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Investigating the Role of Mu Opioid Receptors on Dopamine D1 Receptor Expressing Neurons in the Nucleus Accumbens

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A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Integrative Biology and Physiology

by

Lorna Barrall

2023

ABSTRACT OF THE THESIS

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Lorna Rose Barrall

Master of Science in Integrative Biology and Physiology

University of California, Los Angeles, 2023

Professor Patricia Phelps, Co-Chair

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Mu opioid receptor (MOR) activation has important effects on reward circuits through its modulation of dopamine release in the ventral striatum. Studies suggest that dopamine is also important in chronic pain states where D2/D3 agonists can alleviate pain (Faramarzi et al. 2016). It is also known that MORs in the striatum are highly expressed (Mansour et al. 1995) but it remains unclear to what extent their expression in the striatum contributes to opioid-mediated analgesia.

My dissertation research aimed to determine the role of MORs expressed on D1 receptor containing medium spiny neurons located in the nucleus accumbens shell in opioid reward and opioid reinforcement in chronic pain states. Unpublished research in the Cahill Lab indicated that deletions of MORs from D1 neurons ablated opioid-induced analgesia in the formalin test and in a model of neuropathic pain. In my study, I postulate that MORs on D1- receptor containing neurons in the nucleus accumbens shell are necessary and

sufficient to produce opioid induced analgesia in a chronic pain model. I used a chronic constriction injury model of neuropathic pain that produced mechanical allodynia within a week of nerve injury. I used genetically modified mice where D1 receptor-cre mice were bred with MORloxP mice to ablate MOR from the D1 receptor expressing neurons. Re-expression of MOR into the nucleus accumbens shell was achieved by bilateral injection of AAVDJ-hSyn1-DIO-mCherry-2A-MOR. The control virus, AAVDJ-hSyn-DIO-mCherry, was used to assess the effects of MOR reinsertion by the active virus. Behavioral outcomes were assessed via acute thermal threshold testing using the hot plate test and opioid preference using conditioned place preference tests.

I established that the ablation of MORs from these neurons had no effect on opioid-mediated analgesia in an acute thermal test. I also re-produced previous data that the absence of MORs from D1 receptor expressing neurons prevented opioid-mediated conditioned place preference in both naive and neuropathic pain animals. This study also found no significant differences in the behaviors of animals between the active and control virus conditions. Immunohistochemical experiments were conducted to confirm viral expression and target. Control virus showed expression in the nucleus accumbens shell as expected, however, the active virus showed no expression suggesting that the virus was not active. This study identified that opioid-mediated analgesia in acute threshold tests does not require the expression of MOR in D1 receptor containing neurons. It also identified that MOR in D1 receptor expressing neurons was necessary for opioid-mediated reward and negative reinforcement. However, due to problems with the virus, we were not able to identify site specificity of this effect.

This thesis of Lorna Rose Barrall is approved.

David Glanzman

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Catherine Cahill, Committee Co-Chair

University of California Los Angeles

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Abbreviations

CDC	Centers for Disease Control
D1	Dopamine type 1 receptor
D2	Dopamine type 2 receptor
D3	Dopamine type 3 receptor
D4	Dopamine type 4 receptor
D5	Dopamine type 5 receptor
GABA	γ-Aminobutyric acid
GPCRs	G Protein Coupled Receptors
MOR	Mu opioid receptor
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NMDA	N-Methyl-D-aspartic acid
PAG	Periaqueductal gray
SUD	Substance use disorder
VTA	Ventral tegmental area

Chapter 1: Introduction and Literature Review

Acute Pain

Pain can save our life, or it can destroy its quality. Most of us understand the concept of pain without recognizing its complexity; most of which lies in our nervous systems. Peripheral neurons, the spinal cord, and the brain work in concert to respond to acutely painful stimuli such as a cut, burn, surgery, or illness. Acute pain is evolutionarily beneficial, allowing us to react to and escape dangerous situations (Hughes 2012). Acute pain is defined by its resolution with healing. However, there are instances where prolonged pain can develop into something else entirely. We call this chronic pain.

Acute pain begins at the nociceptors, peripheral neurons such as C fibers or alpha-delta fibers, that transmit information about painful stimuli from our periphery to our central nervous system (McMahon et al. 2014). Nociceptors will synapse in the dorsal horn of our spinal cord on interneurons or directly on ascending pain pathways which consist of wide dynamic range neurons that ascend via various tracts to the brain. Ascending pain pathways, such as the spinoreticular thalamic tract convey peripheral pain information to the brainstem and eventually to the brain for rapid processing and response (McMahon et al. 2014). Structures such as the periaqueductal gray (PAG), amygdala, rostroventromedial medulla, and cortex receive these inputs and will activate our descending pain pathways and endogenous opioid system (McMahon et al. 2014). Regions such as the PAG and rostroventromedial medulla can release endogenous opioid peptides and serotonin, respectively. These two regions along with noradrenergic projections from the locus coeruleus compose our descending pain pathways. Endogenous opioids, serotonin, and noradrenaline released by these descending neuronal projections can inhibit our ascending pain pathways thus reducing our perception of an acute painful stimulus referred to as the

descending noxious inhibitory control or conditioned pain modulation system (McMahon et al. 2014).

Chronic Pain and its Mechanisms

Acute pain, although more complex than previously described, is well understood in comparison to chronic pain. Chronic pain is generally defined as pain that persists for three months or longer and may have various origins and degrees of intensity (Hughes 2012). Importantly, chronic pain will subsist despite healing or may evolve despite evidence of pathology. Many factors play a role in the development of chronic pain, some of the main theories surround mechanisms of central and peripheral sensitization. Peripheral sensitization is a result of the upregulation of inflammatory factors near the site of injury. This build-up of molecules including histamine, nerve growth factor, bradykinin, protons, and others will lower the firing threshold of nociceptive neurons (McMahon et al. 2014). A low threshold may result in allodynia, defined as a painful response to a normally neutral stimulus. It may also result in hyperalgesia, an enhanced or exaggerated response to a normally painful stimulus. Sensitization of nociceptive neurons results from molecular changes. These changes can include the upregulation of receptors or ion channels important in the transduction of pain information.

Another mechanism of peripheral sensitization includes changes in nociceptive neurons surrounding the primary location of pain or injury. The primary location of pain is associated with primary hyperalgesia or an increased pain sensitivity at the site of injury. However, the perceived painful area can be expanded in peripheral sensitization leading to secondary hyperalgesia. The expanded area of decreased pain threshold is due to the increased size of the receptive field, which is an area of the body that when stimulated will activate nociceptors. The mechanisms of secondary hyperalgesia are similar to primary

hyperalgesia with the exception that changes are occurring in nearby, but not directly affected, nociceptors (McMahon et al. 2014).

Central sensitization is another critical molecular mechanism in the development of chronic pain from an acutely painful stimulus. Central sensitization occurs when there is an enhancement or over-excitability in nociceptive pathways in the central nervous system. This hyper-excitable state leads to activation in neurons that under normal conditions are relatively quiescent (Latremoliere and Woolf 2009). There are many physiological mechanisms by which central sensitization can occur including enhanced synaptic transmission, increased neuroplasticity, sensitization of ion channels, and activation of glial cells. Synaptic transmission is enhanced via an increased release of excitatory neurotransmitters such as substance P and glutamate. Enhancement in the release of excitatory neurotransmitters is coupled with a decreased release of inhibitory neurotransmitters such as glycine and GABA. These changes lead to a greater perception of pain by the brain. Over time, changes in the neuronal firing and the molecular environment may lead to density and strength of synapse alterations in nociceptive pathways. Altered synaptic transmission resulting in neuroplasticity is driven by many molecular mechanisms namely, the sensitization of ion channels such as the NMDA receptor. Finally, central sensitization can be characterized by functional alterations in glial cells such as increased release of inflammatory cytokines which may alter perceptions of pain (Latremoliere and Woolf 2009).

Although peripheral and central sensitization can play an important role in the development of chronic pain from an acute injury, they are certainly not the only factors that contribute to this process. Many environmental factors such as early life stress events, genetic variations, and even psychology can predispose some individuals to develop chronic pain while others do not.

Risk Factors for Chronic Pain Development

Both central and peripheral sensitization play an important role in the development of chronic pain but cannot explain the entire pathology. Psychological, genetic, and environmental factors are also important in the development of chronic pain. Certain psychological measures such as the presence of anxiety and depression, high catastrophizing scores, high fear avoidance scores, stress, and high social isolation are correlated with higher rates of chronic pain (Seth and DeGray 2019). These psychological variations may alter an individual's ability to cope with pain, explaining why some individuals develop chronic pain while others do not. In addition to certain psychological measures, poor mental health is a common comorbidity with chronic pain. Studies have found higher rates of depression, anxiety, and sleep disturbance in chronic pain populations (Seth and DeGray 2019). The disruption of an individual's psychology can have quantifiable biological effects. For example, chronic pain patients can show deleterious alterations in the hypothalamic-pituitary-adrenal axis. With a constant stressor, such as chronic pain, alterations in stress hormones, like cortisol can occur which have been shown to aggravate chronic pain (Seth and DeGray 2019).

Certain genetic differences have been associated with a greater predisposition for developing chronic pain. Certain variations in pain signaling pathways, pain sensitivity, and inflammatory pathways could put some individuals at a greater risk for developing chronic pain compared to others. Genes encoding for MOR-1, enzymes that metabolize analgesics, immune system modulators, and melanocortin 1 receptors have been associated with a high proclivity for chronic pain development (Seth and DeGray 2019). Genetic differences have shown alterations in sensory nociceptive thresholds in different mouse strains. Certain genetic variants can show clear differences for various thermal and

mechanical nociceptive assays with 1.2 to 54-fold changes in sensitivity to painful stimuli, further implicating genetics as an important factor in pain perception (Mogil et al. 1999).

An individual's environment and lifestyle factor into their vulnerability to chronic pain development. Some important, and studied environmental changes include the presence of stress, poor quality of sleep, increased occupational stress, and environmental toxins can alter the likelihood of an individual developing chronic pain (Seth and DeGray 2019). Despite associations with these environmental factors, the mechanism by which they modulate chronic pain development is relatively unclear.

Gender also seems to be a risk factor in chronic pain development with women reporting significantly higher levels of chronic pain despite a healthier lifestyle on average and an increased likelihood of seeking medical attention. Numerous hypotheses have been put forth to explain how gender can influence the development of chronic pain. Some current lines of inquiry include the effects of sex hormones and the differential functioning of MORs between genders. While there is no definitive answer, it appears that the varying rates of chronic pain between men and women may be influenced by a combination of biological and psychosocial factors (Seth and DeGray 2019).

Chronic Pain Statistics

Chronic pain is a critical issue, with approximately 20% of Americans aged 18 and older reporting chronic pain within the last three months (Zelaya et al. 2019). Although chronic pain affects people across the population, certain subgroups, including individuals aged 65 and older, those residing in rural areas, and women, appear to be disproportionately affected by this condition (Zelaya et al. 2019). This issue is not unique to Americans as adults in European, Canadian, and Australian populations show similar

rates and reports of chronic pain (Hecke et al. 2013). Although these statistics are alarming, we still have no clear, safe, and effective long-term treatment for chronic pain.

Current Treatments for Chronic Pain

Despite their drawbacks, opioids are still included as a possible treatment option for chronic pain in various protocols and algorithms (Gilron et al. 2006). Approximately 1 in 5 chronic pain patients are on long-term opioids for management of non-cancer chronic pain (Daubresse et al. 2013). While opioid analgesics are widely regarded as the primary treatment for acute pain, their use in chronic pain management remains a subject of controversy due to the limited effectiveness demonstrated in meta-analysis studies (Busse et al. 2018).

Opioid analgesics are unique in their ability to not only dull pain sensations but provide users with a sense of well-being that is often lacking in chronic pain conditions (Trang et al. 2015). Although opioids are incredibly effective at relieving (acute) pain, they come with a host of side effects such as constipation, nausea and vomiting, urinary retention, itch and rash, dry mouth, respiratory depression, hypotension, neurotoxicity, and high abuse potential. The host of side effects of opioid medication has called for alternative therapies to be explored by physicians. Some of the commonly used alternative medications include non-steroidal anti-inflammatories, antidepressants, and even anticonvulsants. Chronic pain can also be medically managed through physical therapy, behavioral therapies such as cognitive behavioral therapy and mind-body therapies, massage, and chiropractic care. If less invasive treatments fail, other interventional therapies such as nerve blocks, epidurals, or spinal cord stimulation may be offered to patients. Support for non-opioid pain management is growing in the medical community, but it doesn't address how the current chronic pain population that regularly takes opioids should be managed

(Cohen et al. 2021). The limited understanding of how to effectively treat individuals with chronic pain on opioid medications warrants further investigation into the impact of opioids in this specific population.

Endogenous Opioid System

Our endogenous opioid system refers to a group of peptides and receptors that modulate nociception, stress response, temperature regulation, respiration, the gastrointestinal system, the endocrine system, memory, mood, and motivation. There are four reported opioid receptors all of which belong to the family of G-protein coupled receptors (GPCR). They include the mu opioid receptor, delta opioid receptor, kappa opioid receptor and nociceptin receptor (previously known as orphanin FQ) receptor. Each of these receptors binds endogenous opioids with varying affinities. The main endogenous opioid peptides include beta-endorphins, enkephalins, and dynorphins. Mu opioid receptors preferentially bind beta-endorphins and enkephalins, delta opioid receptors preferentially bind enkephalin and kappa opioid receptors preferentially bind dynorphin. Nociceptin receptors preferentially bind nociceptin. Opioid peptides are derived from precursor peptide proteins. Proopiomelanocortin produces the largest opioid peptide, ß-endorphin, and other non-opioid-related proteins. Preproenkephalin and preprodynorphin precursors are processed to generate several copies of enkephalin and dynorphin peptides respectively (Higginbotham et al. 2022). The endogenous opioid system alone can have profound physiological effects, but it is also able to modulate other neurotransmitter systems including the serotonin, oxytocin, vasopressin, and dopamine systems. Interactions between endogenous opioids and these systems, generally through opioid receptors, are what allow for adaptive responses to noxious stimuli (Higginbotham et al. 2022).

The endogenous opioid system mediates the effects of both endogenous opioid peptides and exogenous opioid medications. Simple variations in the expression of receptors or endogenous opioid peptides can change an individual's pain threshold or analgesic response to opioid medications can alter the likelihood of developing an opioid use disorder (Higginbotham et al. 2022). Most opioid analgesics used clinically are agonists of the MOR type. The role of the endogenous opioid system in both chronic pain and opioid use disorders will be discussed later in greater detail.

Opioid Receptors

The activation of kappa, mu, and delta opioid receptors results in distinct pharmacological effects of both endogenous and exogenous opioid peptides. For example, MORs when activated by an agonist will produce analgesia, reward, constipation, and respiratory depression, while kappa opioid receptor activation will produce aversive, hallucinogenic, and anxiogenic effects when activated by an agonist (Kieffer et al. 2002). Diverse locations throughout the peripheral and central nervous systems are part of what drives the differential effects of each receptor type. Peripherally, opioid receptors have a wide range of effects including regulation of the neuroendocrine system, immune system, and our nociceptive response system (Higginbotham et al. 2022).

While opioid receptors exhibit varied responses upon activation by an agonist, they share remarkable structural and functional similarities at the molecular level. Opioid receptors are inhibitory G-protein coupled receptors (Gi/Go). When activated these receptors will inhibit neuronal activation via cAMP inhibition (Higginbotham et al. 2022). Activation of the G-protein beta-gamma subunits also activate inwardly rectifying potassium channels, inhibits voltage- gated calcium channels, and enacts other molecular activities that further hyperpolarize the neuron (Higginbotham et al. 2022). Additionally,

activation of opioid receptors can lead to changes in mitogen-activated kinase signaling and influence gene transcription downstream.

The activation of opioid receptors can lead to agonist-biased signaling, wherein the specific ligand binding to the receptor influences the downstream effects within a cell. For example, some agonists preferentially activate G-protein-dependent signaling which is thought to drive the analgesic effects of opioids. Other agonists may activate beta-arrestin dependent signaling which is thought to drive some of the negative effects of opioids like respiratory depression (Higginbotham et al. 2022), albeit this distinct signaling function has been questioned (French et al. 2022).

Opioid receptors are distributed throughout the body, but they exert a significant influence on specific neuronal circuits, notably those involved in pain modulation and reward. Opioids act on pain transmission circuits, particularly the interaction between the PAG and the rostral ventromedial medulla. Additionally, opioids can exert their effects both pre- and post-synaptically within the spinal cord, inhibiting the release of neurotransmitters (such as substance P and calcitonin gene-related peptide) from nociceptive primary afferent neurons. Modulation in these areas will result in a decreased pain perception. One important reward circuit that is affected by opioids is the mesolimbic pathway, which includes the neuronal circuit from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). This pathway is affected by opioids at the level of the midbrain. Typically, GABAergic inhibitory interneurons within the VTA and rostromedial tegmental nucleus inhibit dopaminergic mesolimbic projections. In the presence of opioids, either endogenous or exogenous, the GABAergic interneurons of these midbrain structures will be inhibited resulting in a lowering of the firing rate of inhibitory interneurons of the VTA and rostromedial tegmental nucleus (tail region of the VTA). This process, commonly known as disinhibition, results in a net increase in the firing of VTA dopaminergic neurons. This

pathway, and its activation by exogenous opioids, is incredibly important for both their analgesic and rewarding effects (Ballantyne and LaForge 2007, Taylor et al. 2016). Individuals with variation in strength or composition of this pathway may have an altered likelihood of developing an opioid use disorder.

Dopamine is a part of the catecholamine family of neurotransmitters which also includes norepinephrine and epinephrine. Dopamine is synthesized from its precursor L-3,4- dihydroxyphenylalanine or L-dopa. This synthesis is limited by the enzyme tyrosine hydroxylase, an aromatic amino acid decarboxylase. Dopamine acts on dopamine receptors of which there are five main types: dopamine receptors 1 – 5, which can be further subdivided into inhibitory (D2-4) and excitatory (D1 and D5) receptors (Yang et al. 2020).

Dopamine has many roles such as the production of voluntary movement, reward processing, cognitive perceptions, sleep, mood, attention, memory, and learning. In addition to these many functions, dopamine system disruptions have been implicated in the development of chronic pain and psychiatric illness (Yang et al. 2020). In addition to the mesolimbic circuity, other dopamine projections originating in the midbrain include the mesocortical, nigrostriatal, and tuberoinfundibular pathways (Yang et al. 2020).

Opioid receptors are encoded by specific genes and mRNA sequences. What sets them apart is that the genes for all four types of opioid receptors have multiple exons or coding regions, as well as splice variants. These different splice variants give rise to distinct functions of opioid receptors. However, the precise implications of these variants remain largely unknown due to the numerous possible combinations of exons that can form an opioid receptor. Once the mRNA for an opioid receptor has been finalized, translation, protein folding, and post-translational modifications will occur in the endoplasmic reticulum. Eventually, after these modifications, opioid receptors will be transported to and inserted into the plasma membrane by trans-Golgi networks or secretory vesicles. Once on

the plasma membrane opioid receptors will function similarly to other inhibitory G-protein coupled receptors.

After an agonist binds to an opioid receptor the process of desensitization and internalization can begin. Desensitization occurs upon repeated agonist binding and is characterized by a decrease in the responsiveness of a receptor. Phosphorylation of the receptors by specific protein kinases is one molecular mechanism that can induce desensitization of an opioid receptor (Williams et al. 2013). Additionally, agonist exposure can result in receptor internalization, where the receptor is removed from the plasma membrane. This process begins with the phosphorylation of opioid receptors by G-protein receptor kinases, which triggers the binding of arrestin proteins. Arrestin proteins can lead to a conformational change of the opioid receptor to promote the process of internalization. Clathrins are another protein implicated in the internalization process, specifically in the endocytosis of the receptor. Clathrins are then able to traffic opioid receptors to endosomes where they will either be recycled and returned to the plasma membrane or will be sent to lysosomes for degradation (Williams et al. 2013). The process of endocytosis allows for the acidic environment of the endosomes to remove the agonist from the receptor. Mu opioid receptors belong to class A of GPCRs that undergo recycling and resensitization back to the plasma membrane where they will be active again (Lefkowitz et al. 1997), although new evidence suggests that opioid receptors may also signal via intracellular compartments (Kunselman et al. 2019).

Opioid Receptors and Alterations in Opioid Use Disorder

Opioid receptor function and density throughout the central nervous system are affected by repeated exposure to opioid agonists leading to a greater desensitization of opioid receptors, and thus less responsive to agonist binding. Additionally, the

internalization process may be upregulated or downregulated with repeated exposure leading to fewer active receptors available on the plasma membrane or fewer total available receptors (NIH Report 2021). Changes in the homeostatic mechanisms of the opioid system can affect reward processing centers by altering incentive salience, habit, stress responses, affect, and impulsivity. All of these changes can worsen cycles of opioid abuse and increase the severity of an opioid use disorder (Higginbotham et al. 2022).

Opioid Use Disorder

A state of drug addiction is one in which an individual compulsively consumes a drug with an inability to regulate their consumption. The word addiction is not commonly used in clinical practice and substance use disorder (SUD) is used instead. A SUD can be defined as meeting 3 or more of the 11 symptoms outlined in the DSM-5. Substance use disorders have varying degrees of severity with individuals exhibiting 2-3 symptoms classified as having a mild SUD and individuals showing 6+ symptoms are classified as having a severe SUD (Heilig et al. 2021). Opioid use disorder is a chronic disorder that involves the use of opiates or opioids outside of appropriate clinical applications (Strang et al. 2020). Opioid use disorders and SUDs in general have both psychological and physiological components that drive continued drug use. Physical dependence to a substance speaks to the homeostatic adaptations the body undergoes with repeated drug exposure. Part of this is driven by the activation of an opponent process in which the brain will have an equal and opposite response to drug abstinence as it does to drug exposure. In the case of opioids, when off the drug individuals have higher levels of anxiety, diarrhea, and anhedonia (Evans and Cahill 2016). These homeostatic changes are observed at a symptom level and molecular level. Psychological dependence is another important component of opioid use disorders where a person craves opioids, feels a strong urge to use

them, and experiences relief upon use. There are cases in which an individual has a physical dependence to opioids but does not have a psychological dependence. Prolonged use of opioids will most likely cause physical dependence, but this physical dependence will not necessarily lead to a psychological dependence too (Evans and Cahill 2016). Distinguishing between physical and psychological dependence is important when considering chronic pain patients.

Opioids as mentioned previously are addictive substances, however, individuals who report chronic pain may be more susceptible to abuse. The CDC reported that in 2019, 22.1% of US adults who have chronic pain used a prescribed opioid within the last three months (Dahlhamer et al. 2019). There is some degree of abuse in individuals using opioids for chronic pain treatment with studies finding misuse rates between 0.08% and 81% (Valentino and Volkow 2018). The wide range of reports of abuse of opioids in this population is due to the challenge of truly assessing rates of addiction and abuse. Addiction in this population is commonly referred to as iatrogenic addiction, which is an addiction or dependence that arises as a result of medical treatment or intervention. Iatrogenic addiction has always been a risk with opioid prescribing, but it became a national crisis starting in the 1990s to the present (Kibaly et al. 2021). This shift in prescribing and consequent increases in opioid addiction, overdose and death can be attributed to shifting attitudes about treating pain and the involvement of pharmaceutical companies. In the early 1990s, there was an increased emphasis on the treatment of pain as a human right and reversing the supposed undertreatment of pain. Additionally, during this period pain was promoted as the 5th vital sign, placing it at the same importance as body temperature, pulse, respiration, and blood pressure. This shift in mindset within the medical community was coupled with pharmaceutical companies like Purdue Pharmaceuticals investing hundreds of millions of dollars into promoting opioid medications as non-addicting when

used for treating pain (Vadivelu et al. 2018, Kibaly et al. 2021). These changes in perspectives of opioid medication by the medical community arguably initiated increased prescription of opioid medications and the first wave of the opioid epidemic (Dahlhamer et al. 2019). This first wave of the opioid epidemic is characterized by an increase in opioid prescribing but also overdose deaths. This initial wave was followed by an increase in heroin overdose deaths around 2010, which marks the second wave of the opioid epidemic. Once it was recognized that more liberal opioid prescribing led to increased deaths and increased rates of opioid use disorder, there was a change in attitude and CDC recommended decreased availability of prescription opioids. Physicians became less likely to prescribe opioids but also started refusing to refill existing prescriptions, which forced patients to resort to illicit opioids such as heroin. The second wave of the opioid epidemic was followed by the third. In the third wave of the opioid epidemic, there was an increase in deaths due to fentanyl, a very potent and dangerous synthetic opioid. Users and suppliers made the shift to fentanyl as it is relatively easy to make, and it doesn't require morphine as a precursor like heroin. Additionally, due to the high potency of fentanyl incredibly small amounts can give users their desired high. The transition into the third wave of the opioid epidemic came with serious consequences. Due to fentanyl's high potency, users were at much greater risk of overdose, respiratory depression, and death. The third wave of this epidemic, driven by fentanyl overdose deaths, has been the deadliest, with a large spike in opioid-related deaths starting around 2013 (Dahlhamer et al. 2019). These deaths have only increased during the COVID era (Glober et al. 2020). This increase in opioid use poses a major public health problem as people continue to develop opioid use disorder which is defined by the American Psychiatric Association DSM-5 as a desire to take opioid medications despite social and professional consequences. There are an estimated 16

million individuals worldwide, and 2.1 million individuals in the US, that have an opioid use disorder (Dydyk et al. 2022).

Many factors may contribute to an individual developing an opioid use disorder including genetics, environment, early life stress, and psychology. First, to develop any SUD, an individual's environment must allow for the initiation of drug use and continued access to the substance. Additionally, an individual's genetics or biology may put them at greater risk for opioid use disorder. It is estimated that there is approximately a 50% heritability for opioid use disorders. Additionally, roles such as epigenetics or brain chemistry may make some individuals more susceptible to the development of substance dependence (Dydyk et al. 2022). As reported in many other SUDs, those with pre-existing mental health conditions or exposure to trauma are more likely to develop opioid use disorder.

Risks for Opioid Use Disorder in Chronic Pain Populations

Chronic pain has been associated with changes in the endogenous opioid system, which has effects on how an individual will respond to exogenous opioid medication. As stated earlier, individuals who are receiving opioids for chronic pain treatment have a higher associated risk of abuse when compared to the general population (Ballantyne and LaForge 2007).

Analgesic tolerance is an important consideration in the chronic pain population. Since evidence points to malfunctioning of the endogenous opioid system in chronic pain patients, this will likely affect how they respond to opioid medications. Under normal conditions, tolerance develops with an escalation of opioid dosage and is characterized by medications losing their analgesic efficacy. There are many proposed mechanisms through which tolerance occurs, namely that opioid receptors become desensitized with repeated

exogenous opioid exposure and remain inactive in the plasma membrane. Beta arrestin pathways will then drive the endocytosis and recycling or degradation of receptors. Overall, the development of tolerance will decrease the potency of opioid medications (Higginbotham et al. 2022).

Another important consideration in the chronic pain population is the development of opioid-induced hyperalgesia. This occurs when pain sensitivity increases at the site of injury, or elsewhere, with repeated administration of high doses of opioids. Cellular adaptations cause opioid-induced hyperalgesia that typically involve glutamatergic systems throughout the central and peripheral nervous system. (Higginbotham et al. 2022).

Important Factors in the Development of Opioid Use Disorder

The development of a SUD involves numerous significant factors. However, a few key factors have emerged as particularly influential. These include an individual's psychological characteristics, social and environmental influences, the neurobiology of the person, their genetic predisposition, the initiation of drug use, and the ongoing maintenance of drug use (Ballantyne and LaForge 2007).

Psychological illness can significantly increase the likelihood of an individual developing a SUD. Additionally, psychological illness is a common comorbidity with SUDs. It is estimated that 43% of individuals who are in SUD treatment have symptoms or a diagnosis of mental illness. It is difficult to tease apart whether mental illness resulted from a SUD or if SUDs increase the likelihood of developing a mental illness. Regardless, mental illness can contribute to the development and severity of a SUD (NIH report 2021).

Social and environmental factors are also critical to the development of SUD. Specific risk factors, such as socioeconomic status and childhood stress or trauma, can elevate the probability of an individual developing a SUD. Furthermore, the environment

plays a crucial role in both the initiation and maintenance of drug use. Without experimentation or easy accessibility to drugs, individuals are less prone to developing a SUD (NIH report 2021).

Genetics play a crucial role in determining an individual's susceptibility to developing a substance or opioid use disorder. Genetic variations that particularly impact the rewarding and analgesic effects of opioids are of utmost significance. Substance use disorders also exhibit a substantial heritable risk, estimated at approximately 0.3 to 0.5. High heritability of SUDs means having a family history of substance abuse increases an individual's likelihood of developing a SUD. Many hypothesized genetic variants leave individuals more susceptible to the development of an opioid use disorder, one important is a variation within the cytochrome P450 2D6 gene which is important in the metabolism of opioid medications. Studies have indicated that individuals who possess variants that reduce the metabolism of opioid medications into their active forms have a decreased risk of developing substance abuse or addiction to these substances. Individuals with higher metabolic rates are at a higher risk for opioid use disorder (Ballantyne and LaForge 2007).

The neurobiology of reward systems can also influence the vulnerability of an individual to develop a SUD. Neurobiological changes in the brain's reward pathway are not the only important changes that drive opioid use disorder. On a molecular level, repeated administration of opioids can alter levels of cAMP, an important and ubiquitous secondary messenger, in many cells. These altered levels can drive allostatic changes. Allostatic changes refer to adaptive modifications that occur in the central nervous system (CNS) as a response to chronic stress or repeated exposure to challenging stimuli, such as opioids. Allostasis is the process by which the body maintains stability and adjusts physiological systems to adapt to stressors. When the allostatic load becomes excessive or dysregulated, it can lead to alterations in neurobiology. One location that experiences

allostatic changes is the locus coeruleus. The locus coeruleus is the brain's main noradrenergic hub and is involved in the physical dependence or tolerance associated with repeated opioid administration (Ballantyne and LaForge 2007). Allostatic changes within the locus coeruleus drive some of the negative withdrawal symptoms associated with opioid use disorders.

Mu Opioid Receptors

Mu opioid receptors are the main opioid receptor of focus in my studies. They play an important role in many body systems but are notable for their ability to modulate nociception, stress, temperature, respiration, the gastrointestinal tract, endocrine activities, memory, mood, and motivation. As described previously, they are GPCRs, which are 7 transmembrane-spanning receptors that couple to G-proteins.

Mu opioid receptors are found throughout the central nervous system but there are specific areas that show high expression of MORs. These areas include the caudate, putamen, cortex, thalamus, NAc, hippocampus, and amygdala, which have the highest density in the habenula. Regions such as the PAG and raphe nuclei show moderate concentrations of MORs and the hypothalamus, preoptic area, and globus pallidus show low levels of MORs. Additionally, MORs are in the dorsal horn of the spinal cord. Mu opioid receptors throughout these regions have important functions including respiration, cardiovascular function, reward system functions and reward responses, gastrointestinal functions, analgesic functions, learning and memory functions, locomotor functions, thermoregulation, hormone secretion functions, and immune functions (Allouche et al. 2014).

Mu Opioid Receptors in Pain

Opioids can act at MORs in both the central and peripheral nervous systems to induce analgesic effects. In the peripheral nervous system, endogenous or exogenous opioids binding to MORs on primary or secondary nociceptive neurons in the spinal cord can decrease the activity of these neurons. Decreased activity in these neurons can reduce the transmission of nociception to the brain (Higginbotham et al. 2022). It is important to note that opioid analgesics, via their action at opioid receptors, can lower the subjective feelings of pain without disrupting sensory thresholds (Pasternak et al. 1993).

In the central nervous system, many regions are high in MORs that might be important in the analgesic effects of opioids. This includes the cerebral cortex, limbic structures, and the PAG in the midbrain (Nasseef et al.). Recent studies have looked at changes in the functional connectivity of the brain after oxycodone administration in animals with a MOR knockdown. Large changes in the functional connectivity of the cortex, NAc, pontine reticular nucleus, and PAG were observed. Functional connectivity changes between the PAG and the NAc showed strong alterations in reward, aversion, and pain processing. All these functional changes due to oxycodone could be linked back to the role of MORs in these regions (Nasseef et al. 2019). Additionally, MOR-driven inhibition of GABA neurons located in the PAG and raphe magnus nucleus contribute to the analgesic effect of opioids (Valentino and Volkow 2018).

The role of MORs in the processing of both acute and chronic pain has been extensively studied. In human positron emission tomography (PET) studies, it was found that the MOR availability in the PAG (pain processing) and NAc (reward processing) increased after exposure to an acutely painful stimulus (Zubieta et al. 2001). Another study found that acute pain increased MOR activity in control human subjects while it decreased MOR activity in chronic pain individuals (Porreca et al. 1998) indicating an altered function

of MORs in chronic pain states. This finding was further supported in animal studies of chronic neuropathic pain which found downregulation of MORs in the spinal cord, dorsal root ganglia, and cortex after injury (Hagelberg et al. 2012), although down regulation is not consistently reported in chronic pain models.

Mu opioid receptor function is altered in chronic pain states. Conditions such as fibromyalgia, migraines, neuropathic pain, and lower back pain have all shown decreased MOR binding potential. Studies have shown that patients reporting low back pain show lower levels of the endogenous MOR agonist beta-endorphin. Lower levels of this critical endogenous opioid peptide could alter the response of an individual to both acute and chronic pain.

Additionally, MORs are important in not only the sensory processing of pain but also are critical in the emotional processing of pain. In healthy individuals, it was found that a lower binding affinity of MORs in the cortex correlates with a higher pain sensitivity (DaSilva et al. 2014). Additionally, MOR binding was negatively correlated with a patient's affective pain rating in both control and chronic pain patients (Zubieta et al. 2001). One region where large differences in MOR binding correlated to pain ratings was the NAc (Wanigasekera et al. 2012). These studies along with others implicate MORs as an important part of chronic pain pathology.

Mu Opioid Receptors and Addiction

Mu opioid receptors play a crucial role not only in the analgesic properties of opioid medications but also in the addictive and rewarding effects associated with opioids. Our body's endogenous opioid system is instrumental in regulating mood and overall sense of well-being. It is believed that MORs play a significant role in maintaining our hedonic tone, which is the balance between positive and negative affective states in an organism. (Darcq

and Kieffer 2018). Genetic knockdown models (Oprm 1 deletion variants) have driven our understanding of the roles and functions of MORs. In animals with constitutive, or complete, knockdown of MORs, the analgesic and rewarding effects of morphine are eliminated. These knockdown animals also showed decreased self-administration for other drugs of abuse including alcohol, nicotine, and THC. These results further implicate MORs in the rewarding effects of various drugs of abuse. Mu opioid receptor knockdowns also exhibit a decreased response to natural rewards. Some behaviors that are altered in these animals include reduced maternal infant attachment, impaired social interactions, and reduced motivation to self-administer food and sucrose. These results indicate that MORs are important in various types of rewards (Darcq and Kieffer 2018).

Numerous brain regions are critical for the processing of reward, with two significant ones being the VTA and the NAc. These regions are interconnected by dopaminergic neurons originating from the VTA project to the NAc, forming what is commonly known as the mesolimbic pathway. The mesolimbic pathway has been extensively investigated due to its pivotal role in the processing of rewarding stimuli. Mu opioid receptors are expressed at multiple levels along this pathway including GABAergic interneurons in the VTA and on medium spiny GABAergic neurons originating in the NAc that project back to the VTA (Darcq and Kieffer 2018). GABAergic input to VTA dopaminergic neurons from the rostromedial tegmental nucleus and D2-expressing medium spiny GABAergic neurons in the NAc have also been implicated in the rewarding effects produced by MOR activation (Valentino and Volkow 2018). Overall, MORs play an important role in the actions of the mesolimbic pathway in response to rewarding stimuli.

The hippocampus is another crucial brain region linked to drug use, as it plays a significant role in learning and memory processes. Studies have shown that MOR (MOR) and delta opioid receptor (DOR) agonists in this region facilitate the learning and memory

associated with drug use. As mentioned previously, our endogenous opioid system is an important part of mediating our response to stress. In particular, MORs in the locus coeruleus are essential for regulating our reactions to stressful situations. Inhibition of this noradrenergic hub by MOR agonists promotes relaxation and stress recovery. The stress relief that opioids can elicit may be relevant to the development of opioid use disorders. Moreover, certain physical symptoms related to withdrawal, such as sleep disturbances and hyperarousal, are believed to be mediated by the overactivation of noradrenergic neurons in the locus coeruleus (Valentino and Volkow 2018).

Ventral Tegmental Area

The VTA is a brain region that is important in reinforcement learning, reward, novelty recognition, working memory, and aversion. This region is dense in dopaminergic, GABAergic, and glutamatergic neurons. These populations of neurons are heterogeneous, expressing different receptors, peptides, and proteins. Additionally, these subpopulations show differing action potential durations, firing rates, and input resistances. Differential neuronal populations allow for the complex functions associated with the VTA. Opioid receptors located on these GABAergic interneurons are thought to be important to the analgesic and rewarding effects of opioids (Fields and Margolis 2015).

There are many important projections to and from the VTA. Dopaminergic neurons in the VTA receive glutamatergic inputs from the medial prefrontal cortex, pedunculopontine tegmentum, laterodorsal tegmental nucleus, lateral habenula, PAG, bed nucleus of the stria terminalis, and the dorsal raphe nucleus. These neurons also receive inhibitory GABAergic inputs from the rostromedial mesopontine tegmental nucleus, PAG, dorsal raphe nucleus, lateral hypothalamus, and ventral pallidum. This regulation of dopaminergic neurons by glutamatergic and GABAergic projections is important to the

baseline firing of these neurons, or the dopaminergic tone. In addition to regulation of the dopaminergic neurons in the VTA, GABAergic interneurons of the VTA also have quite intensive regulation. These neurons receive inputs from the medial prefrontal cortex, NAc, lateral habenula, PAG, lateral hypothalamus, dorsal raphe nucleus, and the bed nucleus of the stria terminalis. In addition to dopaminergic projections to the NAc, the VTA also sends projections to the medial prefrontal cortex and lateral habenula (Fields and Margolis 2015). Overall, the VTA serves as a hub for various projections that play crucial roles in regulating its dopaminergic neurons and allowing for nuanced behavioral outputs associated with the VTA.

In addition to the complex regulation of neurons in the VTA, there are also many different sub-populations. The VTA is not a homogenous structure. It is composed of the parafasciculus retroflexus area, parabrachial pigmented area, parainterfascicular nucleus, paranigral nucleus, ventral tegmental tail, and midline nuclei (Holly and Miczek 2015). Each of these regions has different functions and projections throughout the brain. The elaborate organization of the VTA illustrates some of the complexity within this region.

Ventral Tegmental Area and pain

The VTA has been traditionally studied for its association with pleasure and reward. As a major hub for dopaminergic neurons in the brain, projections for the VTA are critical in the encoding of pleasurable stimuli. Interestingly, VTA pathways that encode pleasurable and rewarding stimuli have also been shown to be important for the processing of pain (Higginbotham et al. 2022). In chronic pain patients, plasticity has been shown in the VTA, specifically in dopaminergic neuronal populations. Animal studies further validate this showing that firing patterns of dopaminergic neurons in the VTA are altered in animal models of chronic pain. Clinical interventions for chronic pain at the level of the

VTA have also proven effective. A deep brain stimulation study with cluster headache patients was performed recently. It was found that with stimulation of the VTA, many patients reported decreased headache occurrences and severity (Loggia et al. 2013). From these studies, it is clear that the VTA is an important mediator in the pathogenesis of chronic pain.

Ventral Tegmental Area in addiction and reward

The VTA was studied extensively for its role in reward and motivated behavior. Certain projections from the VTA were associated with either reward or aversion. For example, dopaminergic projections from the VTA to the NAc are generally active in the presence of a rewarding stimulus. Paradoxically, dopaminergic projections from the VTA to the medial prefrontal cortex are shown to be active upon an individual's exposure to an aversive stimulus. Additionally, neuronal projection type seems to be important in the behavior response elicited. For example, glutamatergic projections from the VTA to GABAergic medium spiny neurons (MSNs) in the NAc are shown to be active in aversive events. The complexity of this circuitry points toward the nuance in neuronal firing patterns that contribute to the encoding of motivated behaviors (Fields and Margolis 2015).

Studies have confirmed the importance of the VTA in reward seeking behavior and reinforcement behaviors. The MORs in this region are thought to be a major modulator of observed changes. One study found that the administration of a MOR agonist in the VTA alone produced reinforcement and reward-seeking behavior. This study along with others shows the importance of MORs in the reinforcing behaviors associated with opioid addiction (Higginbotham et al. 2022).

Organization of the Nucleus Accumbens

The NAc is a region located deep within the brain, specifically in the basal forebrain area. It is part of the brain's reward system and plays a crucial role in motivation, pleasure, reinforcement, and addiction. The NAc is divided into two major anatomical regions: the shell and the core. Each of these region's functions to support different roles in the processing of aversive and rewarding stimuli but contributes differentially to goal-directed behaviors. The core connectivity eventually leads to premotor and supplementary motor areas of the cortex, while the shell connects to the prefrontal cortical areas as well as a range of subcortical motor areas, including the extended amygdala and lateral hypothalamus. Dopamine release in the NAc shell is necessary for enabling hippocampal-dependent spatial information to gain control over appetitive learning, whereas dopamine release in the NAc core is important for allowing basolateral amygdala-dependent information to gain control over appetitive learning (Ito et al. 2011). The NAc is part of the ventral striatum, and other regions of the striatum including the caudate and putamen are considered to be part of the dorsal striatum. While structures of the dorsal striatum are typically associated with movement and habit drug-seeking (Everitt and Robbins 2016), the NAc is responsible for motivational learning and connections to additional limbic structures that drive motivation and the processing of emotion.

Within the striatum, there are many neuronal subpopulations. Up to 95% of neurons in the striatum are medium-spiny GABAergic inhibitory neurons. The other 5% are generally GABAergic and cholinergic interneurons. The MSNs in the striatum are generally divided into two subpopulations: dopamine D1 receptor-expressing MSNs and dopamine D2 receptor-expressing MSNs. The D1R MSN population co-expresses other neuropeptides such as dynorphin and substance P. This population is commonly referred to as the direct pathway (Yager et al. 2015). The D2R MSN population co-expresses adenosine 2A receptors

and the endogenous opioid peptide enkephalin. These neurons are commonly referred to as the indirect pathway (Castro and Bruchas 2019). Medium spiny neurons generally have a low baseline firing rate, meaning that larger amounts of a depolarizing signal are required to produce an action potential in these cells. Their low resting membrane potential can be attributed to a high expression of leaky potassium channels. Their decreased excitability results in any produced signals from MSNs having a high fidelity meaning that they are capable of transmitting information with a high degree of accuracy and precision (Castro and Bruchas 2019).

In the NAc, the D1R MSNs generally project to the VTA and substantia nigra whereas D2R MSNs generally project to the ventral pallidum. However, recent studies have shown that these neuronal populations are much more intertwined than expected. For example, some neurons express both D1 and D2 receptors, and some D1 MSNs project to the ventral pallidum. Despite the complexity of these populations, better classifications of each will allow us to better elucidate their functions in opioid use disorder and pain (Yager et al. 2015). Additionally, certain populations of MSNs in the NAc project onto other local MSNs. Meaning, that some D2R MSNs will synapse with other D1R MSNs or D2R MSNs within the NAc. Interestingly, few D1R MSNs project locally to D2R MSNs. Although the implications of this finding have not been thoroughly studied, it points to a local inhibition of the direct pathway by the indirect pathway in the NAc (Castro and Bruchas 2019). The preferential firing of indirect pathway neurons is also illustrated by their higher resting membrane potential compared to direct pathway neurons. This effect however may be balanced by the greater dendritic connections of direct pathway neurons (Castro and Bruchas 2019).

Another interesting feature of NAc organization is the striosomes and matrix. The striosomes compose 10-15% of the striatum with matrices located in between. The signature

of the striosomes is their relatively high MOR, substance P, D1Rs, enkephalin, and prodynorphin expression. The matrix of the striatum is high in calbindin, somatostatin, enkephalin, D2Rs, and choline acetyltransferase (Brimblecombe and Cragg 2016). This patchwork seen throughout the striatum drives the complex communication not only between striatal structures but also in its projections. The core of the NAc shows a similar organization and composition to the dorsal striatum. The main difference lies in where projections are preferentially sent. The NAc core will project back to the VTA while the dorsal striatum projects back to the substantia nigra. Some studies have found that regions within the NAc core relate to phenotypic behaviors. For example, the dorsal region of the core when stimulated facilitates fear extinction whereas the ventral region of the core enhances fear learning (Castro and Bruchas 2019).

This project focuses on the shell of the NAc which is high in striosome composition (Castro and Bruchas 2019). Interestingly, the NAc shell, specifically the medial region, shows some striking differences when compared to the rest of the striatum. Medium spiny neurons in this region show fewer spines and their cell bodies are smaller in diameter when compared to the core. Additionally, these neurons appear to be more excitable than other MSNs in the striatum, especially the population of D2R MSNs. In contrast with other parts of the striatum, it appears that the D1R vs D2R classification of neurons is not overly accurate. Somewhere between 5-30% of MSNs in this area express both D1 and D2 receptors. Additionally, this region shows an expression of D3 receptors, which haven't been found in any other region of the striatum (Castro and Bruchas 2019).

Circuitry of the Nucleus Accumbens

The circuitry of the NAc is complex and a recent circuitry mapping study found 57 brain regions that project to the NAc (Ma et al. 2020). Many of these circuits have not been

clearly defined and many of their functions have yet to be elucidated. However, some projections to the NAc have proven to be important in addiction. These projections include the VTA, basolateral amygdala, prefrontal cortex, hippocampus, mediodorsal thalamus, and lateral hypothalamus. The main outputs from the NAc that are important in reward and addiction projects to the VTA, basolateral amygdala, lateral hypothalamus, and ventral pallidum (Ma et al. 2020).

Mu opioid receptors in the nucleus accumbens

The endogenous opioid system acts as an important modulator of MSN activity within the NAc (Castro and Bruchas 2019). Mu opioid receptors are present in a subpopulation of both D1 and D2 receptor expressing MSNs within the NAc and have been implicated in food reward and the rewarding effects of drugs of abuse (Peciña et al. 2008). The expression of MORs in the NAc and throughout the striatum demarcate a patch (striosome) pattern. They are thought to increase the hedonic value of sensory stimuli and drive our motivation to obtain positively perceived stimuli. Additionally, the distribution of MORs within the NAc has been linked back to the locations of hedonic "hot spots". Injections of a mu-opioid agonist into the medial shell of the NAc, aka the hedonic "hot spot", drove animals to increase their consumption of a natural reward, sucrose, when compared to controls (Peciña and Berridge 2005).

Nucleus Accumbens in reward and addiction

The NAc has been studied extensively for its role in addiction and reward. As a major site where dopamine is released, its neuronal populations are sensitive to any factor that may modulate dopamine transmission, including drugs of abuse.

In self-administration studies of MOR agonists in specific brain regions, a significant increase in self-administration occurred with microinjections into the NAc shell (Fields and Margolis 2015). Interestingly, this effect was not seen in either the NAc core or the dorsal striatum. This implicates the NAc shell as an important location for the rewarding effects of MOR agonists (Fields and Margolis 2015). Additionally, D1 receptors located in the NAc shell proved important in learning paradigms specific to Pavlovian conditioning and spatial short-term memory important specifically to goal-directed behaviors (Di Chiara et al. 2004).

Microdialysis studies have also been conducted that further implicate the NAc in the rewarding effects of many drugs of abuse. Specifically, addictive drugs increase dopamine in the NAc as measured by microdialysis (Di Chiara et al. 2004). These findings are further corroborated by human studies. Brain imaging in humans indicates that psychostimulants increase extracellularly in the NAc. Additionally, self-reported questionnaires measuring liking and high experience from psychostimulants correlated back to the amount of dopamine released in the NAc (Di Chiara et al. 2004). Other studies have implicated dopamine receptors in the NAc directly with the rewarding aspects of amphetamines, cocaine, and nicotine. When D2 or D2/D1 receptor antagonists are injected into the NAc, perceived drug reward as measured through conditioned taste avoidance was abolished (Di Chiara et al. 2004).

Nucleus accumbens and pain

As stated previously, the NAc is important in the processing and encoding of rewarding and aversive stimuli. In chronic pain models, multiple disruptions in normal NAc function have been noted. One of the largest deficits seen is dopamine signaling and dopamine receptor binding potential. It was found in human studies that dopamine receptor binding potential in the NAc could predict the level of pain and self-reported

negative affect (Higginbotham et al. 2022). Additionally, human studies have indicated that activity in the NAc is important in the subjective or affective pain experience and the transition from acute to chronic pain (Schwartz et al. 2014). These findings have been corroborated in animal models. It was found that dopamine transporter activity in the NAc increased in chronic pain states (Higginbotham et al. 2022). Additionally, observed dopamine release in the NAc upon morphine administration was found to be reduced in chronic pain animals. This finding implicates the NAc in one of the many differences in opioid processing between pain models and controls (Higginbotham et al. 2022). In other animal studies, models of chronic pain have shown alterations in excitatory synapses within the NAc indirect pathway. These synaptic modifications have proven to be important in modulated motivation associated with chronic pain states (Schwartz et al. 2014). Projections to the NAc have also proven important in regulating the sensory and affective components of acute pain. Recent studies have indicated that projections from the medial prefrontal cortex to the NAc are important in suppressing responses to acutely painful stimuli (Zhou et al. 2018).

In addition to the molecular and synaptic associated changes in the NAc in chronic pain states, the NAc has also shown differential activity in the progression of neuropathic pain (Kato et al. 2016). In the early phase of neuropathic pain development (17-20 days after sciatic nerve ligation), animals showed increased dopamine levels after the administration of analgesic (pregabalin). In the later phase of neuropathic pain development (31-34 days after sciatic nerve ligation), animals showed no increase in dopamine levels after analgesic administration. Interestingly, early-phase animals showed dopamine increase after consuming a sucrose solution while later-phase animals did not. These results indicate that analgesics and natural rewards differentially modulate

dopamine release, and presumably rewarding effects stimuli, at different periods of chronic pain development (Kato et al. 2016).

Mesolimbic Pathway Disruption in Chronic Pain States

The mesolimbic pathway is critical not only for processing rewarding stimuli, but it also plays a role in the encoding of aversive stimuli. It has been proposed that chronic pain induced changes in the endogenous opioid system, particularly changes in the mesolimbic pathway, may be driving increased opioid abuse within this population. Studies have preliminarily supported that disruption in MORs within mesolimbic circuitry may drive opioid abuse. Additionally, disruptions in dopaminergic tone within the mesolimbic pathway are another proposed mechanism by which chronic pain may alter the rewarding effects of opioids (Higginbotham et al. 2022). Interestingly, in models or conditions of pain, the ability of opioids to trigger dopamine release in the NAc is significantly reduced. This difference in opioid reward in pain patients affects the way they respond to opioid medications (Higginbotham et al. 2022).

This was supported by studies that show MOR agonist administration after peripheral nerve injury decreases the disinhibition of the VTA dopaminergic neurons (Ren et al. 2021). This decreased activity in the VTA would result in less dopamine release into the NAc and thus a decrease in the rewarding effects of opioid medications in chronic pain models. This was somewhat supported in human models. Studies have shown that individuals with chronic pain who have lower activation of MORs in the NAc are at lower risk for opioids. This effect not only correlates to the risk of opioid abuse but also to negative affect and mood alterations commonly associated with chronic pain (Higginbotham et al. 2022).

In addition to MORs, chronic pain will alter dopamine release within the mesolimbic pathway. These changes will lead to alterations in reward processing and responses to rewarding stimuli. In chronic pain animal models, it was found that reward, both natural and drug induced, was impaired. This decrease in reward was associated with disrupted dopamine signaling along the mesolimbic pathway. These findings in animal models were confirmed in human studies which found that decrease in motivation associated with pain was correlated with decreased dopamine release and disrupted function in the NAc. Furthermore, dopamine receptor binding potential in the NAc was correlated to reported negative affect and pain (Higginbotham et al. 2022).

Studies have shown dysfunction of the mesolimbic pathway is associated with not only mood disorder and addiction but also in chronic pain patients (Yang et al. 2020). Specifically, disruptions in mesolimbic reward circuitry were seen in fibromyalgia and burning mouth syndrome patients. Animal studies found microglial activation along the mesolimbic pathway in cases of peripheral nerve injury (Yang et al. 2020). Generally, the mesolimbic pathway works to integrate and process whether an external stimulus is rewarding or aversive and plays an important role in chronic pain pathology.

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Thesis Hypothesis and Objectives

There is evidence that dopamine transmission via mesolimbic circuitry is reduced and likely contributes to the pathology of chronic pain states. This is supported by clinical reports that dopamine receptor agonists alleviate various chronic pain conditions. Mu opioid receptors are present in the ventral striatum and are known for their involvement in positive reinforcement, however, it is unclear what, if any, role MORs expressed on MSNs within the ventral striatum have in modulating neuronal activity associated with ongoing pain. Previous studies reported that morphine injected into the NAc had no effect in modulating thermal sensory thresholds, but morphine injected into this brain region reduced pain behaviors in a model of tonic inflammatory pain (Olmstead and Franklin 1997). Further, genetic deletion of MORs from all GABAergic forebrain neurons did not affect on opioid-mediated antinociception in thermal nociceptive tests, whereas it eliminated heroin self-administration (Charbogne et al. 2017). Similarly, genetically modified mice that only expressed MORs in dynorphin containing dopamine D1 receptor (D1R) MSNs were sufficient to produce opioid self-administration and opioid conditioned place preference, but not to recover opioid analgesia in a thermal nociceptive test (Cui et al. 2014). Given the importance of dopamine circuits in pain and that microinjection of opioids into the NAc reduced nociceptive behaviors in a tonic model, we sought to understand the role of MORs in D1R MSNs in a model of neuropathic chronic pain.

Unpublished data from the Cahill lab shows that genetic deletion of MORs from D1R MSNs reduced opioid analgesia in the formalin model of tonic inflammatory pain as well as reduced opioid reward in both control and chronic pain animals. However, because the deletion was conducted via breeding strategies, it is unknown what brain region may be responsible for these opioid effects. This study aims to better dissect which brain regions

may be responsible for behavioral changes observed in D1 cre+ flMOR/flMOR knockdown animals.

Hypothesis: MORs in D1R MSNs within the NAc shell are responsible for the negative reinforcement of opioids in chronic pain as measured by conditioned place preference.

Objectives:

1. Confirm opioid-induced analgesia is not perturbed in thermal sensory tests of genetically modified animals not expressing MORs in D1R containing MSNs.
2. Determine if the re-expression of MORs in D1R MSNs within the NAc shell is sufficient to produce negative reinforcement in chronic pain states.

Chapter 2: What role do mu opioid receptors play in acute pain responses?

INTRODUCTION

Mu opioid receptors (MORs) have been studied extensively for their importance in reward and analgesia. Constitutive knockout of MORs (throughout the body) shows that this receptor type is responsible for many of the pharmacological effects of opioid analgesics, including analgesia, respiratory depression, constipation, and sedation (Matthes et al. 1996). Knockout of MORs has also shown their importance in opioid reward, where animals will no longer show a conditioned place preference or self-administer opioids. Their exact role in these processes, and what neurons MORs modulate to produce these behavioral phenotypes has yet to be fully understood.

Previous research found that certain populations of MORs have differing effects on behavioral phenotypes (Severino et al.). Studies by Kieffer et al. found that MOR expressing GABAergic forebrain neurons are critical in mediating motivation for both exogenous opioids and natural rewards such as palatable food (Charbogne et al. 2017). In this study, a DLX5/6-Cre driver mouse line was crossed with a MORloxP mouse to genetically ablate MORs from all GABAergic-containing forebrain neurons. In this new mouse, there was a significant decrease in the expression of MORs in the dorsal striatum and NAc, while expression of MOR was intact in the VTA, midbrain, spinal cord, and brainstem. Their results indicate that MORs in these GABAergic neurons do not mediate morphine-induced analgesia, but rather alter the locomotor and motivational effects of MOR agonists. Overall, results from this study point towards differential roles of MORs in forebrain circuits.

A complementary study used an alternative approach to study the role of striatal MORs in opioid reward, where MORs were only expressed in striatal D1-receptor

containing MSNs. Yang and colleagues (Cui et al. 2014) produced a genetically modified mouse where MORs were only expressed in the striatal direct pathway striosome and NAc. They identified that MORs in this population were sufficient for opioid-induced reward as measured by conditioned place preference and intravenous self-administration, and opioid-induced dopamine release via these MSN projections to the VTA. Interestingly, animals with this limited expression of MOR exhibited no opioid-induced analgesia in acute phasic pain assays, while retaining opioid-induced withdrawal.

Previous research found that different populations of MORs throughout the striatum have various roles and functions. Unpublished previous studies (Cahill Lab) demonstrated that animals with ablation of MORs from specific dopamine receptor containing neurons had differential responses to opioids in both pain-naive and chronic pain states. Using the cre-lox system, they deleted MOR from either D1 receptor, D2 receptor or A2A receptor containing neurons using a breeding strategy. The D1Rcre + flMOR/flMOR line targeted MOR expression in mainly direct pathway neurons. The D2Rcre + flMOR/flMOR and A2aRcre + flMOR/flMOR line specifically targeted MORs in the indirect pathway. In D1Rcre + flMOR/flMOR mice, there was a decrease in opioid reward in pain naive and chronic pain animals, whereas there was an increase in opioid reward in D2R cre + flMOR/flMOR and A2aR cre + flMOR/flMOR mice measured by conditioned place preference. Separate cohorts of mice were also subjected to assessment of opioid mediated analgesia using a formalin model of inflammatory pain. The D1Rcre + flMOR/flMOR mice showed a reduction in opioid induced analgesia compared to their wild-type littermates.

In the present study, we asked to what extent ablation of MORs from D1 receptor or A2A receptor containing neurons mediates acute thermal opioid-mediated analgesia as measured by the hot plate test. As with prior studies, a similar breeding strategy was used with the D1R cre +/- flMOR/flMOR and the A2aR cre +/- flMOR/flMOR lines. Similar to

previous studies, we wanted to determine the extent either of these targeted deletions in MOR would result in a differential response to opioid induced analgesia in an acute pain paradigm. We tested this by observing latency to pain behaviors in D1R cre +/- flMOR/flMOR and A2aR cre +/- flMOR/flMOR animals receiving oxycodone or saline in the hot plate test.

METHODS

Mouse Lines

All procedures were authorized by the Institutional Animal Care and Use Committee (IACUC) and comply with the Policies on the Use of Animals in Research. All transgenic mice used in this study were bred by the Animal Breeding Colony.

Adult male and female D1 receptor flMORs and A2a receptor flMORs were generated by breeding flMOR mice (loxP sites flanking exons 2–3 of the *oprm1* gene on a 50:50 C57BL/6J:129Sv background, stock #030074, The Jackson Laboratory) with two Cre driver lines to obtain Cre recombinase on one (D1 receptor cre; stock#030989-UCD and A2a receptor cre; 036158-UCD, MMRRC, NIH, DHHS, 100% C57BL/6J) allele and flMOR on both alleles. Wild type littermates of the same genetic background were used as controls. Mice lacking all MORs (stock #007559, 100% C57BL/6J, The Jackson Laboratory) were bred as heterozygous pairs to generate knock-out (KO) and wild-type (WT) littermates.

Male and female transgenic mice were used between ages 8–32 weeks and 20–36 g of body weight. Animals were maintained on a reverse 12/12 h light/dark cycle with *ad libitum* access to food and water. All behavioral experiments were conducted during the dark cycle.

Hot Plate Tests

Mice were first habituated to the behavioral testing room for one day and were habituated to the hot plate device (at ambient temperature) for 5 minutes. The hot-plate test was used to measure response latencies according to the method described by Chan and Yeung (Chang and Yeung 2006). Harvard Apparatus Ltd. hot plate (Edenbridge, Kent, United Kingdom) was maintained at 52 ± 0.5 °C and surface temperature was continuously monitored with a digital thermometer. The time between the placement of the animal on the hot plate and the occurrence of a pain response was recorded as the response latency. Baselines for each animal were measured, recording how long it took for animals to present with a pain response after being placed on the hot plate. Pain responses included: hind paw licking, jumping, side of cage climbing, and hind paw flicking. Two baseline measurements were taken for each mouse with at least 10 minutes between each test ensuring enough time for recovery after the stimulus and preventing possible tissue damage. The average baseline was calculated for these two measurements. This average baseline was tripled and set as the maximum time for each animal to spend on the hot plate. This maximum time would prevent tissue damage in the event of the analgesic response of mice to oxycodone in the hot plate test. Regardless of the presentation of pain symptoms, mice were removed at their maximum time (3 X baseline average).

Drugs

The Schedule II drug oxycodone was obtained from the NIDA Drug Supply Program (RTI). Oxycodone HCl was used at dosage of a 3.0 mg/kg in a solution with pH 7.4 buffered PBS. Animals were injected with 0.1 mL/10g body weight of a 0.3 mg/mL oxycodone solution or vehicle.

Experimental Design

Hot plate testing assessed any differences in oxycodone induced analgesia between each animal line in an acute pain paradigm. Hot plate tests were performed first by establishing baselines for each animal. Animals were placed on the hot plate device maintained at approximately 52 degrees Celsius and latency to the first pain response was measured. After recording baselines, and calculating maximum time (3 times baseline), mice were given oxycodone or vehicle via an intraperitoneal injection. The next round of hot plate tests was performed 10 minutes after the administration of oxycodone for each subject. Follow-up tests for each subject were performed at 20 minutes, 35 minutes, and 50 minutes post intraperitoneal injection. All testing was done blind to mouse genotype (Cre+/Cre-). All behavioral tests conducted consecutively consisted of all males or females to prevent any changes in response to the opposite sex. Experiments were all performed during the dark state of the reverse light dark cycle between the hours of 10am-3pm.

Statistics

Data are expressed as mean +/- SEM for all groups. Some data were transformed into Percent Maximum possible effect defined as (post drug latency – baseline latency)/baseline latency *100. Two-way ANOVA was used to analyze data obtained from the hot plate testing using Prizm v9 (GraphPad 9.0 software) with further details provided in the results and statistical tables. Following two-way ANOVA was followed by Sidak's post hoc analysis for comparing treatment and genotype. A p-value less than 0.05 was considered significant.

RESULTS

To determine the role of MORs in dopamine D1 receptor and A2a receptor-expressing neurons in opioid-induced analgesia, I tested the effects of oxycodone (3.0 mg/kg, i.p.) in D1R cre +/- flMOR/flMOR and A2aR cre +/- flMOR/flMOR mice in the hot plate test. Ablation of MORs from D1 or A2a receptor-expressing neurons did not affect baseline nociceptive thresholds (Figure 1). The thresholds were not different between wild-type and either MOR genetically modified mouse line (A2aR and D1R). Statistical analysis by 2-way ANOVA showed no significant effect of cre ($F(1,29)=1.146$ $p=0.2932$).

Opioid-mediated antinociception was assessed every 10 minutes for the first 20 minutes and every 15 minutes for 30 minutes following oxycodone injection in D1R cre + flMOR/flMOR and wild-type littermate mice. Oxycodone increased thermal nociceptive thresholds in both cohorts (Figure 2). Statistical analysis by repeated measures 2-way ANOVA revealed a significant effect of time ($F(3.248, 42.23)=45.19$, ****$p<0.0001$), but no significant effect of cre expression ($F(1,13)=0.6475$, $p=0.4355$) and there was no interaction between time and cre observed ($F(4,52)=0.5311$, $p=0.7134$). *Post hoc* analysis using a Sidak multiple comparison tests revealed a significant increase at all time points compared to baseline values. Data was converted to percent maximum possible effect and presented in Figure 2B. Again, data show there was no significant effect of genotype on oxycodone-induced antinociception.

Opioid-mediated antinociception was also assessed every 10 minutes for the first 20 minutes and every 15 minutes for 30 minutes following oxycodone injection in A2aR cre + flMOR/flMOR and wild-type littermate mice. Oxycodone increased thermal nociceptive thresholds in both cohorts (Figure 3). Statistical analysis by repeated measures 2-way ANOVA revealed a significant effect of time ($F(2.760, 44.15)=25.84$, ****$p<0.0001$), but no significant effect of cre expression ($F(1,16)=0.2.199$, $p=0.1575$) and there was no interaction

between time and cre observed ($F(4,64)=0.2083$, $p=0.9329$). *Post hoc* analysis using a Sidak multiple comparison tests revealed a significant increase at all time points compared to baseline values. Data was converted to the percent maximum possible effect and presented in Figure 3B. Again, data show there was no significant effect of genotype on oxycodone-induced antinociception.

Figure 1. Ablation of MORs from D1 or A2a receptor expressing neurons had no effect on thermal nociceptive thresholds. Thermal thresholds were determined on a 52°C hotplate test. Latencies in all genotypes were approximately 10 seconds. Data are expressed as mean +/- SEM for N=7-11 per group.

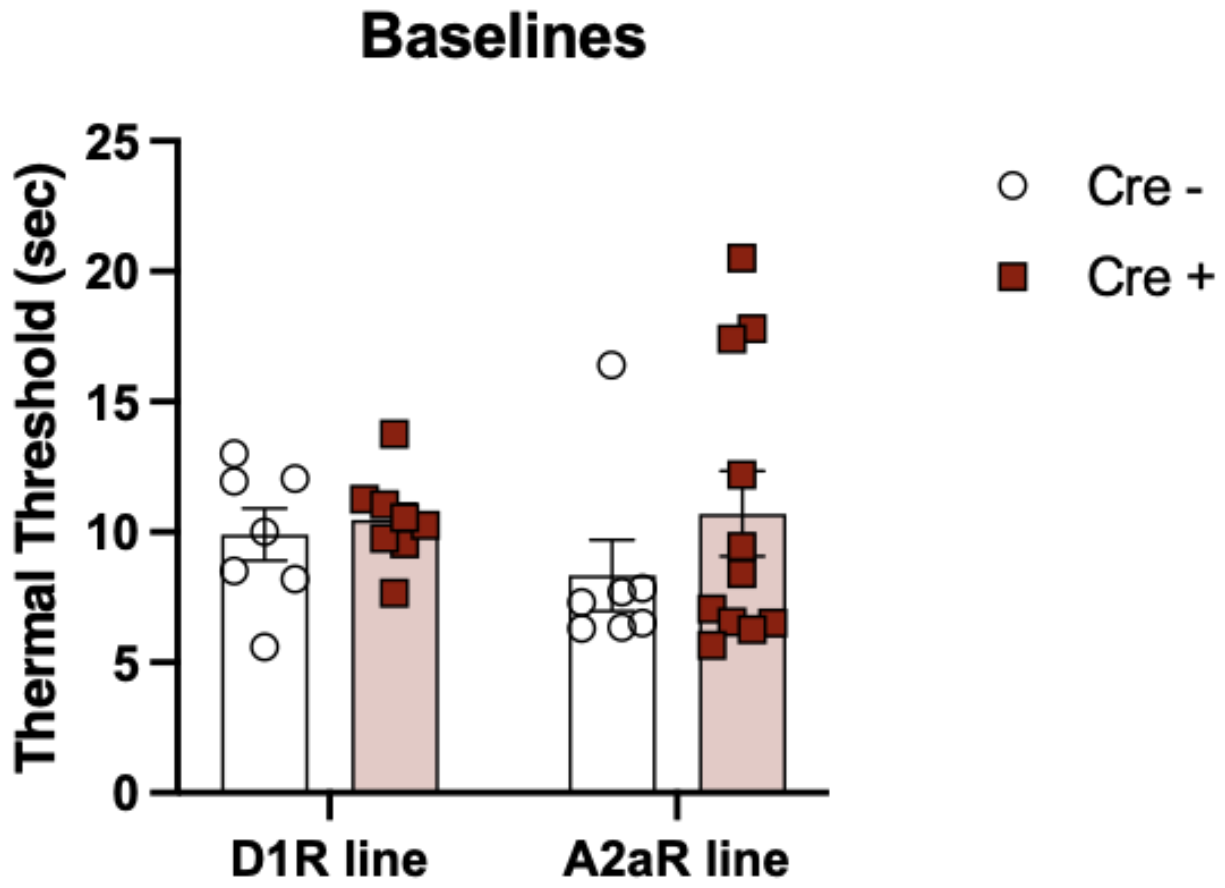


Figure 2. Ablation of MORs from D1 receptor expressing neurons had no effect on oxycodone induced increases in thermal nociceptive thresholds. A. Thermal nociceptive thresholds were determined for 50 minutes following oxycodone injection (0.3 mg/kg, i.p.) in a hot plate test. Oxycodone increased thresholds above baseline values at all time points but there was no effect of genotype. **B.** Thermal threshold latencies of data in panel A were converted to percent maximum possible effect (%MPE). Again, there was no effect of genotype. Data are expressed as mean +/- SEM for N=7-11 per group.

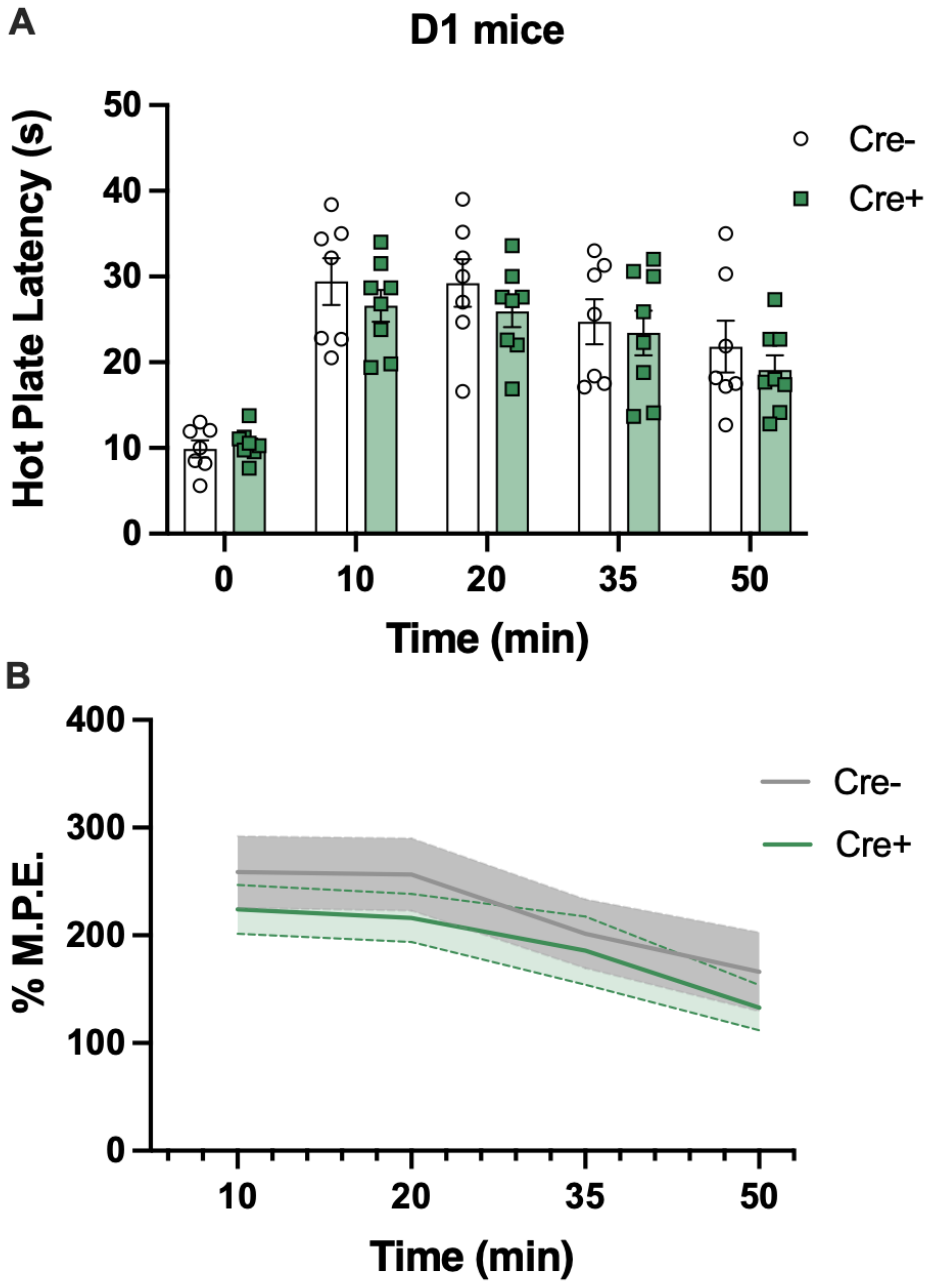
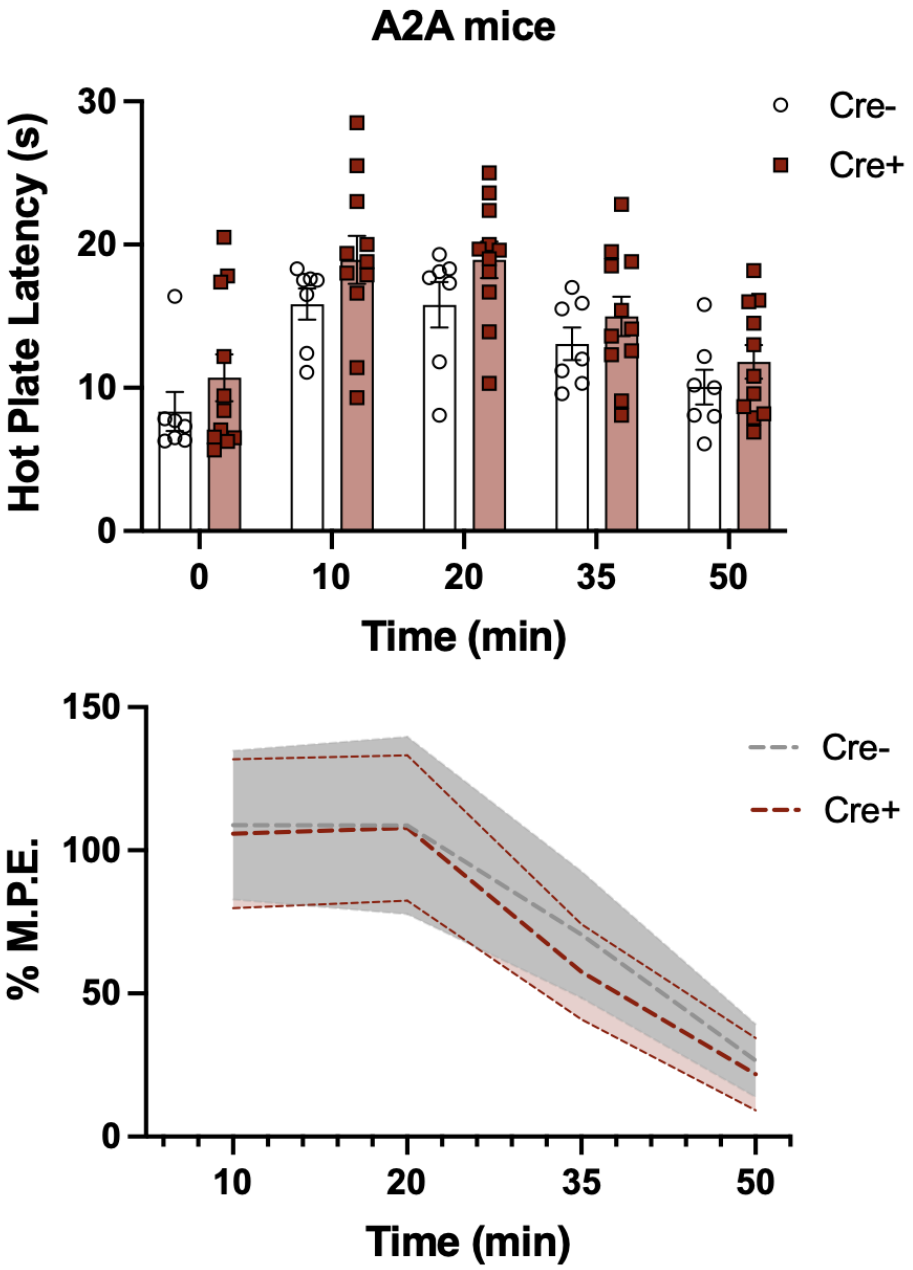


Figure 3. Ablation of MORs from A2a receptor expressing neurons had no effect on oxycodone induced increases in thermal nociceptive thresholds. **A.** Thermal nociceptive thresholds were determined for 50 minutes following oxycodone (0.3 mg/kg, i.p.) injection in a hot plate test. Oxycodone increased thresholds above baseline values at all time points but there was no effect of genotype. **B.** Thermal threshold latencies of data in panel A were converted to percent maximum possible effect (%MPE). Again, there was no effect of genotype. Data are expressed as mean +/- SEM for N=7-11 per group.



DISCUSSION

Results from this study indicate that the absence of MORs from D1 and A2a receptor expressing neurons did not affect baseline nociceptive thermal thresholds. The data also shows that oxycodone induced analgesia is intact in the hot plate test for both D1R cre + flMOR/flMOR and A2aR cre + flMOR/flMOR animals when compared to their Cre- littermates. There is no significant difference in the %MPE or hot plate latency between genotype animals. These results indicate that in acute pain paradigms, specific ablation of MORs do not show differences in analgesic responses when removed from D1 or A2a receptor expressing neurons. This is consistent with previous findings from both Kieffer and Yang who also reported no differences in opioid induced analgesia in acute pain paradigms in D1 specific knockdown animals (Charbogne et al. 2017, Cui et al. 2014). These results indicate that MORs on D1 and A2a receptor expressing neurons are likely not important mediators of opioid induced analgesia in acute thermal nociception. The data further supports that many differences exist in how acute and chronic pain are regulated via opioid analgesics and engage different neuronal processes, as differences in opioid reward were observed in chronic pain paradigms for these genotypes.

Results from this study suggest that oxycodone induced analgesia is mediated by activation of MORs in other neuronal circuits outside of those containing D1 or A2a receptor expressing neurons. As stated in Chapter 1, there are many other locations of MORs outside of those located on D1 or A2a receptor neuronal populations. Some locations that could be mediating opioid induced analgesia in these animals include the dorsal spinal cord and PAG, which contain MORs on neurons that do not express D1 or A2a receptors and thus would not be affected in our genetic models. These results add to our understanding of the complicated behavioral differences between A2aR cre + flMOR/flMOR and D1R cre + flMOR/flMOR animals.

Despite opioid induced analgesia not being altered by conditional deletion of MORs from either of these neuronal populations, MORs in these neurons may be important in other pain modalities or non-sensory affective states. Certain personality traits in humans such as reward responsiveness have correlations with MOR function and endogenous opioid transmission (Wanigasekera et al. 2012). This latter study found that upon administration of a MOR agonist, the amount of opioid induced analgesia was positively correlated to reward responsiveness. Interestingly, the largest post-MOR agonist infusion changes in brain activity occurred in the rostral anterior cingulate cortex (rACC), left orbitofrontal cortex (OFC), right NAc, left caudate, left amygdala and hippocampus, right amygdala, hippocampus, and VTA. Results from this study further implicated the importance of the striatum in the role of pain processing and drove our later questions about the role of MORs in these regions.

Additionally, MORs located on the D1 and A2a expressing neurons have shown some differences in opioid mediated reward responses. Severino and colleagues found differences in opioid induced locomotion, sensitization, and extinction depending on which subpopulations of MORs were ablated (Severino et al. 2020). In D1R cre + flMOR/flMOR animals, there was a decrease in opioid induced locomotion and decreased sensitization to opioid-induced hyperlocomotion. Interestingly, there were no differences in intravenous self-administration (IVSA) of opioids observed in these animals compared to controls (D1R cre- flMOR/flMOR). In the A2aR cre + flMOR/flMOR animals, differential effects were observed. This group showed increased opioid-induced hyperlocomotion and increased sensitization to opioid-induced hyperlocomotion. A2a cre + flMOR/flMOR animals exhibited an increase in drug seeking behaviors after opioid IVSA compared to controls (A2aR cre- flMOR/flMOR).

In conclusion, our experiments showed that there is no significant difference in the analgesic response during an acutely painful stimulus between MOR ablation in D1 and A2a receptor expression neurons, nor was there any effect of genotype on acute thermal nociceptive thresholds. These results aligned with expectations of MOR in dopamine receptor circuits may be more important in chronic pain states rather than acute pain states.

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Chapter 3: What is the role of MORs on D1 receptor expressing neurons within the NAc in opioid induced analgesia, reward, and negative reinforcement?

INTRODUCTION

MOR homeostasis is disrupted in both chronic pain and persistent opioid use states. Studies have begun to explore the function of opioid receptors in various neuronal circuits and brain regions using conditional knockdown approaches. Work by Kieffer and colleagues (2017) indicates that alterations in opioid induced reward, but not opioid induced analgesia, occurs when MORs are knocked down in all forebrain GABAergic neurons (Charbogne et al. 2017). Yang and colleagues (2014) established that re-expression of MORs, on a MOR constitutive knockout background such that MOR was only expressed in D1R MSNs throughout the striatum, restored opioid-induced reward, and dopamine release in the striatum (Cui et al. 2014).

The studies highlighted above suggest that MORs on GABAergic forebrain and D1R MSNs throughout the striatum are responsible for some behaviors associated with opioid induced reward but not analgesia. These studies drove research questions in the Cahill lab about the role that MORs in striatal circuits play in modulating pain. Using the animal models previously studied by the Cahill Lab, opioid induced analgesia was assessed in tonic inflammatory and neuropathic pain models (Severino et al. 2020).

Unpublished studies in the Cahill Lab used D1R cre +/- flMOR/flMOR, D2R cre +/- flMOR/flMOR, and A2aR cre +/- flMOR/flMOR mice to selectively ablate MOR from different neuronal populations found in the striatum. To test whether these specific MOR knockdown mice had any differences in tonic inflammatory pain, animals were subjected to the formalin model of tonic inflammatory pain. During this test, animals were assessed for nociceptive behaviors after an intraplantar injection of formalin over a 50-minute period.

Interestingly, without any drug administration D1R Cre+, D2R Cre+ and A2aR Cre+ animals showed no significant differences in nocifensive behavior compared to wild-type littermates (Cre- animals) in the formalin test. These results indicated that there was no significant difference in the endogenous tone that may modulate nociception in an inflammatory pain model (Figure 4).

The Cahill lab next asked if opioid induced analgesia was perturbed in these genetically modified mice. Formalin testing was done on animals after the administration of oxycodone, a common opioid analgesic. Results demonstrated that oxycodone significantly reduced nocifensive behavior in the D1R cre- flMOR/flMOR mice, however, the absence of MOR in D1R neurons (D1R cre+ flMOR/flMOR mice) prevented opioid-induced attenuation of the formalin-induced pain responses, where nocifensive behaviors were similar to vehicle injected animals (Figure 5). This result indicates a decrease in opioid induced analgesia in an inflammatory pain model when MORs are knocked out from D1R neurons.

The same test in D2R cre + flMOR/flMOR and A2aR cre + flMOR/flMOR mice showed that ablation of MOR from these neuronal populations did not affect pain behavior. However, oxycodone antinociception was significantly enhanced in mice lacking MORs in D2R or A2aR expressing neurons. Together, this data shows that MORs have opposing roles in D1R vs D2R/A2aR containing neurons where oxycodone antinociception was significantly attenuated in mice lacking MORs in D1R expressing neurons and was enhanced in mice lacking MORs from D2R and A2aR co-expressing neurons.

After significant differences in opioid induced analgesia were seen in a model of tonic inflammatory pain, chronic neuropathic pain was then assessed. The next study determined to what extent D1R cre +/- flMOR/flMOR, D2R cre +/- flMOR/flMOR, or A2aR cre +/- flMOR/flMOR mice showed any significant differences in opioid reward in non-pain

states or opioid-induced negative reinforcement in chronic pain states. This question was assessed by inducing a chronic constriction injury (CCI) at the sciatic nerve followed by conditioned place preference (CPP) tests.

In the post-conditioning test, D1R cre - flMOR/flMOR animals that received sham surgery showed no significant difference in time spent in the vehicle or oxycodone conditioning chambers. D1R cre - flMOR/flMOR animals that received a CCI surgery spent significantly more time in the chamber when oxycodone was administered (Figure 6A, 7). D1 cre + flMOR/flMOR sham animals in the post-conditioning test showed a significant aversion to the oxycodone conditioned chamber, whereas those that received CCI surgery showed no significant preference for the oxycodone chamber (Figure 6B).

In the state-dependent test, D1R cre - flMOR/flMOR animals that received sham surgery or CCI surgery spent significantly more time in the oxycodone conditioned chamber (Figure 8A, B). D1 cre + flMOR/flMOR sham animals and CCI animals showed no significant preference for the oxycodone chamber in the state-dependent test (Figure 8A, B). These results indicate that oxycodone place preference, which is normally enhanced in chronic pain states, is absent in D1 cre +flMOR/flMOR animals in both pain-naive/sham and chronic pain states. Similar to the inflammatory pain model, both D2R cre + flMOR/flMOR and A2aR cre + flMOR/flMOR animals that received a CCI surgery showed an enhanced place preference for oxycodone in the post-conditioning and state-dependent test.

From these studies, it was concluded that ablation of MORs from D1R, D2R, and A2aR expressing neurons did not affect baseline nociception. However, ablation of MORs from D1R expressing neurons attenuated oxycodone analgesia in the formalin test and negative reinforcement in chronic pain model, while ablation of MORs from D2R and A2aR

co-expressing neurons showed enhanced oxycodone analgesia and negative reinforcement in chronic pain models.

This study provided some clarification for the role of MORs on specific subpopulations of neurons (D1R, D2R, and A2aR). However, the results are limited due to the lack of specific deletions within a circuit. As discussed previously, D1R expressing neurons are located throughout the striatum and in other brain regions. Alterations in opioid induced analgesia in inflammatory and neuropathic pain models could be occurring in any of these areas. This limitation put many regions into consideration but studies by Franklin and colleagues implicate the NAc as a critical region for opioid induced analgesia.

Franklin and colleagues found that direct injections of morphine into the NAc decreased persistent pain while direct injections of naloxone into the NAc attenuated the antinociceptive effect of systemically administered morphine in the formalin test (Franklin et al. 1989). Additionally, this study found that morphine induced analgesia was blocked by dopamine antagonists. My thesis project implicates MORs in the NAc due to the profound analgesic effects of opioid agonists administered in this area (Franklin et al. 1989). This study, along with those previously mentioned, drove my thesis project to be focused on the NAc as a target for where MORs may be driving altered opioid induced analgesic behaviors.

In the present study, we asked whether re-expression of MORs in D1R MSNs within the NAc shell is sufficient to produce negative reinforcement in chronic pain states. As with prior studies, a breeding strategy was used to establish the D1R cre +/- flMOR/flMOR line and an AAV (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) was used to re-express MORs in all cre+ cells (Severino et al. 2020). Similar to previous studies, we wanted to determine if the re-expression of MORs would result in a differential response to opioid induced analgesia in a neuropathic pain model. We tested this by putting our genetically altered D1R cre +/-

flMOR/flMOR mice through conditioned place preference testing to oxycodone after a CCI or sham surgery.

METHODS

Mouse Line

All procedures were authorized by the Institutional Animal Care and Use Committee (IACUC) and comply with the Policies on the Use of Animals in Research as outlined by the National Institutes of Health and ARRIVE guidelines. All transgenic mice used in this study were bred by the Animal Breeding Colony. D1R cre+ flMOR/flMOR, D2R cre+ flMOR/flMOR were generated by breeding flMOR/flMOR mice (loxP sites flanking exons 2–3 of the *oprm1* gene on a 50:50 C57BL/6J:129Sv background, stock #030074, The Jackson Laboratory) with three Cre driver lines to obtain Cre recombinase on one (D1cre; stock #030989-UCD, D2cre; 032108-UCD) allele and flMOR on both alleles. Control Cre- flMOR/flMOR mice of the same background were generated as littermates from the breeding strategies. Mice lacking all MORs (stock #007559, 100% C57BL/6J, The Jackson Laboratory) were bred as heterozygous pairs to generate knock-out (KO) and wild-type (WT) littermates. Male and female transgenic mice were used between ages 8–32 weeks and 20–36 g of body weight. Animals were maintained on a 12/12 h light/dark cycle with *ad libitum* access to food and water, and experiments were conducted during their active dark phase.

Viral Injection Surgery

All animals were anesthetized with isoflurane (induction at 3% and maintenance at 2.0 – 2.5% in oxygen). An incision was made in the skin over the skull to expose lambda and bregma landmarks to identify coordinates for viral injections. A fine point drill bit was used

to create a hole on each side for bilateral injections, using a beveled 35-gauge needle (Hamilton Company). Each animal had a viral injection of 250nl per side of either AAVDJ-hSyn-DIO-mCherry (packaged in Banghart Lab, made from Addgene 50459), titer: 5.3*10^12 vg/mL (determined by qPCR) or AAVDJ-hSyn1-DIO-mCherry-2A-MOR (packaged by Virovek, made from Addgene 166970), titer 2*10^13 vg/mL into NAcSh (stereotaxic coordinates from Bregma: A/P: + 1.34mm, M/L: ± 0.65mm, D/V: –4.69mm from surface of the skull). Injections were conducted over 5 minutes to minimize tissue damage from the injection rate, and the needle was left in place for 5-7 minutes after the end of the injection and removed slowly to minimize capillary action of the virus coming up the needle track (Figure 9). The skin incision was closed with 5-0 vicryl absorbable sutures and animals recovered for at least 3 weeks to allow adequate time for viral expression before conducting sham or peripheral nerve injuries. Viral injections were later confirmed using immunohistochemistry. All animals received lactated ringers (2 mL), eye gel, and acetaminophen (0.000128 mg/kg) pre-operatively. Post-operative acetaminophen analgesia was also provided in the home cage by mixing acetaminophen in Ensure which was allowed to soak into food pellets.

Chronic Constriction Injury

Animals were assigned into two surgery groups, sham, or chronic constriction injury (CCI). Surgery was performed as described previously (Mosconi and Kruger, 1996). All animals received acetaminophen (0.000128 mg/kg) preoperatively and were anesthetized with isoflurane (induction at 3.0% and maintenance at 1.5 –2.0% in oxygen). An incision was made in the lateral and posterior left thigh followed by blunt dissection to expose the sciatic nerve. For CCI, the nerve was encased with polyethylene tubing (PE20, 2 mm) (Figure 10) as previously described (Liu et al. 2019, Taylor et al., 2015). The control group

(sham) received all surgical procedures except the muscle blunt dissection and nerve manipulation. Post-operative acetaminophen analgesia was also provided in the home cage by mixing acetaminophen in Ensure which was allowed to soak into food pellets. Mice were allowed to recover for 6 days before further experimentation.

Conditioned Place Preference

Conditioned Place Preference testing (CPP) is a Pavlovian conditioning test to measure the motivational reward effects of a drug (Cunningham et al., 2006). This test allows for a within-subject analysis of the time spent in the vehicle compared with a drug-paired conditioning chamber, which allows for paired analysis to assess the rewarding and/or motivational effects of a drug. CPP was conducted using an unbiased, counterbalanced, three-chamber apparatus. Each square box (28 x 28 x 19 cm) was divided into two equal-sized conditioning chambers separated by a guillotine door in addition to a neutral chamber. The two chambers were distinguished with visual and tactile cues so that the animals could discriminate between chambers. The striped chamber had a soft mesh flooring and an ethanol scent cue while the circle chamber had hard mesh flooring and a simple green scent cue. The third chamber contained no wall pattern, wire flooring or scent cues and acted as the neutral chamber.

To counterbalance the groups before the conditioning sessions, animals were placed in the CPP apparatus and allowed free access to all chambers. The time spent in each chamber was recorded over 30 min using an infrared camera attached to a computer running behavioral tracking software (EthoVision; Noldus). The drug paired chamber was assigned such that any innate bias for one chamber over the other was balanced between treatment groups. Preconditioning data was collected after viral injection but before CCI surgery.

During the six total conditioning sessions, animals were confined to only one of the compartments so that they would associate the contextual cues of the compartment with the motivational effects of the stimulus (drug or vehicle, counterbalanced every other day). Conditioning sessions consisted of 3 trials of oxycodone (3.0 mg/kg, i.p.) and 3 trials of saline on alternating days. Animals were confined to the conditioning chambers for 30 minutes.

The CPP assay began 6 d after chronic constriction injury at a time previously shown to demonstrate pain hypersensitivity (Taylor et al., 2014). On the postconditioning day (day 7 after the first conditioning day), no treatment was given to the animals so that they could be tested in a drug-free state. Animals were allowed free access to all chambers of the apparatus and the time spent in the drug-paired chamber measured by motion capture Noldus software was determined over 30 min. Following the post-conditioning day, a state-dependent test was conducted in which all animals received oxycodone (3.0 mg/kg, i.p.) and were allowed to roam all three of the chambers.

Drugs

The Schedule II drug oxycodone was obtained from the NIDA Drug Supply Program (RTI). Oxycodone HCl was used at a dosage of 3.0 mg/kg, on three conditioning days, for each animal. Oxycodone was prepared in 0.1 M phosphate-buffered saline to neutralize the acid salt. Post-surgical pain was managed using acetaminophen at a dosage of 0.1 ml (32 mg/mL) of children's Tylenol per animal (0.000128 mg/kg). Acetaminophen was left in the cage mixed with peanut butter for animals to consume post-operatively.

Von Frey Test

Withdrawal thresholds to a mechanical stimulus applied with calibrated von Frey filaments were measured in sham and CCI animals, as previously described (Taylor et al. 2015). Mice were placed atop a mesh grid floor in a clear acrylic glass enclosure. Von Frey filament (2g force hair) was applied to the plantar surface of the ipsilateral hind paw in an up-down manner. Movement of the hind paw upon application of Von Frey filament was considered a positive response. Each animal was probed 10 times and their score was measured as the number of positive responses out of 10. Baseline measurements were taken for all animals before surgery and 2 weeks postoperatively.

Formalin Test

The formalin test was employed as a model of inflammatory pain involving tissue injury and inflammation whereby an intraplantar subcutaneous injection of formalin (2.5%, 30 µl injection volume) into the mouse's left hind paw. This injection produces a characteristic biphasic nocifensive response characterized by licking and flinching (Kabli and Cahill 2007). Mice were placed in a large plexiglass box (28 cm^3) atop a mirror at a 45-degree angle to help visualize the hind paw at all times. Mice were habituated to the box for 10 minutes prior to injecting the formalin, where they were immediately returned and scoring of pain behaviors commenced. Behavior was evaluated in 5-min intervals for 50 minutes. Following testing, mice were immediately euthanized (Kabli and Cahill 2007).

Immunohistochemistry

Brains were collected from sham and CCI mice after the completion of behavioral data. Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) prior to undergoing intra-cardiac perfusions with 4% paraformaldehyde in saline. Brains were then post-fixed

for 2 h in 4% PFA. They were then transferred to 30% sucrose where they remained until they sunk to the bottom of their tubes. After sitting in sucrose solution, brains were snap-frozen with methylbutane at −30°C and stored at −80°C until further processing. Coronal sections were taken spanning the striatum via cryostat (20 µm thick, Leica) at −20°C, and thaw-mounted on Superfrost charged slides. Antibodies against mCherry and DAPI were used to image mCherry-expressing neurons and cell nuclei, respectively. Slides were removed from the freezer and washed three times with 1X Tris-buffered saline (TBS) (Sigma-Aldrich, Cat # T5912). After the last wash, slides were incubated for 30 min in a blocking solution consisting of 10% donkey serum (Sigma-Aldrich Cat # D9663), 0.5% Triton-X (Millipore Sigma, Cat # 11332481001), and 1X TBS on a shaker at room temperature. The blocking solution was removed, and sections were incubated in a rabbit anti-MOR solution diluted 1:1000 (7TM antibodies #07220319C1C001) in 1X TBS overnight at 4°C. The next day, slides were washed three times with 1X TBS and incubated for 2 h at room temperature in a solution containing 1:1000 diluted secondary goat anti-rabbit Alexa 488 antibody (invitrogen #1812158), 1:1000 diluted rat anti-mCherry antibody tagged with an Alexa 594 fluorophore (invitrogen #2622394), and 10% donkey serum in 1X TBS. Slides were then dried, covered with a DAPI antifade mountant (Thermo Fischer, Cat # P36962), and stored at -20 °C for at least 24 h. Sections were initially imaged using a Leica DMI8 epifluorescent microscope provided by the UCLA IDDRC. The DAPI, GFP, and Cy3 channels were used for DAPI, mu, and mCherry, respectively.

Statistics

Two-way ANOVA was used to analyze data obtained from the Conditioned Place Preference (Pre-Conditioning, Post-Conditioning, and State-Dependent) testing using Prizm v9 (GraphPad 9.0 software) with treatment and surgery as independent variables.

Interactions were reported. The two-way ANOVA was followed by Sidak's post hoc analysis. Von Frey and formalin data were also analyzed by a two-way ANOVA with time and surgery as independent variables. The experiments were not powered to detect sex differences, so mice were grouped together, but sex was identified in figures. Groups were balanced by sex and age as much as possible. A p-value less than 0.05 was considered significant. Experiments were conducted in cohorts ranging from 8-16 mice. Experimental groups were evenly distributed among cohorts and successive experiments were performed to increase N values for each experimental group.

Experimental Design

Mice used for this test were D1R cre + flMOR/flMOR based on a breeding scheme of heterozygous mice that allowed for both double floxed cre deletion of MOR and wild type (cre -) littermates. Some D1R cre - flMOR/flMOR littermates were also used as controls in later testing. These animals once transferred from our breeding colony were allowed at least one week of habituation before their first surgery.

Testing began with the viral re-insertion of MORs into all cre+ cells. This viral construct allowed for re-insertion into only D1R cre+ cells that were previously not expressing MORs. The virus AAVDJ-hSyn1-DIO-mCherry-2A-MOR was injected bilaterally into the NAc shell via stereotaxic methods (Figure 10). Some animals received a control virus, AAVDJ-hSyn-DIO-mCherry, which was only a label for mCherry at the injection site. After viral injection surgery, animals were allowed approximately three weeks of rest to allow for viral re-incorporation of MORs at specific injection sites.

Next, baseline preconditioning data was taken for each animal before introduction of a CCI or sham surgery. Preconditioning data was used to counterbalance any preferred

sides of individual animals. After preconditioning data was collected and analyzed, mice underwent CCI or sham surgeries (Figure 10).

Next, animals began CPP conditioning. This allowed us to assess opioid preference for oxycodone in both pain naive and chronic pain models. Mice were conditioned for 6 days, where they received either saline or oxycodone via i.p. injection If the mouse prefers the drug chamber, it is concluded that the drug is rewarding. We expect that with re-insertion of the MORs into D1R-MSNs of the NAc animals in a chronic pain state will recover a place preference to oxycodone.

To verify the accurate reinsertion of MORs in the appropriate neuronal type and location following our viral injection surgery, immunohistochemistry was employed to visualize the presence of mCherry, which was incorporated into the viral construct.

RESULTS

This experiment used both D1R cre + flMOR/flMOR and D1R cre - flMOR/flMOR littermates. Animals were allowed to habituate to the reverse light/dark vivarium after transfer from the breeding colony for 1 week prior to handling. Mice then underwent extensive handling and habituation to the testing environment prior to experimentation. Next, viral injection surgeries were completed with control or active virus. The control virus (AAVDJ-hSyn-DIO-mCherry) only expresses mCherry, which was later used for visualization via IHC. The active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) allowed for re-insertion of MORs into only D1 cre+ cells that were previously not expressing MORs and contains an mCherry tag.

To determine the extent the active virus may be inducing non-specific or toxic effects, AAVDJ-hSyn1-DIO-mCherry-2A-MOR was injected into the NAc shell bilaterally in D1cre - flMOR/flMOR mice. No viral recombination was expected as there is no cre to turn

on transcription. In context-dependent CPP post-condition testing, D1cre - flMOR/flMOR showed no significant differences in the time spent in the oxycodone-paired chamber compared to the saline compartment, regardless of surgery condition (N=3M, 3F) (Figure 11A). A 2-way ANOVA found no significant differences in surgery ($F(1,8) = 0.02931$, $p=0.8683$) or treatment groups ($F(1,8)=2.819$, $p=0.1317$). Because there was no difference, groups were combined and a student t-test was performed ($t=1.856$, $df=10$, $p=0.0931$). When the data was transformed to the CPP score, which considers their initial preconditioning scores, again none of the groups showed a significant difference from a theoretical value of 0 (Figure 11B). A one sampled t-test showed no difference for sham ($t=0.5096$, $df=2$, $p=0.6610$) or CCI ($t=0.5666$, $df=2$, $p=0.6281$) animals.

In the state-dependent testing, again neither D1R cre - flMOR/flMOR CCI or sham animals showed a preference for the oxycodone conditioned chamber compared to the saline chamber (N=3F for CCI and N=3M for sham (Figure 12 A). A 2-way ANOVA found no difference in surgery ($F(1,8) = 0.02149$, $p=0.8871$) or treatment ($F(1,8)=4.181$, $p=0.0751$). When surgery groups were combined, an unpaired t-test also showed no significant differences ($t=2.041$, $df=10$, $p=0.0685$). When the data was transformed to the CPP score, again none of the groups showed a significant difference from a theoretical value of 0 a CPP score (N=3M, 3F) (Figure 12 B). A one sampled t-test showed no significant differences between sham ($t=0.9336$, $df=2$, $p=0.9336$) or CCI ($t=3.174$, $df=2$, $p=0.866$) animals.

We next asked to what extent the control virus had on oxycodone CPP in D1R cre + flMOR/flMOR mice. There were too few animals per group (N=1) to determine differences in sham and CCI groups (Figure 13).

We next asked to what extent re-expression of MOR in the NAc shell had on oxycodone CPP in both sham and CCI mice. In the post condition test, there was no preference for oxycodone observed (Figure 14A). A 2-way ANOVA showed no effect of

surgical condition ($F(1,22) = 0.00095$, $p=0.9232$) or treatment ($F(1,22)=0.5507$, $p=0.4659$). The transformation of data to a CPP score also revealed no significant differences between groups (sham: $t=1.571$, $df=5$, $p=0.1771$, CCI: $t=0.1588$, $df=6$, $p=0.8790$) (Figure 14B). In the state dependent test, there was no preference for oxycodone (Figure 15A). A 2-way ANOVA showed no effect of surgical condition ($F(1,22) = 0.02457$, $p=0.8769$) or treatment ($F(1,22)=2.610$, $p=01204$). The transformation of data to a CPP score also revealed no significant differences (sham: $t=1.840$, $df=5$, $p=0.1251$, CCI: $t=0.8563$, $df=6$, $p=0.4247$) (Figure 15B).

Using IHC, brains were evaluated for each group. No mCherry expression was found in D1R Cre- and D1R Cre+ animals with active virus (Figure 16). There was mCherry expression found in D1R Cre+ animals that received control virus (Figure 9).

Figure 4. Ablation of MOR from D1R expressing neurons had no effect on nociception induced by formalin. Nocifensive behavior in D1R cre + flMOR/flMOR and D1R cre - flMOR/flMOR animals were assessed in formalin test. Formalin tests were conducted over 50 minutes following injection of formalin (2.5%) subcutaneously in the plantar surface of the left hind paw. Nocifensive behavior was characterized by licking and flinching. No sex differences were detected. Data are expressed as mean +/- SEM for N=9-17 per group.

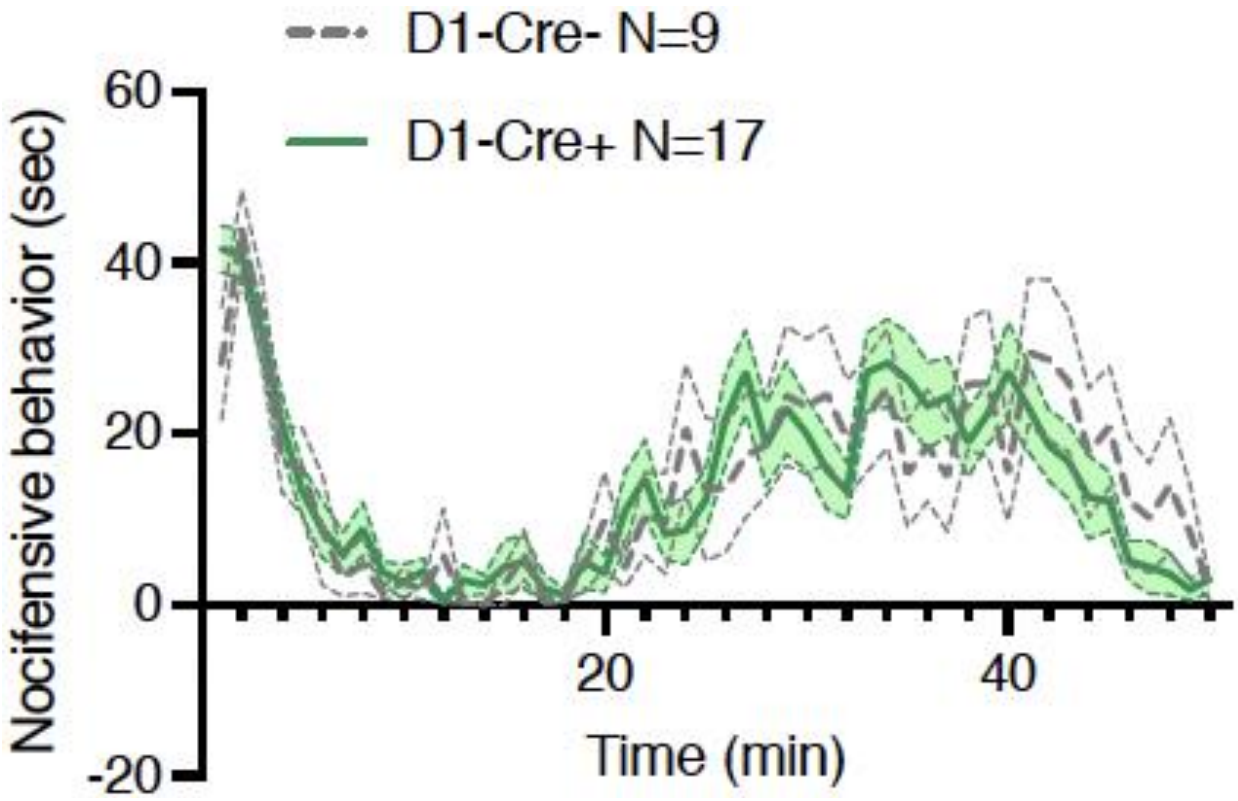


Figure 5. Ablation of MORs from D1R expressing neurons attenuated oxycodone induced antinociception in the formalin test. Oxycodone (0.3 mg/kg, i.p.) was administered to D1R-cre-flMOR/flMOR or D1R-cre+flMOR/MOR male and female adult mice 10 minutes prior to injection of formalin. Nocifensive behavior was measured by licking and flinching after injection of subcutaneous formalin (2.5%) in the plantar surface of the left hind paw. Oxycodone significantly diminished pain behaviors in the Cre - animals, whereas the effects of oxycodone were significantly blunted in the Cre + cohort. No sex differences were detected. Data is expressed as a mean +/- SEM for N=10-13 per group.

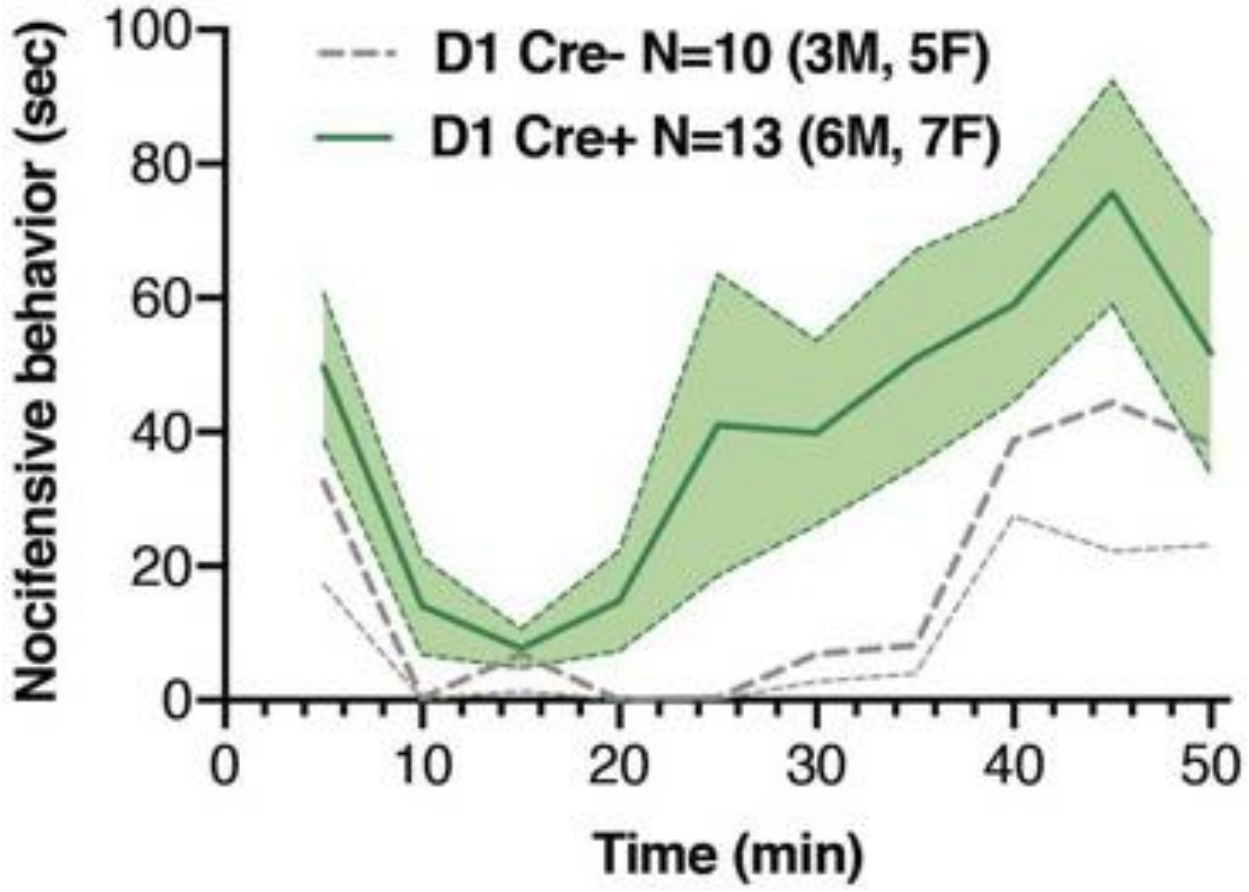


Figure 6. Ablation of MORs from D1R neurons causes a significant aversion to oxycodone chamber in naive pain states and no significant preference in neuropathic pain models in the post-conditioning test. A. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1R cre - flMOR/flMOR that received a sham surgery did not show a preference for the oxycodone chamber over the vehicle (N=5F, 5M). D1R cre - flMOR/flMOR that received a PNI (peripheral nerve injury - aka. CCI surgery) showed a significant preference for the oxycodone chamber over the vehicle chamber (N= 6F, 6M). D1R cre + flMOR/flMOR animals that received a sham surgery showed an aversion for the oxycodone chamber compared to the control (N= 3F, 5M). D1R cre + flMOR/flMOR animals that received a PNI (aka. CCI surgery) showed no significant difference between the time spent in the oxycodone and vehicle chambers (N= 4F, 9M). **B.** A CPP preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and postconditioning day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. D1R cre - flMOR/flMOR animals that received a sham surgery showed no significant difference in CPP score (N=5F, 5M). D1R cre - flMOR/flMOR animals that received a PNI (peripheral nerve injury aka. CCI surgery) showed a significant increase in CPP score (N= 6F, 6M). D1R cre + flMOR/flMOR animals that received sham surgery showed a significant decrease in CPP score (N= 3F, 5M). D1cre + flMOR/flMOR animals that received a PNI (aka. CCI surgery) showed no significant difference in CPP score (N= 4F, 9M). Data is expressed as a mean +/- SEM for N=8-13 per group.

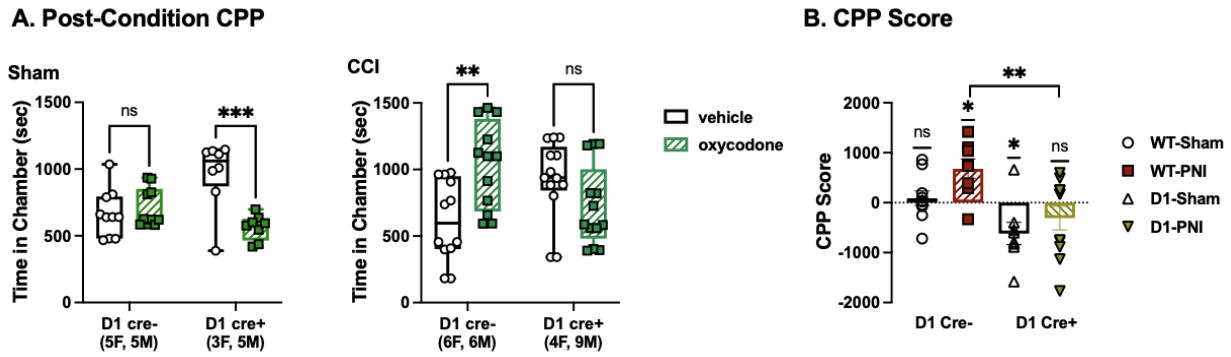


Figure 7: Heat map of state dependent day for D1R cre + flMOR/flMOR and D1R cre - flMOR/flMOR animals with CCI illustrates preference for oxycodone conditioning chamber in D1R cre- animals and limited preference for oxycodone conditioning chamber in D1R cre+ animals. Heat map was taken for the state dependent day where animals received oxycodone (i.p. 0.3 mg/kg) and were allowed access to all three chambers for 30 minutes. This data indicates that animals with MORs deleted from all D1R neurons have less preference for the oxycodone conditioning chamber when compared to controls (D1R cre - flMOR/flMOR).

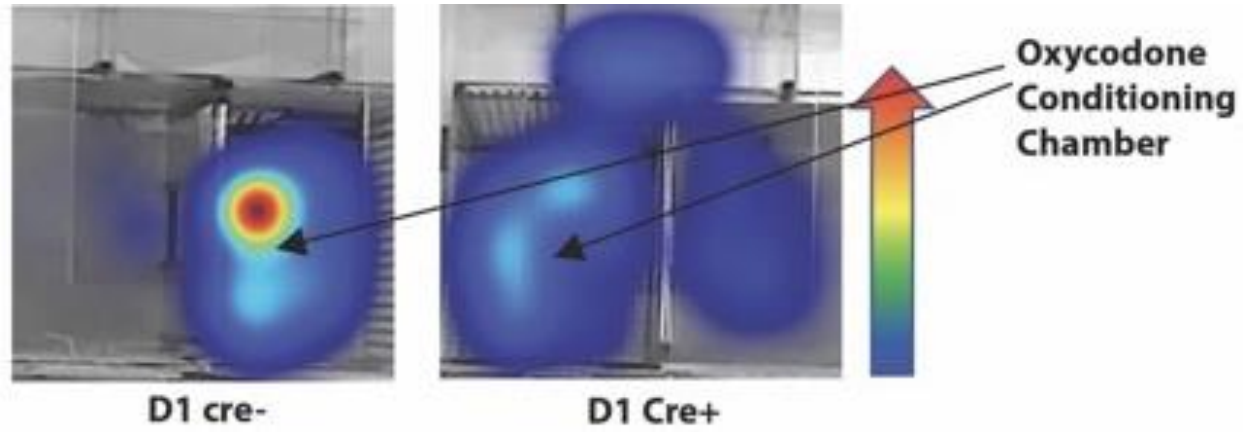


Figure 8: Ablation of MORs from D1R neurons causes no significant difference in CPP score in both naive pain and neuropathic pain models in state dependent test. A. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1cre - flMOR/flMOR that received a sham surgery showed a preference for the oxycodone chamber over the vehicle (N=5F, 5M). D1cre - flMOR/flMOR that received a PNI (peripheral nerve injury - aka. CCI surgery) showed a significant preference for the oxycodone chamber over the vehicle chamber (N= 6F, 6M). D1cre + flMOR/flMOR animals that received a sham surgery showed no preference for the oxycodone chamber compared to the control (N= 3F, 5M). D1cre + flMOR/flMOR animals that received a PNI (aka. CCI surgery) showed no significant difference between the time spent in the oxycodone and vehicle chambers (N= 4F, 9M). **B.** A preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and postconditioning day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. Each animal received an oxycodone injection (i.p. 3 mg/kg) followed by access to all three chambers over 30 minutes. D1cre - flMOR/flMOR that received a sham surgery showed significant increase in CPP score (N=5F, 5M). D1cre - flMOR/flMOR that received a PNI (peripheral nerve injury aka. CCI surgery) showed a significant increase in CPP score (N= 6F, 6M). D1cre + flMOR/flMOR animals that received sham surgery showed no significant difference in CPP score (N= 3F, 5M). D1cre + flMOR/flMOR animals that received a PNI (aka. CCI surgery) showed no significant difference in CPP score (N= 4F, 9M). Data are expressed as mean +/- SEM for N=8-13 per group.

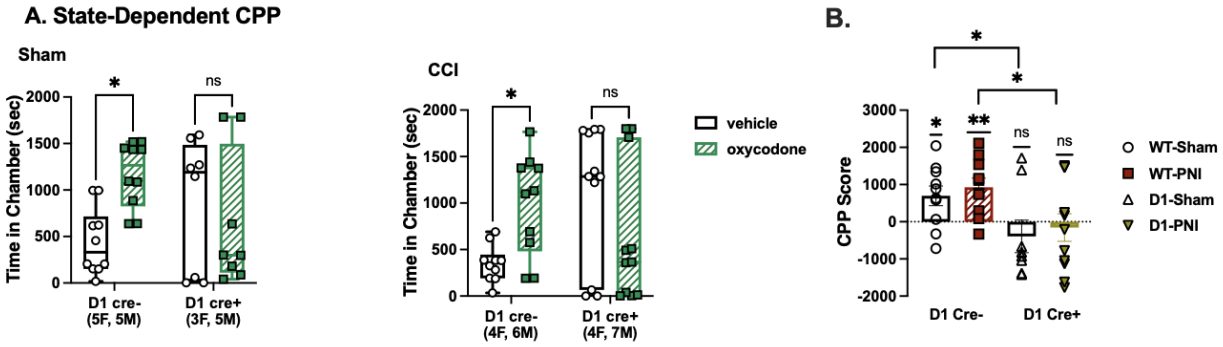


Figure 9: Sample IHC for Control Virus (AAVDJ-hSyn-DIO-mCherry) in D1R cre + flMOR/flMOR animal. Immunohistochemistry against DAPI (blue) and mCherry (yellow) were done in Cre+ animals that received control virus (AAVDJ-hSyn-DIO-mCherry). Below is an example section through the striatum. The expression of mCherry is evident around the anterior commissure, localized generally to the NAc core (N=2).

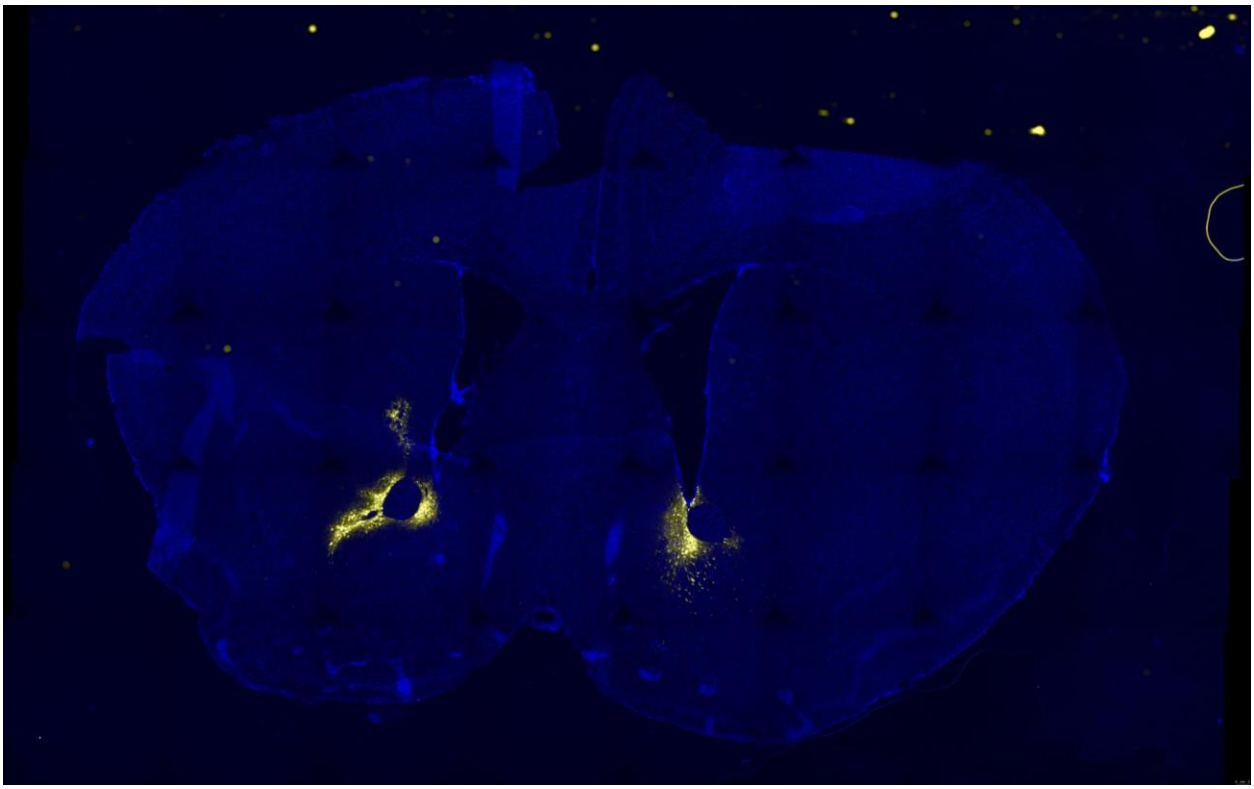


Figure 10: Graphic Representation of Experimental Design. Experiments began with viral injection surgeries where animals received either an active or control virus. After three weeks of recovery, animals underwent pre-conditioning for CPP. Next, animals underwent either a sham or CCI surgery, to induce neuropathic chronic pain. Then CPP testing was done which included 6 days of conditioning followed by a post-conditioning and state dependent test.

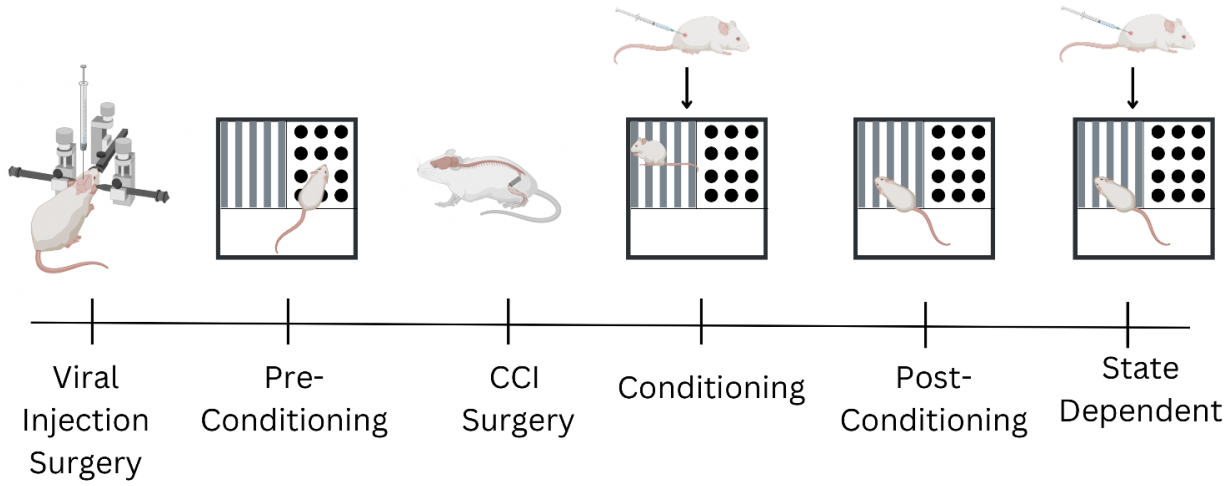


Figure 11: D1cre - flMOR/flMOR animals with active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) showed no significant difference in time spent in the oxycodone conditioned chamber during context dependent test. A. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1R cre - flMOR/flMOR sham animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=3M). D1R cre - flMOR/flMOR CCI animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=3F). **B.** A preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and postconditioning day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1R cre - flMOR/flMOR sham animals that received active virus showed no significant difference in CPP score (N=3M). D1R cre - flMOR/flMOR CCI animals that received active virus showed no significant difference in CPP score (N=3F).

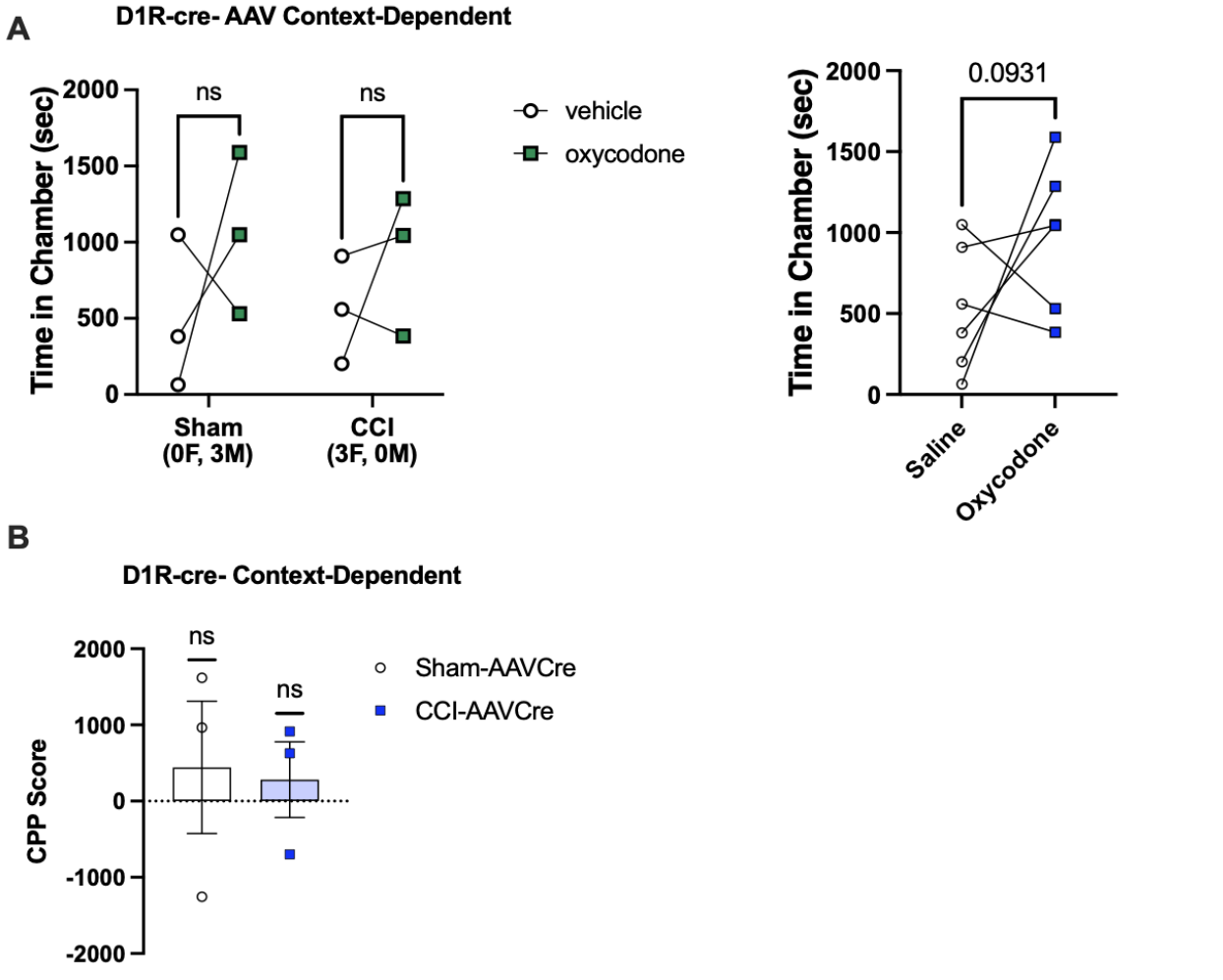


Figure 12: D1cre - flMOR/flMOR animals with active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) showed no significant difference in CPP Score or time spent in chamber during state dependent test. **A.** Time spent in oxycodone and vehicle conditioned chambers was measured after administration of oxycodone (0.3 mg/ kg i.p.) for 30 minutes. D1R cre - flMOR/flMOR sham animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=3M). D1R cre - flMOR/flMOR CCI animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=3F). All animals but one showed a preference for the oxycodone chamber during the state dependent test regardless of surgical assignment (p=0.0685) (N=3F,3M). **B.** A preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and state dependent day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. Time spent in oxycodone and vehicle conditioned chambers was measured after administration of oxycodone (0.3 mg/kg i.p.) for 30 minutes. D1R cre - flMOR/flMOR sham animals that received active virus showed no significant difference in CPP score (N=3M). D1R cre - flMOR/flMOR CCI animals that received active virus showed no significant difference in CPP score (N=3F).

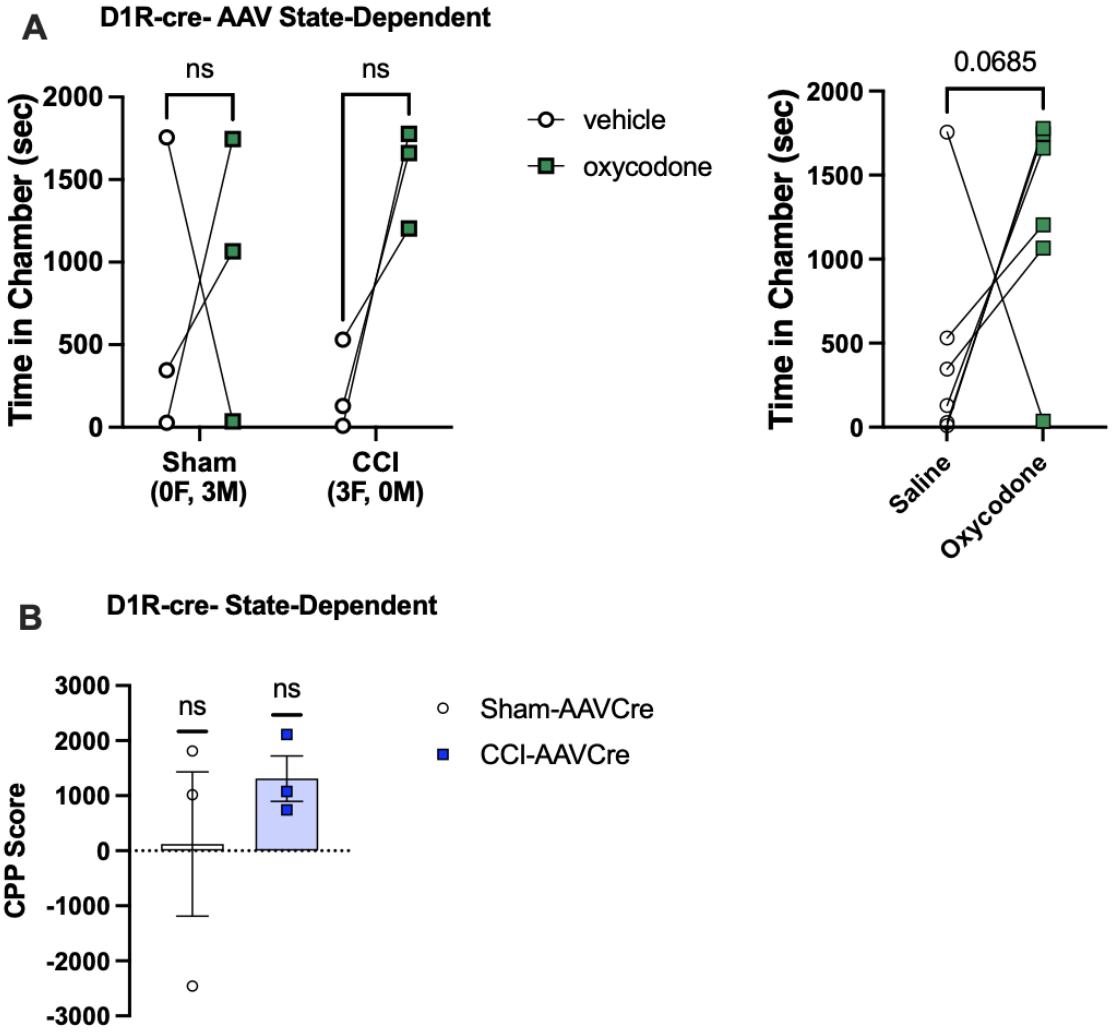


Figure 13: D1R cre + flMOR/flMOR animals with control virus (AAVDJ-hSyn-DIO-mCherry) showed no significant difference in time spent in the oxycodone conditioned chamber during state dependent test or context dependent day. Time spent in oxycodone and vehicle conditioned chambers was measured after administration of oxycodone (i.p. 3 mg/ kg) for 30 minutes in the state dependent test. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes on the context dependent test day. D1R cre + flMOR/flMOR sham animals that received control virus showed no significant difference in time spent in either conditioned chamber (N=1M). D1cre + flMOR/flMOR CCI animals that received control virus showed no significant difference in time spent in either conditioned chamber (N=1M).

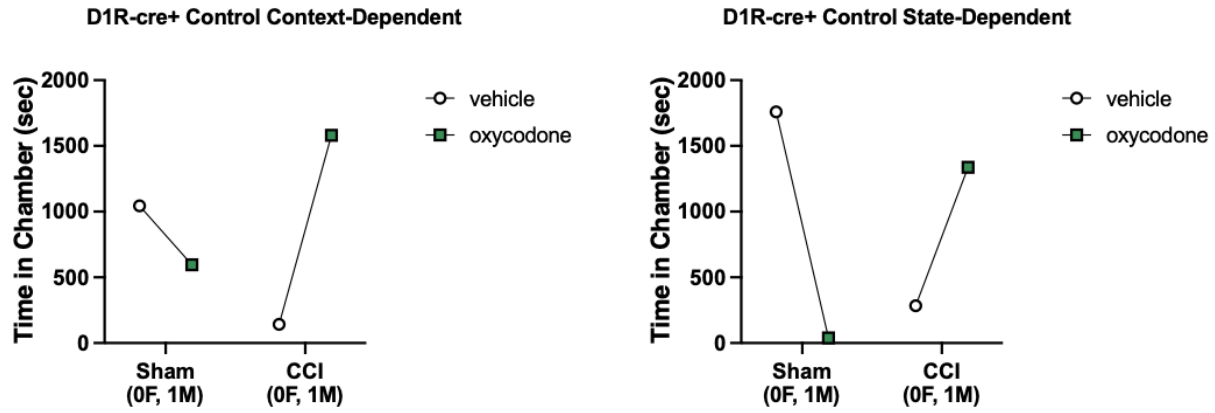


Figure 14: D1R cre + flMOR/flMOR animals with active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) show no significant difference in CPP Score or time spent in chamber during context dependent test. A. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1R cre + flMOR/flMOR sham animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=4M,2F). D1R cre + flMOR/flMOR CCI animals that received active virus showed no significant difference in time spent in either conditioned chamber (N=4F, 3M). **B.** A preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and postconditioning day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes. D1R cre + flMOR/flMOR sham animals that received active virus showed no significant difference in CPP score (N=6). D1R cre + flMOR/flMOR CCI animals that received active virus showed no significant difference in CPP score (N=7).

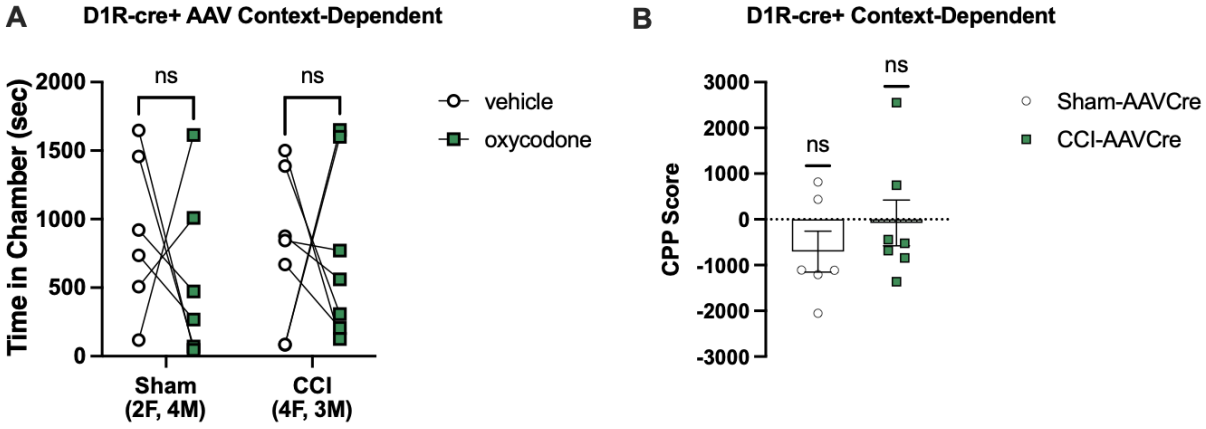


Figure 15: D1R cre + flMOR/flMOR animals with active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) showed no significant difference in time spent in the oxycodone conditioned chamber or CPP score during state dependent test. A. Time spent in oxycodone and vehicle conditioned chambers was measured after administration of oxycodone (0.3 mg/ kg i.p.) for 30 minutes. D1R cre + flMOR/flMOR sham animals that received active virus showed no significant difference in time spent in either conditioned chamber or CPP score (N=2F, 4M). D1R cre + flMOR/flMOR CCI animals that received active virus showed no significant difference in time spent in either conditioned chamber or CPP score (N=4F, 3M). **B.** A preference score for each animal was calculated by taking the difference in time spent on the drug-paired chamber between the preconditioning and state dependent day. Negative scores indicate an aversion to drug paired chamber while positive scores indicate preference for drug paired chamber. Time spent in oxycodone and vehicle conditioned chambers was measured for 30 minutes after administration of oxycodone (0.3 mg/ kg i.p.). D1R cre + flMOR/flMOR sham animals that received active virus showed no significant difference in CPP score (N=6). D1R cre + flMOR/flMOR CCI animals that received active virus showed no significant difference in CPP score (N=7).

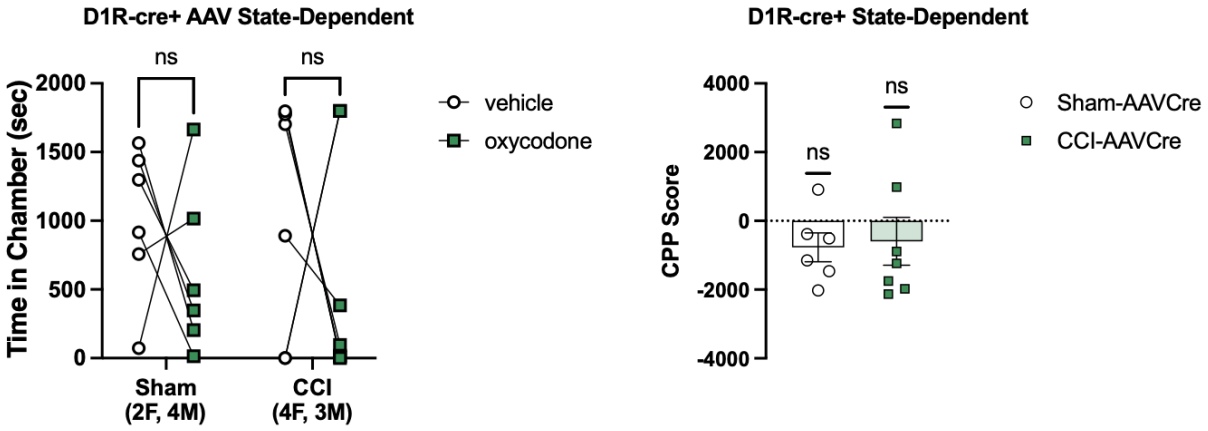
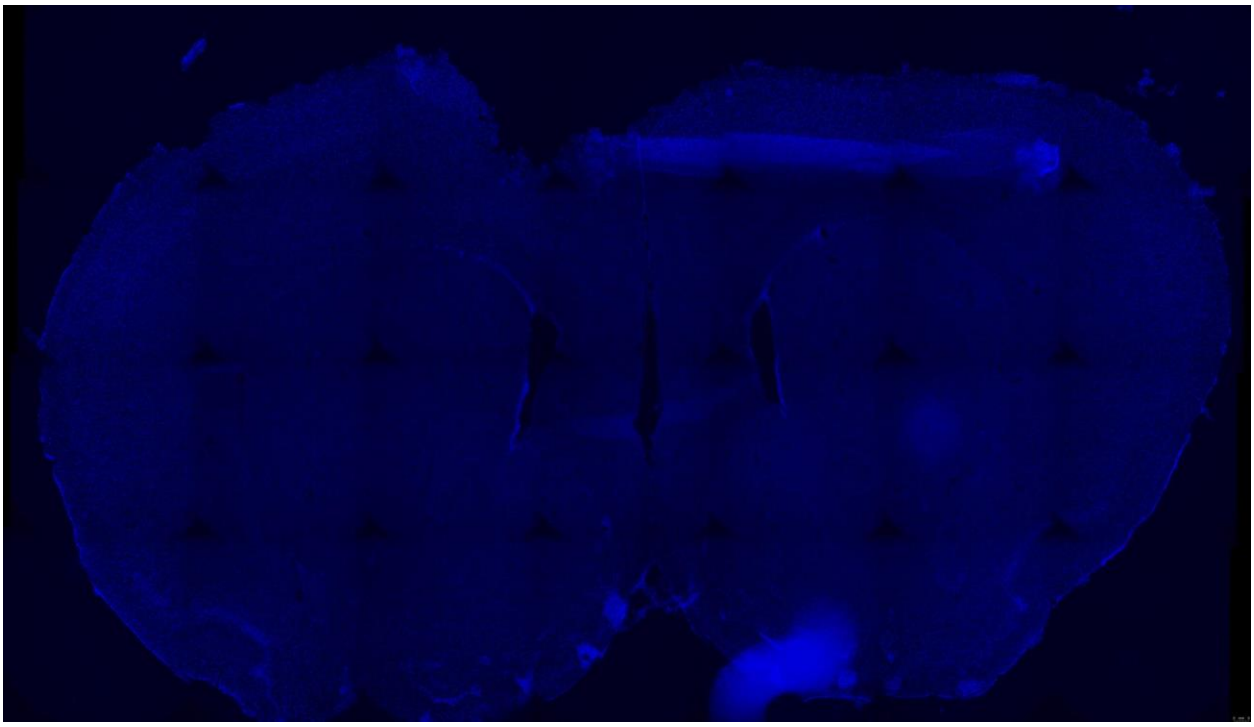


Figure 16: Sample IHC for Active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR) in D1R cre + flMOR/flMOR animal. Immunohistochemistry against DAPI (blue) and mCherry (yellow) were done in Cre+ animals that received active virus. Above is an example section through the striatum. DAPI labeling is seen throughout, but no mCherry labeling was detected (N=4).



DISCUSSION

This study replicated previous unpublished findings from the Cahill Lab showing that animals without MORs in D1R neurons had an aversion to oxycodone measured by conditioned place preference tests, regardless of the pain condition. Additionally, we were able to show in D1R cre- animals that chronic constriction injuries increased preference for opioids compared to pain naive animals, validating the work of previous studies (Cahill et al. 2013). Additionally, my thesis project validates previous findings from our unpublished studies and others that opioid induced analgesia shows no notable sex differences in this mouse model of neuropathic pain (Severino et al. 2020). Based on immunohistochemistry, we confirmed that our active virus resulted in no re-expression of MORs in D1R-expressing neurons in the striatum (Figure 16). The accuracy of the viral injection was validated in control mice via immunohistochemistry where we had strong labeling in the appropriate anatomical site (Figure 9). The expression of our control virus suggests that there was some issue with our active virus, where it may have degraded in shipment. Future experiments are needed to repeat these studies to determine if re-expression of MORs in the NAc will recover our behavioral phenotype. Moreover, viral expression confirmation is needed prior to performing other behavioral studies.

The active virus used in this study has yet to be published elsewhere but the plasmid (Addgene 166970) used to make the active virus was used in other studies. Banghart and colleagues used a similar viral construct (AAVDJ-Syn1-FLEX-mCh-T2A-FLAG-hMOR-WPRE) to assess the neural basis of opioid induced respiratory depression (OIRD). This virus rescued MORs in Oprm1 Cre/Cre mice in the lateral parabrachial nucleus, a region important in opioid induced respiratory depression. Researchers re-expressed MORs in this region and induced OIRD upon administration of morphine in animals that previously had decreased symptoms present, due to a lack of MORs. Similarly,

they confirmed injection sites using immunohistochemistry (Liu et al. 2022). This study confirms that a similar viral construct can rescue and re-express MORs, showing that a similar virus has success in other animal models and experiments. Discussions with the Banghart lab lead our research group to the conclusion that the virus was likely degraded in transit as signals for the control virus were also lower than expected (Figure 9). Future experiments are planned to more thoroughly investigate why animals showed no expression of virus.

Despite our lack of success with this viral construct, the underlying questions asked in this project require further exploration. In a recent study by Narita and colleagues, researchers explored the effects of activating D1R expressing or D2R expressing neurons via optogenetics in the NAc. After microinjections of D1 or D2 agonists they were able to decrease nociceptive behaviors associated with nerve injury induced thermal allodynia. Upon optogenetic activation of D1R expressing neurons, signs of nerve induced allodynia were reported. Similar results were found when D2R expressing neurons were optogenetically inhibited. This study concluded that activation of D1R expressing neurons and inhibition of D2R expressing neurons in the NAc can relieve signs of neuropathic pain in mice (Sato et al. 2022). These results align with unpublished studies from our lab which indicate that removal of MORs from D1R expressing neurons significantly attenuates opioid-induced analgesia. Current findings implicate the D1R expressing neuronal subpopulation as important in the symptoms of chronic pain, but further work must be done to see alterations in the NAc specifically are responsible for changes in opioid induced analgesia.

The NAc has also been implicated in the pathology of chronic pain in human subjects. Apkarian and colleagues performed fMRI studies in humans with sub-acute back pain to explore possible mechanisms of brain reorganization during the process of pain

chronification. In this longitudinal study, researchers found a greater connectivity between the NAc and prefrontal cortex in subjects with sub-acute back pain. Interestingly, this increased functional connectivity was correlated with pain persistence (Baliki et al. 2012). These results suggest that the NAc is important in the pathology of chronic pain in humans. This study provides further support for studying the nucleus accumbens role in chronic pain in animal models, since mechanisms are likely conserved. A better understanding of the molecular pathologies in this region may allow for targeted drug development or other medical interventions to treat chronic pain.

Additionally, future research should consider the diversity within the NAc shell itself when developing targeted genetic alterations. In a recent study by Bruchas and colleagues, researchers explored the role of distinct dynorphin neuronal populations in the NAc (Al-Hasani et al. 2015). This group photostimulated dynorphin neurons within various regions of the NAc and assessed animal behavior. Dynorphin neurons located within the ventral shell drove aversion behavior while the dorsal shell dynorphin neurons drove preference and reward behaviors when stimulated (Al-Hasani et al. 2015). Anatomical specificity in behavioral response has been further implicated with identification of opioid hedonic hotspots located in the rostral dorsal quadrant of the medial NAc shell (Castro and Berridge 2014). Findings from these studies and others suggest that certain anatomical regions of the NAc are responsible for different behavioral phenotypes concerning aversion, reward, and opioid preference. These studies further implicate the NAc as an important and highly specific region that mediates both chronic pain and opioid induced analgesia.

Overall, my thesis project replicated results of previous unpublished studies from the Cahill lab. Despite the viral construct not properly working, more experiments should be conducted to further investigate the role of MORs on D1R expressing neurons in the NAc shell.

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Future Directions

Since the active viral construct showed no recombination in D1R Cre+ flMOR/flMOR animals, more investigations must take place to determine why this occurred. Validation of our existing virus will be done in D1R Cre+ and D1R Cre+ flMOR/flMOR animals. Bilateral viral injection surgeries, with the active virus (AAVDJ-hSyn1-DIO-mCherry-2A-MOR), will be performed using methods outlined in Chapter 3. This experiment will allow us to assess if the virus did not recombine due to some unforeseen genetic interaction in D1 Cre+ flMOR/flMOR as all previous studies with this virus looked at Cre+ animals only. Additionally, the Banghart Lab has generously sent additional virus from a new batch. This virus will also be bilaterally injected in D1R Cre+ and D1R Cre+ flMOR/flMOR animals using methods outlined in Chapter 3. With extra care and handling we hope that this virus will have little to no degradation. This experiment will also allow us to see if the previous virus was faulty due to mishandling. The Banghart lab has also offered to perform viral injections in their D1R Cre+ mouse line to confirm its validity with no chance of viral degradation due to transport. Hopefully, these studies will allow us to understand why the viral construct did not work in my thesis project.

With a better understanding of why the virus failed, experiments can proceed to address the hypothesis in my thesis project. Hopefully, issues with the viral construct can be resolved and similar tests can be run using the same experimental paradigm. Additionally, other genetically manipulated animals may assess this question to gain a better clarity on the role of MORs on D1R expressing neurons in the nucleus accumbens.

Authorship Acknowledgements

Chapter 1: Lorna Barrall drafted text which was edited by Dr. Catherine Cahill.

Chapter 2: Dr. Catherine Cahill and Lorna Barrall conceived the experimental question. Lorna Barrall designed and conducted all experiments. Lorna Barrall drafted text which was edited by Dr. Catherine Cahill. Dr. Catherine Cahill and Dr. Merel Dagher graphed data and helped with statistical analysis.

Chapter 3: Jacob Alderete, Anthony Tanzillo, Hazem Nasef and Jason Miller conducted unpublished studies presented in the Chapter 3 introduction. Dr. Catherine Cahill graphed data and helped with statistical analysis. Dr. Catherine Cahill and Lorna Barrall conceived the experimental question for the thesis project. Lorna Barrall designed and conducted all experiments. Viruses were provided by Dr. Matthew Banghart (UCSD). Emily Ellis, Katrina Winata, and Ryan Dannis assisted with surgeries and behavior tests for all experiments. Dr. Catherine Cahill, Dr. Merel Dagher, Gabby Sigal and Raniya Feroz performed immunohistochemistry assays. Lorna Barrall drafted text which was edited by Dr. Catherine Cahill. Dr. Catherine Cahill and Dr. Merel Dagher graphed data and helped with statistical analysis.

Future Directions: Lorna Barrall drafted text which was edited by Dr. Catherine Cahill.