maintaining behavior. It is likely that some of these factors were conditioning factors resulting from the extensive associations of previous drug effects with the test environment. Because at the time of these progressive ratio tests the mice were aging (reaching approximately 9 months of age) and had extensive behavioral and pharmacological histories, it was impossible to continue to test them to isolate these potential factors and would have to be left for future researchers. Also, a limitation of the present studies was that only one type of progressive ratio schedule was tested. There are multiple determinants of break points under progressive ratio schedules including the initial FR used, step size, and criteria for defining breakpoint (Stafford and Branch,

1998) that could not be investigated in these studies given the primary aim of examining "volitional" intake of oxycodone in Tat-expressing mice.

In conclusion, in addition to previous mouse intravenous self-administration reports, these results suggest that oxycodone can also be volitionally consumed via the oral route in mice at behaviorally active levels. Overall, this methodology provides a useful, noninvasive technique enabling the study of the determinants of oxycodone self-administration in mice the duration of which may only be limited by a mouse's natural lifespan.

2.2. Oxycodone physical dependence in C57BL/6J mice

Physical dependence upon oxycodone was induced in C57BL/6J mice after nine days of its b.i.d. subcutaneous administration as inferred by naloxone-precipitated somatic signs of opiate-like withdrawal syndrome. In previous reports, morphine-dependent rhesus monkeys demonstrated cross-dependency to oxycodone (Beardsley et al., 2004), and naloxone precipitated disruptions of lever pressing maintained by intra-cranial self-stimulation in rats chronically-treated with oxycodone suggestive of dependence (Wiebelhaus et al., 2016). Thus, oxycodone demonstrates opiate-like dependence effects across species including mice, rats and rhesus monkeys. The signs and their patterns of direction observed in C57BL/6J mice of the current study are similar to those reported in previous studies with other strains of mice that used oxycodone regimens involving continuous drug delivery (Mori et al., 2013; Raehal and Bohn, 2011) or repeated injections (Bhalla et al., 2015) to induce dependence. The regimen to induce physical dependence in this study was adapted from a previous study that found physical dependence upon morphine to be induced in mice (Muldoon et al.,

2014). After naloxone-precipitated withdrawal in morphine-treated wildtype mice, similar numbers of paw tremors but fewer numbers of jumps were observed in comparison to the present study's results with oxycodone. Moreover, unlike morphine, naloxone precipitated similar degrees of withdrawal in both wildtype and beta-arrestin-2 knockout mice made dependent on oxycodone via osmotic pumps (Raehal and Bohn, 2011). These differences between morphine and oxycodone, in addition to clinical psychopharmacological and analgesic effects (Curtis et al., 1999; Wightman et al., 2012; Zacny and Lichtor, 2008), suggest that these two mu-opioid agonists have different pharmacological profiles that warrant further investigation into oxycodone's specific abuse-related effects on behavior. Unlike previous studies that only reported tests after one dose of naloxone to precipitate withdrawal, a wide range of naloxone doses (0.1 to 10 mg/kg) was evaluated in the present study. A naloxone dose-dependent increase in withdrawal severity was observed. The C57BL/6J strain of mice has been observed to consistently display characteristic effects of opiate dependence (e.g., naloxone-precipitated jumping) across various methods of inducing physical dependence upon morphine, although at times with less intensity than some other strains such as Swiss-Webster mice (Kest et al., 2002). The regimen used in the present study therefore provides a novel method to measure physical dependence-related effects of oxycodone in future behavioral studies.

3. Chapter III

Studies described in Chapter III reported the ability for ASR to serve as a measure of oxycodone dependence in C57BL/6J mice using a novel oxycodone dependence regimen. In addition to startle, habituation, but not PPI demonstrated

withdrawal-specific effects. A lack of nonspecific effects of the tetracycline derivative doxycycline supported these findings. These results support the use of the described procedure for evaluating oxycodone dependence-related effects. Moreover, the established regimen and procedure add another methodological layer to evaluate oxycodone dependence-related effects in mice expressing the neurotoxic HIV-1 Tat protein.

Chronic (nine-day), b.i.d. subcutaneous administration of oxycodone to C57BL/6J mice fed a REG-chow diet resulted in a significant increase in the ASR, but did not affect habituation or PPI. Naloxone-precipitated withdrawal, however, resulted in a significant decrease in ASR and habituation, but not PPI. These results with oxycodone mimic findings in rats where chronic morphine, via implantation of a 75-mg morphine pellet, increased ASR whereas naloxone-precipitated withdrawal decreased ASR (Mansbach et al., 1992). However, other researchers have found opposite effects in rats under withdrawal from morphine. In these latter studies, however, morphine dependence was induced via an acute intraperitoneal injection of morphine (1-10 mg/kg) and spontaneous as well as naloxone-precipitated withdrawal resulted in significant increases in ASR (Harris and Gewirtz, 2004). The authors suggested the observed increases in ASR reflect a state of anxiety as a component of morphine withdrawal syndrome as it is listed as part of the syndrome in humans. We attempted to replicate these findings, albeit in mice, and found spontaneous withdrawal (3-h after acute morphine injection) increased ASR in a morphine dose-dependent manner but an acute naloxone injection 3-h post-morphine decreased ASR (see Figure 32). Moreover, in our hands, acute morphine alone attenuated ASR.

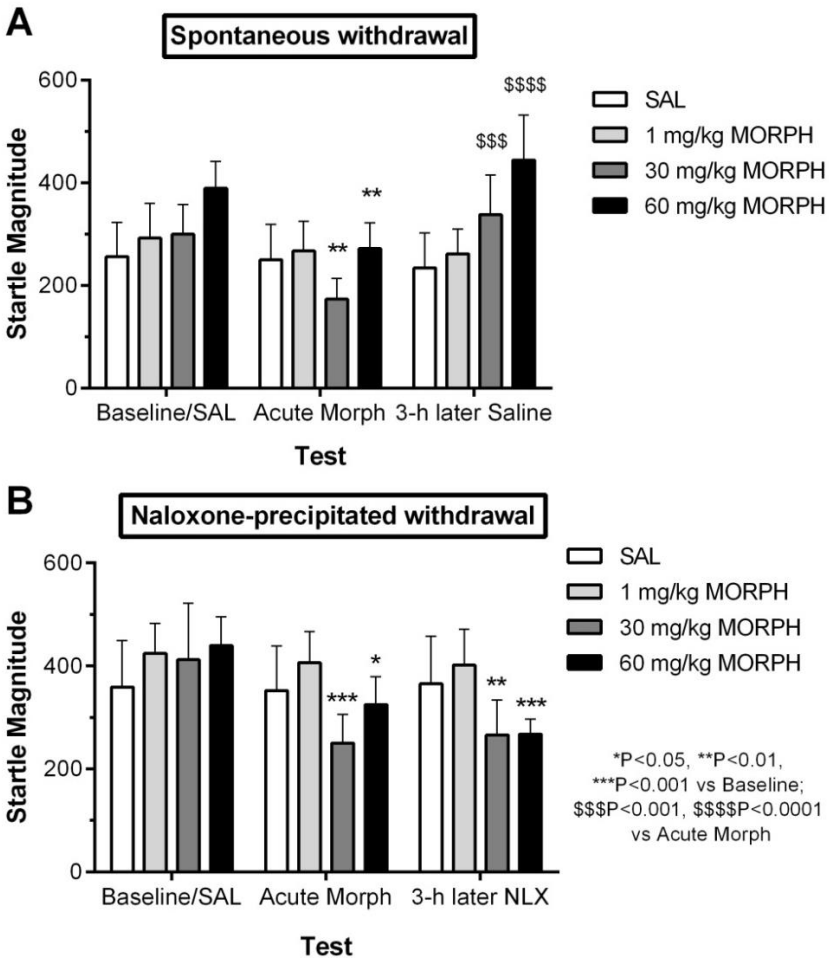

Figure 32. Spontaneous withdrawal (A) and naloxone-precipitated withdrawal (B) from acute morphine on the acoustic startle response in C57BL/6J mice.
N=8/group. Data represents the mean (± S.E.M.) startle magnitude for 13 STIM trials during a 30-min test session.

Differences in these results from previous studies in rats may be due to differences in methodology (i.e., species, morphine/naloxone doses, and time after acute morphine). Importantly, however, our results showing decreased ASR after naloxone-precipitated withdrawal from morphine mimic what was observed after naloxone-precipitated withdrawal in oxycodone-treated mice. Together, these results suggest that mu-opioid agonist dependence-related effects may be reliably measured using acoustic startle response procedures.

Acoustic startle and its related measure, PPI, are typically found to be altered by dopamine agonists or NMDA antagonists such as amphetamine or ketamine, respectively. Mu-opioid agonists are not as well known to affect these measures directly, where variable effects have been reported, although morphine has shown to increase PPI in healthy human volunteers and naloxone has been demonstrated to block amphetamine-induced PPI deficits in rats suggesting a role of the opioid system in regulating these effects (Quednow et al., 2008; Swerdlow et al., 1991). Recently, rats trained to self-administer intravenous morphine demonstrated increased startle 1-h but not 3-h after self-administration sessions, whereas PPI was only disrupted after 3-h post-session (Lee et al., 2016). In sum, these effects support the role of the opioid system in regulation of startle reflexes as well as sensorimotor gating and further characterization of its role is warranted.

3.1. Effects of doxycycline on acoustic startle

Control experiments in C57BL/6J mice fed a diet infused with the tetracycline derivative doxycycline essentially replicated our previous findings with REG-fed C57BL/6J mice. Doxycycline treatment alone did not alter ASR, habituation, or PPI up to 58 days of DOX exposure, suggesting a lack of effect of doxycycline on these measures. Moreover, while there was no significant effect of chronic oxycodone on ASR in DOX-fed mice, naloxone-precipitated withdrawal significantly reduced ASR as well as habituation. This demonstrates that doxycycline does not interfere with the development or expression of oxycodone dependence as measured by acoustic startle and further supports the use of this procedure to characterize oxycodone abuse-related effects in the mouse. Interestingly, doxycycline and similar tetracycline derivatives have shown to

affect behavioral effects of other drugs of abuse, such as ethanol. For example, C57BL/6J mice supplied a DOX-containing diet had reduced ethanol consumption values and an increased sensitivity to its motor-impairing effects (McIver et al., 2012). Administration of a semisynthetic tetracycline, tigecycline, also reduced ethanol consumption and ethanol withdrawal in mice, and administration of minocycline suppresses morphine reward as measured by conditioned place preference, which together suggests antibiotics may differentially interact with drugs of various classes (Bergeson et al., 2016; Hutchinson et al., 2008; Martinez et al., 2016). Further commentary of doxycycline's effects and consideration of its anti-inflammatory properties in the expression of these effects is discussed later in this chapter.

4. Chapter IV

The results from Chapter IV extended the developed oral oxycodone self-administration procedure and the physical dependence regimen to HIV-1 Tat-expressing mice. Tat transgenic mice readily self-administered oral oxycodone and were susceptible to naloxone-precipitated withdrawal effects as measured by acoustic startle. Under the conditions tested, HIV-1 Tat expression altered oxycodone abuse-related effects and consideration of the limitations of the studies in this regard are discussed below. Nevertheless, results from this chapter demonstrate the potential of HIV-1 Tat altering oxycodone abuse-related effects which support recent reports involving morphine (Fitting et al., 2016).

4.1. Oral oxycodone self-administration in Tat transgenic mice

Results from the oral self-administration study replicate earlier findings with C57BL/6J mice where, as oxycodone concentration increased, the number of deliveries obtained decreased. This was observed in both Tat(-) and Tat(+) mice, regardless if Tat expression was actively being induced or not (i.e., Pre-DOX versus DOX or Post-DOX phases of the study). Once Tat expression was induced or Tat was still likely present (i.e., during DOX and Post-DOX phases in Tat(+) subjects), numbers of liquid deliveries of oxycodone increased for Tat(+) mice relative to both their Pre-DOX baseline and to Tat(-) mice. Importantly, Tat(+) mice maintained significantly greater numbers of deliveries of 1 mg/ml oxycodone during their Post-DOX phase as compared to Tat(-) mice during their Pre-DOX phase. This suggests, among several possibilities, that Tat expression may have either increased the reinforcing efficacy of oxycodone or reduced sensitivity to oxycodone (e.g., via a greater degree of general tolerance or adaptation to oxycodone's potential aversive effects) resulting in an increased maintenance of behavior. This is the first report of self-administration of a drug in Tat-expressing mice, therefore limitations of the interpretation of these results must be considered. At the present time, the only preclinical reports of interactions of drug self-administration and HIV-1 are those of cocaine i.v. self-administration in HIV-1 transgenic rats which express seven of the nine viral proteins (Reid et al., 2001). In the first report, HIV-1 transgenic rats displayed a significant leftward shift in the cocaine, but not heroin, dose-response curve as compared to Fischer 344 rats in an intravenous self-administration model suggesting an increased sensitivity to cocaine’s rewarding properties (McIntosh et al., 2015). Although there was a leftward shift in heroin’s dose-response curve for

HIV-1 transgenic rats, the lack of statistical significance was suggested by the authors to be due to its negligible dopaminergic involvement than what is found with cocaine. In the second report, it was found that while HIV-1 transgenic rats did not show any difference from control animals in cocaine self-administration, there was significantly enhanced firing of medial prefrontal cortex pyramidal neurons from cocaine-exposed HIV-1 transgenic rats as compared to Fischer 344 rats and to saline-yoked control animals (Wayman et al., 2016). In the present study, consumption of oxycodone in Tat transgenic mice reached behaviorally active levels as some subjects exhibited stereotypic behaviors including hyperlocomotion and biting/chewing of levers. To maintain simplicity of design and interpretation, and to avoid the disruptive effects of removing pre-session food that were observed in C57BL/6J mice, Tat transgenic mice were tested only under post-prandial conditions throughout the study. Therefore, at the present time it cannot be concluded that oxycodone was serving as a positive reinforcer in Tat transgenic mice or whether the reinforcing efficacy of oxycodone was altered by Tat expression. The increased number of deliveries seen mainly in Tat(+) mice, however, suggests that Tat expression did have an effect on oral oxycodone self-administration. One possible explanation for the increased responding maintained by oxycodone could be due to an increased or more rapid tolerance to oxycodone's subjective effects than Tat(-) mice that resulted in an increased responding to maintain a level of effect. In fact, an increased tolerance to morphine's antinociceptive effects was reported recently in Tat-expressing mice, which supports the plausibility of Tat altering inherent properties of opioids (Fitting et al., 2016). Moreover, in HIV-1 transgenic rats, an increased tolerance to methamphetamine-induced hyperthermia was

observed (Kass et al., 2010). Therefore, it is possible that the observed increase in oxycodone oral self-administration is due to an increased tolerance to oxycodone in Tat-expressing mice, although other possible mechanisms are possible.

4.2. Acoustic startle measures in Tat transgenic mice

In general, Tat expression did not alter acoustic startle or habituation up to 58 days of DOX exposure. However, PPI was notably decreased in Tat-expressing mice after 16-d on DOX that was maintained over time. This supports previous findings where Tat exposure significantly reduced PPI, suggesting deficits in sensorimotor gating are observed after Tat exposure and reflect sensorimotor gating deficits reported in HAND patients (Fitting et al., 2006a; Minassian et al., 2013; Moran et al., 2014; Paris et al., 2015). Acute oxycodone administration significantly decreased ASR in DOX-fed Tat(-) and Tat(+) mice (although it was not significantly altered at the lowest dose, 9 mg/kg, for Tat(+) mice). This was the first evidence to show oxycodone has acute effects on acoustic startle. These acute effects of oxycodone are interesting as they were only found for ASR and virtually had no effect on habituation or PPI. Therefore, under the conditions tested, Tat expression did not interact with acute oxycodone to worsen PPI deficits seen with Tat expression alone. This may be due to Tat expression alone inducing PPI deficits only after 16-d of DOX exposure. In the present study, acute oxycodone was administered and tested only after 7-d of DOX exposure, therefore an interaction of acute oxycodone and Tat expression may be time-sensitive and longer durations of DOX exposure may be necessary to demonstrate an interactive effect with oxycodone. This lack of effect was also seen in REG-fed Tat transgenic mice chronically

administered oxycodone, suggesting chronic oxycodone does not have acoustic startle-altering effects on its own.

In saline-treated control mice, PPI deficits were not evident in Tat(+) mice after DOX exposure as previously seen in the timecourse study. This may be due to differences in testing parameters such as time between repeated testing as well as repeated injections prior to testing for the chronic study. Repeated injections, handling, as well as testing nocturnal rodents during the light phase, have all demonstrated to alter behavior in both rats and mice (Izumi et al., 1997; Longordo et al., 2011). Both the timecourse study and the chronic saline experiment would need to be replicated to first determine the replicability of outcomes from both experiments before manipulating the DOX exposure length prior to saline administration to determine if these effects are due to duration of Tat expression.

Naloxone-precipitated withdrawal in Tat transgenic mice replicated findings with C57BL/6J mice, in which acoustic startle and habituation was significantly decreased as a result of naloxone injections. This was observed for both REG- and DOX-fed Tat(-) and Tat(+) mice, and was dependent on naloxone dose where the lowest dose (0.1 mg/kg) increased ASR and higher doses (1 and 10 mg/kg) decreased ASR. While in general no significant differences between Tat(-) and Tat-expressing mice occurred, an attenuated severity of naloxone-precipitated withdrawal effects, particularly for habituation, was observed for Tat-expressing mice suggesting that Tat expression reduced the expression of dependence-related effects. The degree of physical dependence upon an opioid has been inferred to be correlated with the severity of withdrawal symptoms produced after administration of an antagonist, such as naloxone

(Blasig et al., 1973; Geary and Wooten, 1985; Heishman et al., 1989). Therefore, attenuated withdrawal effects in Tat-expressing mice may be due to a resistance to physical dependence. This is contrary to the original hypothesis that Tat expression worsens oxycodone abuse-related effects; however, recent findings have shown a similar pattern of behavior in Tat-expressing mice in which morphine physical dependence was assessed (Fitting et al., 2016). In that study, Tat transgenic and C57BL/6J mice were implanted with a placebo or 75-mg morphine pellet and morphine-induced antinociceptive tolerance as well as somatic signs of withdrawal produced after a 1 mg/kg naloxone challenge were measured. Tat-expressing mice were found to have both an increased antinociceptive tolerance and decreased severity of morphine physical dependence. It was suggested that this attenuated dependence effect was possibly due to the use of an insufficient dose of naloxone (1 mg/kg) to precipitate withdrawal, and that higher doses may increase the severity of a withdrawal effect. This is indeed what was observed in the present study, in which only the highest dose of naloxone (10 mg/kg) was able to significantly decrease habituation in Tat-expressing mice. However, in comparison to the effects seen at 1 mg/kg naloxone, the evidence does not strongly support this conclusion as the severity of decreased habituation in Tat-expressing mice was not different between 1 mg/kg and 10 mg/kg doses of naloxone. The effects of Tat expression on dependence-related measures of other drugs of abuse have not yet been evaluated. Importantly, however, physical dependence is just one phenomenon indicative of abuse liability and therefore does not reflect the entire pharmacological profile of oxycodone or its alteration by HIV-1 Tat

expression. Further expansion of this point is discussed in the summary of chapters below.

5. Chapter V

5.1. Oxycodone's effects on locomotor activity and its sensitization in C57BL/6J mice

Results from Chapter V showed that oxycodone induces mu-opioid agonist prototypic increases in locomotor activity and is able to induce locomotor sensitization in C57BL/6J mice. The tetracycline derivative doxycycline does not interfere with oxycodone's hyperactivity effects, further providing evidence of a lack of nonspecific effects on oxycodone's behavioral effects. In the oxycodone locomotor sensitization study with C57BL/6J mice, the time to peak effect after an acute injection of 9 mg/kg oxycodone (~6-min) did not change from Day 1 to Day 8, however, recovery toward baseline levels accelerated. That is, on Day 8, mice rapidly decreased distance traveled after reaching peak effects, whereas mice maintained near-peak effects longer on Day 1. Oxycodone locomotor sensitization has been previously found in mice, but reporting of time-sensitive shifts were not mentioned (Liu et al., 2005; Niikura et al., 2013). In the Liu et al. (2005) study, time to peak effect after an acute injection of 5 mg/kg oxycodone was approximately 30-min. This relatively large difference from the 6-min peak effect in the present study may be due to both dose (5 versus 9 mg/kg) and mouse strain (Kunming versus C57BL/6J). Differences in mouse strain can affect opioid responsiveness and their abuse-related effects (Elmer et al., 2010; Kest et al., 2002; Metten et al., 2009), and therefore may explain why time to drug peak effect is sooner in

the present study than in the Liu et al. (2005) study. Still, the rapid decrease in distance traveled on Day 8 may suggest that there is alteration of the pharmacokinetic profile of oxycodone after repeated administration.

5.2. Oxycodone's effects on locomotor activity in Tat transgenic mice

HIV-1 Tat expression in mice showed less locomotor activity and fewer stereotypy counts as compared to Tat(-) control mice up to 58 days of DOX exposure. This supports previous findings of motor impairments with this mouse model of neuroAIDS (Hahn et al., 2015), and also corresponds to those reported in HIV-associated neurocognitive disorders (Antinori et al., 2007). Acute administration of oxycodone produced significant increases in locomotor activity and stereotypy counts in both Tat(-) and Tat(+) mice. Relative to a saline baseline, the time spent in the center area of the chamber under acute oxycodone conditions was reduced in both Tat(-) and Tat(+) mice, but to a lesser degree in Tat-expressing mice. That is, Tat expression interacted with oxycodone's acute effects to result in greater center time than was observed in Tat(-) mice. The time in center is an indirect index of anxiety-related behavior (Lipkind et al., 2004; Prut and Belzung, 2003), and previous studies have shown Tat expression alone results in reduced time in open field tests and greater time in dark regions in the light/dark assay, suggesting a greater anxiety-related behavior after Tat expression is induced (Hahn et al., 2015; Paris et al., 2013). In the present study, Tat(+) mice did have a reduced time in center under saline conditions than Tat(-) mice, although the effect was not statistically significant (data not shown). Anxiety-like behavior is not typically observed after acute administration of opioids, although cessation of chronic opioid administration sufficient to produce withdrawal is reported to

produce anxiety-like behavior (Harris and Aston-Jones, 1993; Schulteis et al., 1998; Zhang and Schulteis, 2008). Therefore, the acute effects of oxycodone on decreasing center time were unexpected; however, mice were injected 25-min prior to a 30-min acoustic startle test before being tested in locomotor activity and this may have promoted reduced center time rather than as an effect of oxycodone alone. Omitting startle tests prior to locomotor activity tests and conducting other kinds of anxiety-related behavioral tests under oxycodone administration conditions would help clarify its potential effects on anxiety-like behavior.

Chronic oxycodone significantly increased locomotor activity as well as stereotypy counts in Tat transgenic mice, regardless of chow type provided or genotype. The effect was somewhat blunted in DOX-fed Tat(+) mice (i.e., Tat-expressing), although nonsignificantly so, and may reflect a dampening effect by Tat expression's on locomotor activity in general. Center time, however, was not affected by chronic oxycodone administration. Precipitated withdrawal with naloxone dose-dependently suppressed locomotor activity and stereotypy as expected, while increasing center time. An increase in center time was unexpected, as it indicates a lack of anxiety-related effects that is in conflict with other literature demonstrating withdrawal increases anxiety-related behavioral effects (Harris and Aston-Jones, 1993; Schulteis et al., 1998; Zhang and Schulteis, 2008). Similar to the acute oxycodone study, however, the chronic study was in conjunction with prior acoustic startle testing and may have interfered with any expression of anxiety-related behavior in locomotor activity tests. Moreover, the locomotor chambers and procedures used may not be sensitive to anxiety-related behavioral effects (e.g., testing was conducted in darkened chambers).

5.3. Opioid locomotor sensitization in Tat transgenic mice

Tat expression attenuated the development to and the expression of morphine and oxycodone locomotor sensitization. In the morphine sensitization study, Tat expression was induced and maintained for 12-weeks prior to morphine tests. Acute administration of 32 mg/kg morphine on Day 1 resulted in similar increases in locomotor activity in Tat(-) and Tat(+) mice. However, when the same dose of morphine was given prior to locomotor activity tests on Days 2-4, a reduced expression of sensitization was observed for Tat(+) mice but not Tat(-) mice. When morphine was given again on Days 8 and 11, however, Tat(+) mice had surpassed or achieved the level of total distance traveled observed as of Tat(-) mice. Together, these results suggested that Tat expression reduces the development of morphine sensitization.

In the oxycodone sensitization study, REG-fed Tat transgenic mice were first tested under a cumulative oxycodone dosing regimen to establish a pre-Tat expression baseline. Interestingly, there was already a reduced sensitivity to the highest two doses of oxycodone in Tat(+) mice. This effect may be attributable to a mild level of Tat protein being present, as the Tat promotor has been found to have some constitutive activity in this mouse model (Bruce-Keller et al., 2008; Fitting et al., 2012; Fitting et al., 2010). Once mice were placed on DOX chow for two weeks, the cumulative oxycodone dosing regimen was repeated. There was sensitization to the two highest doses of oxycodone (10 and 33 mg/kg) for Tat(-) mice, but only at the highest dose (33 mg/kg) for Tat(+) mice suggesting Tat expression reduced oxycodone locomotor sensitization. The results from locomotor sensitization tests with morphine or oxycodone using Tat transgenic mice indicate that Tat expression reduces, but does not abolish, the

development of opioid sensitization. Similarly, attenuation of cocaine locomotor sensitization was reported in Tat-expressing mice as well as in ovariectomized rats that received intra-accumbal injections of Tat (Harrod et al., 2008; Paris et al., 2014b). However, in transgenic rats expressing seven of the nine viral proteins, greater sensitization to methamphetamine-induced effects was observed suggesting other viral proteins may drive contrary effects on abuse-related effects of drugs (Kass et al., 2010; Liu et al., 2009). In sum, the results from the present study further provide evidence for Tat expression altering abuse-related effects of opioids.

6. Is a reduced sensitivity to opioids in Tat-expressing mice sufficient to conclude a lack of abuse liability?

The results from Chapter IV and V indicated three major findings: (i) Tat expression increases oral self-administration of oxycodone, (ii) Tat expression attenuates oxycodone dependence-related effects as measured by acoustic startle and habituation, and (iii) Tat expression slows the development or expression of morphine and oxycodone locomotor sensitization. Together, along with recent reports in this mouse model showing reduced physical dependence (Fitting et al., 2016), the evidence presented suggest that Tat expression appears to reduce opioid sensitivity rendering the measurement of abuse-related effects challenging. This interpretation is unexpected as there are many reports demonstrating the co-exposure of opioids and HIV-1 Tat results in worsened neuronal damage or toxicity, increases pro-inflammatory cytokine or chemokine release, and increases microglial activation (Bruce-Keller et al., 2008; El-Hage et al., 2005; Fitting et al., 2014; Gurwell et al., 2001). Moreover, increased morphine antinociceptive tolerance in Tat-expressing mice suggests a greater sensitivity

to opioids, although the underlying mechanisms behind tolerance and physical dependence may be independent of one another (Christie et al., 1987). Blunted sensitivity to opioids, specifically morphine, has been demonstrated by Tat-expressing mice as well as by rats given microinjections of another neurotoxic viral protein, gp120, into the periaqueductal grey region (Chen et al., 2011; Fitting et al., 2012). In the gp120 study, this effect was blocked by administration of an antagonist for the CXC chemokine receptor 4 (CXCR4) suggesting the role of this receptor in gp120-induced modulation of morphine sensitivity. Potential mechanisms behind the observed effects in the studies presented in this dissertation are discussed in the next section.

Conversely, could Tat expression directly reduce abuse-related effects of opioids in general? In most theories of addiction, the presence and severity of a withdrawal syndrome upon cessation of drug intake is sufficient to classify a drug as having abuse liability and the withdrawal syndrome itself can promote further abuse of the drug. Therefore, one interpretation of the attenuated opioid abuse-related effects seen in the present acoustic startle and locomotor activity studies and in the Fitting et al. (2016) study might be due to Tat expression blunting abuse-related effects. Though, if this were the case, one would assume there would be substantial epidemiological data in HIV-infected individuals demonstrating reduced drug misuse after HIV infection, however no such evidence to support that claim has been reported. The majority of clinical literature investigating opioid and HIV interactions are centered on the treatment of HIV-related pain. Interestingly, HIV-infected individuals with a history of drug misuse report greater levels of pain proceeded by greater use of prescription opioids and require a higher morphine dosage to treat pain (Kaplan et al., 2000; Tsao et al., 2007).

Moreover, Tat expression did promote increased oral self-administration in the present studies. Therefore, it is unlikely that Tat expression is actively reducing the reinforcing properties of oxycodone or other opioids, and rather the choice of laboratory procedure to measure abuse-related effects may or may not detect changes in the sensitivity to opioids. Moreover, these changes may be reflected in opposite directions than predicted insofar as a blunted sensitivity to oxycodone may have been the underlying cause of increased oral self-administration observed in Tat-expressing mice.

7. Potential mechanisms of oxycodone-Tat interactions

The majority of preclinical studies that have begun to examine opioid and HIV-1 Tat interactions typically use the prototypic mu-opioid agonist, morphine. From these studies, the underlying mechanisms of these interactions have been suggested to involve neuroinflammation (i.e., increase in pro-inflammatory cytokine and chemokine release) via glial cell activation. It is fairly well known that opioids have immunomodulatory effects, and morphine-induced glial activation has shown to have a role in reinstatement of morphine conditioned place preference via prevention of morphine-CPP after administration of the glial cell inhibitor ibudilast, also known as AV-411 (Schwarz et al., 2011). In fact, ibudilast and other glial cell modulators such as minocycline have been shown to attenuate abuse-related effects of opioids as well as other drugs of abuse (Habibi-Asl et al., 2009; Hutchinson et al., 2009; Hutchinson et al., 2008; Snider et al., 2013; Snider et al., 2012). Together, it has been suggested that these glial cell inhibitors may be a potential treatment option for both opioid and psychostimulant abuse (Beardsley and Hauser, 2014; Cooper et al., 2016). However, caution should be noted as these glial modulators are mostly nonspecific inhibitors and

therefore possess multiple effects at various targets which may have opposing or undesirable side effects. Additionally, in the present studies, HIV-1 Tat expression altered abuse-related effects of oxycodone which, under the conditions tested, appeared as an overall reduction in some measures (acoustic startle and locomotor sensitization) and an increase in others (oral self-administration). It would therefore be interesting to measure these abuse-related effects in Tat-expressing mice after administration of a glial cell inhibitor. First, however, these effects would need to be further characterized to clarify if Tat expression is altering abuse-related effects due to a reduced opioid sensitivity. If glial cell activation is the underlying mechanism, administration of a glial inhibitor would attenuate or reverse these effects. Interestingly, glial cell inhibition has indeed begun to be evaluated at as a potential therapeutic for HIV-associated neurocognitive impairments. These evaluations have been performed primarily with minocycline, and while cognitive improvement was not observed in HIV-infected individuals, levels of lipid biomarkers for oxidative stress were reduced (Sacktor et al., 2011; Sacktor et al., 2014). In the present studies, the Tat transgenic mouse model utilized the tetracycline derivative doxycycline to induce Tat expression in Tat(+) mice. Doxycycline itself possesses anti-inflammatory properties and according to one report has a greater anti-inflammatory activity than minocycline (Leite et al., 2011), suggesting differences in their actions that may or may not translate to altering Tat-induced effects. Although this is one disadvantage of the mouse model, as doxycycline may be blunting some Tat-specific effects, it did not completely mask Tat's effects in these studies, nor in previous studies observing Tat-induced behavioral impairments.

Recent studies have begun to characterize the effects of HIV-1 Tat on the functional profile of the mu-opioid receptor to understand the mechanism behind the observed reduced morphine efficacy in previous Fitting et al. (2016 and 2012) studies. Using [^{35}S]GTPγS autoradiography, it was observed that HIV-1 Tat exposure decreased [^{35}S]GTPγS binding via reduced E_{max} values rather than a decrease in potency and that this decrease was not a result of decreased mu-opioid receptor (MOR) levels (Hahn et al., 2016). Importantly, these effects were dependent on length of Tat exposure and were brain region-specific insofar as the nucleus accumbens and amygdala were most sensitive to Tat-induced alterations in MOR signaling. To further explain how Tat might be altering morphine efficacy, the authors examined the role of β-arrestin-2 (βarr2), a regulatory protein which has been implicated in morphine tolerance via increased desensitization of the MOR (Bohn et al., 2000), and found that βarr2 protein expression levels were significantly increased by Tat and showed an enhanced association with MOR which indicates decreased MOR functional availability due to possible desensitization of the MOR. This mechanism for Tat-morphine interactions would explain why a reduced morphine efficacy was then observed in the Fitting et al. (2012 and 2016) studies, but would this hold true for oxycodone? Oxycodone has been shown to have a lesser degree of MOR activation than morphine in several brain regions as measured by GTPγS binding in mice and rats (Lemberg et al., 2006; Nakamura et al., 2013; Thompson et al., 2004). Furthermore, in a study examining multiple MOR agonists (morphine, oxycodone, fentanyl, and methadone) on antinociceptive tolerance in wildtype and βarr2 knockout mice, only morphine antinociceptive tolerance was blocked in βarr2 KO mice (Raehal and Bohn, 2011). Together, this evidence further

demonstrates that MOR agonists are not identical in their pharmacological effects. Therefore, the Tat-oxycodone effects observed in the present studies may be through a mechanism that is independent of βarr2 regulation. The similarity in behavioral responses in the present Tat-oxycodone and previous Tat-morphine studies, but notable differences in the role of βarr2 in the pharmacology of these two opioids, may suggest an alternative underlying mechanism in which morphine and oxycodone converge within the context of HIV-1 Tat expression. Still, oxycodone's active metabolite, oxymorphone, may be driving the behavioral effects and therefore might not represent the effects of oxycodone itself. In conclusion, investigation of the role of βarr2 in Tat-oxycodone effects is essential to further clarify the mechanisms behind the observed behavioral effects seen in the present studies.

8. Future Directions

Results from these studies suggest multiple future directions, but the most important directions that would complement the present studies are discussed below. Importantly, these immediate directions would be essential prior to pharmacotherapy screening, such as testing the effects of glial cell inhibitor administration on oxycodone abuse-related effects or their effects on opioid-Tat behavioral interactions.

In the present self-administration studies, determining whether oxycodone served as a positive reinforcer was met with challenges, as described earlier. Therefore, the immediate future direction for this procedure would be to clearly identify oxycodone's reinforcing efficacy in oral self-administration. One way this might be achievable would be to utilize a concurrent fixed ratio schedule of reinforcement to examine self-administration of oxycodone and water. That is, responding on one lever at a certain

ratio requirement would result in an oxycodone liquid delivery and responding on a second lever at the same, or different, ratio requirement would result in a water delivery. This technique has been used to evaluate ethanol and phencyclidine as an oral reinforcer in rats (Carroll, 1982; Roehrs and Samson, 1981; Samson and Doyle, 1985) and this type of choice procedure in preclinical research advances the inference of a drug serving as a positive reinforcer beyond that of an active/inactive lever choice procedure (for review, Banks and Negus, 2012), as was used in the present oral oxycodone self-administration studies, and would complement the type of abuse liability assessment procedures used in clinical studies (i.e., choice procedures with drug versus money or an alternative reinforcer). Once oxycodone's oral reinforcing effects are demonstrated, further characterization of HIV-1 Tat expression on the modulation of these effects can be examined.

Oxycodone dependence-related effects were measured by acoustic startle and its related measure habituation. However, there is a need to further characterize the dependence regimen used and its effects in startle procedures. First, the acute effects of oxycodone must be investigated in C57BL/6J mice. These effects were only studied in DOX-fed Tat transgenic mice, and therefore may limit the interpretation of how acute oxycodone alone affects startle. Moreover, if Tat-expressing mice are showing reduced dependence-related effects due to reduced opioid sensitivity, then this should be surmountable by administering higher doses of oxycodone. Using a dosing regimen with higher doses of oxycodone, it would be hypothesized that a greater degree of severity of naloxone-precipitated withdrawal effects would be observed as compared to those observed using the present dosing regimen.

9. Conclusions

The studies within this dissertation have added new methodologies for characterizing oxycodone's abuse-related effects in the mouse and have further characterized a mouse model of neuroAIDS in reference to the clinically relevant opioid, oxycodone. Oxycodone was shown to be volitionally, orally self-administered, demonstrated physical dependence-related effects, and engendered locomotor sensitization in C57BL/6J mice. Acoustic startle and its related measure, habituation, proved to be useful for demonstrating oxycodone dependence-related effects in a sensitive, objective, and quantifiable manner. Together, these procedures enable new approaches for exploring biological and environmental determinants of the abuse-related effects of oxycodone.

HIV-1 Tat expression in transgenic mice was able to modulate oxycodone's abuse-related effects in interesting ways. Tat expression increased oral self-administration of oxycodone, attenuated oxycodone dependence-related effects as measured by acoustic startle and habituation, and slowed the development or expression of morphine and oxycodone locomotor sensitization. Recent studies with morphine support the present findings insofar as an attenuated sensitivity to morphine suggested by reduced morphine efficacy and dependence in Tat-expressing mice may be occurring via Tat-induced mu-opioid receptor desensitization. These reported results need to be replicated with oxycodone, but, in general, they support the hypothesis that HIV-1 Tat expression alters sensitivity to opioids that may affect their overall abuse and accelerated neuropathology in opioid-dependent, HIV-infected individuals.

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