THE BEHAVIORAL ROLE OF MU OPIOID RECEPTORS IN GLUTAMATERGIC NEURONS

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DEDICATION

To my incredibly supportive family, friends, and mentors.

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Mu opioid receptors (MORs) mediate the analgesic and rewarding effects of opioids. Most research has focused on MORs in GABAergic neurons; however, MORs are also in glutamatergic neurons and their role in opioid-related behaviors was unclear. Our lab previously showed that MORs inhibit glutamate transmission from vesicular glutamate transporter 2 (vGluT2)-expressing thalamostriatal synapses. The behavioral relevance of MORs in vGluT2-expressing neurons was unknown; therefore, I utilized a conditional MOR knockout mouse with MORs deleted in vGluT2-expressing neurons (MORflox-vGluT2cre). MORflox-vGluT2cre mice have disrupted opioid reward, locomotor stimulation, and withdrawal, compared to cre-recombinase negative littermate controls. However, other MOR-mediated behaviors, including opioid-induced antinociception, alcohol reward, and palatable substance consumption are intact. MORs are expressed in vGluT2 neurons in several reward-related brain regions, including the thalamus and lateral habenula (LHb). To determine whether MORs in these brain regions modulate opioid-related behaviors, an adeno-associated viral (AAV) vector encoding cre-recombinase was stereotaxically injected into the thalamus or LHb of MORflox mice to specifically delete MORs in these brain regions. Opioid reward and locomotor stimulation remained intact in both thalamic and LHb MOR knockout mice; however, basal locomotor activity was increased in LHb MOR knockout mice. Sucrose consumption was also intact in LHb MOR knockout mice. Interestingly, in LHb MOR KO mice opioid withdrawal-induced paw shakes were increased, while withdrawal-induced jumping was completely ablated. Our lab previously showed that MORs inhibit glutamate transmission from the anterior insular cortex (AIC), which is disrupted by in vivo alcohol exposure. To

determine the role of AIC MORs, AIC MORs were deleted with AAV vectors. AIC MOR knockout mice had intact opioid, sucrose, and alcohol reward, but had increased basal locomotor activity. MORs in glutamatergic neurons are critical mediators of opioid reward; however, the specific glutamatergic neurons mediating the rewarding effects of opioids remains to be determined.

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LIST OF ABBREVIATIONS

AIC	Anterior insular cortex
AAV	Adeno-associated virus
CPP	Conditioned place preference
Ctrl	Control
DA	Dopamine
DID	Drinking in the dark
DOR	Delta opioid receptor
GPCR	G protein coupled receptor
i.p.	Intraperitoneal
KO	Knockout
KOR	Kappa opioid receptor
LHb	Lateral habenula
MOR	Mu opioid receptor
NAc	Nucleus accumbens
NOR	Nociceptin opioid receptor
oEPSC	Optically-evoked excitatory postsynaptic currents
REML	Restricted maximum likelihood
RMTg	Rostromedial tegmental area
s.c.	Subcutaneous
vGluT2	Type II vesicular glutamate transporter
VTA	Ventral tegmental area
2BC	Two-bottle choice

CHAPTER 1

Introduction

1.1 Opioid epidemic

Opioids are a frequently abused class of drug that includes many commonly prescribed pain relievers (i.e. morphine, oxycodone), synthetic opioids (i.e. fentanyl, carfentanyl), and the illicit substance, heroin. It is estimated as much as 11% of the United States' population has misused opioids during their lifetime (Saha et al., 2016). In 2019, there were nearly 50,000 deaths due to opioid-related overdoses (CDC, 2020; Skolnick, 2018). Despite their high abuse potential, opioids are the standard treatment for chronic pain, which affects approximately 20% of adults in the United States (Dahlhamer et al., 2018; Von Korff, 2013). Approximately one quarter of people prescribed opioids abuse them and 10% transition to using the illicit opioid, heroin (Carlson et al., 2016; Vowles et al., 2015). The ongoing opioid epidemic in the United States has occurred in three distinct waves. The first wave began as opioids were prescribed more frequently due to pharmaceutical companies marketing opioids as a safe, non-addictive treatment for pain (Van Zee, 2009). As opioids were prescribed at greater rates, abuse of and overdoses due to prescription opioids increased (CDC, 2011; Van Zee, 2009). In the 2010's the second wave was characterized by an increase in heroin overdose-related deaths (Rudd et al., 2014). The third wave began in 2013 with an increase in overdose-related deaths due to synthetic opioids (Gladden et al., 2016; Mattson et al., 2021; Prekupec et al., 2017). These synthetic opioids are a particularly concerning problem because they are significantly more potent than other opioids (Prekupec et al., 2017). Additionally, these synthetic opioids are often added to other drugs of abuse (i.e. cocaine, methamphetamine, heroin) and illegitimately sold counterfeit pills (Prekupec et al.,

2017). Because of the lack of oversight and quality control in these illegally sold drugs, people often unknowingly consume lethal doses of synthetic opioids (Prekupec et al., 2017). In addition to the potentially deadly effects of opioid abuse, chronic opioid abuse results in maladaptive neurobehavioral changes including increased risk-taking, impaired working memory, and impaired cognitive performance (Baldacchino et al., 2012).

1.2 Mu opioid receptor signaling

Opioids bind to and exert their effects through opioid receptors, which are categorized into one of four subtypes: mu-opioid receptors (MORs), delta-opioid receptors (DORs), kappa-opioid receptors (KORs), and non-traditional nociceptin opioid receptors (NORs) (Granier et al., 2012; Henderson & McKnight, 1997; Le Merrer et al., 2009; Manglik et al., 2012; Wu et al., 2012). Opioid receptors are members of Class A, rhodopsin-like, G protein coupled receptors (GPCRs). GPCRs are seven transmembrane receptors that interact with heterotrimeric G proteins, comprised of α, β, and γ subunits, in the plasma membrane. Opioid receptors couple to the inhibitory $G_{i/o}$ class of G proteins (Allouche et al., 2014; Stein et al., 2003). In the inactive state, GDP is bound to the Gαi subunit. Opioid receptors are activated by both exogenous opioid drugs and endogenous opioids peptides: endorphins (MOR), enkephalins (MOR, DOR), dynorphins (KOR), and nociceptin (NOR). When an exogenous opioid or endogenous opioid peptide binds to the opioid receptor, the GDP bound to the Gαi subunit is exchanged for GTP, causing the G proteins to dissociate from the opioid receptor. The Gαi subunit and Gβγ dimer then act on different intracellular signaling pathways.

Opioid receptors inhibit neural activity and neurotransmission both presynaptically and postsynaptically by signaling through both G protein (Gαi and Gβγ) pathways, as well as

the MAPK and arrestin signaling pathways (for review, see (Al-Hasani & Bruchas, 2011)). The Gαi subunit inhibits adenyl cyclase, reducing cyclic AMP formation (Minneman & Iversen, 1976; Taussig et al., 1993). The Gαi subunit also inactivates G-protein gated inward rectifying potassium channels, which results in hyperpolarization (Ippolito et al., 2002; Torrecilla et al., 2002). The Gβγ dimer binds to and inhibits voltage-dependent calcium channels (Bourinet et al., 1996).

The regulation of MORs following receptor activation varies according to the MOR agonist (see (Bailey & Connor, 2005), for review). In general, following receptor activation, MORs are phosphorylated by G protein-coupled receptor kinase, which recruits β-arrestin 1 and 2 (Bohn et al., 2000; Schulz et al., 2004). This may lead to receptor internalization; although, this depends on the MOR agonist (Bailey & Connor, 2005; Groer et al., 2007). The phosphorylated, arrestin-bound MORs continue to signal through various MAPK signaling pathways (Kramer & Simon, 2000). Through MAPK signaling, MORs can modulate gene expression, including those involved with cellular differentiation, proliferation, and apoptosis (Raman et al., 2007).

1.3 Behavioral roles of mu opioid receptors

MORs are the primary target of many opioids and mediate many opioid-related behaviors. The behavioral role of MORs was determined by classical studies using constitutive MOR knockout mice (for review, see (Kieffer & Gaveriaux-Ruff, 2002)). In the absence of administered exogenous MOR agonists, behavioral differences between MOR knockout and wild-type mice are thought to be controlled by endogenous MOR signaling. There are conflicting findings regarding whether endogenous MOR signaling is involved in locomotor activity. Some studies found no effect of MOR deletion (Becker et

al., 2000; Sora et al., 1997); however, other studies found that locomotor activity was reduced in MOR knockout mice (Matthes et al., 1996; Tian et al., 1997). Conflicting results were also obtained regarding whether endogenous MOR signaling mediates nociception, with some studies reporting no effect of MOR deletion (Fuchs et al., 1999; Matthes et al., 1996; Matthes et al., 1998) and others reporting increased (LaBuda et al., 2000; Matthes et al., 1998; Qiu et al., 2000; Sora et al., 1997) or decreased nociception (Sora et al., 1999). These conflicting findings are likely due to the use of different behavioral assays, experimental designs, and methods of MOR deletion in these studies. Endogenous MOR signaling is also involved in mating behaviors in male mice; although, mating behavior in female MOR knockout mice has not been tested (Tian et al., 1997). Endogenous MOR signaling regulates stress and emotional responses, including anxiety and depression (Filliol et al., 2000; LaBuda et al., 2000). Specifically, MOR knockout mice show decreased anxiety and depressive-like behaviors and reduced stress-induced analgesia (Filliol et al., 2000; LaBuda et al., 2000).

MOR knockout mice have altered responses to opioid drugs, as well as several other drugs of abuse. Many studies have demonstrated the role of MORs in opioid-induced analgesia, as opioid-induced analgesia is abolished in MOR knockout mice (Fuchs et al., 1999; Kitanaka et al., 1998; Loh et al., 1998; Matthes et al., 1996; Mizoguchi et al., 1999; Qiu et al., 2000; Schuller et al., 1999; Sora et al., 1999; Sora et al., 1997). Opioid reward is also abolished in MOR knockout mice, as well as opioid-induced locomotor stimulation, a phenomenon commonly associated with many drugs of abuse (Becker et al., 2000; Matthes et al., 1996; Sora et al., 2001; Tian et al., 1997). MORs also mediate several of the negative and potentially dangerous effects of opioids, including opioid withdrawal and respiratory depression (Dahan et al., 2001; Matthes et al., 1996; Matthes et al., 1998; Morin-Surun et al., 2001). MORs are involved in mediating the effects of

other drugs of abuse. Marijuana and alcohol reward is abolished in MOR knockout mice (Ghozland et al., 2002; Hall et al., 2001; Roberts et al., 2000). Alcohol-induced locomotor stimulation is reduced in MOR knockout mice, while the development of alcohol dependence is accelerated (Ghozland et al., 2005; Hall et al., 2001). Although studies have found conflicting results, marijuana withdrawal and alcohol-induced anxiolysis may also be modulated by MORs (Ghozland et al., 2005; Ghozland et al., 2002; LaBuda & Fuchs, 2001; Lichtman et al., 2001). These classical studies using total MOR knockout mice were imperative to understanding the behavioral relevance of MORs; however, studies utilizing microinjections of MOR agonists, conditional MOR knockout mice, and brain-region specific MOR knockdowns have demonstrated that MORs in different neuronal populations mediate distinct behaviors.

Studies have utilized intracranial microinjections of MOR agonists to determine site-specific roles of MORs; these studies have been extensively reviewed in (Le Merrer et al., 2009). Here, significant findings of these studies, relating to site-specific MOR-mediated effects of reward and opioid withdrawal, are described. Results of MOR agonist microinfusion studies vary based on sub-regional specificity and behavioral assays used. In animal models, conditioned place preference (CPP) and self-administration methodologies are used to access the rewarding properties of abused drugs and natural rewards. Due to the role of midbrain dopamine (DA) transmission in reward, most research has focused on the ventral tegmental area (VTA) and nucleus accumbens (NAc). MORs in the following brain regions are involved with the rewarding effects of palatable food: VTA, NAc, ventral pallidum, and amygdala. Hypothalamic MORs are also involved in regulating feeding behaviors; however, hypothalamus MORs primarily mediate feeding associated with nutrient and energy requirements. MORs in the VTA, NAc, amygdala, hippocampus, periaqueductal gray, and lateral hypothalamus

are involved in mediating the rewarding effects of opioids. Inactivation of MORs in these brain regions reduces the rewarding effects of other drugs of abuse. Specifically, MORs in the VTA (cocaine, alcohol, nicotine), NAc (cocaine, alcohol), amygdala (cocaine, alcohol), hippocampus (alcohol), and ventral pallidum (cocaine) contribute to the rewarding effects of non-opioid drugs of abuse. The site-specific roles of MORs in mediating reward suggest that they may also be involved in key stages of addiction, including withdrawal symptoms.

Following development of opioid dependence by prolonged, repeated exposure, opioid withdrawal occurs following a period of abstinence or by precipitation through administration of an opioid receptor antagonist. Opioid withdrawal involves somatic (i.e. changes in body temperature, diarrhea, tremors, nausea) and motivational (i.e. anhedonia, anxiety, stress, dysphoria) components (Frenois et al., 2005). In animal models, the somatic component of opioid withdrawal is assessed by the appearance of physical withdrawal symptoms, while the motivational component is assessed by environmental associative learning (conditioned place aversion), suppression of conditioned operant responding for food, and intracranial self-stimulation (Frenois et al., 2005). MORs in the NAc, VTA, and amygdala are involved with the motivational component of opioid withdrawal but play a relatively minor role in the somatic component. In contrast, MORs in the periaqueductal gray and locus coeruleus are heavily involved in the somatic component of withdrawal, but only minorly involved with the motivational aspect of withdrawal. MORs in the hypothalamus also play a minor role in the somatic, but not motivational, component of withdrawal. These micro-infusion studies provided the first evidence that MORs in different brain regions mediate distinct behaviors; however, these studies are unable to differentiate pre- and post- synaptic MORs, cell-type specific effects, or neurocircuit-specific effects.

Recently, studies have utilized genetic approaches and viral constructs to conditionally delete MORs in specific cell types and brain regions. It is well established MORs in GABAergic neurons that project to VTA DA neurons are crucial for opioid reward (Johnson & North, 1992; Lecca et al., 2011; Matsui & Williams, 2011). However, MORs are located in many other brain regions and cell-types (Kalyuzhny et al., 1996). Utilizing a conditional MOR knockout mouse, the Kieffer lab determined MORs in GABAergic forebrain neurons mediate opioid-induced locomotor stimulation, motivation to obtain heroin and palatable food (increased operant responding, breaking point, and cue-induced reinstatement), and alcohol reward. In contrast with total MOR knockout mice, GABAergic forebrain MOR knockout mice have intact opioid analgesia, reward, and withdrawal. MORs are primarily deleted in the striatum of these GABAergic MOR knockout mice; however, the striatum is a heterogenous region, primarily comprised of D1 (direct pathway, striatonigral neurons) and D2 (indirect pathway, striatopallidal neurons) DA receptor expressing- GABAergic medium spiny neurons, along with a small population of cholinergic interneurons. Other studies have investigated cell-type specific effects of striatal MORs. Re-expressing MORs in D1-expressing striatal neurons of total MOR knockout mice restores opioid-induced locomotor activity (Cui et al., 2014). Likewise, mice with MORs deleted in D1-expressing neurons have reduce opioid-induced locomotor activity (Severino et al., 2020). Re-expression of MORs in D1-expressing striatal neurons restored opioid reward and partially restored motivation to obtain opioids (Cui et al., 2014). In contrast, deleting MORs in D1-expressing neurons did not affect opioid reward or motivation (Severino et al., 2020). It is possible MORs in other neurons can compensate for MOR deletion in D1-expressing neurons, resulting in intact opioid reward and motivation in D1- MOR knockout mice. MORs in D1-expressing striatal neurons do not appear to be involved with opioid analgesia or withdrawal, as re-

expression of MORs in these neurons did not rescue these behaviors (Cui et al., 2014). Deleting MORs in adenosine A2A-expressing neurons or cholinergic interneurons enhances opioid-induced locomotor activity and opioid motivation but does not affect opioid reward (Severino et al., 2020). Although A2A receptors are primarily found in D2-expressing medium spiny neurons in the striatum (Schiffmann et al., 2007), deleting MORs in D2-expressing neurons did not affect opioid locomotor activity, reward, or motivation (Severino et al., 2020). Therefore, effects of deleting MORs in A2A-expressing neurons is likely due to a neuronal population that expresses MORs and A2A, but not D2. It is important to note that while the goal of these MOR knockout mice was to investigate cell-type specific roles of striatal MORs, the behavioral effects could also be mediated by MORs in D1-expression, A2A-expressing, or cholinergic neurons in extrastriatal brain regions.

Although most research to date has focused on the behavioral role of MORs in GABA neurons, recent studies have identified roles of MORs in glutamatergic neuronal populations in opioid-induced locomotor activity, opioid reward, and opioid withdrawal. Mice with MORs deleted in a subset of glutamatergic neurons that express the type II vesicular glutamate transporter (vGluT2) lack opioid-induced locomotor stimulation, have disrupted opioid reward, and have altered withdrawal-related behaviors (Reeves et al., 2021; Zhang et al., 2020) (see Chapter 2). Another study utilized conditional MOR knockout mouse to investigate the role of MORs in a glutamatergic brain region with a high density of MORs, the medial habenula (Boulos et al., 2020). Medial habenula MOR knockout mice showed reduced opioid withdrawal-related behaviors and withdrawal-associated conditioned place aversion. Opioid-induced locomotor activity, opioid analgesia, opioid reward, and palatable food reward was intact in medial habenula MOR knockout mice.

Several studies have used RNA interference to knockdown MOR expression in the VTA. Knockdown of MOR expression in the VTA disrupts opioid-induced locomotor stimulation, opioid reward, alcohol reward (Lasek et al., 2007; Zhang et al., 2009). MORs in the VTA also mediate behavioral adaptations to stress, as social stress-induced maladaptive behavioral changes are prevented by knockdown of VTA MOR expression (Johnston et al., 2015). Other studies have used viral vectors in combination with genetic approaches to investigate cell-type specific effects of MORs in brain regions of interest. A study found that MORs in parvalbumin-expressing interneurons in the prelimbic cortex are involved in morphine-induced locomotor stimulation and sensitization (Jiang et al., 2021). MORs in GABAergic interneurons in the hippocampus mediate stress-induced memory impairment (Shi et al., 2020). These studies highlight the importance of considering regional and cell-type heterogeneity in MOR-mediated behaviors and demonstrate MORs in small sub-populations of neurons can greatly influence opioid-related behaviors.

1.4 Aims and hypotheses

The ongoing opioid crisis presents a severe health exigency; therefore, it is imperative to understand the mechanisms of these opioid drugs and the mechanisms and opioid receptor systems on which these drugs act. Although the role of MORs in GABAergic neurons in opioid-related behaviors has been well established, research regarding the behavioral role of MORs in glutamatergic neurons is lacking. The central hypothesis of this dissertation is: MORs in glutamate neurons mediate opioid-related behaviors, including reward. The aims of this dissertation were to (1) characterize the behavioral roles of MORs in a subset of glutamatergic neurons that express vGluT2; (2) determine

the role of MORs in three glutamatergic brain regions (AIC, thalamus, and LHb) in opioid-related behaviors.

CHAPTER 2

Mu opioid receptors in vGluT2-expressing glutamatergic neurons modulate opioid reward

2.1 Introduction

The abuse of opioids, such as oxycodone, is on the rise in the United States, with more than 47,000 deaths due to opioid overdose in 2017 (Centers for Disease Control and Prevention, 2018). Approximately one quarter of people prescribed opioids for pain management abuse them (Vowles et al., 2015). Chronic opioid abuse results in neurobehavioral changes including increased risk-taking, impaired working memory, and impaired cognitive performance (Baldacchino et al., 2012). MORs mediate the rewarding and analgesic effects of commonly prescribed and abused opioids (Kieffer & Gaveriaux-Ruff, 2002). It is well established that MORs modulate opioid reward through inhibition of GABA transmission (Fields & Margolis, 2015). The classical model of opioid reward implicates MOR-mediated depression of GABA release from VTA GABAergic interneurons, which synapse onto local VTA DA neurons. MOR activation decreases the inhibitory tone to VTA dopamine neurons, resulting in increased DA release in the NAc (Fields & Margolis, 2015). This model is supported by the fact that knocking down MOR expression in the VTA blocks the rewarding effects of opioids (Zhang et al., 2009). Others have suggested a revised model where MORs on GABAergic inputs from other brain regions to VTA DA neurons may mediate behavioral responses to opioids (Matsui & Williams, 2011). In addition, other studies indicate that MORs expressed in forebrain GABA neurons also modulate opioid reward (Charbogne et al., 2017; Cui et al., 2014; Severino et al., 2020).

MORs also regulate glutamate transmission in multiple neurocircuits, including those involved in drug abuse, but their specific role in opioid reward is unknown (Birdsong et al., 2019; Blomeley & Bracci, 2011; Hoffman & Lupica, 2001; Jiang & North, 1992; Margolis & Fields, 2016; Martin et al., 1997; Wamsteeker Cusulin et al., 2013; Zhu & Pan, 2005). It is increasingly clear that glutamate transmission is a critical component of opioid reward-related behaviors. The illicit opioid, heroin, increases extracellular glutamate in the NAc; an effect not seen with feeding-related behaviors (Kalivas, 2009; LaLumiere & Kalivas, 2008). Within the NAc and VTA, acute opioid exposure depresses glutamate release but enhances NMDA glutamate receptor function. Morphine fails to promote dopamine neuron activation following inhibition of VTA AMPA and NMDA receptors (Barrot et al., 2012; Marion Jalabert et al., 2011). This treatment also inhibits the development of morphine CPP without affecting locomotor activity (Harris et al., 2004).

We previously showed that MOR activation inhibits multiple glutamatergic inputs to the striatum, a critical brain region controlling behavioral responses to drugs of abuse. In these studies, we found that MOR activation inhibits glutamate transmission at vGluT2-expressing thalamic inputs to striatal medium spiny neurons (Munoz et al., 2018). vGluT2-expressing neurons are a subclass of glutamate neurons widely expressed throughout the brain (Fremeau et al., 2004). vGluT2-expressing neurons in multiple brain regions modulate reward-related behaviors (Faget et al., 2018; Root et al., 2018; Tooley et al., 2018; Wang et al., 2015). Given our findings that MORs regulate glutamate transmission at vGluT2-expressing synapses and the involvement of vGluT2 neurons in reward-related neurocircuitry, we decided to investigate the role of MORs in vGluT2

neurons in behavioral responses to opioids. Here we report on the generation of a mutant mouse that selectively lacks MOR expression within vGluT2 neurons and its behavioral responses to the prescription opioid analgesic, oxycodone.

2.2 Materials and Methods

2.2.1 Animals

MORflox-vGluT2cre mice were bred and genotyped in-house from conditional MOR knockout mice (MORflox) and vGluT2cre progenitors (vGlut2Cre: JAX #016963). MORflox mice were generously donated by Dr. Jennifer Whistler (UC Davis) and have been previously characterized (Goldsmith et al., 2013; Munoz et al., 2018). All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine and all guidelines for ethical treatment and care for experimental animals established by the NIH (National Institutes of Health, Maryland, USA) were followed. Experiments were conducted on cre-recombinase expressing mice [MORflox-vGluT2cre (+), Cre+ (KO)] and littermate cre negative controls [MORflox-vGluT2cre (-), Cre- (Ctrl)]. All mutant mice used in these studies were backcrossed to C57BL/6J mice for a minimum of 7 generations. Mice were group-housed on a standard 12-hour light/dark cycle (lights on 0700hr), with the exception of mice used for drinking and food consumption studies, which were single-housed on a reverse 12-hour light/dark cycle (lights on 1800 hr), with at least one week acclimation before testing. Food and water were available ad libitum for all mice. Male and female mice were between 8-12 weeks of age at the start of experiments and tested in only one experiment, except where otherwise indicated.

2.2.2 Materials

Oxycodone hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in saline (0.9% w/v) for in vivo injections and in reverse-osmosis water for drinking studies. DAMGO was purchased from Bachem (Torrance, CA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or ThermoFisher (Waltham, MA).

2.2.3 Measurement of MOR Expression

Tissue dissection, RNA extraction, and reverse transcription (RT): Adult male mice were deeply anesthetized via isoflurane and whole brains were rapidly excised. Following the Franklin and Paxinos atlas (*The Mouse Brain*, Elsevier, 2007), sections containing thalamus or prefrontal cortex (including medial prefrontal, orbitofrontal, and anterior insular cortices) were made using a stainless steel mouse brain matrix (Zivic Instruments, Pittsburgh, PA) and were rapidly dissected and collected from each mouse. The dissected tissues were immediately homogenized, and RNA was isolated using the RNeasy Plus Universal Mini kit (Qiagen; Cat No: 73404) according to the manufacturer's protocol. RNA concentrations were determined by Nanodrop (ThermoFisher) and 1μg of total RNA was converted to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (ThermoFisher; Cat No: 4368814).

mRNA quantification: We used quantitative polymerase chain reaction (qPCR) to determine MOR gene expression. The qPCR primers used for the MOR gene (mus musculus opioid receptor, μ1 [Oprm1], transcript variant MOR-1C, mRNA) were developed and ordered from Integrated DNA Technologies (Coralville, IA, USA). The forward primer (5′- CTCCTGGCTCAACTTGTCCCACGT -3′) targeted a portion of exon 1 and the reverse primer (5′- ACAGTGATGATGAGGACCGGCATG -3′) targeted a portion of exon 3. The specificity of the primers was verified through PCR using thalamus cDNA

and the PCR product was subcloned via the TOPO TA cloning kit (ThermoFisher, Cat No: 450071) to confirm the sequence. qPCR was conducted by using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA; Cat No: 1725270) on an CFX Connect Real-Time PCR Detection System (Bio-Rad). The relative amount of each transcript was determined via normalization across all samples to the endogenous control, GAPDH. RNA samples from each individual animal were run in triplicate. To quantify the relative expression levels of the genes for each mouse genotype, we calculated the difference (ΔCt) between the cycle threshold of Oprm1 and the housekeeping gene, GAPDH. From these data, the ΔΔCt ([ΔCtOprm1(Cre+)–ΔCtOprm1(Cre-)]) was computed and converted to a relative quantitative (RQ) value using the formula $2^{-\Delta\Delta Ct}$.

2.2.4 Corticosterone quantification

Mice were injected with either saline (10 mg/mL i.p.) or oxycodone (10 mg/kg i.p.) and returned to their home cage. 30 minutes later, mice were briefly anesthetized with isoflurane and decapitated to collect trunk blood. Trunk blood was centrifuged at 4°C at 1200 rpm for 5 minutes and plasma was collected for corticosterone analysis. To measure corticosterone levels, enzyme-linked immunosorbent assay (ELISA) corticosterone kit (Enzo: Cat No. ADI-900-097) was used according to the kit's instructions. Samples were ran in duplicate with correction at 580 nm.

2.2.5 Stereotaxic Surgery

Male MORflox-vGluT2cre mice and littermate controls, at postnatal day 62-80, were anesthetized with isoflurane and stereotaxically injected with the adeno-associated viral (AAV) vector, AAV9.hSyn.ChR2(H134R)-eYFP (Addgene) to drive expression of the photosensitive cation channel, channelrhodopsin2 (ChR2), in thalamic neurons. Bilateral

injections were made into the thalamus (coordinates: A/P: -1.6, M/L: ±0.35, D/V: −3.5) (100 nl/injection, 25 nl/min infusion rate). Mice were allowed to recover for at least 2 weeks before electrophysiological recordings.

2.2.6 Brain Slice Preparation for Electrophysiology

Brains slices were prepared for electrophysiological recordings as previously described (Munoz et al., 2018). Animals were deeply anesthetized with isoflurane before decapitation. The brain was quickly removed and placed into ice-cold cutting solution (194mM sucrose, 30mM NaCl, 4.5mM KCl, 1mM $MgCl_2$, 26mM $NaHCO_3$, 1.2mM NaH_2PO_4, 10mM Glucose) saturated with a mixture of 95% O_2/5% CO_2, and sliced to a thickness of 280 μm on a VT1200S vibratome (Leica, Germany). Slices containing the dorsal striatum were transferred to an artificial cerebrospinal fluid (aCSF) solution containing (124mM NaCl, 4.5mM KCl, 1mM $MgCl_2$, 26mM $NaHCO_3$, 1.2mM NaH_2PO_4, 10mM Glucose, 2mM $CaCl_2$; adjusted to 310–320 mOsm, continuously bubbled with 95% O_2/5% CO_2,) at 32 °C for 1 hour before being moved to room temperature. Immediately before recording, slices were transferred to a recording chamber continuously perfused with aCSF solution that was saturated with 95% O_2/5% CO2 and held at 30 °C.

2.2.7 Electrophysiology

Optically-evoked excitatory postsynaptic currents (oEPSCs) in dorsal striatal medium spiny neurons were produced in brain slices using 470-nm blue light (5-ms) delivered via field illumination through the microscope objective. Light intensity was adjusted to produce stable oEPSCs of 200–600 pA amplitude prior to experimental recording. oEPSCs were evoked every 30s. Prior to recording, brain slices were imaged via an Olympus MVX10 microscope (Olympus Corporation of America, Center Valley, PA) to

verify ChR2-eYFP expression. Whole cell recordings of oEPSCs were carried out at 29–32 C° and aCSF was continuously perfused at a rate of 1-2 ml/min. Recordings were performed in the voltage clamp configuration using a Multiclamp 700B amplifier and a Digidata 1550B (Molecular Devices, San Jose, CA). Slices were visualized on an Olympus BX51WI microscope. Medium spiny neurons were identified by their size, membrane resistance, and capacitance. Picrotoxin (50 μM) was added to the aCSF for recordings to isolate excitatory transmission. Patch pipettes were prepared from filament-containing borosilicate micropipettes (World Precision Instruments, Sarasota, FL) using a P-1000 micropipette puller (Sutter Instruments, Novato, CA), having a 2.0-3.5 MΩ resistance. The internal solution contained (in mM): 120 CsMeSO3, 5 NaCl, 10 TEA-Cl, 10 HEPES, 5 lidocaine bromide, 1.1 EGTA, 0.3 Na-GTP and 4 Mg-ATP (pH 7.2 and 290-310 mOsm). Neurons were voltage clamped at -60 mV for the duration of the recordings. Data were acquired using Clampex 10.3 (Molecular Devices). Series resistance was monitored and only cells with a stable series resistance (less than 25 MΩ and that did not change more than 15% during recording) were included for data analysis. Recordings were made 2–7 hrs after euthanasia.

2.2.8 Behavior Experiments

The following behavioral assays were performed in both male and female mice; studies were sufficiently powered to detect sex differences. For clarity of focusing on genotype, data with no statistically significant sex differences are collapsed by sex. Mice involved in behavior experiments were habituated to handling for at least three days prior to testing. Mice were acclimated to the behavior room with a white noise machine for at least 30 minutes before testing.

Open-field locomotor activity: Mice were placed in the center of unlit open-field chambers (16” X 16”; Omnitech, Columbus, OH) and recorded for 20 minutes. Mice were acclimated to the chamber on day 1. On day 2 mice were habituated to injections by giving them a saline injection (10 mL/kg i.p.) before being placed in the chamber. Saline testing occurred on day 3; mice were given a saline injection (i.p.) immediately prior to being placed in the chamber. On day 4, mice were injected with oxycodone (5 mg/kg i.p.) before being immediately placed in the chamber. Locomotor activity was measured by recording the number of beam breaks in horizontal directions by Fusion software.

Oxycodone conditioned place preference (CPP): Mice underwent a modified protocol of oxycodone CPP (Niikura et al., 2013). Mice underwent pretesting in a 3-chambered apparatus (Omnitech), consisting of two contextually distinct chambers with different wall patterns and floor textures, connected by a center, neutral chamber. Mice were injected with saline (10 mL/kg i.p.) before being placed into the center chamber and allowed to freely explore the entire apparatus for 20 minutes. Duration of time spent on each side of the apparatus was measured using Fusion software (Omnitech). Initial side preference was determined by comparing the amount of time spent on each side of the apparatus during the pretest. For conditioning sessions, a biased design was used, where any mouse that showed an initial side preference greater than 200 seconds was given oxycodone in its initially non-preferred side. The side of the drug-paired chamber was counterbalanced across genotypes. Conditioning sessions, where mice were confined to one of the contextually distinct chambers, occurred twice a day for three days. In the mornings, mice were injected with saline (10 mL/kg i.p.) and immediately placed into one side of the apparatus for 5 minutes. 4 hours later, mice were injected with oxycodone (0.05, 0.5 or 5 mg/kg i.p.) and placed into the other side of the apparatus for 5 minutes.

The day after the last conditioning session, mice were tested for conditioned place preference, using the same protocol as the pretest. Oxycodone-paired side preference was determined by comparing the time spent in the oxycodone-paired side during testing to baseline, as well as subtracting the duration of time spent in the saline-paired side from the duration of time spent in the oxycodone-paired side.

*Ethanol CP*P: Ethanol (EtOH) CPP was performed as previously described for oxycodone CPP, except there were 4 conditioning sessions for saline and ethanol (3 g/kg i.p.).

Light/dark box: Light/dark box inserts were inserted into open-field locomotor chambers (Omnitech). The light area was set to 400 lux. 3 hours into the dark cycle, mice were acclimated to the behavior room, dimly lit with red light. Mice were placed into the light area, an inch away from the doorway, and facing away from the doorway. Locomotor activity and time spent in the light and dark areas were measured by recording the number of beam breaks in horizontal directions by Fusion software.

Naloxone-precipitated withdrawal: Mice used to test naloxone-precipitated withdrawal had previously undergone CPP with 0.5 mg/kg oxycodone. Three days following the completion of CPP testing, oxycodone was administered using a modified dose ramping protocol (10 mg/kg to 40 mg/kg over 8 days), previously demonstrated to produce oxycodone dependency in C57BL/6J mice (Enga et al., 2016). Mice were subcutaneously injected twice daily with 10 mg/kg oxycodone on day 1, 20 mg/kg oxycodone on day 2, and 30 mg/kg oxycodone on days 3-7. All doses were administered subcutaneously during the light cycle, approximately 7 hours apart. Mice were habituated to empty cages used for withdrawal testing on the morning of day 7 for 10

minutes. On day 8, the day of testing, a final dose of oxycodone (40 mg/kg, s.c.) was administered to all mice. This slightly higher dose was chosen to ensure sufficient oxycodone levels were maintained for the remainder of behavioral testing. Approximately 60 minutes following the final oxycodone injection, mice were given a saline injection (10 mL/kg, i.p.) and placed into a clean home cage with bedding removed to observe opioid withdrawal-related behaviors. Following the ten-minute observation period, mice were returned to their home cage for approximately 90 minutes. Mice were then treated with the MOR antagonist, naloxone (5 mg/kg, i.p.), to precipitate opioid withdrawal and observed for another ten minutes in the same modified home cage. Opioid withdrawal-related behaviors were assessed by the same trained, blinded observer. The number of paw shakes, wet dog shakes, and jumps were counted. Ptosis, body tremor, teeth chattering, piloerection, and diarrhea were assessed on a yes/no basis with a point given for each five-minute interval that the behavior occurred during. A global withdrawal score was calculated to give all withdrawal behaviors proportional weighting using the equation: (global withdrawal score=jumps*0.8+wet dogs shakes*1+diarrhea*1.5+paw shakes*0.35+ptosis*1.5+teeth chattering*1.5+body tremor*1.5+piloerection*1.5) (Maldonado et al., 1996).

Nociception/shock-flinch: Mice first received a saline injection (10 mL/kg s.c.). 30 min later, they were placed into startle response chambers (San Diego Instruments, San Diego, CA) that contained shock grids and received a 3 min habituation period in which 3, 115 dB (40 ms) acoustic startle pulses were given at the end of each 60 sec period. The purpose of these 115 dB pulses is to familiarize mice to the loud startle pulses and to minimize large startle responses to low intensity startle pulses. Following this, mice were exposed to a startle/shock threshold session which consisted of 5 blocks of different auditory or sensory (shock) stimuli with the auditory stimuli ranging from 85-115

dB in 10 dB increments and the shock intensities (0.08, 0.1, 0.2, 0.4, and 0.6 mA). Each block contained an auditory and shock intensity in a randomized order. The auditory stimuli lasted 40 ms and the shocks lasted 0.5 sec. The intertrial intervals were 30 sec. In total, each block contained 9 stimuli presentations. An accelerometer detects changes in force due to jumping/flinching and the output is an excitation voltage change in millivolts (mV). One week later this test was repeated after mice received an oxycodone injection (3 mg/kg s.c.).

Sucrose/quinine two-bottle choice (2BC): Mice were habituated to drinking from self-made double ball-bearing sipper tubes, similar to those previously described (Thiele et al., 2014), filled with reverse-osmosis water for 23 hours. Mice were given access to two sipper tubes for 23 hours, beginning 11 hours into the dark cycle: one filled with reverse-osmosis water and the other filled with either sucrose or quinine solution. Mice were counterbalanced across genotypes into groups receiving sucrose or quinine first. Following 6 testing sessions, a 1-week washout period with no drinking sessions was conducted before switching substances. Increasing concentrations of sucrose (0.5%, 1%, 2% w/v) or quinine (0.03 mM, 0.1 mM, 0.3 mM) were given after two sessions at the previous concentration. Sipper tubes were presented on alternating sides of the home cage to account for side-preference. Preference for sucrose or quinine over water was calculated as the percentage of total fluid consumed that contained sucrose or quinine solutions.

Oxycodone 2BC: Mice were habituated to drinking from sipper tubes as described above. Every other day following habituation, mice were presented with both a sipper tube filled with oxycodone solution and a sipper tube filled with reverse-osmosis water for 24 hours at a time, in their home cages, beginning 11 hours into the dark cycle. This

was repeated every other day. Experiments with transgenic mice were performed with increasing concentrations of oxycodone given after 4 sessions at the previous concentration (0.1 mg/mL, 0.3 mg/mL, 1 mg/mL, 3 mg/mL). Sipper tubes were presented on alternating sides of the home cage to account for side-preference. Preference for oxycodone over water was calculated as the percentage of total fluid consumed that contained oxycodone solution.

Food consumption: The amount of food in the home cage was weighed every 24 hours, 11 hours into the dark cycle, for 5 days. The amount of food consumed each day was calculated by subtracting the amount of food remaining from the previous day's food weight.

2.2.9 Data Analysis

Experimenters were blinded to genotype during all stages of data collection. Data are presented as the mean +/- SEM. Data were analyzed using GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). The level of significance was set at $p<0.05$ for all analyses. Statistically significant individual data point outliers were identified using the ROUT method with Q=1% and excluded. Some data were excluded on the basis of technical errors, such as leaking drinking tubes or equipment failures that occurred during a measurement session. Normal distribution was assessed prior to statistical analysis. Two-tailed unpaired t-tests and two-tailed paired t-tests were used to analyze normally distributed data. Mann-Whitney U tests were used to analyze non-normally distributed data. For data with multiple groups and/or repeated measures, ANOVA or restricted maximum likelihood (REML) with Sidak's post hoc tests were used. REML was used if data points were missing due to removal of outliers or experimental exclusion.

2.3 Results

2.3.1 MORflox-vGluT2cre mice lack MOR expression in vGluT2-expressing neurons

To ablate MOR expression specifically within vGluT2-expressing neurons, we bred conditional MOR knockout mice (MORflox) with mice that express cre-recombinase in vGluT2-expressing neurons (vGluT2cre), similar to previous studies (Goldsmith et al., 2013; Munoz et al., 2018). We assessed cre-expressing vGluT2 neuron MOR knockout mice and littermate controls, identified here as Cre+ (KO) and Cre- (Ctrl), respectively. The thalamus is a region with a high density of vGluT2-expressing neurons (Fremeau et al., 2004). MOR mRNA expression was significantly decreased in the thalamus of Cre+ (KO) mice, compared to Cre- (Ctrl) controls (**Figure 1A**, unpaired t-test: $p=0.0005$, $t_4=10.42$). MOR mRNA levels were also assessed in the cortex, a region with low vGluT2-expression (Fremeau et al., 2004). In cortex, MOR expression was not significantly different between genotypes (**Figure 1B**, Mann-Whitney test, $p=0.19$). To functionally assess the knockout of MORs in vGluT2-expressing neurons in Cre+ (KO) mice, we investigated a vGluT2-expressing synapse we had previously shown to exhibit MOR-mediated inhibition of glutamate transmission [21]. Our previous work demonstrated that MORs inhibit glutamate transmission at vGluT2-expressing thalamostriatal synapses but did not reveal if it was specifically MORs within the vGluT2 neurons themselves that mediated that inhibition [21]. To address this question, as well as to functionally assess MOR knockout, we performed whole cell patch clamp electrophysiological recordings from dorsolateral striatal medium spiny neurons and specifically stimulated vGluT2-expressing thalamic inputs. To accomplish this, we injected AAV9.hSyn.ChR2(H134R)-eYFP into the thalamus of Cre- (Ctrl) and Cre+ (KO) animals (**Figure 1C**) and used optical stimulation to evoke excitatory postsynaptic currents (oEPSCs) in medium spiny neurons. Following application of the MOR agonist,

DAMGO (300 nM, 5 min), oEPSCs were decreased in Cre- (Ctrl) but not Cre+ (KO) mice (**Figure 1D-E**, unpaired t-test: p=0.04, t_9=2.41). Overall, these results are supportive evidence that MORs are deleted from vGluT2-expressing neurons in Cre+ (KO) mice.

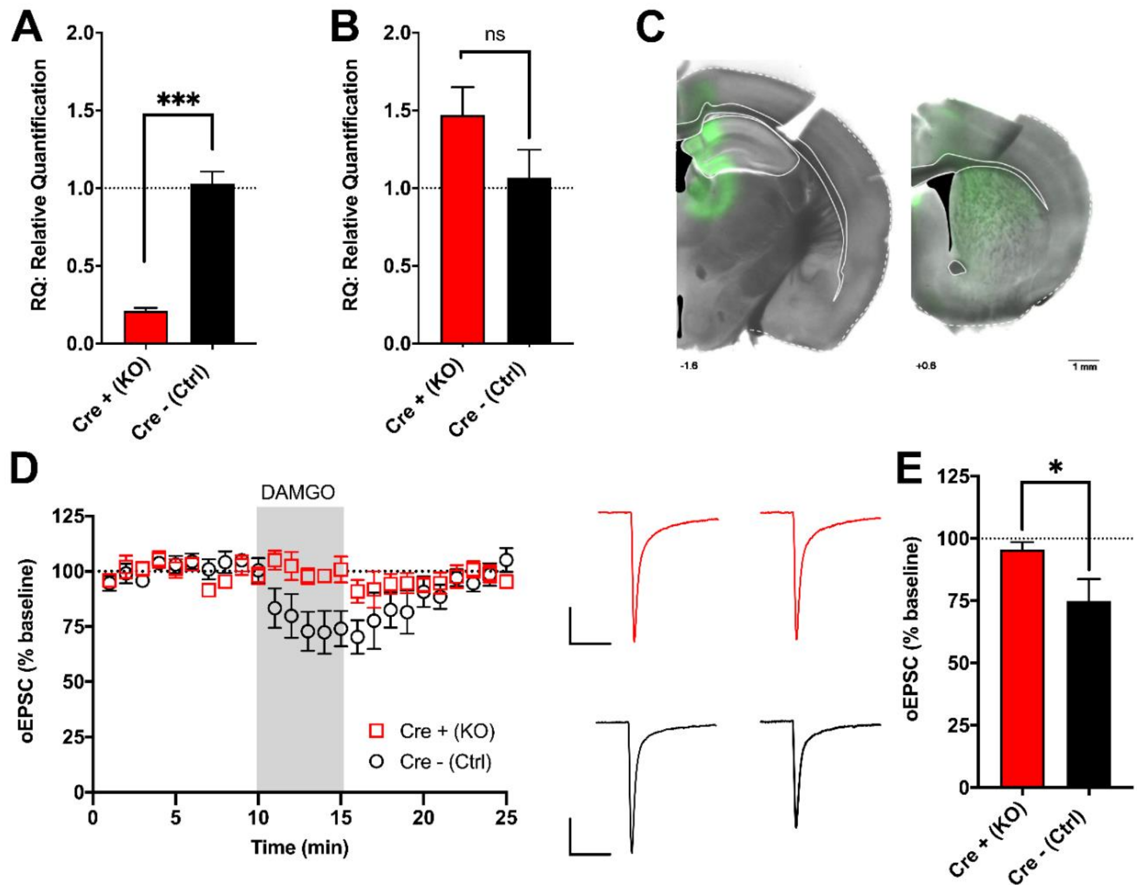


Figure 1. MORflox-vGluT2cre mice lack MOR-expression in vGluT2-expressing neurons. (A) Male Cre+ (KO) animals had less MOR (Oprm1) mRNA expression in the thalamus than Cre− (Ctrl) littermate controls (*n* = 3, 3). (B) There were no differences in cortical MOR mRNA expression between male Cre+ (KO) and Cre− (Ctrl) animals (*n* = 3, 3). (C) Representative image showing eYFP expression in male Cre+ (KO) mouse injected with AAV.ChR2.eYFP into the thalamus with expression of ChR2 in the terminal fields within dorsal striatum. (D) In male Cre− (Ctrl) mice, the MOR agonist, DAMGO (300 nM, 5 min), reduced optically evoked excitatory postsynaptic currents (oESPCs) in response to thalamic input stimulation in dorsal striatal medium spiny neurons; however, DAMGO failed to inhibit glutamate release in male Cre+ (KO) mice (*n* = 6, 5). Also depicted are representative oEPSC traces of baseline (average of 1-10 min, left) and DAMGO treatment (average of 12-17 min, right). Scale bars = 100 pA, 50 ms. (E) Following application of DAMGO, male Cre− (Ctrl) mice had decreased average oEPSC magnitudes compared with baseline (12-17 min vs. baseline); whereas male Cre+ (KO) mice showed no change from baseline. * $p < 0.05$; *** $p < 0.001$. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).

2.3.2 MORflox-vGluT2cre mice lack oxycodone-induced conditioned place preference and locomotor stimulation

MORs are involved in the locomotor and rewarding effects of opioids (Kieffer & Gaveriaux-Ruff, 2002). Low doses of opioids produce locomotor stimulation (Katsuura & Taha, 2010). Therefore, we examined the locomotor activity of MORflox-vGluT2cre mice following an injection of either saline (10 mL/kg i.p.) or oxycodone (5 mg/kg i.p.). The two genotypes had differential responses to treatment (**Figure 2**; two-way ANOVA: treatment, $p<0.0001$, $F(1,35)=95.82$; genotype, $p<0.0001$, $F(1,35)=59.71$; interaction, $p<0.0001$, $F(1,35)=64.98$). While there were no differences in basal locomotor activity between Cre+ (KO) and Cre- (Ctrl) mice following saline injection, Cre- (Ctrl) mice had a greater locomotor response to oxycodone than Cre+ (KO) mice (Sidak's test: saline $p=0.99$; oxycodone $p<0.0001$). Cre- (Ctrl) mice showed oxycodone-induced locomotor stimulation ($p<0.0001$), while Cre+ (KO) mice did not ($p=0.45$).

To measure oxycodone reward in these mice, we used conditioned place preference (CPP), a well-validated measure of drug reward (Cunningham et al., 2006). Following conditioning with 5 mg/kg oxycodone, Cre- (Ctrl) mice spent more time in the oxycodone-paired side, while Cre+ (KO) mice spent less time in the oxycodone-paired side during the drug-free test (**Figure 3A**, two-way ANOVA: genotype $p<0.0001$, $F(1,33)=44.86$; test $p=0.54$, $F(1,33)=0.38$; interaction $p<0.0001$, $F(1,33)=39.78$) (Sidak's test: baseline vs. test, Cre+ (KO) $p=0.0005$; Cre- (Ctrl) $p<0.0001$). Assessments of side preference showed that Cre- (Ctrl) mice displayed CPP for the oxycodone-paired side, while Cre+ (KO) mice showed conditioned place aversion (**Figure 3B**, Mann-Whitney test, $p<0.0001$). These data suggest that Cre+ (KO) mice may find oxycodone aversive,

unlike Cre- (Ctrl) mice, which find oxycodone rewarding. However, an alternative hypothesis is that Cre+ (KO) mice are more sensitive to the effects of oxycodone and would find a lower dose of oxycodone rewarding; therefore, we tested two lower oxycodone doses (0.5 and 0.05 mg/kg) in separate cohorts of mice. Following conditioning with 0.5 mg/kg oxycodone, Cre- (Ctrl) mice spent more time in the oxycodone paired side, while Cre+ (KO) mice did not change the amount of time spent in the oxycodone-paired side (**Figure 3C**, two-way ANOVA: test, $p=0.0023$, $F(1,27)=11.28$; genotype, $p<0.0001$, $F(1,27)=28.04$; interaction, $p=0.0012$, $F(1,27)=13.21$) (Sidak's test, baseline vs. test, Cre+ (KO) $p=0.97$; Cre- (Ctrl) $p=0.0001$). Cre- (Ctrl) mice acquired CPP for the oxycodone-paired side, while Cre+ (KO) mice did not (**Figure 3D**, unpaired t-test: $p<0.0001$, $t_{27}=4.80$). Following conditioning with 0.05 mg/kg oxycodone, both Cre- (Ctrl) and Cre+ (KO) mice spent slightly more time in the oxycodone-paired side (**Figure 3E**, two-way ANOVA: test, $p=0.015$, $F(1,28)=6.72$; genotype, $p=0.22$, $F(1,28)=1.59$, interaction, $p=0.602$, $F(1,28)=0.28$). While there was a main effect of test, post hoc analyses failed to reveal a significant effect of test for either genotype (Sidak's test: baseline vs. test, Cre+ (KO) $p=0.26$; Cre- (Ctrl) $p=0.082$), suggesting that both Cre+ (KO) and Cre- (Ctrl) mice acquire little to no oxycodone CPP at this dose. An assessment of CPP score also revealed no genotype difference (**Figure 3F**, unpaired t-test: $p=0.54$, $t_{28}=0.62$). Altogether, these data suggest that Cre+ (KO) mice have disrupted opioid reward and are not hypersensitive to the rewarding effects of oxycodone.

Because MORs are also involved in mediating the rewarding effects of ethanol (Kieffer & Gaveriaux-Ruff, 2002), we hypothesized that Cre+ (KO) mice would also have disrupted ethanol reward. Following conditioning with 3 g/kg EtOH (i.p.), both Cre+ (KO) and Cre- (Ctrl) mice spent more time in the EtOH-paired side (**Figure 4A**, two-way ANOVA:

Genotype, p=0.86, F (1, 28) = 0.033, Session, p=0.0002, F (1, 28) = 19.07, Interaction, p=0.28, F (1, 28) = 1.24) (Sidak's post hoc tests. Session: Cre+ (KO) p=0.031, Cre- (Ctrl), p=0.0029)). Both Cre+ (KO) and Cre- (Ctrl) mice acquired CPP for ethanol (3 g/kg i.p.), suggesting that ethanol reward is intact in Cre+ (KO) mice (**Figure 4B, u**npaired t-test: p=0.76, t=0.30, df=28). These results suggest that MORs on vGluT2-expressing neurons are not involved with alcohol reward.

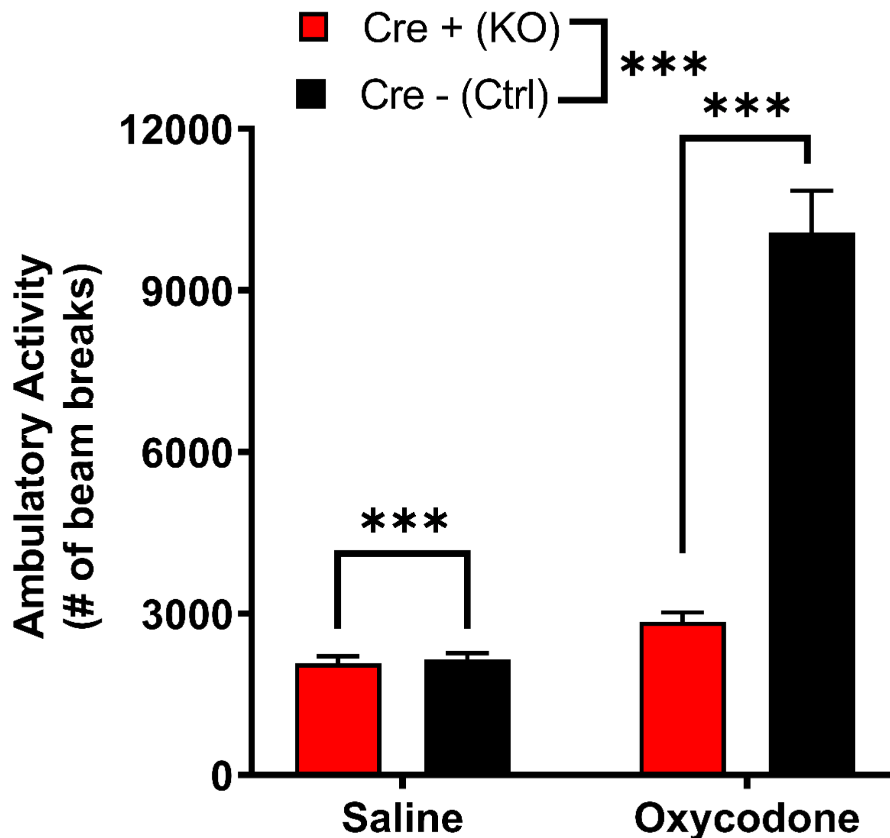
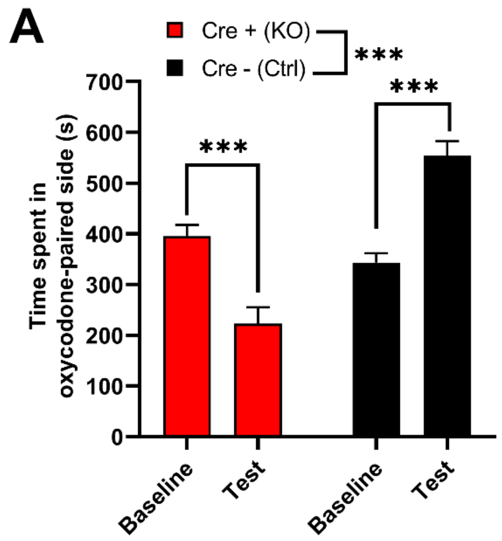


Figure 2. MORflox-vGluT2cre mice lack oxycodone-induced locomotor stimulation. There were no baseline differences in locomotor activity in male and female Cre+ (KO) and Cre− (Ctrl) mice, but Cre+ (KO) mice lacked the oxycodone-induced locomotor stimulation seen in controls [*n* = 17 (8M/8F) Cre+ (KO), 21 (10M/11F) Cre− (Ctrl)]. No sex differences were detected. Data are collapsed across sex. *** *p* < 0.001. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).


A
Cre + (KO)
Cre - (Ctrl)
***
Time spent in
oxycodone-paired side (s)
700
600
500
400
300
200
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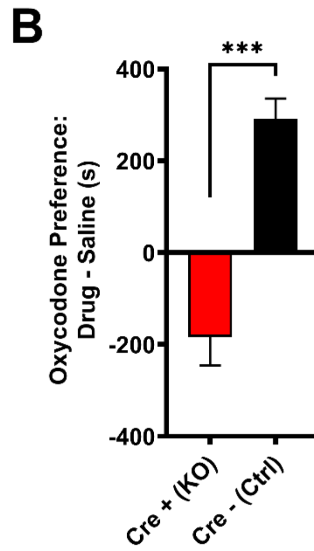
Test
Baseline
Test


B
***
Oxycodone Preference:
Drug - Saline (s)
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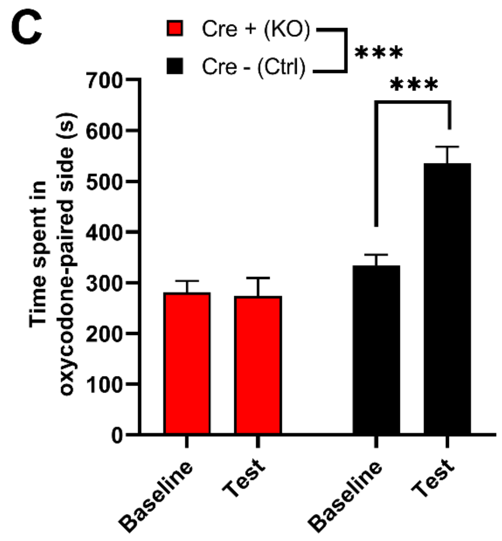
200
0
-200
-400
Cre + (KO)
Cre - (Ctrl)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Cre + (KO)
Cre - (Ctrl)
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Time spent in
oxycodone-paired side (s)
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Baseline
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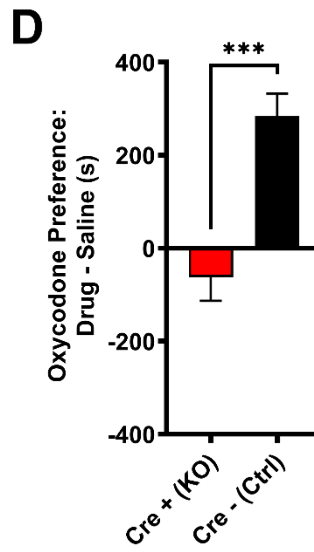
D
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Oxycodone Preference:
Drug - Saline (s)
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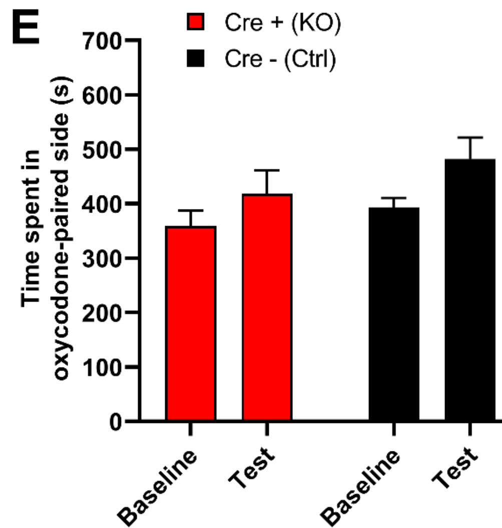
Cre + (KO)
Cre - (Ctrl)


E
Cre + (KO)
Cre - (Ctrl)
Time spent in
oxycodone-paired side (s)
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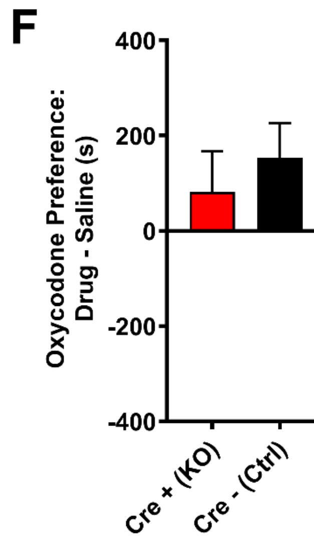
F
Oxycodone Preference:
Drug - Saline (s)
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200
0
-200
-400
Cre + (KO)
Cre - (Ctrl)

Figure 3. MORflox-vGluT2cre mice lack oxycodone-induced conditioned place preference. (A) Following conditioning with 5 mg/kg oxycodone, male and female Cre+ (KO) mice spent less time in the oxycodone-paired side, whereas Cre− (Ctrl) mice spent more time in the oxycodone-paired side [*n* = 18 (9M/9F) Cre+ (KO), 17 (9M/8F) Cre− (Ctrl)]. No sex differences were detected. (B) Cre+ (KO) mice acquired conditioned place aversion for 5mg/kg oxycodone, whereas Cre− (Ctrl) mice acquired conditioned place preference. (C) Following conditioning with 0.5mg/kg oxycodone, Cre− (Ctrl) mice spent more time on the oxycodone-paired side, whereas Cre+ (KO) did not change the amount of time spent in the oxycodone-paired side [*n* = 16 (8M/8F) Cre+ (KO), 13 (5M/8F) Cre− (Ctrl)]. (D) Cre− (Ctrl) mice acquired conditioned place preference for 0.5mg/kg oxycodone, whereas Cre+ (KO) mice did not. (E) Following conditioning with 0.05mg/kg oxycodone, neither Cre− (Ctrl) nor Cre+ (KO) mice spent significantly more time in the oxycodone-paired side, although there was a significant overall impact of test [*n*=16 (8M/8F) Cre+ (KO), 14 (7M,7F) Cre− (Ctrl)]. (F) Cre− (Ctrl) and Cre+ (KO) mice did not differ in their conditioned place preference scores for 0.05mg/kg oxycodone. No sex differences were detected. Data are collapsed across sex. ***$p < 0.001$. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2crenegative (−).

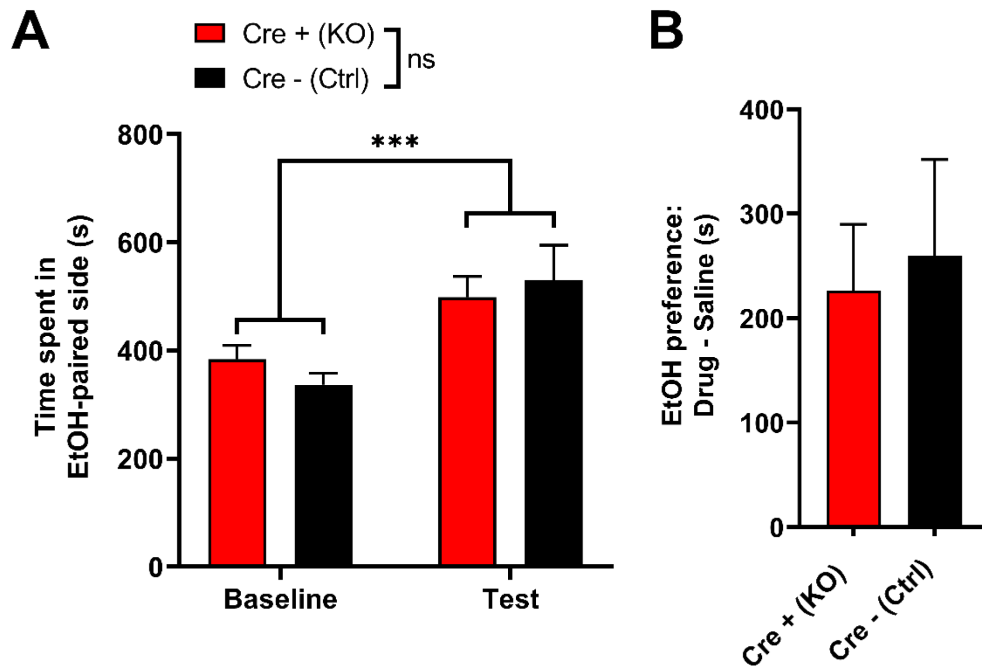


Figure 4. MORflox-vGluT2cre mice acquire ethanol CPP. A) Following conditioning with 3 g/kg ethanol, Cre- (Ctrl) and Cre+ (KO) mice spent more time in the EtOH-paired. B) Cre- (Ctrl) and Cre+ (KO) mice acquired conditioned place preference for 3 g/kg EtOH [n=18 Cre+ (KO), 12 Cre- (Ctrl)]. No sex differences were detected. Data collapsed across sex. ***$p<0.001$. Error bars indicate +/- SEM. Cre+ (KO)= MORflox-vGluT2cre positive (+); Cre– (Ctrl)= MORflox-vGluT2cre negative (−).

2.3.3 MORflox-vGluT2cre mice have normal anxiety and stress responses

Cre+ (KO) mice acquired conditioned place aversion, rather than preference, for a higher dose of oxycodone (5 mg/kg i.p.), suggesting higher doses of oxycodone may be aversive, rather than rewarding, for these mice. As a possible explanation for this phenomenon and because MORs mediate anxiety-like behaviors (Filliol et al., 2000), we hypothesized that Cre+ (KO) mice may have increased anxiety, compared to Cre– (ctrl) mice. We utilized the light-dark box test to measure basal anxiety in these mice (Bourin & Hascoët, 2003). There were no genotype differences in time spent in the light or dark area (**Figure 5A**; two-way ANOVA: Area, $p=0.11$, $F(1,16)=2.9$, Genotype, $p=0.44$, $F(1,16)=0.63$, Interaction $p=0.72$, $F(1,16)=0.13$) or entries into either area (**Figure 5B**; two-way ANOVA: Area, $p=0.72$, $F(1,16)=0.13$, Genotype, $p=0.25$, $F(1,16)=1.42$, Interaction, $p=0.87$, $F(1,16)=0.026$). These data suggest basal anxiety is unaltered in Cre+ (KO) mice. Because of the genotype differences in oxycodone-induced locomotor stimulation, we were unable to test oxycodone-induced anxiety using light-dark box assessment, as locomotor differences are a confounding factor in this assessment. Another possible explanation for the aversive effect of oxycodone in Cre+ (KO) mice is that these mice have an enhanced stress response to oxycodone. MORs modulate opioid-induced increases in corticosterone levels, the main glucocorticoid involved in the rodent stress response (Joëls et al., 2018; Roy et al., 2001); therefore, we measured the plasma concentration of corticosterone following an injection of either saline (10 mg/mL i.p.) or oxycodone (10 mg/kg i.p.). Mice injected with oxycodone had higher corticosterone levels than mice injected with saline (**Figure 5C**; two-way ANOVA: Treatment, $p=0.0038$, $F(1,29)=9.9$, Genotype, $p=0.31$, $F(1,29)=1.1$, Interaction, $p=0.93$, $F(1,29)=1.07$); however, there were no genotype differences. Female mice had higher oxycodone-induced corticosterone levels than males, regardless of genotype (**Figure 5D**; two-way ANOVA: Sex, $p=0.041$, $F(1,19)=4.8$, Genotype, $p=0.38$, $F(1,19)=0.81$,

Interaction, p=0.16, F(1,19)=2.1). Sample sizes were too low to detect significant sex differences in light-dark box testing and saline-induced corticosterone levels. Altogether, these results suggest MORflox-vGluT2cre mice do not have altered anxiety or stress responses.

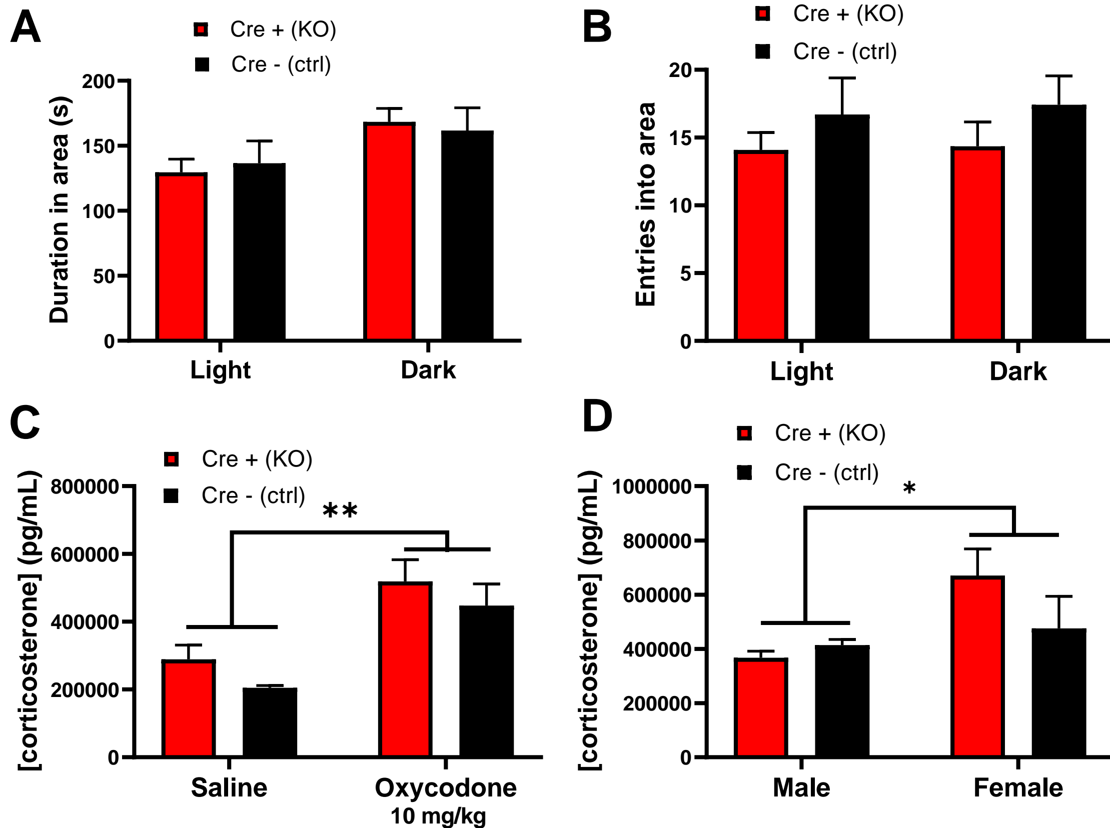


Figure 5. MORflox-vGluT2cre mice have normal anxiety and stress responses. (A) There were no differences in time spent in or (B) entries into the light and dark areas between Cre+ (KO) and Cre- (ctrl) mice. [n= 11 (6M/ 5F) Cre+ (KO), 7 (2M/ 5F) Cre- (ctrl)]. (C) Oxycodone (10 mg/kg i.p.) increased corticosterone levels in both Cre+ (KO) and Cre- (ctrl) mice. (D) Female mice had higher levels of oxycodone-induced corticosterone than males, regardless of genotype. [n= saline: 6 (2M/ 4F) Cre+ (KO), 4 (2M/ 2F) Cre- (ctrl); oxycodone: 14 (7M/ 7F) Cre+ (KO), 9 (4M/ 5F) Cre- (ctrl)]. * $p < 0.05$, ** $p < 0.01$. Error bars indicate ± SEM. Cre+ (KO)= MORflox-vGluT2cre positive (+); Cre– (Ctrl)= MORflox-vGluT2cre negative (−).

2.3.4 MORflox-vGluT2cre mice have reduced oral oxycodone consumption

To further characterize oxycodone reward in MORflox-vGluT2cre mice, we used an assessment of 24 hr two-bottle choice (2BC) between orally consuming oxycodone or water. Cre+ (KO) mice consumed less oxycodone (**Figure 6A,** two-way ANOVA: Genotype p<0.0001, F(1,31)=40.83; Concentration p<0.0001, F(1.29, 40.07)=47.95; Interaction p<0.0001, F(3, 93)=22.79) (Sidak's test: 0.1 mg/ml, p=0.99; 0.3 mg/ml, p=0.020; 1 mg/ml, p<0.001; 3 mg/ml, p=0.0011) and showed decreased preference for oxycodone, compared to Cre- (Ctrl) mice (**Figure 6B**, two-way ANOVA: Genotype p=0.00020, F(1,31)=18.19; Concentration p<0.0001, F(2.62, 81.08)=141.30; Interaction p<0.0001, F(3, 93)=28.08) (Sidak's test: 0.1 mg/ml, p=0.92; 0.3 mg/ml, p=0.14; 1 mg/mL p<0.0001; 3 mg/mL p=0.0056). Cre- (Ctrl) control mice showed decreased preference for 3 mg/mL oxycodone concentration, compared to other concentrations (Sidak's test: 0.1 vs. 3: p<0.0001; 0.3 vs. 3: p<0.0001; 1 vs. 3: p<0.0001). Cre+ (KO) mice displayed significantly decreasing preferences for each higher concentration, except between 1 mg/ml and 3 mg/ml (Sidak's test: 0.1 vs. 0.3, p=0.0003; 0.1 vs. 1, p<0.0001; 0.1 vs. 3, p<0.0001; 0.3 vs. 1, p<0.0001; 0.3 vs. 3, p<0.0001; 1 vs. 3: p=0.4982). There were no sex differences in oxycodone consumption or preference (**Figure 8 A-B, Table 1**). Total fluid consumption did not significantly differ between genotypes (**Figure 7A**; two-way ANOVA: Genotype, p=0.16, F (1, 31) = 2.088, Concentration, p=0.032, F (1.582, 49.03) = 4.052, Interaction, p=0.17, F (3, 93) = 1.732). Females drank significantly more fluid overall than males and there were significant differences in the amount of fluid consumed at each of the concentrations (**Figure 8C, Table 1**). Altogether, these data suggest that Cre+ (KO) mice do not find oxycodone as rewarding as Cre- (Ctrl) mice; however, an alternative hypothesis is that Cre+ (KO) mice have altered preferences for rewarding substances or are possibly more sensitive to the bitter taste of oxycodone.

Therefore, a separate group of mice underwent two series of 2BC: one with escalating concentrations of sucrose (0.5%, 1%, 2% w/v), a naturally rewarding substance, and the other with escalating concentrations of quinine (0.03 mM, 0.1 mM, 0.3 mM), a naturally aversive, bitter substance. In both series the preference was tested against water and mice were counterbalanced in which order they underwent each series with a one-week washout period between each series. There were no genotype differences in sucrose consumption (**Figure 6C**; two-way ANOVA: genotype, p=0.48, F(1,31) = 0.52; concentration, p<0.0001, F(1.163, 36.06) = 526.60; interaction, p=0.43, F(2, 62) = 0.86), preference (**Figure 6D**; two-way ANOVA: genotype, p=0.58, F(1,31) = 0.32; concentration, p<0.0001, F(1.036, 32.10) = 43.04; interaction, p=0.62, F(2, 62) = 0.49), or total volume of fluid consumed during sucrose dinking sessions; although, there was a significant genotype x sucrose concentration interaction (**Figure 7B**; two-way ANOVA: Genotype, p=0.22, F (1, 31) = 1.55; Concentration, p<0.0001, F (1.751, 54.27) = 69.14; Concentration x Genotype, p=0.0083, F (2, 62) = 5.18) (Sidak's post hoc tests. Genotype: 0.5% p=0.25; 1% p=0.071; 2% p=0.99) and significant effects of sex (**Figure 9D-F, Table 1**). There were also no genotype differences in quinine consumption (**Figure 6E**; two-way ANOVA genotype, p=0.090, F(1,31) = 3.069; concentration, p=0.0006, F(1.591, 49.31) = 9.87; interaction, p=0.44, F(2, 62) = 0.84), preference (**Figure 6F**; two-way ANOVA: genotype, p=0.22, F(1,31) = 1.60; concentration, p<0.0001, F(1.149, 35.62) = 53.61; interaction, p=0.62, F(2, 62) = 0.48), or total volume of fluid consumed during quinine dinking sessions (**Figure 7C**). There were significant effects of sex on total fluid consumption during quinine drinking (**Figure 8G-I, Table 1**; two-way ANOVA: Genotype, p=0.39, F (1, 31) = 0.78, Concentration, p=0.32, F (1.93, 59.84) = 1.14, Concentration x Genotype, p=0.030, F (2, 62) = 3.72). Cre+ (KO) and

Cre- (Ctrl) mice also did not differ in food consumption, but females consumed more food than males (**Figure 9A**; two-way ANOVA: Genotype, p=0.46, F (1, 33) = 0.57; Sex, p<0.0001, F (1, 33) = 60.46; Interaction, p=0.56, F (1, 33) = 0.35) (Sidak's post hoc tests. Genotype: Female p=0.56, Male p=0.99; Sex: Cre+ (KO) p<0.0001, Cre- (Ctrl) p<0.0001). Baseline weights were recorded for all mice involved in behavior experiments, before beginning testing. Cre+ (KO) mice weighed slightly less than Cre- (Ctrl) mice (**Figure 9B**; (two-way ANOVA: Genotype, p<0.0001, F (1, 277) = 21.21; Sex, p<0.0001, F (1, 277) = 938.9; Interaction, p=0.58, F (1, 277) = 0.32) (Sidak's post hoc tests. Genotype: Female p=0.0095, Male p=0.0006; Sex: Cre+ (KO) p<0.0001, Cre- (Ctrl) p<0.0001). Altogether, these results indicate that the genotype differences in oral oxycodone consumption are not due to general differences in sensitivity to naturally rewarding or aversive substances and further suggest that MORs on vGluT2cre-expressing neurons are specifically involved with opioid reward.

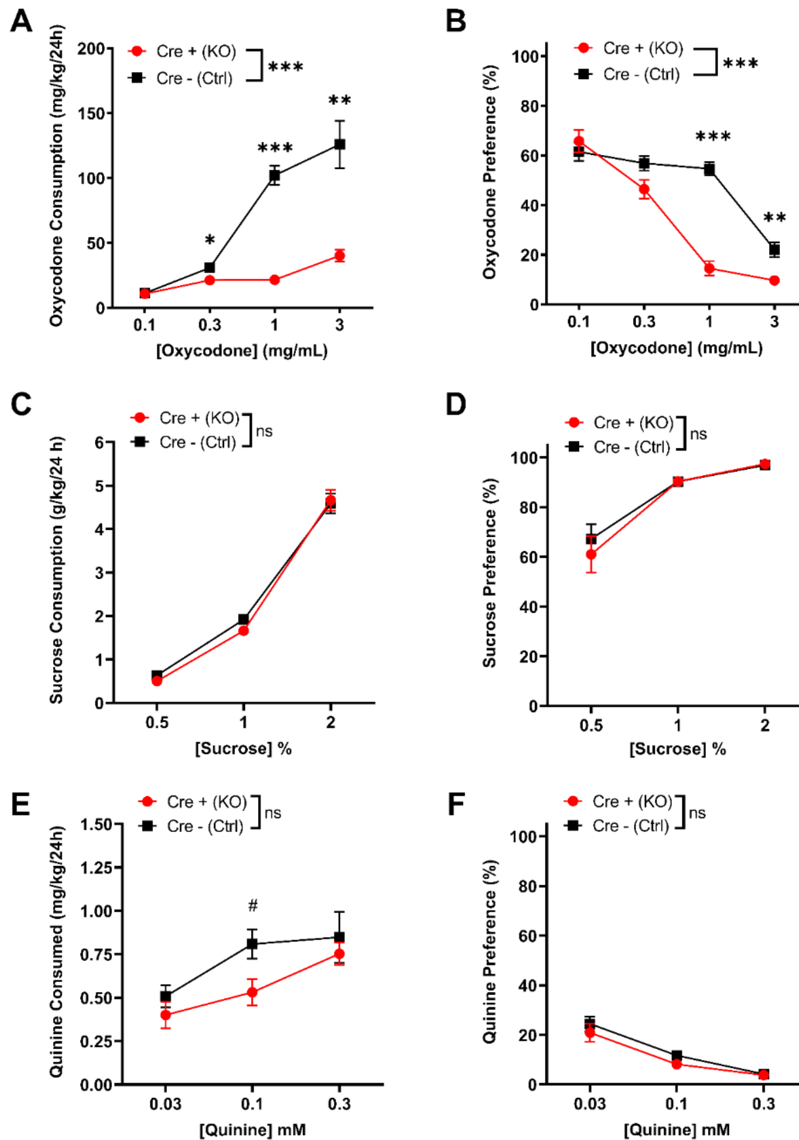


Figure 6. MORflox-vGluT2cre mice have reduced oral oxycodone consumption and but unchanged sucrose and quinine consumption. (A) Cre+ (KO) mice drank less oxycodone solution than Cre− (Ctrl) controls at 0.3, 1, and 3 mg/ml concentrations [*n* = 16 (8M/8F) Cre+ (KO); *n* = 17 (9M/8F) Cre− (Ctrl)]. No sex differences were detected. (B) Cre+ (KO) mice showed less preference for oxycodone solution over water than Cre− (Ctrl) controls at 1 and 3mg/ml concentrations. No sex differences were detected. (C) There were no differences in sucrose consumption or (D) preference between genotypes [*n* = 16(9M/7F) Cre+ (KO), 17(8M/9F) Cre− (Ctrl)]. E) There were no statistically significant differences in quinine consumption or (F) preference between genotypes [*n*=16(9M/7F) Cre+ (KO), 17(8M/9F) Cre− (Ctrl)], although there was a trend at 0.1 mM quinine. Data presented here are collapsed across sex for clarity. Findings where sex differences were identified may be found in **Figure 8** and **Table 1**. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. # $p = 0.06$. Error bars indicate ± SEM. Cre+ (KO) = MORfloxvGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).

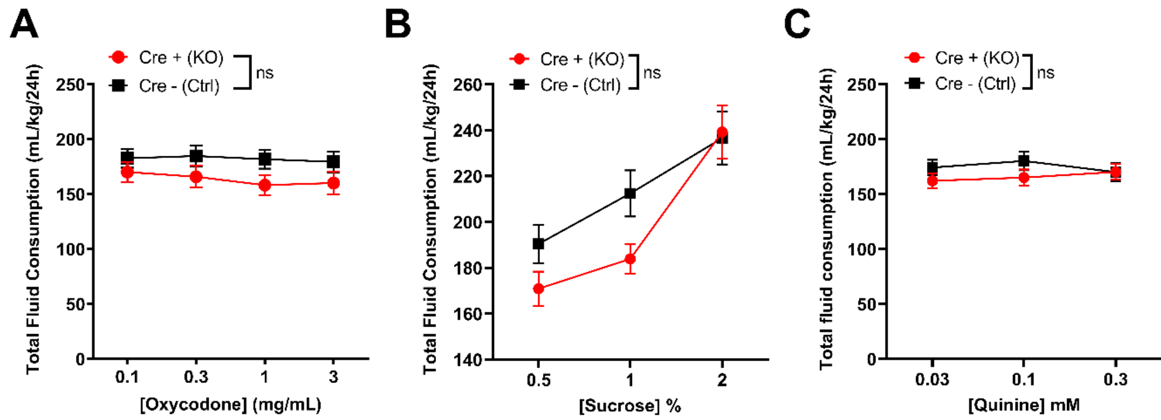


Figure 7. MORflox-vGluT2cre mice have normal fluid consumption. (A) There were no differences between genotypes in total fluid consumption during oxycodone drinking sessions [n=16 (8M/8F) Cre+ (KO); n=17 (9M/8F) Cre- (Ctrl)]. (B) There were no genotype differences in fluid consumption during sucrose drinking sessions; although, there was a significant genotype x sucrose concentration interaction [n=16(9M/7F) Cre+ (KO), 17(8M/9F) Cre- (Ctrl)]. (C) There were no differences between genotypes in fluid consumption during quinine drinking sessions (two-way ANOVA: Genotype, $p=0.39$, $F(1, 31) = 0.78$, Concentration, $p=0.32$, $F(1.93, 59.84) = 1.14$, Concentration x Genotype, $p=0.030$, $F(2, 62) = 3.72$). n=16(9M/7F) Cre+ (KO), 17(8M/9F) Cre- (Ctrl). Data collapsed across sex. Findings where sex differences were identified may be found in **Figure 8** and **Table 1.** Error bars indicate ± SEM. Cre+ (KO)= MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (-).

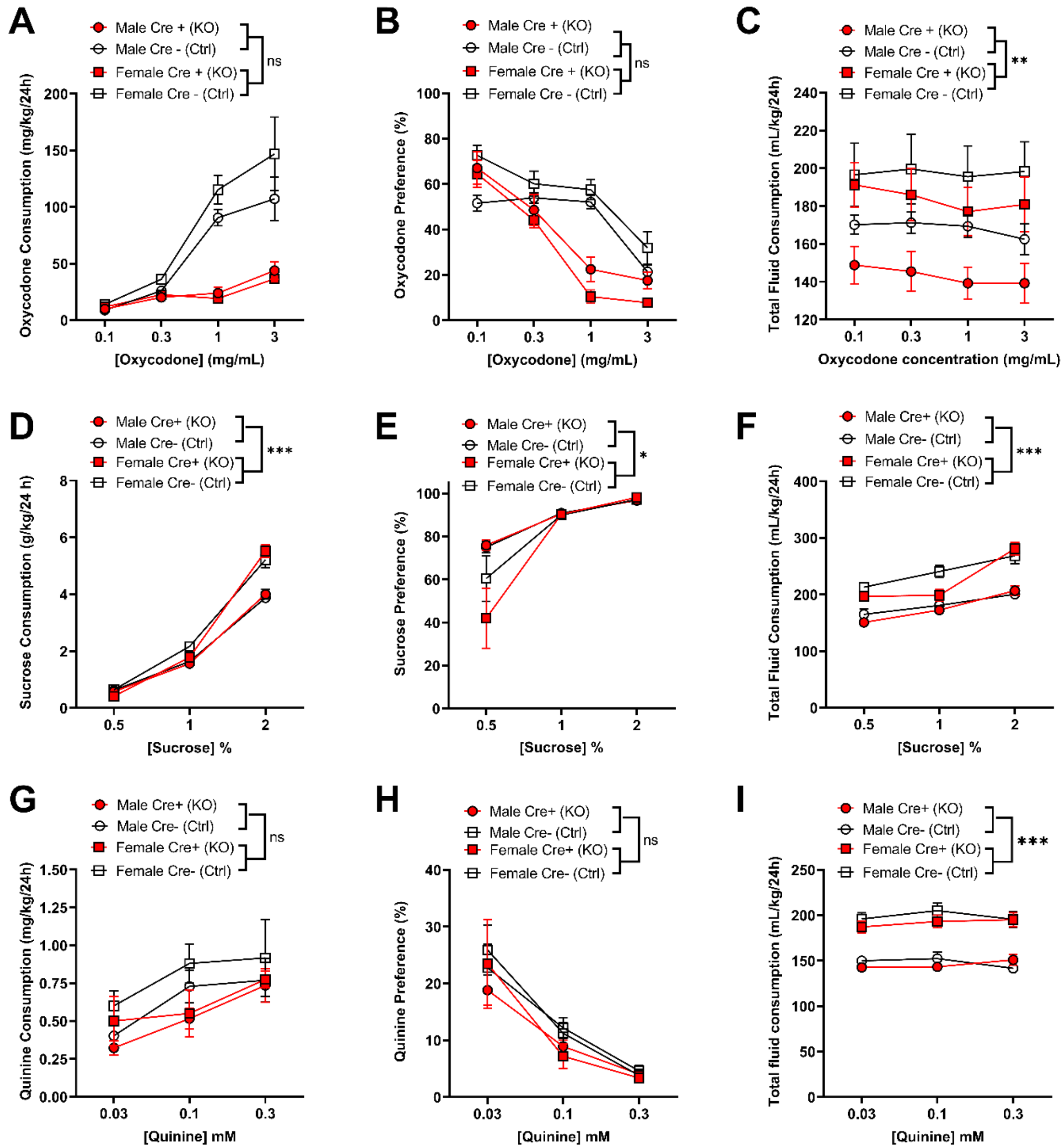


Figure 8. Sex differences in oxycodone, sucrose, and quinine two-bottle choice. Associated with **Table 1**. (A-C) Assessments of sex and genotype differences in oral oxycodone consumption [n=16 (8M/8F) Cre+ (KO); n=17 (9M/8F) Cre- (Ctrl)]. Females consumed significantly more fluid than males. (D-F) Assessments of sex and genotype differences in oral sucrose consumption [n=16(9M/7F) Cre+ (KO), 17(8M/9F) Cre- (Ctrl)]. Females consumed significantly more sucrose and total fluid than males but had lower preference for sucrose than males. G-I) Assessments of sex and genotype differences in oral quinine consumption [n=16(9M/7F) Cre+ (KO), 17(8M/9F) Cre- (Ctrl)]. Females consumed significantly more fluid than males. *p < 0.05, **p < 0.01, *** p < 0.001. Error bars indicate ± SEM. Cre+ (KO)= MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (–).

	Concentration	Sex	Genotype	Concentration x Sex	Concentration x Genotype	Sex x Genotype	Concentration x Sex x Genotype
Oxycodone							
mg/kg	F (1.28, 37.18) = 47.70	F (1, 29) = 1.85	F (1, 29) = 45.54	F (3, 87) = 0.33	F (3, 87) = 22.78	F (1, 29) = 2.75	F (3, 87) = 1.11
	P<0.0001	P=0.19	P<0.0001	P=0.81	P<0.0001	P=0.11	P=0.35
Pref.	F (2.58, 74.78) = 117.4	F (1, 29) = 0.28	F (1, 29) = 18.69	F (3, 87) = 2.22	F (3, 87) = 24.11	F (1, 29) = 6.90	F (3, 87) = 0.60
	P<0.0001	P=0.60	P=0.0002	P=0.092	P<0.0001	P=0.014	P=0.62
Total Fluid	F (1.57, 45.46) = 3.84	F (1, 29) = 9.13	F (1, 29) = 2.82	F (3, 87) = 0.63	F (3, 87) = 1.69	F (1, 29) = 0.24	F (3, 87) = 0.35
	P=0.038	P=0.0052	P=0.10	P=0.60	P=0.18	P=0.63	P=0.79
Sucrose							
mg/kg	F (1.40, 40.59) = 1200	F (1, 29) = 31.99	F (1, 29) = 0.26	F (2, 58) = 40.40	F (2, 58) = 3.68	F (1, 29) = 0.25	F (2, 58) = 1.10
	P<0.0001	P<0.0001	P=0.62	P<0.0001	P=0.031	P=0.61	P=0.34
Pref.	F (1.048, 30.39) = 56.69	F (1, 29) = 6.32	F (1, 29) = 0.75	F (2, 58) = 8.75	F (2, 58) = 1.20	F (1, 29) = 0.87	F (2, 58) = 1.49
	P<0.0001	P=0.018	P=0.39	P=0.0005	P=0.31	P=0.36	P=0.23
Total Fluid	F (1.70, 49.16) = 84.09	F (1, 29) = 49.92	F (1, 29) = 1.85	F (2, 58) = 5.51	F (2, 58) = 7.47	F (1, 29) = 0.42	F (2, 58) = 2.67
	P<0.0001	P<0.0001	P=0.18	P=0.0064	P=0.0013	P=0.52	P=0.078
Quinine							
mg/kg	F (1.58, 45.90) = 9.028	F (1, 29) = 1.85	F (1, 29) = 2.68	F (2, 58) = 0.22	F (2, 58) = 0.83	F (1, 29) = 0.20	F (2, 58) = 0.055
	P=0.0012	P=0.18	P=0.11	P=0.80	P=0.44	P=0.66	P=0.95
Pref.	F (1.15, 33.38) = 52.39	F (1, 29) = 0.084	F (1, 29) = 1.42	F (2, 58) = 1.16	F (2, 58) = 0.42	F (1, 29) = 0.0052	F (2, 58) = 0.048
	P<0.0001	P=0.77	P=0.24	P=0.32	P=0.66	P=0.94	P=0.95
Total Fluid	F (1.94, 56.12) = 1.13	F (1, 29) = 76.49	F (1, 29) = 0.69	F (2, 58) = 0.53	F (2, 58) = 3.48	F (1, 29) = 0.18	F (2, 58) = 0.27
	P=0.33	P<0.0001	P=0.41	P=0.59	P=0.038	P=0.67	P=0.77

Table 1. Statistical analyses of sex differences in oral consumption of oxycodone, sucrose, and quinine. Associated with **Figure 8**. Assessments of consumption and preference (Pref) of each substance and total fluid intake associated with each substance. All data analyzed with three-way ANOVA with genotype, sex, and substance concentration as factors. Significant differences are marked in shaded cells.

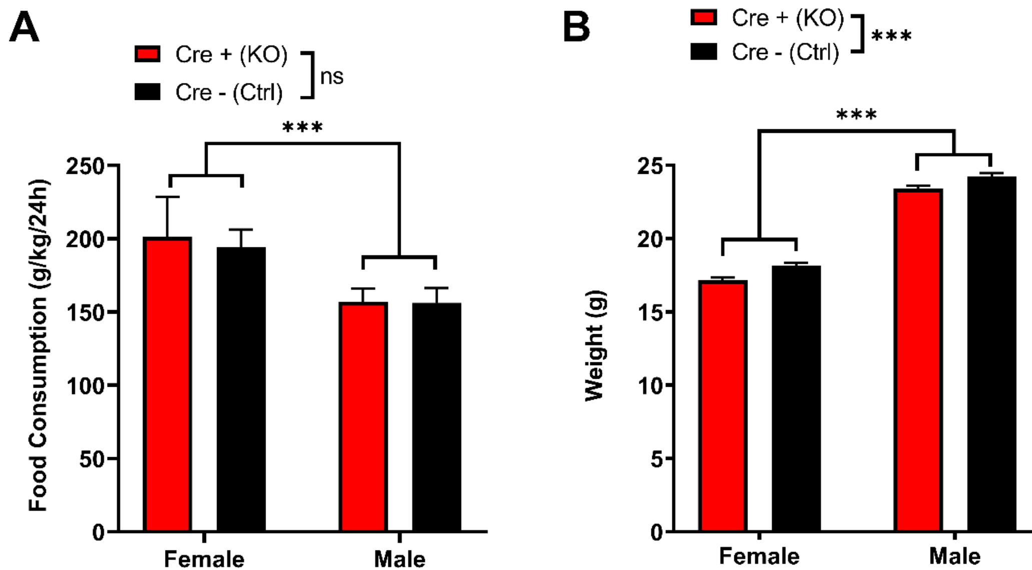


Figure 9. Morflox-vGluT2cre mice have reduced body weight but unchanged food consumption. (A) Food consumption between Cre+ (KO) and Cre- (Ctrl) mice. Female mice of both genotypes consumed more food than [n=16 (8M/8F) Cre +(KO), 21 (9M/12F) Cre- (Ctrl) mice]. (B) Cre+ (KO) mice weighed less at baseline (~8 wks old) than Cre- (Ctrl) mice. Females weighed less than males in both genotypes [n=139 (70M/69F) Cre+ (KO), 142 (69M/73F) Cre- (Ctrl)]. ***p < 0.001. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (–).

<u>2.3.5 MORflox-vGluT2cre mice have intact oxycodone-induced antinociception</u>

Because MORs are also involved in antinociception (Kieffer & Gaveriaux-Ruff, 2002), we measured responses to painful stimuli using the shock-flinch test. We used this method to measure antinociception because the locomotor stimulation- and straub tail- inducing effects of oxycodone cause it to be difficult to obtain accurate and reliable measurements from traditional pain assessment tests, such as the hot plate, tail flick, and Hargreaves methodologies. There were no differences in baseline (saline 10 mL/kg s.c.) responses to shocks of various intensities between genotypes (**Figure 10A**; REML: genotype, p=0.53, F(1,34)=0.41; shock intensity, p<0.0001, F(3.27,105.50)=79.88; interaction, p=0.79, F(4,129)=0.43). Male mice were significantly more sensitive to shock

treatments (**Figure 11A**; REML: Shock intensity, $p<0.0001$, $F(4,36)=83.9$, Sex, $p=0.0019$, $F(1,9)=18.91$, Genotype, $p=0.36$, $F(1,9)=0.92$, Shock intensity x Sex, $p=0.0034$, $F(4,36)=4.77$, Shock intensity x Genotype, $p=0.79$, $F(4,36)=0.42$, Sex x Genotype, $p=0.57$, $F(1,9)=0.36$, Shock intensity x Sex x Genotype, $p=0.45$, $F(4,10)=1.0$). Following an oxycodone injection (3 mg/kg s.c.), responses to shocks decreased equally in both male and female Cre- (Ctrl) and Cre+ (KO) animals (**Figure 10B, 11B**; REML: genotype, $p=0.3508$, $F(1,34)=0.90$; shock intensity, $p<0.0001$, $F(4,132)=17.41$; interaction, $p=0.054$, $F(4,132)=2.39$). These results indicate that pain responses and oxycodone-induced antinociception are intact in Cre+ (KO) animals, suggesting that MORs on vGluT2-expressing neurons are not involved in nociception or the antinociceptive effect of oxycodone.

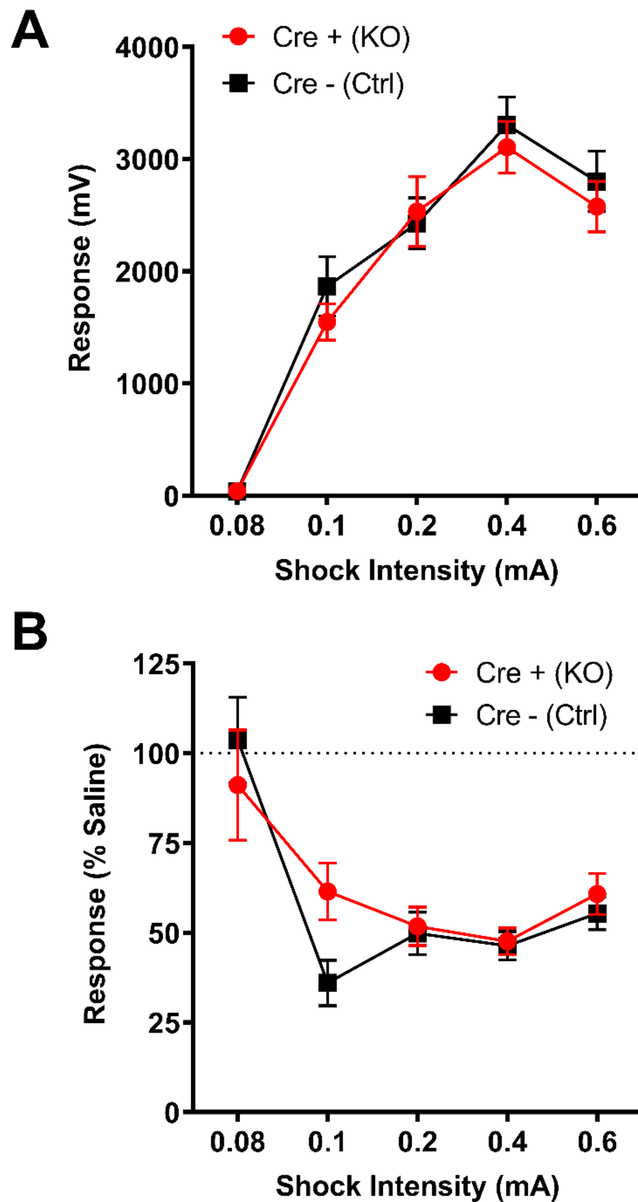


Figure 10. MORflox-vGluT2cre mice have intact oxycodone-induced antinociception. (A) Cre+ (KO) and Cre− (Ctrl) mice have equivalent nociceptive responses to a range of shock stimuli mice [n = 17 (9M/8F) Cre+ (KO), 18(8M/10F) Cre− (Ctrl)]. (B) A 3mg/kg oxycodone treatment produces equivalent antinociception in both Cre+ (KO) and Cre− (Ctrl) mice; although, there was a trend at the 0.1 mA shock intensity for Cre+ (KO) mice to be less sensitive to oxycodone treatment ($p = 0.099$). Data presented here are collapsed across sex for clarity. Sex differences are presented in **Figure 11.** Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−). mV = millivolts.

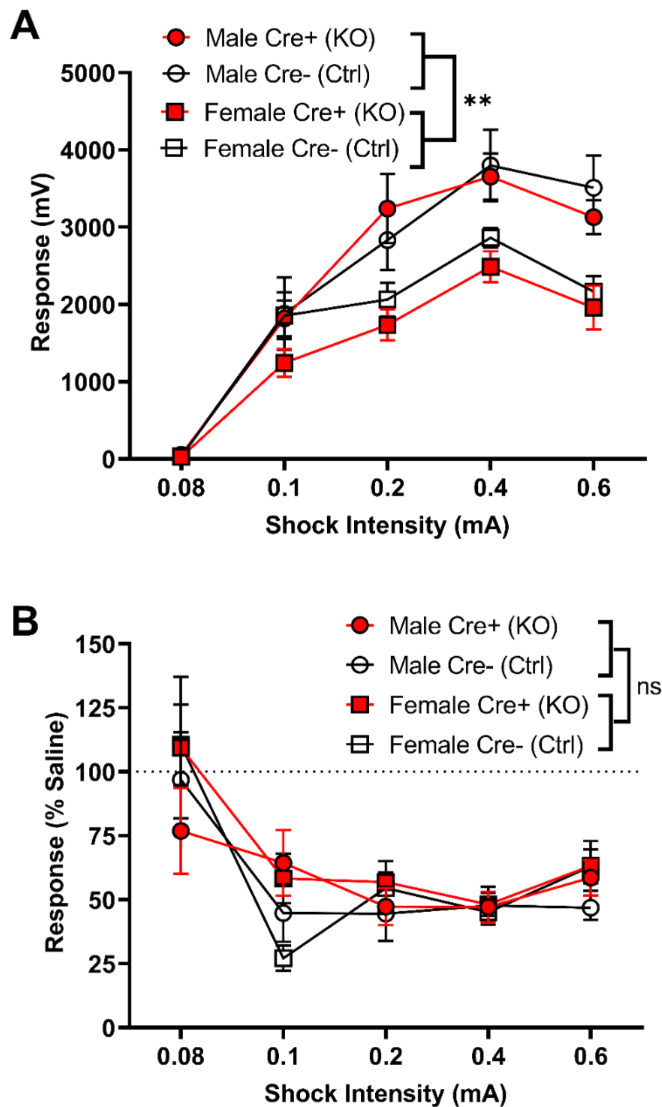


Figure 11. Male mice are more sensitive to shock stimuli than female mice. n=17 (9M/8F) Cre+ (KO), 18(8M/10F) Cre- (Ctrl) for all panels. (A) There are no genotype differences in baseline responses to shock stimuli, but male mice are more sensitive to shock stimuli than female mice [n=17 (9M/8F) Cre+ (KO), 18(8M/10F) Cre- (Ctrl)]. (B) Behavioral responses to oxycodone treatment were not impacted by the sex or genotype of mice. There was a significant effect of shock intensity. **p < 0.01. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).

2.3.6 MORflox-vGluT2cre mice show baseline oxycodone withdrawal-related responses and no increase in withdrawal responses following naloxone treatment.

To determine if Cre+ (KO) mice differ from Cre- (Ctrl) mice in the development of oxycodone dependency or naloxone-precipitated withdrawal, we used a dose-ramping schedule followed by an assessment of baseline opioid withdrawal-like behaviors (saline, 10 mL/kg i.p.) and a subsequent assessment following a naloxone injection (5 mg/kg i.p.) (**Figure 12A**). Assessment of global withdrawal score revealed a main effect of treatment, but not genotype (two-way ANOVA: genotype, $p=0.64$, $F(1,27)=0.23$; treatment, $p<0.0001$, $F(1,27)=25.90$), although there was a significant interaction between treatment and genotype ($p<0.0001$, $F(1,27)=25.93$) (**Figure 12B**). Surprisingly, following saline injection, Cre+ (KO) mice had a higher global withdrawal score than Cre- (Ctrl) mice (Sidak's test: saline, $p=0.0093$). Following naloxone administration, there was an increase in the global withdrawal score of Cre- (Ctrl) mice, but there was no change in Cre+ (KO) mice (Sidak's test: Cre+ (KO), $p>0.99$; Cre- (Ctrl) $p<0.0001$). Additionally, the global withdrawal score following naloxone-precipitated withdrawal was higher for Cre- (Ctrl) mice than Cre+ (KO) mice (Sidak's test: $p=0.011$). Measures of the individual withdrawal behaviors are displayed in **Figure 13**. These data indicate that Cre+ (KO) mice show responses typically associated with opioid withdrawal, an hour following oxycodone treatment, prior to naloxone-precipitated withdrawal, suggesting that MORs on vGluT2-expressing neurons are involved in opioid withdrawal-related behaviors.

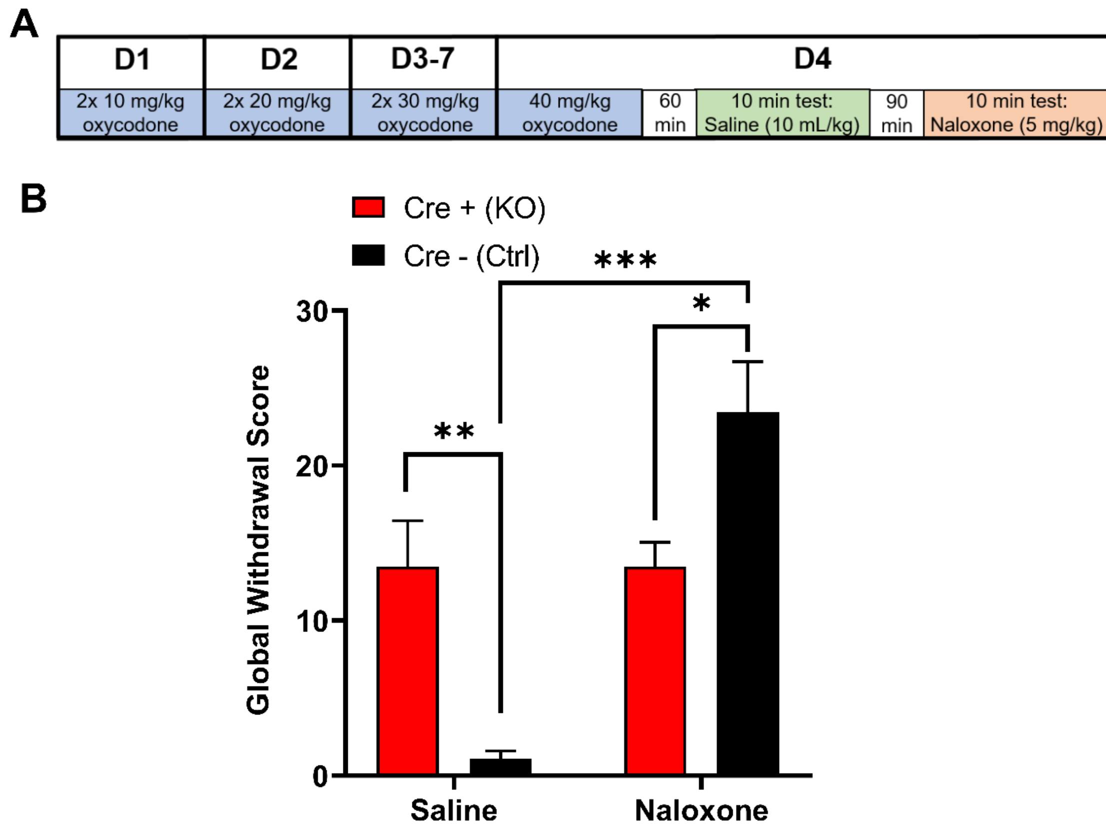


Figure 12. MORflox-vGluT2cre mice have altered oxycodone withdrawal-related behaviors. (A) Schematic of experiment schedule. (B) Global withdrawal scores for mice following a saline injection and 1.5 hrs later, a naloxone injection [(n = 16(8M/8F) Cre+ (KO),13(5M/8F) Cre− (Ctrl)]. Cre+ (KO) mice had a higher global withdrawal score following saline injection than Cre− (Ctrl) mice but had a lower global withdrawal score following naloxone injection than Cre− (Ctrl) mice. No sex differences were detected. Data are collapsed across sex. Data for individual withdrawal-related behaviors are presented in **Figure 13**. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).

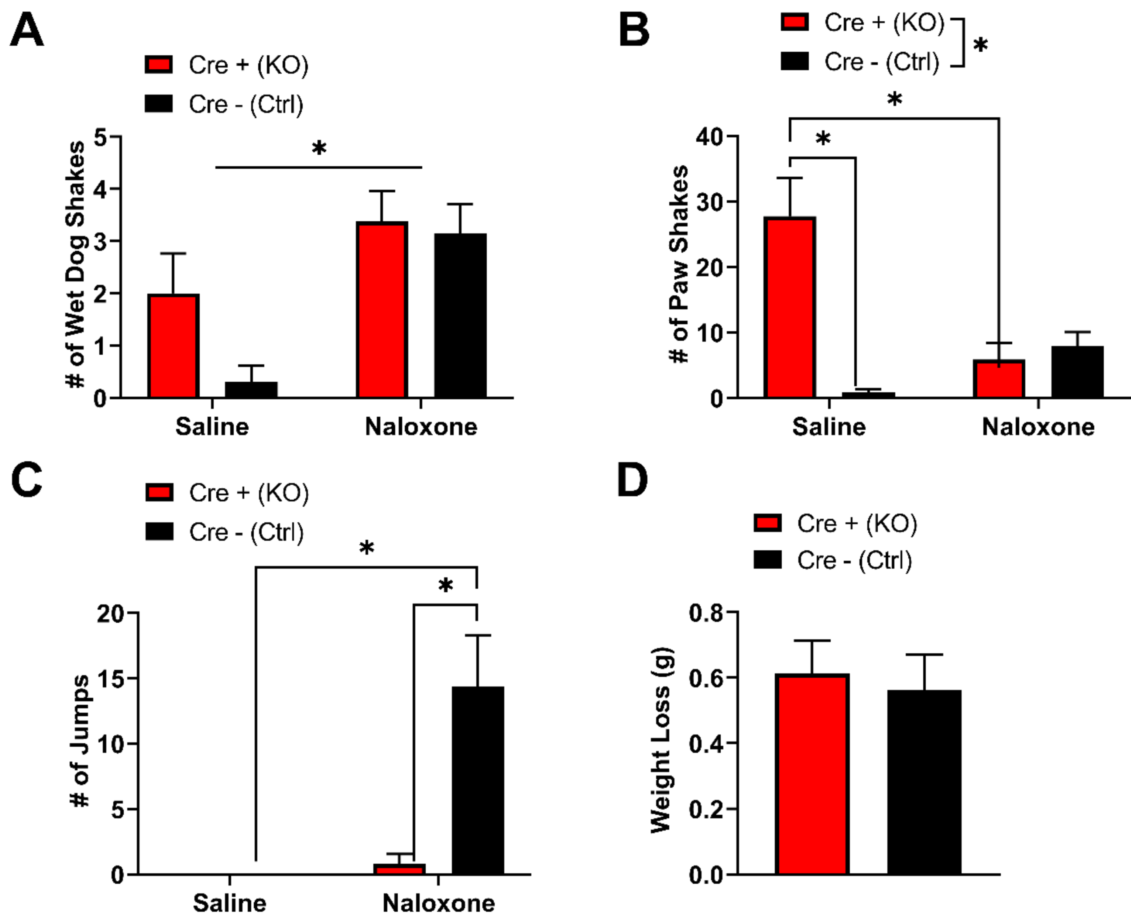


Figure 13. Individual oxycodone withdrawal-related behaviors. n=16(8M/8F) Cre+ (KO), 13(5M/8F) Cre- (Ctrl) for all panels. (A) Naloxone treatment produced an increase in wet dog shakes in oxycodone-dependent Cre- (Ctrl) mice, but not in Cre+ (KO) mice. (two-way ANOVA: Genotype, p=0.1057, F (1, 27) = 2.803; Treatment, p=0.0028, F (1, 27) = 10.80; Treatment x Genotype, p=0.2621, F (1, 27) = 1.312) (Sidak's post-hoc, Treatment: Cre+ (KO), p=0.23; Cre- (Ctrl), p=0.012). (B) Oxycodone-dependent Cre+ (KO) mice displayed more paw shakes than Cre- (Ctrl) following saline treatment, but naloxone treatment reduced these. (two-way ANOVA: Genotype, p=0.0034, F (1, 27) = 10.33; Treatment, p=0.0457F (1, 27) = 4.390; Treatment x Genotype, p=0.0004, F (1, 27) = 16.54) (Sidak's post hoc tests. Genotype: Saline p<0.0001, Naloxone p=0.91; Treatment: Cre+ (KO) p=0.0002, Cre- (Ctrl) p=0.35). (C) Naloxone treatment increased jumping in oxycodone-dependent Cre- (Ctrl) mice, but not in Cre+ (KO) mice. (two-way ANOVA: Genotype, p=0.0009, F (1, 27) = 14.05; Treatment, p=0.0003, F (1, 27) = 17.61; Treatment x Genotype, p=0.0009, F (1, 27) = 14.05) (Sidak's post-hoc tests. Genotype: Saline p>0.9999, Naloxone p<0.0001; Treatment, Cre + (KO) p=0.93, Cre– (Ctrl) p<0.0001). (D) Oxycodone-dependency did not result in differences in weight loss between genotypes. (Unpaired t-test, p=0.74, t=0.3425, df=27). No sex differences were detected. Data are collapsed across sex. *p < 0.05. Error bars indicate ± SEM. Cre+ (KO) = MORflox-vGluT2cre positive (+); Cre– (Ctrl) = MORflox-vGluT2cre negative (−).

2.4 Discussion

The major finding of this study is that MORs found on a subset of glutamatergic neurons that express the glutamate transporter, vGluT2, modulate opioid reward. In the absence of these vGluT2 neuron MORs, oxycodone reward is ablated. In addition, these vGluT2 neuron MORs mediate the locomotor stimulatory effect of opioid treatment, a behavioral response common to many drugs of abuse (Wise & Bozarth, 1987), as well as opioid withdrawal-related behaviors. Many studies find that MOR-mediated regulation of GABA transmission is critical for opioid reward, opioid-induced locomotion, and opioid self-administration (Fields & Margolis, 2015). Therefore, the current data suggest that there may be complex interplay between MOR-mediated regulation of GABA and glutamate transmission. While MOR expression has been documented in glutamatergic neurons and MOR-mediated regulation of glutamate transmission has been reported for many brain regions (Birdsong et al., 2019; Blomeley & Bracci, 2011; Hoffman & Lupica, 2001; Jiang & North, 1992; Margolis & Fields, 2016; Martin et al., 1997; Wamsteeker Cusulin et al., 2013; Zhu & Pan, 2005), our findings demonstrate a role for MORs expressed in a specific subpopulation of glutamate neurons in modulating opioid reward.

Total MOR KO mice lack opioid-induced locomotor stimulation and opioid CPP, fail to show opioid withdrawal symptoms following naloxone-precipitated withdrawal, and lack morphine-induced antinociception (Kieffer & Gaveriaux-Ruff, 2002). Similar to the total MOR KO, our Cre+ (KO) mice also lack opioid-induced locomotor stimulation, have reduced opioid consumption, and show little to no CPP for low doses of oxycodone ($\leq$0.5 mg/kg). However, the Cre+ (KO) mice acquire conditioned place aversion, rather than a lack of preference, for a higher dose of oxycodone (5 mg/kg) and oxycodone-induced antinociception remains intact in these mice. Cre+ (KO) mice do not have a lack of

opioid withdrawal-like behaviors like the total KO mice; instead, the Cre+ (KO) mice display withdrawal-like responses following the induction of oxycodone dependence, prior to naloxone-precipitated withdrawal. These data could mean that MORs on vGluT2-expressing neurons may specifically modulate certain withdrawal responses. It is possible Cre+ (KO) mice experience a negative affective state in response to high doses of oxycodone, which may partially explain why these mice acquire conditioned place aversion for 5 mg/kg oxycodone. Alternatively, these mice could experience withdrawal at a faster rate than Cre- (Ctrl) mice. MOR KO mice have disrupted ethanol reward and do not acquire ethanol CPP, whereas Cre+ (KO) mice do acquire ethanol CPP, suggesting that MORs in vGluT2 neurons do not mediate ethanol reward (Ben Hamida et al., 2019; Kieffer & Gaveriaux-Ruff, 2002). These data also suggest the behavioral effects of MOR genetic deletion from vGluT2-expressing neurons is specific to opioids, although additional drugs of abuse should be tested in the future. Food and sucrose consumption are also not affected in Cre+ (KO) mice and they weigh less than their Cre– (Ctrl) littermates. In comparison, total MOR KO mice have decreased palatable food intake and increased body weight (Awad et al., 2019; Charbogne et al., 2017; Han et al., 2006; Ostlund et al., 2013). In forebrain GABAergic neuron-specific MOR KO mice, opioid-induced locomotor stimulation was ablated, similar to our Cre+ (KO) mice (Charbogne et al., 2017). However, the GABA neuron MOR KO mice had increased opioid self-administration, intact opioid CPP, and reduced ethanol CPP, which all contrast with our results here (Ben Hamida et al., 2019; Charbogne et al., 2017). A recent study also assessed opioid reward and aversion using mutant mice that lack MOR expression in the medial habenula. In these mice, behavioral responses to morphine were intact, but naloxone-induced withdrawal-related aversion was disrupted, further contrasting with our findings (Boulos et al., 2020). Altogether, our results suggest

that MORs in vGluT2-expressing neurons have a critical role in modulating opioid reward.

One of the specific challenges moving forward is the identification of the specific vGluT2 neuron MORs that mediate opioid reward. vGluT2 is expressed in primarily glutamatergic neurons and is predominantly expressed in the thalamus, amygdala, hypothalamus, cerebellum, and brainstem (Fremeau et al., 2004). vGluT2 is also expressed in subpopulations of neurons in the VTA, ventral pallidum, and certain regions of cortex (Fremeau et al., 2004; Yamaguchi et al., 2007). Activation of vGluT2-expressing neurons within the VTA causes place preference, suggesting a role for vGluT2 VTA neurons in reward (Wang et al., 2015). VTA vGluT2 neurons also play a role in aversion signaling, given that a subset of VTA vGluT2 neurons responds to aversive stimuli (Root et al., 2018). Studies have shown that vGluT2 VTA neurons drive aversion through local input to GABAergic interneurons in the VTA, as well as projections to the lateral habenula (LHb) (Qi et al., 2016; Root et al., 2014). MORs regulate both GABA and glutamate transmission in LHb, suggesting this may be a potential site of MOR action in our behavioral effects (Margolis & Fields, 2016). Activation of vGluT2 neurons in the ventral pallidum is aversive; these neurons innervate the LHb and rostromedial tegmental nucleus (RMTg), as well as VTA GABA and DA neurons (Faget et al., 2018; Tooley et al., 2018). MORs in VP regulate behavioral responses to aversive stimuli, however it is important to note that our Cre+ (KO) mice do not show a difference in behavioral responses to quinine, an aversive substance (Inui & Shimura, 2017). However, others have shown that blockade of vental pallidum MORs induces morphine conditioned place aversion (Skoubis & Maidment, 2003). vGluT2 neurons in the lateral hypothalamus that project to VTA DA neurons, specifically those that project to the ventral NAc medial shell, are involved in encoding aversive stimuli (de

Jong et al., 2019). It is possible that MORs in these aversion processing vGluT2-expressing brain regions mediate opioid reward by inhibiting these aversion-encoding pathways. Glutamatergic projections from the rostral intralaminar thalamus to the dorsal striatum are involved in modulating reward through striatal dopamine release and we have demonstrated that these synapses express and are modulated by MORs (Cover et al., 2019; Munoz et al., 2018). It is possible that MORs regulate any of these sets of glutamatergic neurons to produce our observed behaviors.

It is important to note that while our intent was to study the behavioral role of MORs in glutamatergic neurons, our study is limited by the fact that vGluT2 is co-expressed in neurons that are known to release other neurotransmitters, that would not normally be considered classical “glutamatergic” neurons. For example, vGluT2 is expressed in VTA dopamine neurons, which co-release dopamine and glutamate; however, this represents a small population of vGluT2 neurons in the VTA (Morales & Margolis, 2017). In the brainstem, vGluT2 is expressed in catecholaminergic neurons (Stornetta et al., 2002). vGluT2 is also expressed in cholinergic spinal cord motor neurons, which synapse onto muscles and cause them to contract (Herzog et al., 2004). In addition, vGluT2 can be found in GABAergic neurons in the anteroventral periventricular nucleus; a brain region mainly involved in sex-specific physiology and behaviors (Ottem et al., 2004). Given that most of these brain regions or neuronal subtypes are not part of what are usually considered reward neurocircuits, we do not think these are the likely sites of our behavioral effects. However, it is nonetheless possible that MORs in any one of these neural populations could mediate the effects seen in Cre+ (KO) mice. Future work is needed to determine which vGluT2-expressing neurocircuits are regulated by MORs and how MORs in these neurocircuits mediate opioid reward.

In conclusion, these MORflox-vGluT2cre mice are a valuable tool to dissect the role of MORs in a subset of glutamatergic neurons that express vGluT2. Our findings challenge the concept that MORs in GABA neurons are the principal drivers of opioid reward; although, they do not suggest that MORs in GABA neurons are not critical. We have demonstrated that there are vGluT2-containing neurocircuits that are involved in opioid reward and MORs expressed in these neurons mediate this reward. Although these mice have helped to reveal the role of these MORs in this class of glutamatergic neuron, additional work is needed to identify the specific vGluT2 populations and brain regions responsible for these effects. While this task will be challenging, it may ultimately lead to novel combinatorial pharmacological therapeutics for treating opioid abuse and addiction.

CHAPTER 3

The behavioral role of mu opioid receptors in three glutamatergic brain regions: medial thalamus, anterior insular cortex, and lateral habenula

3.1 Introduction

It is well established that MORs, the primary target of many opioid drugs, mediate many opioid-related behaviors, including reward, antinociception, locomotor stimulation, locomotor sensitization, and withdrawal (Kieffer & Gaveriaux-Ruff, 2002; Matthes et al., 1996; Sora et al., 1997). MORs also contribute to the rewarding effects of other drugs of abuse (i.e. alcohol, marijuana, cocaine) and natural rewards (i.e. social interactions, sucrose, high fat food) (Kieffer & Gaveriaux-Ruff, 2002). The classical model of opioid reward establishes MORs on GABAergic neurons as the primary drivers of opioid reward. According to the classical model of opioid reward, opioid-induced increases in midbrain DA transmission is indirectly mediated by MORs in GABAergic VTA interneurons and VTA-projecting RMTg neurons (M. Jalabert et al., 2011; Johnson & North, 1992). Activation of these MORs reduces inhibitory input to VTA DA neurons, disinhibiting these DA neurons and increase DA release from the VTA (Fields & Margolis, 2015; M. Jalabert et al., 2011; Johnson & North, 1992). However, studies utilizing conditional MOR knockout mice have demonstrated MORs in other brain regions and cell types mediate opioid-related behaviors (see section 1.1.3). For example, a group used a conditional GABAergic forebrain MOR knockout mouse to characterize the behavioral role of MORs in this specific neuronal population (Ben Hamida et al., 2019; Charbogne et al., 2017). Like total MOR knockout mice, deleting MORs in GABA forebrain neurons inhibited opioid-induced locomotor stimulation (Charbogne et al., 2017; Kieffer & Gaveriaux-Ruff, 2002). In contrast to total MOR

knockout mice, opioid reward, analgesia, and withdrawal were intact in GABA forebrain MOR knockout mice, while motivation to self-administer heroin and palatable food were increased. These studies demonstrate that MORs in different neuronal populations can mediate distinct behaviors.

Although, most research has focused on the behavioral relevance of MORs in GABA neurons, recent studies have shown that MORs in glutamatergic neurons also mediate opioid-related behaviors. Recently, a conditional vGluT2 MOR knockout mouse (MORflox-vGluT2cre) was utilized to identify the role of MORs in vGluT2-expressing glutamatergic neurons. Deleting MORs in vGluT2-expressing neurons disrupted opioid reward, locomotor stimulation, and withdrawal but did not affect alcohol reward, natural reward (sucrose and food), or opioid analgesia (Reeves et al., 2021; Zhang et al., 2020). These were the first studies to identify a critical role of MORs in glutamatergic neurons in modulating opioid reward and withdrawal; however, it is unknown which vGluT2-expressing neuronal populations mediate these opioid behaviors. Therefore, we sought to identify glutamatergic brain regions involved with opioid-related behaviors, specifically investigating the behavioral role of MORs in the thalamus, anterior insular cortex (AIC), and LHb.

Our lab has previously shown that presynaptic MORs inhibit glutamate transmission to the dorsal striatum (Munoz et al., 2018; Muñoz et al., 2020). While the dorsal striatum receives glutamatergic inputs from several brain regions, MORs specifically inhibit glutamate transmission from vGluT2-expressing thalamic and AIC inputs. Thalamic inputs to the dorsal striatum arise primarily from the intralaminar and midline thalamic

nuclei (Smith et al., 2004; Unzai et al., 2017). Intralaminar thalamic nuclei are involved with reward and drug abuse; specifically, intralaminar thalamic projections to the dorsal striatum mediate drug seeking and craving (Cover et al., 2019; Li et al., 2018). The thalamus also has high vGluT2 expression; therefore, it is possible that MORs in the thalamus mediate some of the effects seen in conditional vGluT2 MOR knockout mice (see Chapter 2). Because the thalamus has high vGluT2 expression and is involved in reward and drug-related behaviors and neural circuits, we hypothesized deleting thalamic MORs would disrupt opioid reward. Presynaptic MORs also inhibit glutamate transmission from the AIC to the dorsal striatum (Munoz et al., 2018). Unlike MOR-mediated inhibition of thalamostriatal synapses, MOR-mediated inhibition of AIC- striatal synapses is ablated by in vivo alcohol exposure (Munoz et al., 2018). Therefore, we sought to determine the role of AIC MORs in alcohol- and opioid- related behaviors.

Because MORs in both glutamate and GABA neurons are involved in mediating opioid reward, opioid reward likely involves a complex interplay between MOR-mediated modulation of GABA and glutamatergic transmission. One possibility is the MOR-containing LHb-RMTg-VTA neurocircuit. Both RMTg and VTA GABA neurons have been implicated in mediating opioid reward via VTA DA disinhibition; therefore, the LHb may be a location of interplay between MOR-mediated modulation of GABA and glutamate transmission to mediate opioid reward (Fields & Margolis, 2015; Johnson & North, 1992; Matsui et al., 2014; Matsui & Williams, 2011). The LHb is involved in reward processing, specifically encoding aversive stimuli, negative reward, and reward-prediction errors, and is implicated in drug abuse (Baker et al., 2016; Jhou et al., 2009; Lammel et al., 2012; Matsumoto & Hikosaka, 2007; Stamatakis & Stuber, 2012; Tan et al., 2012; Ullsperger & von Cramon, 2003). Activation of LHb neurons inhibits DAergic VTA

neurons, an effect that is mediated by GABA (Christoph et al., 1986; Ji & Shepard, 2007; Matsumoto & Hikosaka, 2007). The LHb is primarily glutamatergic; therefore, LHb-induced inhibition of midbrain DA transmission is likely mediated by intermediary GABAergic neurons, rather than directly by LHb GABA release. Two possibilities for these intermediary neurons are VTA and RMTg GABA neurons, which send GABAergic projections to and inhibit VTA DA neurons (Hong et al., 2011; Johnson & North, 1992). Activation of the LHb is aversive; this effect is mediated by an increase in LHb neurotransmission to the RMTg and VTA (Hong et al., 2011; Jhou et al., 2009; Lammel et al., 2012; Stamatakis & Stuber, 2012; Tan et al., 2012). Like the LHb, VTA DA neuron-projecting RMTg neurons are activated in response to aversive stimuli, suggesting the LHb-RMTg-VTA circuit is involved in processing aversion and reward (Jhou et al., 2009). It is possible that MORs promote opioid rewad by inhibiting aversion-encoding LHb projections to the VTA and RMTg. LHb MORs may also regulate locomotor activity, as LHb activation suppresses motor behavior, possibly via LHb projections to the RMTg, a region involved with motor response inhibition (Hikosaka, 2010; Jhou et al., 2009; Lavezzi et al., 2015).

MORs are present in and modulate neural activity in several reward- and aversion-related glutamatergic brain regions; however, the behavioral role of MORs in specific brain regions is not well studied. The goal of this study was to identify behavioral roles of MORs in several drug abuse and reward-related glutamatergic brain regions. To accomplish this goal, we site-specifically deleted MORs by stereotaxically injected an AAV vector encoding cre-recombinase into MORflox mice into the target brain region (medial thalamus, AIC, or LHb). To characterize the behavioral role of MORs in these brain regions, we used a variety of behavioral assays, including those to access reward,

locomotor activity, and opioid withdrawal. In this study, we identify a role of AIC and LHb MORs in locomotor activity and LHb MORs in opioid withdrawal-related behaviors.

3.2 Materials and Methods

3.2.1 Animals

MORflox mice were generously donated by Dr. Jennifer Whistler (UC Davis) and have been previously characterized (Goldsmith et al., 2013; Munoz et al., 2018). All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine and all guidelines for ethical treatment and care for experimental animals established by the NIH (National Institutes of Health, Maryland, USA) were followed. All mutant mice used in these studies have been backcrossed to C57BL/6J mice for a minimum of 7 generations. Mice were group-housed on a standard 12-hour light/dark cycle (lights on 0700hr), with the exception of mice used for drinking studies, which were single-housed on a reverse 12-hour light/dark cycle (lights on 1800 hr), with at least one week acclimation before testing, and mice used for food consumption studies. Food and water were available ad libitum for all mice, except where otherwise indicated. Male and female mice were between 5-8 weeks of age at the time of surgery. Mice were 14-18 weeks of age at the start of experiments.

3.2.2 Materials

Oxycodone hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in saline (0.9% w/v) for in vivo injections.

3.2.3 Stereotaxic Surgery

Male and female MORflox mice underwent surgery at 5-8 weeks of age. Mice were anesthetized with isoflurane and stereotaxically bilaterally injected with an AAV vector encoding cre-recombinase and GFP (Cre (KO)) or GFP only (GFP (Ctrl)) in the medial thalamus, AIC, or LHb. Details of viral injections, including viral vector, injection volume, injection rate, and coordinates are included in **Table 2**. All viral vectors were obtained from Addgene. Transfection was allowed to occur for at least 8 weeks to ensure complete MOR deletion in the target region. Following behavioral experiments, GFP expression in the target brain region was confirmed. Mice with low, unilateral, or misplaced GFP expression were excluded from analyses, unless otherwise indicated.

	Medial thalamus	**Anterior insular cortex**	**Lateral habenula**
Cre (KO) AAV	pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40	pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40	pENN.AAV.hSyn.HI.eGFP-Cre.WPRE.SV40
GFP (Ctrl) AAV	pENN.AAV.CamKII0.4.eGFP.WPRE.rBG	pENN.AAV.CamKII0.4.eGFP.WPRE.rBG	pENN.AAV.hSyn.HI.eGFP.WPRE.bGH
Injection volume (nL)	100	50	50
Injection rate (nL/min)	25	25	12.5
Coordinates			
A/P	-0.5	-0.5	-1.2
M/L	±0.3	±0.3	±0.5
D/V	-0.42	-0.42	-3.55

Table 2. Details of stereotaxic viral injections to delete MORs in target brain regions

3.2.4 Behavior Experiments

The following behavioral assays were performed in both male and female mice. For clarity of focusing on genotype, data with no statistically significant sex differences or trends are collapsed by sex. Mice involved in behavior experiments were habituated to

handling for at least three days prior to testing. Mice were acclimated to the behavior room with a white noise machine for at least 30 minutes before testing.

Open-field locomotor activity: Mice were placed in the center of unlit open-field chambers (16” X 16”; Omnitech, Columbus, OH) and recorded for 20 minutes. Mice were tested at the same time each day for two consecutive days. Locomotor activity was measured by recording the number of beam breaks in horizontal and vertical directions by Fusion software.

Oxycodone conditioned place preference (CPP): Mice underwent a modified protocol of oxycodone CPP (see Section 2.2.8 for details), except 3 mg/kg i.p. oxycodone was used.

Oxycodone-induced locomotor sensitization: Mice underwent a 5-day protocol to induce oxycodone locomotor sensitization. On day 1, mice were habituated to the open-field locomotor chamber (Omnitech) for 60 minutes. Saline testing occurred on day 2; mice were habituated to the open-field locomotor chamber for 15 minutes. Mice were then briefly removed from the locomotor chamber and administered saline (10 mg/mL i.p.) before being returned to the chamber and for 60 minutes. Days 3-5 were identical to day 2, except mice were administered oxycodone (10 mg/kg i.p.). Locomotor activity was measured by recording the number of beam breaks in horizontal directions by Fusion software. Development of oxycodone-induced locomotor sensitization was defined as an increase in oxycodone-induced locomotor activity on day 5 (last oxycodone injection), compared to day 3 (first oxycodone injection).

Naloxone-precipitated withdrawal: Oxycodone dependence was induced using a modified dose ramping protocol (see Section 2.2.8 for details). Approximately 2 hours after the final oxycodone injection, withdrawal was precipitated by administering naloxone (5 mg/kg i.p.). Mice were immediately placed into empty open-field locomotor chambers for a 10-minute observation period, during which withdrawal-related behaviors were assessed (see Section 2.2.8 for details).

EtOH drinking in the dark (DID): Mice were acclimated to drinking reverse-osmosis water from custom-built home cage lickometers (similar to (Godynyuk et al., 2019)) for 2 weeks, followed by 4 weeks of EtOH (20% w/v in reverse osmosis water) drinking (5 drinking sessions/ week). A modified DID binge-like drinking model was used to encourage high levels of alcohol drinking (Thiele & Navarro, 2014). Lickometers were inserted 3 hours into the dark cycle for 2 hrs on days 1-4 each week and 4 hrs on day 5 each week. During drinking sessions, home cage water bottles were removed. To measure the amount of fluid consumed, lickometers were weighed immediately before and after each drinking session. No drinking sessions occurred on days 6-7 each week. Mice were weighed weekly after the final drinking session each week, and weekly weights were used to normalize fluid consumption.

Ethanol intermittent access: Mice that had previously undergone EtOH DID were tested in intermittent access alcohol drinking following a 2-week washout period. 10 hours into the dark cycle, home cage water bottles were removed and replaced with a custom-built home cage lickometers containing EtOH (20% w/v) for 24 hrs. Drinking sessions occurred every other day, 6 days per week. Lickometers were weighed before and after each drinking session to measure the volume of EtOH consumed. Mice were weighed

weekly following the final drinking session for the week and weekly weights were used to normalize fluid consumption.

Sucrose consumption: Mice were food deprived for 23 hrs. Following the food deprivation period, mice were weighed, placed in an empty home cage and given ad libitum access to sucrose pellets for 60 minutes. The amount of sucrose pellets consumed was calculated by subtracting the weight of the remaining pellets following the free access period from the initial weight.

3.2.5 Data Analysis

Experimenters were blinded to viral treatment during all stages of data collection. Data are presented as the mean +/- SEM. Data were analyzed using GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). The level of significance was set at $p<0.05$ for all analyses. Because of small sample sizes, no outliers were excluded. Some data were excluded on the basis of technical errors, such as leaking drinking tubes or equipment failures that occurred during a measurement session. Normal distribution was assessed prior to statistical analysis. Two-tailed unpaired t-tests were used to analyze normally distributed data. No data was non-normally distributed. For data with multiple groups and/or repeated measures, ANOVA or REML with Sidak's or Tukey's post hoc tests were used. REML was used if data points were missing due to experimental exclusion.

3.3 Results

3.3.1 Deleting medial thalamus MORs does not affect oxycodone reward

Presynaptic MORs modulate glutamate transmission from vGluT2-expressing thalamic synapses in the dorsal striatum (Munoz et al., 2018; Reeves et al., 2021). Thalamic input

to the dorsal striatum is primarily from midline and intralaminar nuclei, which have been implicated in reward and drug abuse (Cover et al., 2019; Smith et al., 2004). We sought to determine whether MORs in these dorsal striatal-projecting thalamic nuclei were involved with mediating the rewarding effects of oxycodone. These thalamic nuclei are primarily located in the medial region of the thalamus; therefore, we bilaterally injected an AAV vector encoding cre-recombinase into the medial thalamus of MORflox mice (Thal Cre (KO)) to specifically delete MORs in thalamic nuclei that target the dorsal striatum. Controls were littermates bilaterally injected with an AAV vector encoding GFP in the medial thalamus (Thal GFP (ctrl) (**Figure 14A**). Following an 8-week transfection period, we assessed oxycodone reward in these mice using CPP. Following conditioning with oxycodone (3 mg/kg i.p.), both Cre (KO) and GFP (ctrl) mice acquired equivalent levels of place preference for the oxycodone-paired side (**Figure 14B**, unpaired t-test: p=0.61, t_{11}=0.53). Additionally, locomotor activity was compared during the final saline and oxycodone conditioning sessions. There were no differences in locomotor activity between Cre (KO) and GFP (ctrl) mice in saline or oxycodone sessions and oxycodone induced locomotor stimulation in both groups (**Figure 14C**, two-way ANOVA: genotype p=0.61, (F1, 11) = 0.28; treatment p<0.0001, F(1,11)=492.4; interaction p=0.061, F(1, 11)=4.35). These data suggest that medial thalamus MORs are not involved with opioid reward or locomotor activity.

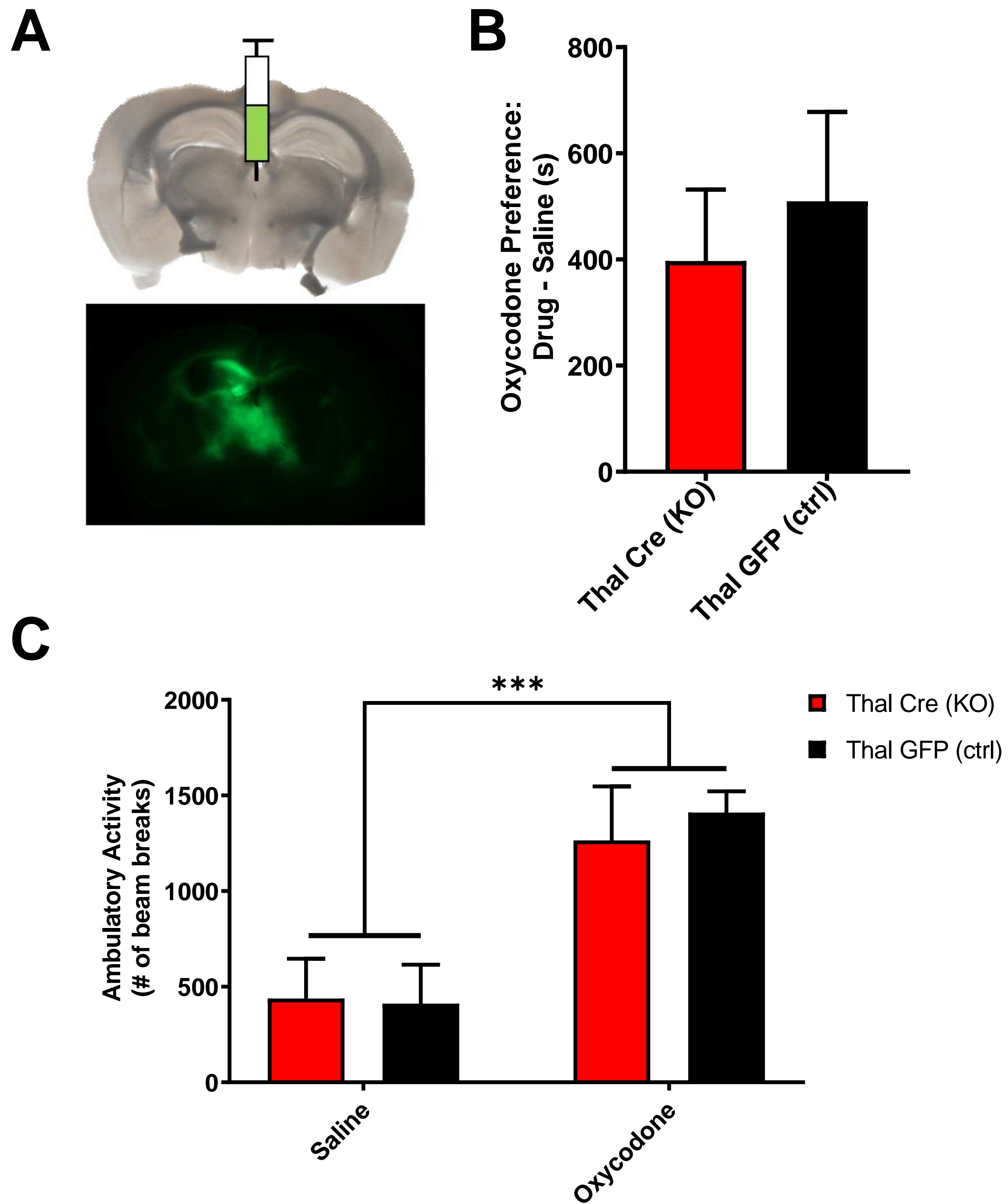


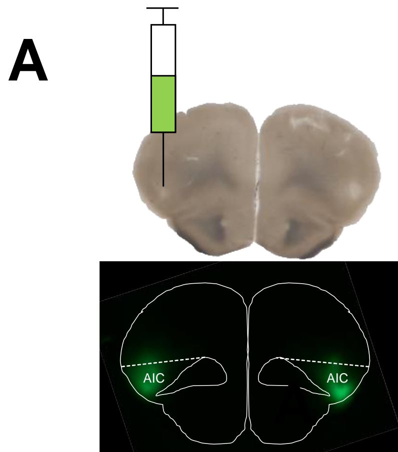
Figure 14. Deleting medial thalamus MORs does not disrupt oxycodone reward or locomotor activity. (A) Representative image showing GFP expression in the medial thalamus of a GFP (ctrl) mouse. (B) Both Cre (KO) and GFP (ctrl) mice acquired place preference for 3 mg/kg oxycodone. (C) There were no differences in locomotor activity during the final saline or oxycodone conditioning sessions between Cre (KO) and GFP (ctrl) mice. Oxycodone caused locomotor stimulation in both groups. [*n* = 7 (4M/3F) Thal Cre (KO), 6 (3M/3F) Thal GFP (ctrl)]. No sex differences were detected. Data are collapsed across sex. *** $p < 0.001$. Error bars indicate ± SEM. Thal Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 medial thalamus injection; Thal GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG medial thalamus injection.

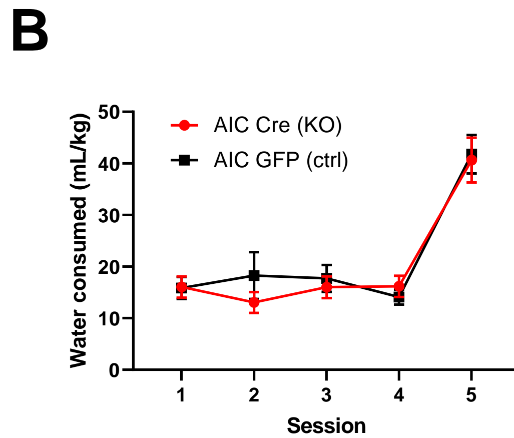
3.3.2 Deleting AIC MORs does not affect alcohol consumption

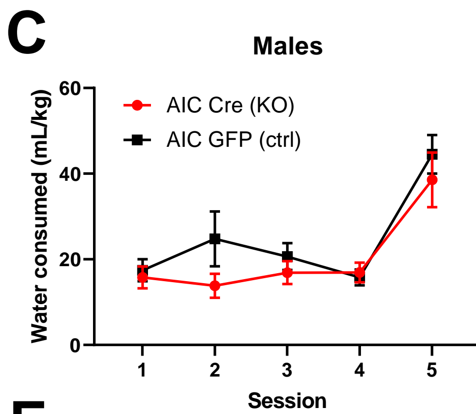
In addition to thalamostriatal synapses, presynaptic MORs also modulate AIC- dorsal striatum synapses. Our lab previously identified MOR-mediated long-term depression of glutamate transmission from AIC inputs in the dorsal striatum (Munoz et al., 2018). This MOR-mediated long-term depression is ablated by in vivo exposure to alcohol, including voluntary drinking using the DID model of binge-like drinking (Munoz et al., 2018). Therefore, we hypothesized AIC MORs are involved with alcohol drinking. To test this hypothesis, we stereotaxically injected viral vectors encoding cre-recombinase (AIC Cre (KO)) or GFP only (AIC GFP (ctrl) into the AIC of MORflox mice (Figure A), similar to what was described in section 3.3.1. We used a modified DID procedure to assess alcohol drinking in mice with AIC MORs deleted. Each week of testing consisted of 5 days of daily drinking sessions (D1-4 2hrs, D5 4hrs to encourage higher drinking levels), followed by 2 days of abstinence. During the first 2 weeks of testing, mice were habituated to drinking water from the lickometers. Stable drinking levels were reached after the first week; therefore, the second week of water drinking was used to access basal water consumption. There were no differences in basal water consumption between AIC Cre (KO) and GFP (ctrl) mice (**Figure 15B**, two-way ANOVA: session $p<0.0001$, $F(2.7,62.9)=48.56$; virus $p=0.69$, $F(1, 23)=0.17$; interaction $p=0.6$, $F(4,92)=0.69$). Because sex differences are often reported in alcohol drinking studies (Middaugh et al., 1999), we separately examined all drinking data in males and females. There were no sex-specific differences in water consumption between AIC Cre (KO) and GFP (ctrl) mice (**Figure 15C-D**, two-way ANOVA, males: session $p<0.0001$, $F(2.3,29.7)=21.87$; virus $p=0.26$, $F(1,13)=1.4$; interaction $p=0.42$, $F(4,52)=1$; females: session $p<0.0001$, $F(2.4,19.3)=31.31$; virus $p=0.49$, $F(1,8)=0.52$; interaction $p=0.95$, $F(4,32)=0.17$).

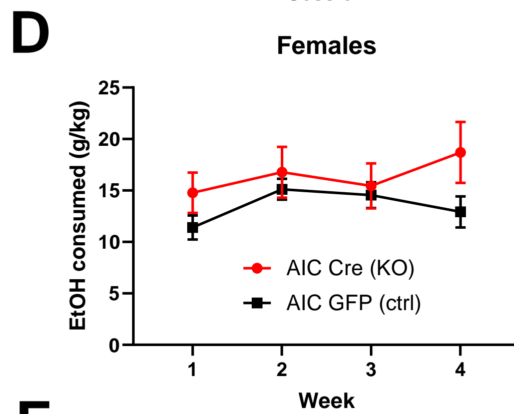
For the next 4 weeks, lickometers were filled with 20% EtOH to investigate alcohol drinking. The amount of EtOH consumed per session (**Figure 15E**, REML: session $p<0.0001$, $F(5.2,119.8=28.23)$; virus $p=0.96$, $F(1,23)=0.0029$; interaction $p=0.22$, $F(19,436)=1.24$) or week (**Figure 15F**, two-way ANOVA: week $p=0.15$, $F(2.1,48.8)=1.95$; virus $p=0.98$, $F(1,23)=0.00093$; interaction $p=0.023$, $F(3,69)=3.39$) was equal in AIC Cre (KO) and GFP (ctrl) mice. There was as significant week x virus interaction effect, but post hoc analyses did not reveal any significant differences between AIC Cre (KO) and GFP (ctrl) mice in any week (Sidak's test, Cre (KO) vs. GFP (ctrl): wk1 $p>0.99$, wk2 $p=097$, wk3 $p=0.99$, wk4 $p=0.82$). EtOH drinking in female mice remained consistent across the 4 weeks (two-way ANOVA: week $p=0.093$, $F(2,15.7=2.79)$; virus $p=0.25$, $F(1,8)=1.54$; interaction $p=0.16$, $F(3,24)=1.89$); however, EtOH drinking decreased in male mice over time (**Figure 15G-H**, two-way ANOVA: week $p<0.0001$, $F(2.7,35.5)=21.59$; virus $p=0.5$, $F(1,13)=0.49$; interaction $p=0.0029$, $F(3,39)=5.54$). In male mice, there was a significant week x virus interaction, but post hoc analyses did not identify any significant differences between AIC Cre (KO) and GFP (ctrl) mice in any week (Sidak's test, Cre (KO) vs. GFP (ctrl): wk 1 $p=0.73$, wk 2 $p=0.66$, wk 3 $p=0.92$, wk 4 $p=0.97$). We also investigated longer bouts of alcohol drinking to see if differences between groups occurred when higher amounts of alcohol were consumed. Following a two-week washout period, mice that had previously undergone EtOH DID underwent 24hr intermittent EtOH access, where mice were given access to EtOH-containing lickometers for 24hrs, every other day. There were no differences in EtOH consumption using 24hr intermittent EtOH access (**Figure 16A**, REML: session $p<0.0001$, $F(2.4,59.2)=14.9$; virus $p=0.92$, $F(1,25)=0.011$; interaction $p=0.31$, $F(9,220)=1.18$). There were also no sex-specific difference in EtOH consumption (**Figure 16B-C**, REML, males: session $p=0.0003$, $F(1.8,26.5)=11.94$; virus $p=0.68$, $F(1,15)=0.18$, interaction $p>0.99$, $F(9,131)=0.19$; females: session $p=0.022$, $F(1.8,14.3)=5.2$; virus $p=0.65$, $F(1,8)=0.22$;

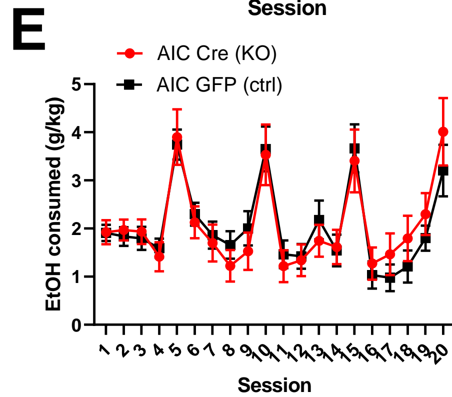
interaction $p=0.14$, $F(9,70)=1.56$). Altogether, these data suggest that deleting AIC MORs are not involved with alcohol consumption.

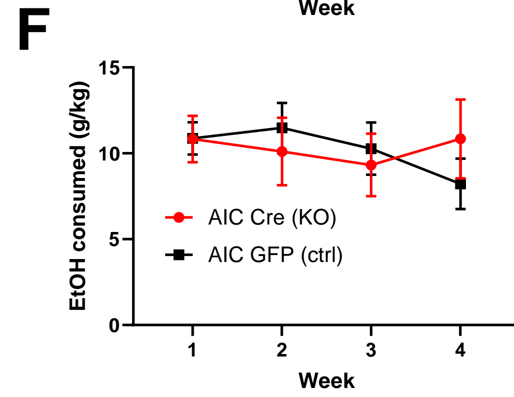

A
AIC
AIC

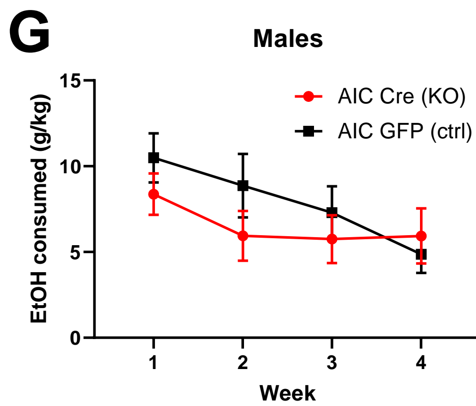

B
AIC Cre (KO)
AIC GFP (ctrl)
Water consumed (mL/kg)
50
40
30
20
10
0
1
2
3
4
5
Session


C
Males
AIC Cre (KO)
AIC GFP (ctrl)
Water consumed (mL/kg)
60
40
20
0
1
2
3
4
5
Session


D
Females
AIC Cre (KO)
AIC GFP (ctrl)
EtOH consumed (g/kg)
25
20
15
10
5
0
1
2
3
4
Week


E
AIC Cre (KO)
AIC GFP (ctrl)
EtOH consumed (g/kg)
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Session


F
AIC Cre (KO)
AIC GFP (ctrl)
EtOH consumed (g/kg)
15
10
5
0
1
2
3
4
Week


G
Males
AIC Cre (KO)
AIC GFP (ctrl)
EtOH consumed (g/kg)
15
10
5
0
1
2
3
4
Week

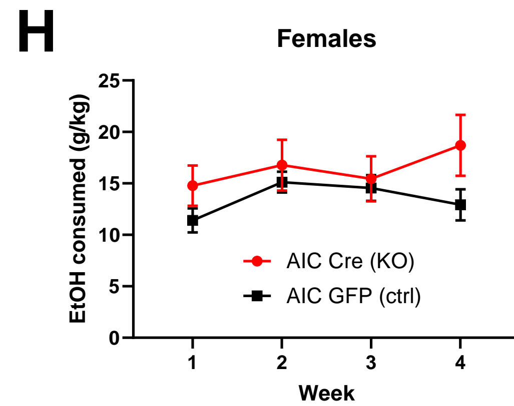

H
Females
AIC Cre (KO)
AIC GFP (ctrl)
EtOH consumed (g/kg)
25
20
15
10
5
0
1
2
3
4
Week

Figure 15. Deleting AIC MORs does not affect alcohol drinking using a DID procedure. (A) Representative image showing bilateral GFP expression in the AIC of a GFP (ctrl) mouse. (B) Water consumption did not differ between Cre (KO) and GFP (ctrl) mice. (C) Water consumption in male mice; no differences between Cre (KO) and GFP (ctrl) mice were detected. (D) Water consumption in female mice; no differences between Cre (KO) and GFP (ctrl) mice were detected. (E) EtOH consumption did not differ between Cre (KO) and GFP (ctrl) mice. (F) The weekly sum of EtOH consumed by Cre (KO) and GFP (ctrl) mice is equal. (G) Weekly sum of EtOH consumed by male mice; no differences between Cre (KO) and GFP (ctrl) mice were detected. (H) Weekly sum of EtOH consumed by female mice; no differences between Cre (KO) and GFP (ctrl) mice were detected. [n = 13 (8M/5F) AIC Cre (KO), 12 (7M/5F) AIC GFP (Ctrl)]. Error bars indicate ± SEM. AIC Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 AIC injection; AIC GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG AIC injection.

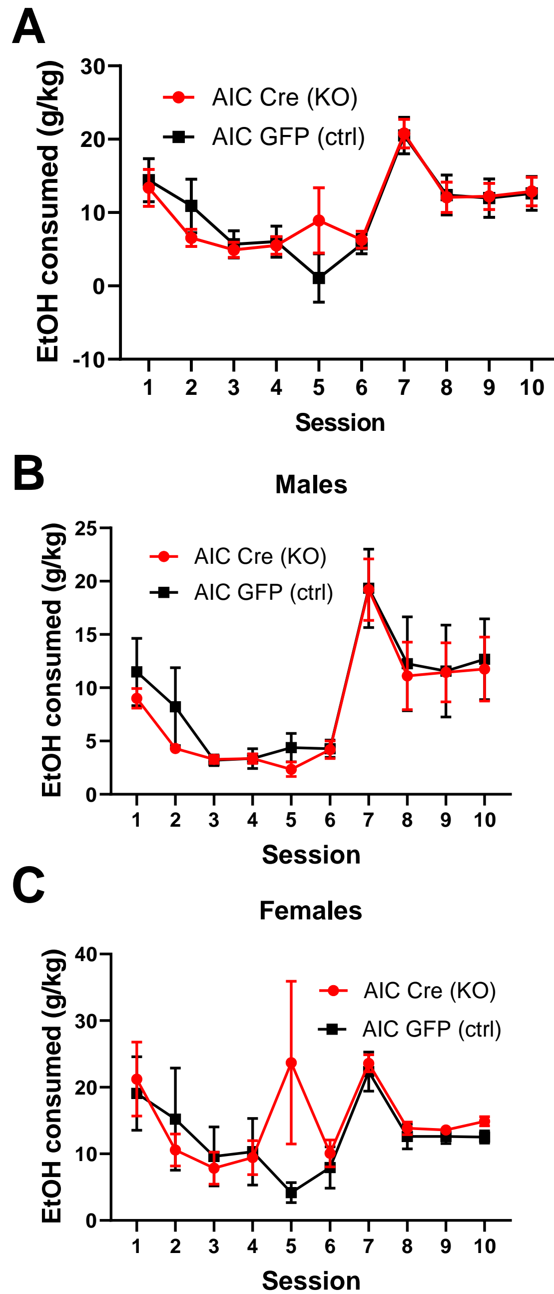


Figure 16. Deleting AIC MORs does not affect alcohol drinking using an intermittent access procedure. (A) EtOH consumption does not differ between Cre (KO) and GFP (ctrl) mice. (B) No differences in EtOH consumption between Cre (KO) and GFP (ctrl) mice were detected in males (C) or females. [*n* = 13 (8M/5F) AIC Cre (KO), 12 (7M/5F) AIC GFP (Ctrl)]. Error bars indicate ± SEM. AIC Cre (KO) = pENN.AAV.CamKII.HI.GFPCre.WPRE.SV40 AIC injection; AIC GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG AIC injection.

3.3.3 Deleting AIC MORs causes hyperlocomotion

MORs are involved in mediating a wide variety of behaviors. Likewise, the AIC is also implicated in a wide variety of behaviors (Benarroch, 2019). Because AIC MORs could potentially be involved in an abundance of behaviors, we utilized a behavioral battery of tests to determine the role of MORs in opioid- and reward-related behaviors. Mice were single housed one week prior to behavioral experiments. Mice underwent the following behavioral tests in order: open-field locomotor assessment, sucrose consumption, oxycodone CPP, oxycodone locomotor sensitization, and oxycodone withdrawal. Because differential locomotor activity is a confounding factor in many opioid- and reward- related behavioral tests, we first examined open-field locomotor activity. AIC Cre (KO) mice had slightly increased locomotor activity, compared to GFP (ctrl) mice (**Figure 17A**, unpaired t-test: $p=0.044$, $t_{14}=2.21$). There were no differences in number of rearing episodes (**Figure 17B**, unpaired t-test: $p=0.81$, $t_{14}=0.25$), which is often used as an indication of exploratory behavior (Lever et al., 2006). There were also no differences in time spent in the outside or inside zone of the open-field chamber, a measure used to access anxiety (Seibenhener & Wooten, 2015) (**Figure 17C-D**, two-way ANOVA: zone $p<0.0001$, $F(1,28)=23.44$; virus $p=0.9$, $F(1,28)=0.017$; interaction $p=0.34$, $F(1,28)=0.95$). These data suggest that AIC MORs are involved with regulating locomotor activity, but not exploration or anxiety.

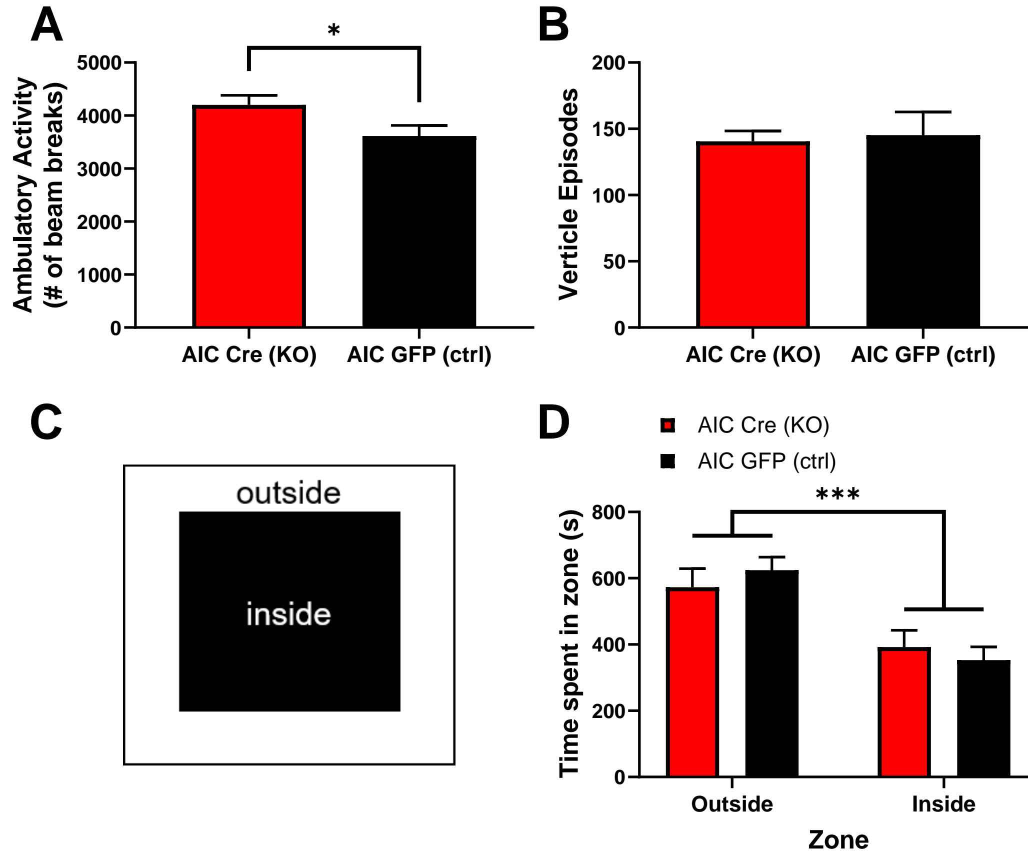


Figure 17. Deleting AIC MORs causes hyperlocomotion. (A) Cre (KO) mice had increased locomotor activity in an open-field assessment, compared to GFP (ctrl) mice (B) Quantity of rearing episodes did not differ between Cre (KO) and GFP (ctrl) mice. (C) Outside and inside zones of the open-field chamber, used to access anxiety. (D) Both Cre (KO) and GFP (ctrl) spent more time in the outside of the open-field chamber than in the inside [(*n* = 8(4M/4F) Cre (KO), 8(4M/4F) GFP (Ctrl)]. No sex differences were detected. Data are collapsed across sex. * $p < 0.05$; *** $p < 0.001$. Error bars indicate ± SEM. AIC Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 AIC injection; AIC GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG AIC injection.

3.3.4 Deleting AIC MORs does not disrupt the rewarding effects of sucrose or oxycodone

MORs mediate the rewarding effects of natural rewards (i.e. sucrose, food), and opioids (Kieffer & Gaveriaux-Ruff, 2002). To access the potential role of AIC MORs in modulating natural rewards, we measured sucrose consumption following 23hrs of food

deprivation. There were no differences in body weight between AIC Cre (KO) and GFP (ctrl) mice, but male mice weighed more than females (**Figure 18A**, two-way ANOVA: sex $p<0.0001$, $F(1,12)=145$; virus $p=0.23$, $F(1,12)=1.58$; interaction $p=0.76$, $F(1,12)=0.095$). We first determined that food consumption did not differ between AIC Cre (KO) and GFP (ctrl) mice; although, there was a significant effect of sex (**Figure 18B**, two-way ANOVA: sex $p=0.016$, $F(1,12)=7.81$; virus $p=0.24$, $F(1,12)=1.52$; interaction $p=0.87$, $F(1,12)=0.03$). Following 23hrs of food deprivation, mice were given unlimited access to sucrose pellets for one hour. The amount of sucrose pellets consumed during this period did not differ between viral groups or sexes (**Figure 18C**, two-way ANOVA: sex $p=0.95$, $F(1,12)=0.004$; virus $p=0.39$, $F(1,12)=0.8$; interaction $p=0.77$, $F(1,12)=0.09$). These data suggest the naturally rewarding effects of sucrose and food are not affected by deleting AIC MORs.

We have previously demonstrated MORs in certain neuronal populations specifically modulate opioid reward, but not natural rewards or alcohol reward (Reeves et al., 2021); therefore, we investigated the role of AIC MORs in opioid reward using CPP. During the final conditioning sessions, AIC Cre (KO) and GFP (ctrl) showed similar locomotor activity; locomotor activity was increased during the oxycodone conditioning session (**Figure 18D**, two-way ANOVA: treatment $p<0.0001$, $F(1,10)=80.74$; virus $p=0.72$, $F(1,10)=0.14$; interaction $p=0.39$, $F(1,10)=0.82$). This contrasts our open-field locomotor data showing increased locomotor activity in AIC Cre (KO) mice. The lack of locomotor differences during the saline conditioning session is likely due to the short duration (5 min) of the CPP conditioning session. Following conditioning, both Cre (KO) and GFP (ctrl) mice acquired place preference for the oxycodone-paired side (**Figure 18E**, unpaired t-test: $p=0.63$, $t_{14}=0.49$), suggesting opioid reward is intact in Cre (KO) mice. Overall, these data suggest AIC MORs do not mediate the rewarding effects of sucrose,

food, or opioids. Although there appears to be no role of AIC MORs in the acute oxycodone-induced locomotor stimulation (**Figure 17D**), repeated oxycodone administration results in sensitization to the locomotor stimulatory effect of oxycodone. Sensitization to the effects of drugs of abuse is thought to underlie long-term behavioral changes, including drug craving, compulsivity, and relapse (Kalivas et al., 1998; Robinson & Berridge, 2001). To access the role of AIC MORs in the development of oxycodone locomotor sensitization, we used a modified 5-day protocol (D1-habituation, D2-saline, D3-5 10 mg/kg oxycodone i.p.) to induce oxycodone locomotor sensitization (Kumar et al., 2016). Because of locomotor differences between AIC Cre (KO) and GFP (ctrl) mice, oxycodone-induced locomotor activity was normalized to baseline locomotor activity (saline). There were no differences in locomotor activity using the oxycodone sensitization protocol (**Figure 18F**, two-way ANOVA: session $p=0.018$, $F(1.3,18.2)=6.09$; virus $p=0.92$, $F(1,14)=0.01$; interaction $p=0.41$, $F(2,28)=0.92$). Both Cre (KO) and GFP (ctrl) mice developed sensitization to oxycodone's locomotor stimulatory effect, as indicated by increased locomotor activity with repeated oxycodone administration (Tukey's test, D1 vs. D2 $p=0.60$, D1 vs. D3 $p=0.045$, D2 vs. D3 $p=0.0089$).

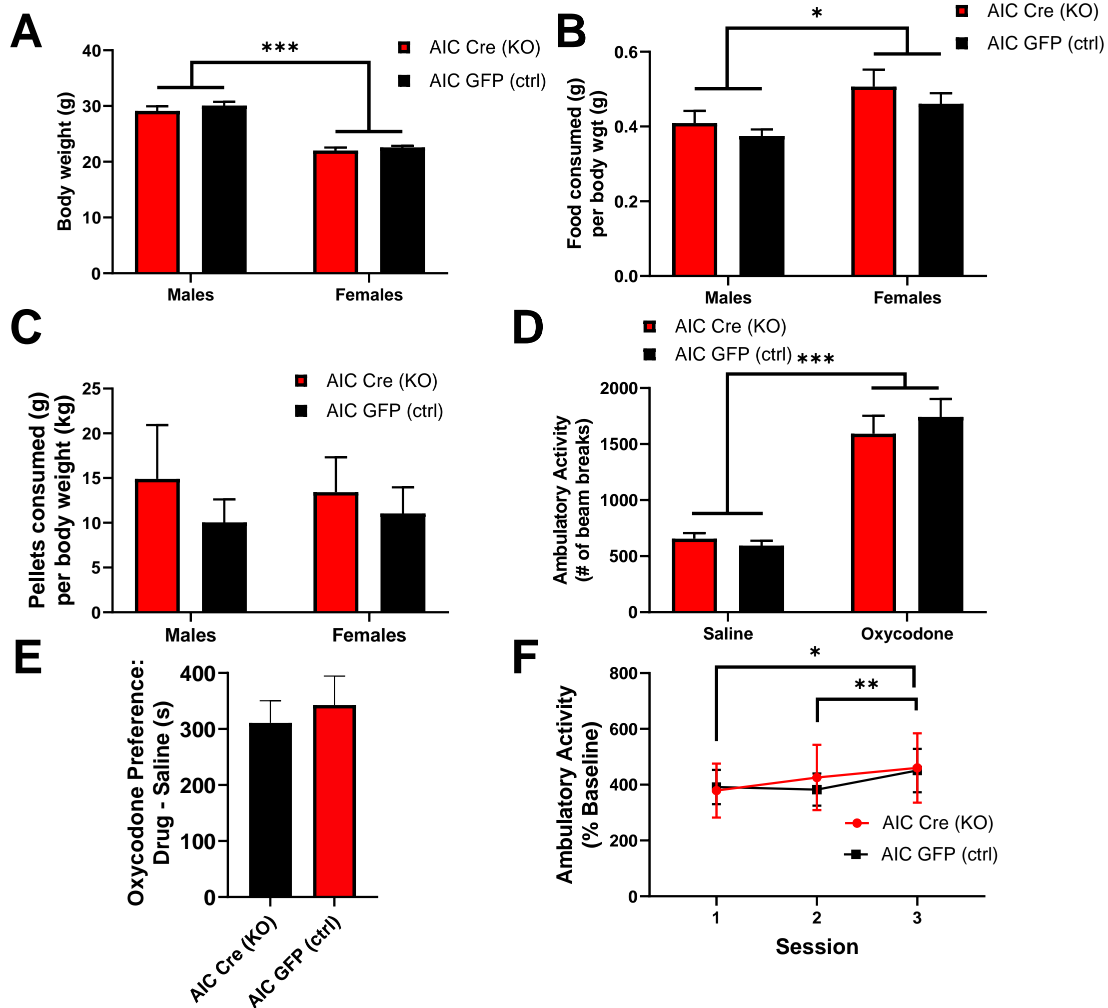


Figure 18. Deleting AIC MORs does not affect sucrose or opioid reward. (A) Male mice weighed more than females, regardless of viral group. (B) Female mice consumed more food, normalized by body weight, than males, regardless of viral group. (C) Following acute food deprivation, Cre (KO) and GFP (ctrl) mice consumed equal amounts of sucrose, normalized by body weight. No sex effects were detected. (D) Cre (KO) and GFP (ctrl) mice displayed similar locomotor activity during the final CPP conditioning sessions. Oxycodone induced locomotor stimulation in both groups. (E) Both Cre (KO) and GFP (ctrl) mice acquired place preference for oxycodone. (F) Both Cre (KO) and GFP (ctrl) mice developed sensitization to the locomotor stimulatory effects of oxycodone. [(n = 8(4M/4F) AIC Cre (KO), 8(4M/4F) AIC GFP (Ctrl)]. No sex effects were detected for oxycodone CPP or sensitization studies; data is collapsed by sex for these studies. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate ± SEM. AIC Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 AIC injection; AIC GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG AIC injection.

3.3.5 Deleting AIC MORs does not affect opioid withdrawal-related behaviors

Recently, a role for MORs in several glutamatergic neuronal populations has been identified in mediating opioid withdrawal-associated behaviors (Boulos et al., 2020; Reeves et al., 2021; Zhang et al., 2020). We induced oxycodone dependency using a dose-ramping protocol of oxycodone administration (Reeves et al., 2021). Approximately 2 hrs after the final dose of oxycodone, oxycodone withdrawal was precipitated by administration of the opioid receptor antagonist, naloxone (5 mg/kg i.p.). The global withdrawal score, used to access overall withdrawal severity while equally weighting all withdrawal-associated scored behaviors, did not differ between Cre (KO) and GFP (ctrl) mice (**Figure 19A**, unpaired t-test $p=0.21$, $t_{12}=1.32$). The number of individual withdrawal-associated behaviors (wet dog shakes, paw shakes, jumps) were equal between groups (**Figure 19B**, two-way ANOVA: behavior $p<0.0001$, $F(2,42)=12.25$; virus $p=0.4$, $F(1,42)=0.71$; interaction $p=0.51$, $F(2,42)=0.68$). These data suggest that AIC MORs are not involved with oxycodone withdrawal-associated behaviors.

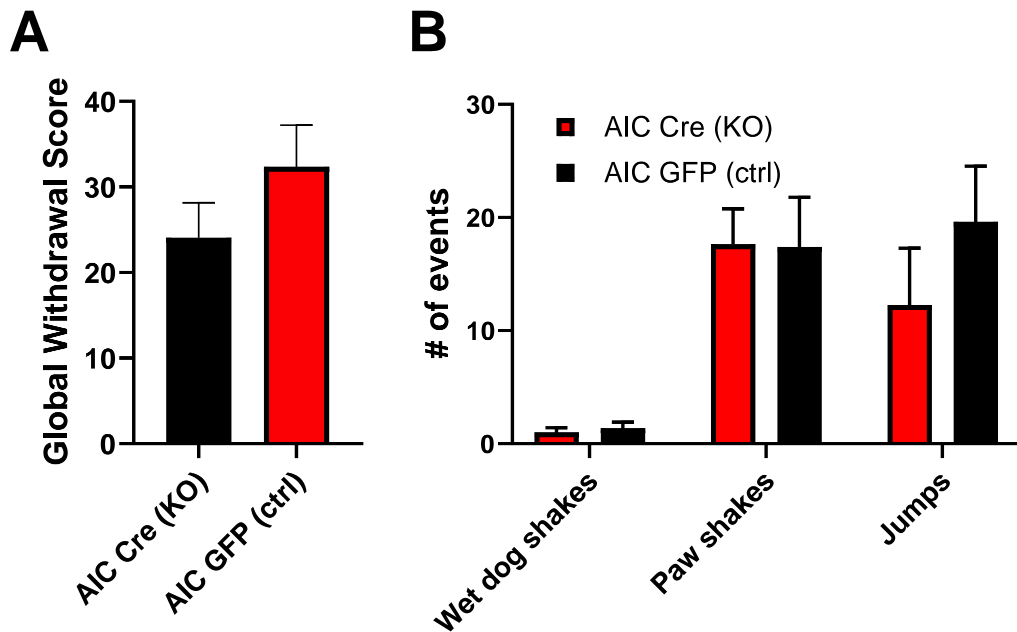


Figure 19. Deleting AIC MORs does not affect naloxone-precipitated oxycodone withdrawal-associated behaviors. (A) Naloxone-precipitated global withdrawal score is equal in Cre (KO) and GFP (ctrl) mice. (B) Quantity of individual naloxone-precipitated withdrawal-associated behaviors, including wet dog shakes, paw shakes, and jumps, do not differ between Cre (KO) and GFP (ctrl) mice. [(n = 8(4M/4F) Cre (KO), 8(4M/4F) GFP (Ctrl)]. No sex effects were detected for oxycodone CPP or sensitization studies; data is collapsed by sex for these studies. *$p < 0.05$; *** $p < 0.001$. Error bars indicate ± SEM. AIC Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 AIC injection; AIC GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG AIC injection.

3.3.6 Deleting LHb MORs causes hyperlocomotion

LHb MORs were deleted using the method previous described in our medial thalamus and AIC MOR knockout studies (**Figure 20A**). LHb Cre (KO) and LHb GFP (ctrl) mice underwent a battery of behavioral assessments, similar to what was previously described for AIC MOR KO mice (open-field locomotor, sucrose consumption, oxycodone CPP, oxycodone locomotor sensitization, oxycodone withdrawal). LHb Cre (KO) and GFP (ctrl) mice remained group housed to avoid additional stress or behavioral effects caused by single housing. One LHb GFP (ctrl) mouse (female) was removed

from analysis due to misplaced GFP expression. Two LHb Cre (KO) (1 male, 1 female) mice were removed from analyses due to unilateral LHb GFP expression. In an open-field locomotor activity assessment, LHb Cre (KO) mice displayed hyperlocomotion, compared to controls (**Figure 20B**, unpaired t-test: $p=0.02$, $t_{10}=2.76$). There were no differences in quantity of exploratory rearing (**Figure 20C**, unpaired t-test: $p=0.40$, $t_{10}=0.89$). LHb Cre (KO) and GFP (ctrl) mice spent more time in the outside of the open-field chamber, compared to the center, and no effects were detected between groups (**Figure 20D**, two-way ANOVA: zone $p<0.0001$, $F(1,10)=41.38$; virus $p=0.37$, $F(1,10)=0.90$; interaction $p=0.71$, $F(1,10)=0.14$). Altogether, these results suggest LHb MORs are involved in regulating locomotor activity but are not involved in exploration or anxiety open-field assessments.

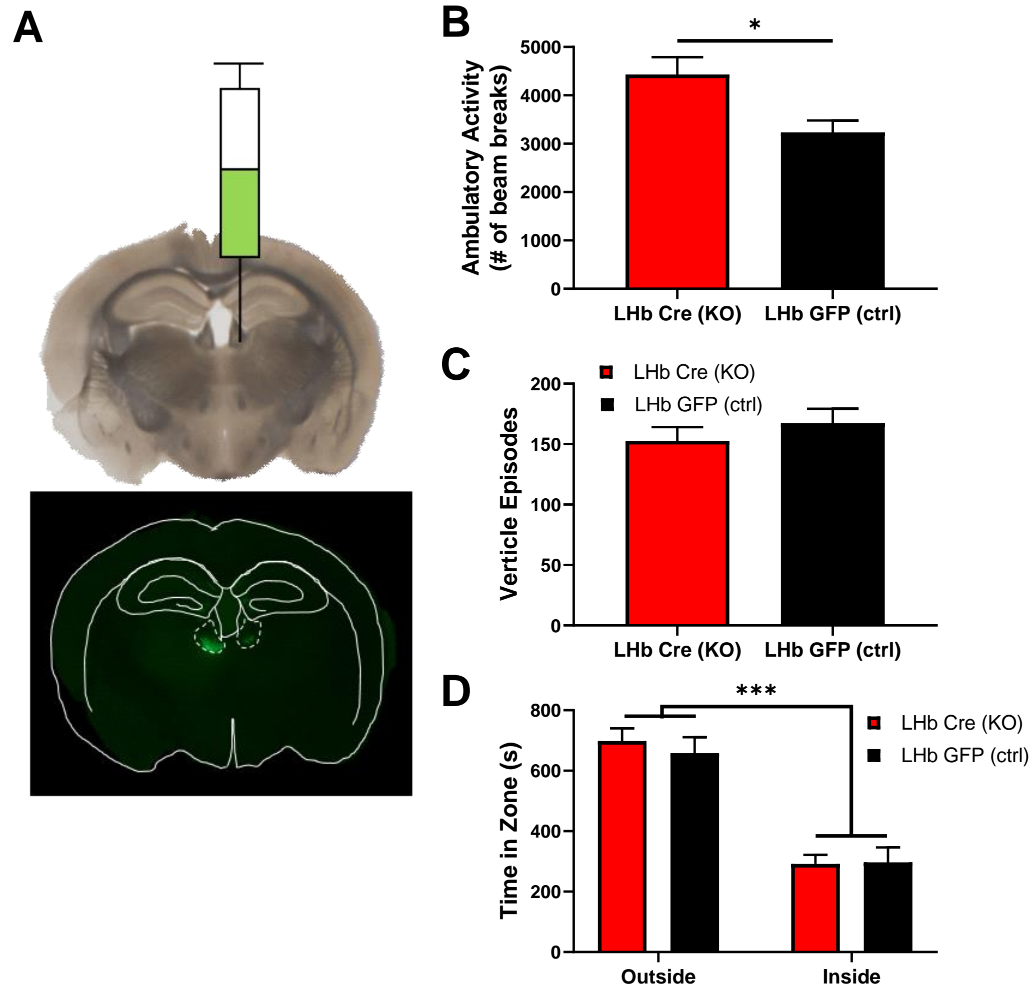


Figure 20. Deleting LHb MORs causes hyperlocomotion. (A) Representative image showing bilateral GFP expression in the LHb of a Cre (KO) mouse. (B) Cre (KO) mice had increased locomotor activity in an open-field assessment, compared to controls. (C) The number of rearing episodes did not differ between LHb Cre (KO) and GFP (ctrl) mice. (D) Both Cre (KO) and GFP (ctrl) spent more time in the outside of the open-field chamber than in the center. [(n = 6(4M/2F) Cre (KO), 6(4M/2F) GFP (Ctrl)]. No sex differences were detected. Data are collapsed across sex. * $p < 0.05$; *** $p < 0.001$. Error bars indicate ± SEM. LHb Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 LHb injection; LHb GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG LHb injection.

3.3.7 Deleting LHb MORs does not disrupt the rewarding effects of sucrose or oxycodone

The LHb provides major glutamatergic input to the reward pathway (VTA, RMTg), is activated by aversive stimuli, and is inactivated by rewarding stimuli. In opposition to the effects of drugs of abuse and natural rewards, LHb activation indirectly reduces midbrain DA transmission (Ji & Shepard, 2007). MOR agonists inhibit the LHb (Margolis & Fields, 2016); therefore, it is possible LHb MORs modulate reward by inhibiting the "aversion-encoding" LHb. To investigate the potential role of LHb MORs in sucrose reward, we measured sucrose pellet consumption following a 23hr period of food deprivation. There were no differences in body weight between LHb Cre (KO) and GFP (ctrl) mice, but there was a significant sex effect (**Figure 21A**, two-way ANOVA: sex $p<0.0001$, $F(1,8)=49.06$; virus $p=0.83$, $F(1,8)=0.051$; interaction $p=0.17$, $F(1,8)=2.32$). The amount of sucrose pellets consumed was normalized to body weight. Sucrose consumption did not differ between Cre (KO) and GFP (ctrl) mice and there was no significant effect of sex (**Figure 21B**, two-way ANOVA: sex $p=0.32$, $F(1,8)=1.14$; virus $p=0.29$, $F(1,8)=1.31$; interaction $p=0.32$, $F(1,8)=0.89$). These data suggest LHb MORs do not mediate the rewarding effects of sucrose.

We utilized oxycodone (3 mg/kg i.p.) CPP to investigate the potential role of LHb MORs in opioid reward. During CPP conditioning sessions, there was a trend for differential locomotor activity between Cre (KO) and GFP (ctrl) mice (**Figure 21C**, two-way ANOVA: treatment $p<0.0001$, $F(1,10)=58.76$; virus $p=0.14$, $F(1,10)=2.62$; interaction $p=0.23$, $F(1,10)=1.66$). Oxycodone (3 mg/kg i.p.) induced hyperlocomotion in Cre (KO) and GFP (ctrl) mice. Although not statistically significant, there was a trend for Cre (KO) mice having higher levels of locomotor activity during saline conditioning, compared to GFP (ctrl) mice (Sidak's test: saline $p=0.10$). This trend is in agreement with our open-field

locomotor assessment (**Figure 20B**) showing that Cre (KO) had higher locomotor activity, compared to controls. Following conditioning, both Cre (KO) and GFP (ctrl) mice acquired place preference for the oxycodone-paired side (**Figure 21D**, unpaired t-test p=0.61, t_{10}=0.52), suggesting deleting LHb MORs does not disrupt opioid reward. We also investigated the development of sensitization to the locomotor stimulatory effect of oxycodone, an important component of drug abuse. Because LHb Cre (KO) and GFP (ctrl) mice have differential basal locomotor activity, oxycodone-induced locomotor activity was normalized to locomotor activity during the saline session. Repeated exposure to oxycodone (10 mg/kg i.p.) resulted in locomotor sensitization, as evidenced by increased oxycodone-induced locomotor activity with repeated oxycodone administration (Tukey's test: D1 vs. D2 p=0.0002, D1 vs. D3 p=0.0007, D2 vs. D3 p=0.012). There were no differences in oxycodone locomotor sensitization between LHb Cre (KO) and GFP (ctrl) mice (**Figure 21E**, two-way ANOVA: session p=0.0003, F(1.2,11.9)=25.3; virus p=0.44, F(1,10)=0.64; interaction p=0.95, F(2,20)=0.05). Altogether, these data suggest LHb MORs are not involved with mediating the rewarding effects of sucrose or opioids.

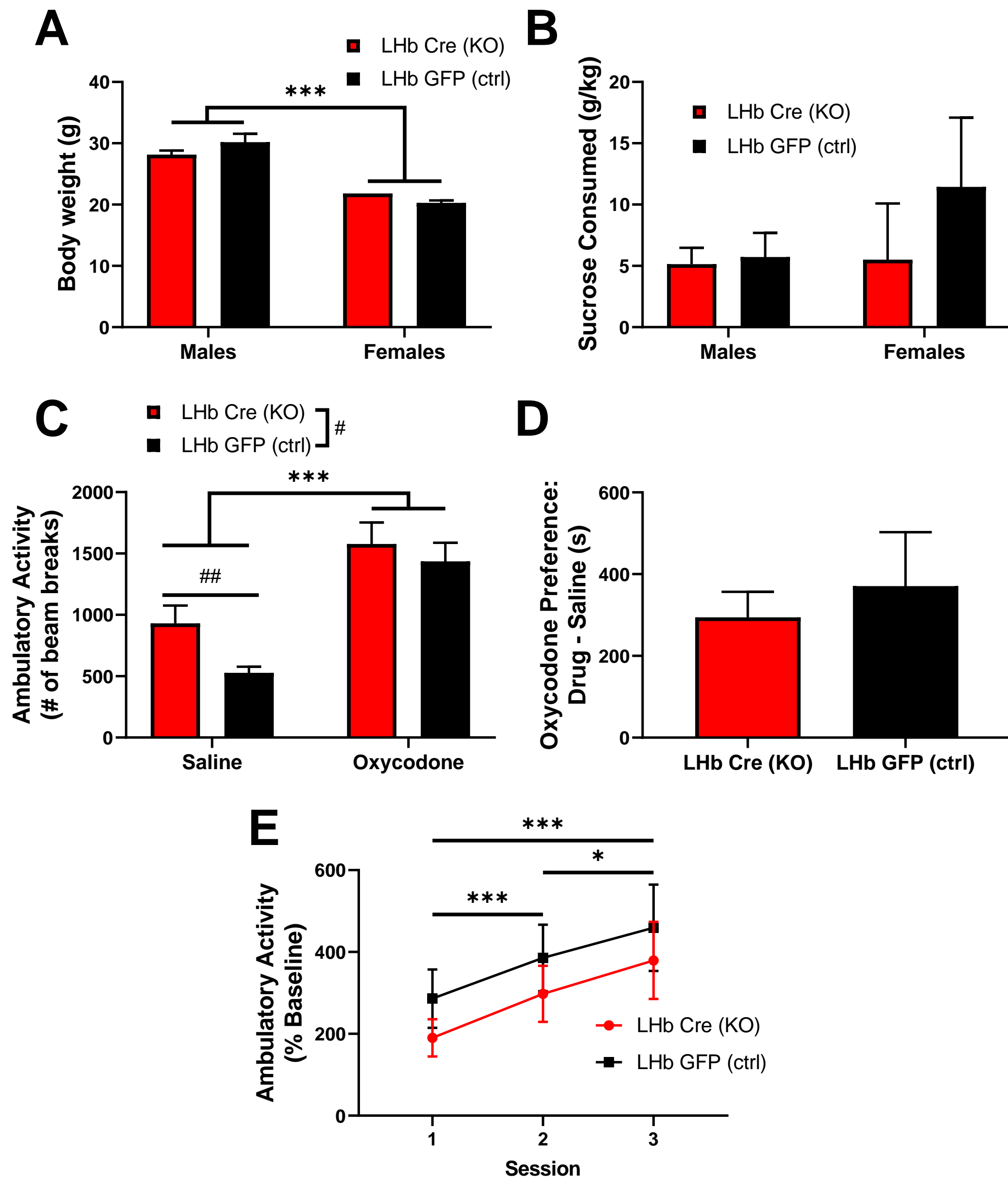


Figure 21. Deleting LHb MORs does not affect sucrose or opioid reward. (A) Male mice weigh more than females. (B) Following a single 23 hr food deprivation period, Cre (KO) and GFP (ctrl) mice consume equal amounts of sucrose, normalized to body weight. No sex effects were detected. (C) Locomotor activity during final saline and oxycodone CPP conditioning sessions. Locomotor activity was higher during oxycodone conditioning than during saline conditioning. During saline conditioning, there was a trend for higher locomotor activity in Cre (KO) mice, compared to GFP (ctrl). (D) Both Cre (KO) and GFP (ctrl) mice acquire place preference for oxycodone. (E) Both Cre (KO) and GFP (ctrl) mice develop sensitization to the locomotor stimulatory effects of oxycodone. [(*n* = 6(4M/2F) Cre (KO), 6(4M/2F) GFP (Ctrl)]. No sex effects were detected for oxycodone CPP or sensitization studies; data is collapsed by sex for these studies. # $p = 0.14$; ## $p = 0.1$; * $p < 0.05$; *** $p < 0.001$. Error bars indicate ± SEM. LHb Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 LHb injection; LHb GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG LHb injection.

3.3.8 Deleting LHb MORs alters opioid withdrawal-related behaviors

Opioid withdrawal is associated with decreased DA transmission from the midbrain, an effect that is also achieved by activation of the LHb (Clerke et al., 2021; Meye et al., 2017). In general, withdrawal from opioids and other drugs of abuse enhances LHb activity (Clerke et al., 2021; Zhang et al., 2005). Preventing or reversing withdrawal-induced LHb potentiation reduces aversive withdrawal symptoms (Clerke et al., 2021). We investigated opioid withdrawal-related behaviors in oxycodone dependent LHb MOR knockout mice and controls. Oxycodone withdrawal was precipitated by administration of naloxone (5 mg/kg i.p.). To access overall severity of withdrawal symptoms, a global withdrawal score that combines and gives equal weight to all accessed withdrawal behaviors, was calculated. Global withdrawal score was equal between LHb Cre (KO) and GFP (ctrl) mice (**Figure 22A**, unpaired t-test: $p=0.42$, $t_{10}=0.83$), but there was a trend for females to have higher global withdrawal scores than males (**Figure 22B**; two-way ANOVA: sex $p=0.096$, $F(1,8)=3.56$; virus $p=0.62$, $F(1,8)=0.27$; interaction $p=0.3$, $F(1,8)=1.23$). Interestingly, although global withdrawal score was equal between groups, individually quantified withdrawal-associated behaviors (wet dog shakes, paw shakes, and jumps) differed between Cre (KO) and GFP (ctrl) mice (**Figure 22C**, two-way ANOVA: behavior $p<0.0001$, $F(2,30)=24.29$; virus $p=0.013$, $F(1,30)=6.97$; interaction $p<0.0001$, $F(1,30)=24.76$). The quantity of wet dog shakes did not differ between groups, but Cre (KO) displayed more paw shakes than GFP (ctrl) mice (Sidak's test: wet dog shakes $p>0.99$, $t_{30}=0.18$; paw shakes $p<0.0001$, $t_{30}=7.04$). Strikingly, oxycodone withdrawal-associated jumping was completely ablated in Cre (KO) mice ($p=0.039$, $t_{30}=2.64$). These data suggest LHb MORs are involved in mediating specific withdrawal-associated behaviors, including paw shakes and jumping.

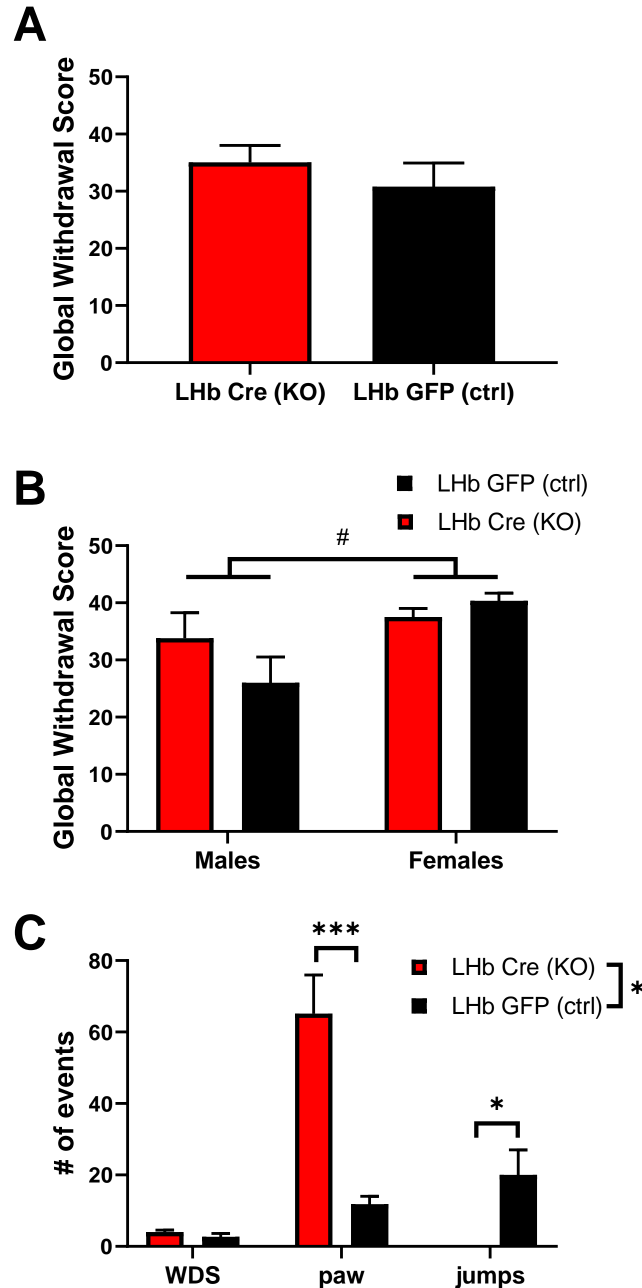


Figure 22. Deleting LHb MORs alters naloxone-precipitated oxycodone withdrawal-related behaviors. (A) Naloxone-precipitated global withdrawal score was equal in Cre (KO) and GFP (ctrl) mice. (B) There was a trend that female mice have higher naloxone-precipitated global withdrawal scores than males. (C) In mice undergoing naloxone-precipitated oxycodone withdrawal, Cre (KO) and GFP (ctrl) mice displayed equal quantities of wet dog shakes. Cre (KO) mice displayed more paw shakes than controls. Withdrawal-associated jumping was completely ablated in Cre (KO) mice. No sex effects were detected for individual withdrawal behaviors; data is collapsed by sex. [(*n* = 6(4M/2F) Cre (KO), 6(4M/2F) GFP (Ctrl)]. #p = 0.096; *p < 0.05; *** p < 0.001. Error bars indicate ± SEM. LHb Cre (KO) = pENN.AAV.CamKII.HI.GFP-Cre.WPRE.SV40 LHb injection; LHb GFP (Ctrl) = pENN.AAV.CamKII0.4.eGFP.WPRE.rBG LHb injection.

3.4 Discussion

In this study, we investigated the role of MORs of three glutamatergic brain regions: medial thalamus, AIC, and LHb. Deleting medial thalamus, AIC, or LHb MORs did not affect oxycodone reward. These regional MOR deletions did not affect locomotor activity during saline or oxycodone CPP conditioning sessions. However, deleting AIC or LHb MORs caused hyperactivity in an open-field locomotor assessment. This suggests a role of endogenous MOR signaling in the AIC and MOR in regulating locomotor activity, specifically in preventing excessive activity. Alcohol reward was also unaffected by deleting AIC MORs; although, alcohol reward was not tested in medial thalamus or LHb MOR knockout mice. In assessments of physical oxycodone withdrawal-associated symptoms, deleting AIC or LHb MORs did not affect the overall severity of oxycodone withdrawal. However, deleting LHb MORs altered specific oxycodone withdrawal-associated behaviors, including paw shakes and jumping. The major findings of these study were (1) AIC and LHb MORs are involved with regulating locomotor activity and (2) LHb MORs mediate specific opioid withdrawal-associated behaviors. It is important to note, while we were able to confirm GFP expression in medial thalamus and AIC controls (GFP (ctrl)), GFP expression was absent in medial thalamus and AIC MOR knockouts (Cre (KO)); therefore, these experiments must be repeated. Because it was unclear if cre-recombinase expression was achieved in medial thalamus and AIC Cre (KO) animals, the remainder of this discussion will focus on the results from deleting LHb MORs.

Deleting LHb MORs caused hyperactivity but did not affect oxycodone-induced locomotor activity or the development of oxycodone locomotor sensitization. A role for the LHb in locomotor activity has been established by several studies. Activation of the LHb reduces locomotor activity through indirect inhibition of midbrain DA neurons

(Hikosaka, 2010). It is possible this effect is mediated by increased activation of the RMTg, which inhibits locomotor activity (Jhou et al., 2009; Lavezzi et al., 2015). MOR activation is inhibitory; therefore, deleting LHb MORs would be expected to increase LHb activity by removing endogenous MOR inhibition. However, deleting LHb MORs caused hyperactivity, while other studies have found activating the LHb cause hypolocomotion. Deleting LHb MORs likely causes long-range changes in neural activity throughout reward neurocircuitry, including LHb-projecting regions. Therefore, it is possible these changes induced by deleting LHb MORs could result increased activation of RMTg-projecting LHb neurons. Additional studies are needed to identify specific LHb synapses modulated by MORs.

The LHb is also prominently involved with processing rewards (Graziane et al., 2018). However, we found that deleing LHb MORs did not affect sucrose or opioid reward. We used assessed sucrose consumption and used CPP to access opioid reward; however, motivational aspects are also an important component of reinforcing substances. Studies have shown deleting MORs in certain neuronal populations alters motivation to obtain opioids and palatable food reward, but does not affect opioid reward, as measured by CPP (Charbogne et al., 2017; Severino et al., 2020). To access the role of LHb MORs in motivation to obtain sucrose pellets and opioids, operant self-administration can be used, including assessments of breaking point and cue-induced reinstatement.

The LHb encodes aversive information and may be involved with the aversive effects of drug withdrawal (Clerke et al., 2021; Graziane et al., 2018; Sánchez-Catalán et al., 2017). We found that deleting LHb MORs did not affect the overall severity of opioid withdrawal-associated symptoms but did alter specific withdrawal behaviors. Paw shakes were increased in mice with deleted LHb MORs and withdrawal-induced jumping

was completely ablated. Additional experiments are needed to interpret these effects. Withdrawal increases LHb activation, which is associated with hypolocomotion; therefore, ablation of jumping in LHb MOR knockouts could represent hypolocomotion. The LHb is also involved with depressive-like and escape behaviors (Lecca et al., 2017), suggesting ablation of withdrawal-induced jumping could represent or reduced escape behaviors. We assessed the somatic component of opioid withdrawal; however, MORs also mediate the motivational, aversive component of withdrawal (see Section 1.1.3). A recent study found deleting MORs in a subset of neurons in the medial habenula attenuated naloxone conditioned place aversion and physical withdrawal symptoms in morphine-dependent mice. Therefore, additional studies will examine naloxone conditioned place aversion in oxycodone-dependent mice.

It is important to note that our intent of this study was to identify behavioral roles of MORs in glutamatergic brain regions, the use of stereotaxic intracranial viral injections deletes MORs in all types of cells in the target region. Although the brain regions investigated are primarily glutamatergic, it is possible MORs in other cell types may also mediate behaviors. In the LHb, a small population of GABA neurons has been identified that is involved with stress-induced motivational responses (Zhang et al., 2018). Additionally, viral vector diffusion could delete MORs in neighboring brain regions and potentially mediate behaviors. Indeed, some of our LHb MOR knockout mice showed small amounts of GFP expression in the hippocampus, medial habenula, and paraventricular thalamic nucleus. Future studies will test the behavioral effect of deleting MORs in these neighboring brain regions to ensure the effects seen in LHb MOR knockout mice are specific to the LHb.

In conclusion, we have demonstrated MORs in LHb neurons modulate locomotor activity and certain opioid withdrawal-related behaviors. Additional work is needed to determine whether LHb MORs are involved in motivational aspects of reward or opioid withdrawal. Because we were unable to detect GFP expression in medial thalamus and AIC MOR knockout mice, our experiments assessing the role of MORs in these regions must be repeated to ensure MORs were deleted from these regions. Additionally, the studies investigating the role of MORs in other glutamatergic neuronal populations, such as the lateral hypothalamus, are needed to fully understand how MORs throughout glutamatergic neurocircuits mediate behaviors.

CHAPTER 4

Conclusion

4.1 General Summary

The research in this dissertation investigated the behavioral relevance of MORs in distinct glutamatergic neuronal populations. To characterize the role of MORs in a subset of glutamatergic neurons that express vGluT2, we used conditional MOR knockout mice (MORflox-vGluT2cre). We utilized stereotaxic viral intracranial injections to specifically delete MORs in several glutamatergic brain regions of interest: medial thalamus, AIC, and LHb. These brain regions were chosen due to being primarily glutamatergic, modulated by MORs, and involved with reward and drug abuse.

Deleting MORs in vGluT2-expressing neurons disrupts opioid reward, locomotor stimulation, and withdrawal, but does not affect alcohol reward, sucrose consumption, or opioid-induced antinociception

MORs regulate glutamate transmission, but the behavioral role of MORs in glutamatergic neurons has been poorly explored. We utilized a conditional MOR knockout mouse (MORflox-vGluT2cre) to characterize the behavioral role of MORs in vGluT2-expressing neurons. In Cre+ (KO) mice, specific MOR deletion in vGluT2-expressing neurons was confirmed via qPCR. To functionally confirm MOR knockout in vGluT2 neurons, we used whole cell patch clamp electrophysiology. MOR activation inhibited optically stimulated glutamate transmission from vGluT2-expressing thalamostriatal synapses in Cre- (ctrl) mice; this effect was completely ablated in Cre+ (KO) mice.

To access the role of MORs in vGluT2 neurons, we used a variety of behavioral assays, including those used to measure reward, anxiety, locomotor activity, nociception, and withdrawal. These behavioral assays were chosen due to the known role of MORs in mediating these behaviors (Kieffer & Gaveriaux-Ruff, 2002). Opioid reward was measured using CPP, a test of environmental association, and a more translational self-administration model, 2BC drinking. Cre+ (KO) mice do not acquire conditioned place preference for a low doses (0.05, 0.5 mg/kg) of oxycodone, but acquire conditioned place aversion for a higher dose (5 mg/kg); whereas, control mice acquire conditioned place preference for all tested doses. In 2BC consumption assessment, Cre+ (KO) mice consume less and have reduced preference for oxycodone over water, compared to controls. These data suggest deleting MORs in vGluT2-expresing neurons disrupts opioid reward and may enhance opioid aversion at higher doses. MORflox-vGluT2cre mice also lack oxycodone-induced locomotor stimulation, but have no basal differences in locomotor activity, compared to controls. Other MOR-mediated behaviors were unaffected by deleting MORs in vGluT2-expressing neurons. Although MORs are also involved in mediating the rewarding effects of other drugs of abuse, including alcohol, deleting MORs in vGluT2-expressing neurons does not affect alcohol reward. Deleting MORs in vGluT2 neurons also does not affect consumption of the natural reward, sucrose. These results suggest MORs in vGluT2 neurons may be specifically involved in opioid reward; however, other drugs of abuse (i.e. marijuana, cocaine) and natural rewards (i.e. high fat diet, social interaction) still need to be tested.

Additionally, opioid-induced antinociception, anxiety, and stress responses did not differ in Cre+ (KO) mice, compared to controls. Oxycodone-dependent vGluT2 MOR knockout mice had altered withdrawal-like responses, compared to controls. Interestingly, MORflox-vGluT2cre mice display baseline withdrawal-like responses following the

development of oxycodone dependence that are not seen in controls. In addition, unlike controls, withdrawal-like responses do not increase following challenge with the opioid antagonist, naloxone. Additional experiments are needed to understand why deleting MORs in vGluT2 neurons causes basal withdrawal-like responses, with no change in withdrawal-like responses following naloxone administration. Overall, these data reveal that MOR modulation of glutamate transmission is a critical component of opioid reward, locomotor stimulation, and withdrawal.

Effects of deleting MORs in specific glutamatergic brain regions: thalamus, AIC, and LHb

We stereotaxically injected an AAV vector encoding cre-recombinase into the medial thalamus, AIC, or LHb of MORflox mice to specifically delete MORs in these brain regions. We recently identified a role of MORs in vGluT2-expressing neurons in modulating opioid reward (see Chapter 2); however, it is unknown which vGluT2 neuronal populations mediate this effect (Reeves et al., 2021). The thalamus has high vGluT2 expression, is modulated by MORs, and is involved with reward and drug abuse (Cover et al., 2019; Li et al., 2018; Munoz et al., 2018; Smith et al., 2004). To determine if thalamic MORs mediate opioid reward, we used a viral approach to delete medial thalamus MORs, specifically targeting striatum-projecting intralaminar and midline nuclei. Deleting thalamic MORs did not affect opioid reward and locomotor stimulation. We have recently shown MORs modulate glutamate transmission from the AIC (Munoz et al., 2018), a brain region that has begun receiving much attention for its role in drug abuse, reward processing, and decision making (Benarroch, 2019; Huettel, 2006; Koob & Volkow, 2016). Deleting AIC MORs did not affect alcohol or sucrose consumption, opioid reward, opioid-induced locomotor stimulation or sensitization, or opioid withdrawal. However, deleting AIC MORs did result in basal hyperlocomotion.

The vGluT2-expressing LHb is known for encoding aversive stimuli and sends projections to the classical opioid reward circuitry (RMTg, VTA) (Baker et al., 2016; Balcita-Pedicino et al., 2011; Ji & Shepard, 2007; Matsumoto & Hikosaka, 2007). We investigated the role of LHb MORs in opioid-related behaviors using a viral approach to delete LHb MORs. Deleting LHb MORs resulted in basal hyperlocomotion, but did not affect opioid reward, opioid-induced locomotor stimulation, or opioid locomotor sensitization. Deleting LHb MORs did not affect overall quantity of withdrawal-like responses but did alter responses. LHb MOR knockout mice had increased withdrawal-induced paw shakes, but ablated withdrawal-induced jumping, compared to controls. Additional experiments are needed to understand how deleting LHb MORs alters opioid withdrawal-like responses. Overall, we characterized previously unidentified brain region-specific roles of MORs in modulating basal locomotor activity (AIC, LHb) and opioid withdrawal-associated behaviors (LHb).

4.2 Significance

Our data challenges the classical model of opioid reward, that MORs in GABA neurons are the primary drivers of opioid reward. We have found that MORs in glutamate neurons also play a critical role in opioid reward, as well as opioid-induced locomotor stimulation and withdrawal. Because MORs in both GABA and glutamate neurons are both involved with opioid reward, the rewarding effects of opioids are likely mediated through a complex interplay between GABA and glutamate neurons. Additionally, we have shown that MORs in different glutamate neuronal populations (vGluT2-expressing, AIC, LHb) mediate differential opioid-related behaviors. These data demonstrate the need to characterize brain region- and neurocircuit-specific effects of MORs to fully understand how MORs mediate opioid-related effects. The research in this dissertation

has advanced the field of opioid addiction by increasing the understanding of how MOR modulation of glutamate transmission mediates opioid behaviors. By identifying specific MOR-modulated brain regions and neurocircuits that mediate unwanted opioid effects (i.e. reward, tolerance, withdrawal), it is possible new molecular targets could be identified for the development of prevention and treatment of opioid abuse. As technology and drug delivery methods advance, it is possible an opioid drug could be developed that specifically target MORs that modulate antinociception, but not reward, avoiding the negative consequences and high abuse potential of opioids.

4.3 Limitations

Our studies identifying the behavioral role of MORs in vGluT2-expressing neurons utilized a conditional MOR knockout in all vGluT2-expressing neurons. As previously discussed, these MORflox-vGluT2cre mice are useful in identifying the role of MORs in a subset of glutamatergic neurons; however, it is unknown which vGluT2 neurons are involved in these MOR-mediated behaviors. Another limitation of these mice is that vGluT2 is highly expressed during development; therefore, it is possible MORs are deleted in neurons that do not express vGluT2 in adult mice, including non-glutamatergic neurons. One region that expresses vGluT2 only during development is the cortex. We showed MOR expression was not reduced in the cortex of MORflox-vGluT2cre mice, suggesting MORs were not deleted from these neurons during development. Nevertheless, it is a possibility that must be considered. Additionally, vGluT2 is expressed in some neurons that co-express other neurotransmitters, including GABA and DA. It is possible the behavioral effects seen in MORflox-vGluT2cre mice are due to disruptions of MOR-mediated inhibition of these co-expressed neurotransmitters. It is unlikely these neurons mediate the effects seen in MORflox-vGuT2cre mice, as these

neurons represent small subpopulations or are located in brain regions not typically associated with reward or opioid withdrawal. Nevertheless, it is a possibility that these neurons play a role in the behavioral alterations observed in MORflox-vGluT2cre mice.

Studies characterizing the role of MORs in specific glutamatergic brain regions utilized an AAV vector encoding cre-recombinase and the fluorescent protein marker, GFP. Although, an 8-week transfection period was used to ensure complete MOR deletion in the target region, the quantity of MOR deletion was not directly tested. GFP expression was confirmed in the target region following behavioral testing, with the exception of medial thalamus and AIC MOR knockouts. For thalamic and AIC MOR knockouts, we were unable to detect GFP expression in Cre (KO) mice; therefore, it is unclear whether expression of cre-recombinase was achieved using this viral vector. If cre-recombinase expression was not achieved, MORs would not have been deleted in the target brain region of Cre (KO) mice. Therefore, all experiments in thalamic and AIC MOR knockouts need to be repeated and proper cre-recombinase expression should be confirmed for all Cre (KO) mice following behavioral testing. For LHb MOR knockouts, mice with poor or misplaced GFP expression were excluded from analyses. To ensure complete MOR deletion, following behavioral experiments, RNA should be isolated from the target brain region to quantify MOR expression using qPCR. It is possible for some mice to have reduced MOR deletion due to inefficient transfection or reduced viral volume due to the syringe being partially occluded. The remaining MOR expression in the target region could prevent or attenuate the effects seen in mice with complete MOR deletions. Additionally, viral transfection can spread to neighboring brain regions. MOR deletion in neighboring brain regions could mediate behavioral effects, rather than MORs deleted in the target region. As a control for this, mice injected with the viral vector in brain regions neighboring the target region should also be behaviorally assessed. Additionally,

because our research has utilized mice, it is unknown whether our findings apply to other species (i.e. rats, primates, humans).

4.4 Future Directions

Additional follow-up experiments are needed to fully interpret some of our behavioral findings. MORflox-vGluT2cre mice acquired conditioned place aversion to a higher dose of oxycodone (5 mg/kg) but not lower doses, suggesting this higher dose of oxycodone is aversive to MORflox-vGluT2cre mice. This dose of oxycodone is commonly used in opioid conditioned place preference assessments and is rewarding to wildtype mice (Reeves et al., 2021). It is unknown why MORflox-vGluT2cre mice find this dose of oxycodone aversive. Additionally, MORflox-vGluT2cre mice display baseline withdrawal-like responses following oxycodone dependency, prior to naloxone-precipitated withdrawal. It is possible MORflox-vGluT2cre mice experience spontaneous opioid withdrawal at a faster rate than controls, possibly due to differences in oxycodone metabolism. Future studies should characterize the timeline of spontaneous opioid withdrawal and oxycodone metabolism in these mice. Another possibility is MORflox-vGluT2cre mice experience aversive withdrawal-like responses or a negative affective state following higher doses of oxycodone, which may explain why MORflox-vGluT2cre mice acquire conditioned place aversion to a higher dose of oxycodone.

Our studies have characterized behavioral responses to the opioid, oxycodone, and in some cases, alcohol. Other drugs of abuse, including other opioids (i.e. morphine, heroin) should be tested. MORs modulate several behaviors that we have not tested, including social interactions, respiration, and mating behaviors. Future studies should investigate potential roles of MORs in glutamatergic neurons in these behaviors.

Additionally, the behavioral role of MORs in other glutamatergic neuronal populations should also be tested. Stereotaxically injecting an AAV encoding cre-recombinase into MORflox mice deletes MORs in all neurons in the target brain region; therefore, this approach cannot be used to determine the role of MORs in glutamate neurons in heterogenous brain regions, comprised of multiple cell types (i.e. VTA, ventral pallidum, lateral hypothalamus). To determine the role of MORs in glutamate neurons in heterogenous brain regions, an AAV encoding cre-recombinase under the control of the vGluT2 promotor will be used.

Another important step is to identify specific glutamatergic synapses that are modulated by MORs, as well as the mechanisms of these effects (i.e. presynaptic MORs, direct effects of postsynaptic MORs, multisynaptic effects). This is a large task, as MORs are found in glutamatergic neuronal populations throughout the nervous system and regional, cell-type, input-, and projection- specific effects are possible. Additionally, drug exposure and stress can alter MOR signaling. To accomplish this goal, a combinational approach utilizing stereotaxic viral injections, optogenetics, and whole-cell patch clamp electrophysiology will be used to identify synapse-specific MOR modulation of glutamate transmission, as previously demonstrated by the Atwood lab (Munoz et al., 2018; Muñoz et al., 2020). Behavioral effects of MORs in these MOR-modulated glutamatergic neurocircuits can be identified using viral and genetic approaches (i.e. chemogenetics and optogenetics). Fully undercovering the complex circuitry of synapse-specific MOR modulation of glutamate transmission and opioid-related behaviors may lead to the identification of new therapeutic targets and strategies to treat and prevent opioid abuse.

4.5 Final Discussion

The work presented in this dissertation provides novel evidence that MORs in specific glutamatergic neuronal populations modulate opioid-related behaviors, including reward, locomotor activity, and withdrawal. Using a conditional MOR knockout mouse, we identified roles of MORs in a subset of glutamatergic neurons that express vGluT2 in modulating opioid reward, locomotor stimulation, and withdrawal. Furthermore, we identified roles of MORs in specific glutamatergic brain regions in locomotor activity (AIC, LHb) and opioid withdrawal (LHb). Overall, this research has increased our understandings of the mechanisms and neurocircuitry involved in opioid-related behaviors, specifically opioid reward and withdrawal, critical components of opioid abuse. I hope that this research will contribute to the identification of novel therapeutic targets and strategies for the treatment and prevention of opioid use disorder.

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CURICULUM VITAE
Kaitlin C. Reeves

Education

- Indiana University
  (August 2017- October 2021)
    - Program: Indiana University BioMedical Gateway (IBMG) Program
    - Degree: PhD in Medical Neuroscience
    - 3.9 GPA
    - Relevant coursework:
        - Experimental methods and animal models for neuroscience disorders
        - Synaptic neurotransmission and plasticity
        - Neurochemistry and neurodevelopment
        - Systems neuroscience and neuroanatomy
        - Intracellular signal transduction
        - Statistical inference and biostatistics
        - Genetics and molecular biology
        - Cellular basis of systems biology
        - Experimental design and grant writing
        - Biochemistry

- Indiana University- Purdue University, Indianapolis (IUPUI)
  (August 2013- May 2017)
    - Bachelor of Arts in Biology
    - Minors- Chemistry, Psychology
    - 3.8 GPA
    - Relevant coursework:
        - Abnormal psychology
        - Social psychology
        - Child & adolescent psychology
        - Immunology- lecture and laboratory
        - Cell biology- lecture and laboratory
        - Microbiology- lecture and laboratory
        - Human anatomy and physiology- lecture and laboratory
        - Organic chemistry- lecture and laboratory
        - Genetics and molecular biology
        - Analytical chemistry
        - Data analysis using spreadsheets

Professional Experience

- Indiana University School of Medicine (IUSM)
    - *PhD candidate in Dr. Brady Atwood's Lab*
      (January 2018- present)
        - Research Focus: Cell-type and brain-region specific contributions of opioid receptors in opioid reward
        - Created a transgenic mouse model to delete mu opioid receptors in a subset of glutamatergic neurons

        - Utilized a battery of mouse behavioral assays to characterize opioid-related behaviors in the transgenic mouse model
        - Performed stereotaxic intracranial viral injections to delete mu opioid receptors in specific brain regions
        - Collaborated with the university's behavioral phenotyping core to obtain data using new behavioral assays for the lab: pre-pulse inhibition and shock-flinch antinociception
        - Developed new techniques for the lab: mouse tail flick and plantar paw withdrawal assessment of antinociception, conditioned place preference for opioids
        - Utilized interesectional virus intracranial injection approach to express hTau into dorsolateral striatum-projecting anterior insular cortex neurons for a collaboration with a lab studying the role of tau protein in neurodegenerative diseases
        - Trained and mentored 12 students and staff on a variety of mouse behavioral and surgical techniques
    - *Graduate Student in Dr. William Truitt's* Lab
      (March 2018 - May 2018)
        - Assisted lab members in performing various social interaction behavioral experiments in rats
        - Prepared samples for lab members by slicing, mounting, and staining rat brains
    - *Graduate Student in Dr. Christopher Robinson's Lab*
      (Oct. 2017 - Dec. 2017)
        - Developed an in-vitro model to test the effect of hormones on enteric virus infection in HeLa cells that the lab continues to use
        - Utilized qPCR to obtain data that hormones affect viral infection, providing rational for a graduate student's research

- Health and Science Innovations
    - *Research Assistant, Program Coordinator, Mentor*
      (Jan. 2017 - Aug. 2017)
        - Aided in design and revision of experimental protocol regarding the correlation of ADHD symptom severity and estrogen levels
        - Collaborated with Pearson to obtain specialized quantitative ADHD diagnostic tool
        - Presented experimental protocol and rational to community members and members of the organization
        - Submitted research protocol to IRB for approval
        - Trained, supervised, and directed 4 staff members and approximately 20 volunteers
        - Developed biology and chemistry workshops for high school students
        - Mentored and advised approximately forty high school students, regarding their research projects
        - Planned and coordinated a summer research program for high school students interested in science and medicine
        - Engaged the community, networked, and promoted the organization
        - Presented a workshop and demonstration on various laboratory equipment and techniques to high school students

- Clinical and Translational Support Laboratory (CTSL) of Indiana CTSI
    - *Laboratory Technician*
      (September 2014 - January 2016)
        - Utilized protocol instructions to properly assemble supplies for various studies
        - Processed confidential medical information into data entry system
        - Processed samples in accordance to the protocol's specifications
        - Utilized Excel to accurately input billing information

- Indiana University- Purdue University, Indianapolis (IUPUI)
    - *Laboratory Assistant of Dr. Jiliang Li*
      (January 2016 - May 2016)
        - Analyzed electron microscopy images of cultured cells to obtain data for a graduate student's research
        - Maintained mouse colony utilizing gel electrophoresis and PCR to genotype mice
        - Cultured and sub-cultured cells in a sterile environment to avoid contamination
    - *Bepko Learning Center Academic Success Coach*
      (January 2015 - May 2015)
        - Mentored and organized regular meetings with students on academic probation to set goals, develop personalized action plans, and discuss progress
        - Acquired and distributed information regarding campus and professional resources to students

Publications

- Grecco GG, Mork BE, Huang JY, Metzger CE, Haggerty DL, **Reeves KC**, Gao Y, Hoffman H, Katner SN, Masters AR, Morris CW, Newell EA, Engleman EA, Baucum AJ, Kim J, Yamamoto BK, Allen MR, Wu Y, Lu H, Sheets PL, Atwood BK. Prenatal methadone exposure disrupts behavioral development and alters motor neuron intrinsic properties and local circuitry. *eLife*. (2021). PMID: 33724184
- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Gao Y, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons modulate opioid reward. *Addiction Biology*. (2020). PMID: 32686251
- Haggerty DL, Grecco GG, **Reeves KC**, Atwood BK. Adeno-associated viral vectors in neuroscience research. *Molecular Therapy Methods & Clinical Development*. (2019). PMID: 31890742
- Grecco, GG, Haggerty DL, **Reeves KC**, Gao Y, Maulucci D, Atwood BK. Prenatal opioid exposure reprograms the behavioral response to future alcohol reward. *Submitted to Neuropsychopharmacology.*
- Reeves KC, Shah N, Muñoz B, Atwood BK. Opioid receptor-mediated synaptic plasticity in the central nervous system. *In preparation*.

Skills

- Stereotaxic surgical procedures in mice and rats
- Behavioral assays and analysis in mice
    - Conditioned place preference (CPP)
    - Operant conditioning
    - Social interaction/recognition
    - Tail flick, thermal plantar, hot plate, and shock-flinch model of antinociception
    - Open field locomotor assessment
    - Light-dark box anxiety test
    - Pre-pulse inhibition
    - Two-bottle choice drinking
    - Opioid withdrawal
    - Opioid locomotor sensitization
- Perfusion, slicing, mounting, and staining of brain tissue
- DNA, RNA, and protein purification and quantification, including gel electrophoresis, blots, PCR, and qPCR
- Immunohistochemistry
- Enzyme-linked immunosorbent assay (ELISA)
- Experimental design in neuroscience and microbiology
- Sterile culturing and sub-culturing of cell cultures
- Data and statistical analysis using Excel, GraphPad Prism, and SPSS

Awards and Honors

- Trainee Professional Development Award, Society for Neuroscience, 2020
- Pre-doctoral NIAAA training grant, T32 AA07462, 2019 - present
    - Genetic Aspects of Alcoholism
- Medical Neurosciences Graduate Program Travel Award, IUSM, 2019
- Paul & Carole Stark Fellowship Recipient, 2018 - 2019
- Bepko Scholars and Fellows Recipient, 2013 - 2021
- Mentor of the Year Award, Health and Science Innovations, 2017
- IUPUI Honors College Scholar, 2017

Professional Memberships

- Member of International Narcotics Research Conference (INRC), 2019 - present
- Member of Society for Neuroscience (SfN), 2019 - present
- Member of Research Society on Alcoholism (RSA), 2019 - present
- Member Alpha Lambda Delta/Phi Eta Sigma (ALD/PES) honor society, 2014 - 2017

Teaching Experience

- Indiana University School of Medicine (IUSM)
    - *Instructor*: graduate-level course
      (September 2019)
        - Course: Synaptic Plasticity in the Central Nervous System

        - Lecture: mechanisms of long-term potentiation with an emphasis on postsynaptic changes in AMPA receptor function.

- Indiana University- Purdue University Indianapolis (IUPUI)
    - *Chemistry and Biology Tutor*
      (January 2015 - May 2016)
        - Developed academic strategies to improve self-sufficiency of students
        - Provided instruction and mentoring to students based on their needs
    - *Chemistry Recitation Leader*
      (August 2014- December 2014)
        - Redirected students to encourage higher level learning, critical thinking, and group discussion
        - Prepared learning activities, instructions, and assessment tools

Abstracts and Presentations

Talks

- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors in vGluT2-expressing glutamatergic neurons modulate opioid reward. Seminar presented at *Stark Neurosciences Research Institute, Indiana University School of Medicine*. (2021, January). Virtual seminar.
- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons modulate opioid reward. Oral presentation given at *International Narcotics Research Conference*. (2020, July). Virtual conference.
- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman H.J, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons modulate opioid aversion. Seminar given at *Indiana University- Purdue University Indianapolis (IUPUI) Addiction Neuroscience Seminar*. (2020, March). Indianapolis, IN.
- **Jones KC**, Kube MJ, Haggerty DL, Fritz BM, Munoz B, Yin F, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons regulate aversion to oxycodone. Oral presentation given at *Stark Summer Science Symposium*. (2019, July). Indianapolis, IN.

Posters

- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors in vGluT2-expressing glutamatergic neurons modulate opioid reward. *Greater Indiana Society for Neuroscience*. (2021, April). Virtual meeting.
- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors in vGluT2-expressing glutamatergic neurons modulate opioid reward. *Society for Neuroscience Global Connectome*. (2021, January). Virtual meeting.
- **Reeves KC**, Kube MJ, Grecco GG, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors on vGluT2-expressing

glutamatergic neurons modulate opioid reward. *Stark Summer Science Symposium*. (2020, October). Virtual symposium.

- **Reeves KC**, Kube MJ, Haggerty DL, Fritz BM, Muñoz B, Yin F, Haggerty DL, Hoffman HJ, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons regulate aversion to oxycodone. *Society for Neuroscience (SfN) annual meeting*. (2019, October). Chicago, IL.
- **Jones KC**, Kube MJ, Haggerty DL, Fritz BM, Muñoz B, Yin F, Atwood BK. Mu opioid receptors on vGluT2-expressing glutamatergic neurons regulate aversion to oxycodone. *Gill Symposium*. (2019, September). Bloomington, IN.
- **Jones KC**, Fritz BM, Muñoz B, Atwood BK. Synapse-specific contributions of striatal mu opioid receptors in opioid and alcohol abuse. *Greater Indiana SfN meeting*. (2019, March). Indianapolis, IN.
- **Jones KC**, Fritz BM, Muñoz B, Atwood BK. Synapse-specific contributions of striatal mu opioid receptors in opioid and alcohol abuse. *Gill Symposium*. (2018, September). Bloomington, IN.
- **Jones KC**, Fritz BM, Muñoz B, Atwood BK. Synapse-specific contributions of striatal mu opioid receptors in opioid and alcohol abuse. *Indiana University School of Medicine Graduate Programs Poster Showcase*. (2018, August). Indianapolis, IN.
- **Reeves KC**, Orr R. Clinical and Translational Support Laboratory. *Life-Health Sciences Internship poster session*. (2015, April). Indianapolis, IN.