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Akash Shanmugam April 10, 2023

A Tale of Two Opioids:

Comparing the Potency of Methadone and Morphine in Analgesia and Abuse Liability in Female Mice

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Abstract

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The potential use of methadone as an analgesic has long been debated due to its potent antinociceptive properties and lower abuse liability when compared to canonical opioids such as morphine, the current gold standard of first-line analgesia. While both morphine and methadone induce activation of the central analgesic and reward circuits through stimulation of the mu-opioid receptor (MOR), recent research has suggested that differences in the abuse liability between methadone and morphine may be mediated by a heteromer formed by MOR and the galanin 1 receptor (GalR1). Methadone and morphine have similar potency for the MOR alone, but methadone is much less potent at activating the heteromer. Importantly, GalR1 and MOR co-expression appears to be mostly limited to the reward circuitry, whereas neurons that comprise the central analgesic circuits express either MOR or GalR1, suggesting a potential mechanism for the lower abuse liability of methadone. However, very few studies have directly compared morphine- and methadone-induced reward and analgesia. The purpose of this study was to compare the potencies of methadone and morphine in inducing analgesia (using von Frey and hot plate assays) and abuse liability (using conditioned place preference; CPP) in female mice. RNAScope in situ hybridization was used to characterize the co-expression of MOR and GalR1 mRNA in GABAergic neurons in the rostromedial tegmental nucleus (RMTg) following the CPP paradigm to determine whether intermittent opioid exposure potentially alters heteromer abundance in a key node of the brain reward circuit. We found that while methadone required a

higher dose than morphine to induce a CPP, both drugs required similar doses to induce analgesia in both mechanical and thermal antinociception tests. Neither drug induced changes in MOR or GalR1 mRNA, nor the degree of co-expression, following CPP administration. These results are consistent with the idea that the MOR-GalR1 heteromer is critical for opioid-induced reward but not analgesia and support further consideration of methadone as a first line analgesic and the MOR-GalR1 heteromer as a target for opioid misuse therapies.

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Introduction

Background on Opioid Epidemic

Since the 1990s, the United States has grappled with the opioid epidemic, a major public health crisis that took the lives of 50,000 people in 2019 (CDC, 2019). The COVID-19 pandemic has exacerbated the impact of this epidemic by isolating those who struggle with opioid abuse and reducing access to treatment programs, as the CDC reported a 30% increase in deaths related to drug overdoses in 2020 compared to the previous year (Kariisa et al., 2022). While this crisis has affected all demographics, women are especially vulnerable to opioid misuse because they experience dramatically higher rates of long-term opioid use and prescription overdoses (Campbell et al., 2010; Unick et al., 2013). Opioid misuse and the subsequent risk for overdoses often begin with prescription drugs given in a clinical setting (Omidian et al., 2022). Indeed, when compared to men who struggled with opioid abuse, higher percentages of women reported experiencing an opioid for the first time as a legitimate prescription for pain (Cicero et al., 2008). After reporting chronic pain to their physicians, women were prescribed opioids more often than men, even in situations such as headaches where the opioid has no reported efficacy in ameliorating symptoms (Cicero et al., 2009). While these findings demonstrate a need for improvement in clinical practice guidelines around pain management in women, a greater understanding of opioid abuse liability in female rodent models could also help address addiction in this demographic.

The Effects of Morphine and Other Opioids on the Reward and Analgesic Circuits

Morphine, a commonly prescribed opiate, has historically been considered the gold standard for the control of severe pain (Pergolizzi et al., 2008). However, morphine and other opioids are often abused for the pleasurable feelings they produce (Kosten and George, 2002). The neurobiology of the hedonic feelings associated with opioid abuse liability is rooted in the mesolimbic reward circuit, a dopaminergic pathway in the brain (Bozarth and Wise, 1981; Xi and Stein, 2000). The dopaminergic cell bodies originate in the ventral tegmental area (VTA) of the midbrain, primarily projecting to the nucleus accumbens (NAc), basolateral amygdala (BLA), and medial prefrontal cortex (mPFC) among other target regions in the limbic forebrain (Koob, 1992; Lammel et al., 2008). The rewarding effects of opioids involve the dopaminergic transmission from the VTA (Nestler, 2005). Systemic morphine administration potentiates the strength of local field potentials in the VTA, and when acutely injected into the VTA, morphine facilitates intracranial electrical self-stimulation reward (Ahmadi-Soleimani et al., 2018; Wolfswinkel and van Ree, 1985). Through c-Fos immunohistochemistry, heroin has been shown to activate the NAc-projecting dopaminergic neurons in the VTA and increase dopamine release in the NAc, highlighting the VTA-NAc pathway (Corre et al., 2018). This pathway's role in the reinforcing properties of opioids has been confirmed, as either optogenetic or chemogenetic silencing of these dopaminergic neurons blocks heroin self-administration (Galaj et al., 2020; Corre et al., 2018). Thus, it is generally accepted that the VTA-NAc dopaminergic pathway plays a critical role in the abuse liability of both prescription and non-prescription opioids.

Opioids like morphine exert their effects on this circuit through the activation of the mu-opioid receptor (MOR). MOR knockout mice lack both morphine-induced analgesia and reward-seeking behaviors (Matthes et al., 1999; David et al., 2008). When compared to other opioid

receptors, activation of the MOR produces the strongest analgesic actions but also the highest reward and reinforcement (Gruber et al., 2007). MOR agonists inhibit GABAergic neurons that project onto and tonically inhibit dopaminergic neurons in the VTA, and this disinhibition causes the dopamine release associated with the rewarding effects of opioids (Kelle et al., 1980; Gysling and Wang, 1983; Steffensen et al., 2006). MOR agonists specifically inhibit GABAergic inputs to VTA dopaminergic neurons at presynaptic sites, indicating that MORs are located on axon terminals as confirmed by electron microscopic immunohistochemistry (Zhang et al., 2015). MOR activation in the VTA also activates regions that receive dopaminergic afferents, including the NAc, BLA, and ACC, demonstrating that MOR agonists engage the mesolimbic reward circuit (Campos-Jurado et al., 2019). Structural assessments of the agonist-receptor efficacy show that various MOR agonists preferentially engage distinct transmembrane helices of the MOR and induce different conformational changes in the receptor, which are thought to contribute to different efficacies of MOR agonists (Ricarte et al., 2021). MORs are expressed throughout the brain, and as such, the VTA receives GABAergic inputs from both local interneurons and distal brain areas (Erbs et al., 2015; Beier et al., 2015). The local GABAergic interneurons in the VTA were initially assumed to regulate the disinhibition of dopaminergic neurons by opioids, but the rostromedial tegmental nucleus (RMTg) is now thought to represent a significant region that sends dense opioid-sensitive GABAergic inputs into the VTA (Johnson and North, 1992; Matsui and Williams, 2011). MORs are enriched in the RMTg, with ~70% of GABAergic neurons containing mRNA for the receptor in the rat (Galaj et al., 2020). The inhibition from opioid activation is largely evoked from the RMTg, and substantially less from other contributors of GABAergic input from the NAc and local VTA neurons (Matsui et al, 2014). While stimulation of RMTg neurons elicits a complete suppression of dopaminergic

activity, morphine depresses inhibition of dopaminergic neurons after RMTg stimulation (Lecca et al., 2012). Reducing GABA release from the RMTg attenuates opioid-induced excitation of dopaminergic neurons, and selective inactivation of RMTg neurons blocks dopaminergic neural activity in the VTA after local infusions of morphine, demonstrating the RMTg's critical role in the reward circuit (de Guglielmo et al., 2014; Jalabert et al., 2011). Although the mesolimbic reward circuit is likely the largest contributor to the hedonic feelings associated with opioid use, dopamine-deficient mice, mice locally treated with dopamine receptor antagonists in the NAc, and chemical lesioning of dopaminergic terminals in the NAc have all failed to completely block opioid reward-seeking behaviors, suggesting the presence of other non-dopaminergic opioid reward pathways (Hnasko et al., 2005; Pettit et al., 1984; Gerrits and van Ree, 1996). Despite some conflicting evidence, the VTA-NAc dopaminergic circuit remains a key substrate for the neurobiological basis of opioid reward.

Opioid activation of MORs throughout the central nervous system is also thought to be a major mechanism of opioid-induced analgesia. In the midbrain, MOR agonists can produce antinociceptive effects through the periaqueductal gray (PAG), where the sum effect is activation of descending inhibitory neurons (Depaulis et al., 1987; Pathan and Williams, 2012). GABAergic inhibition of cells in the PAG has been shown to block nociceptive transmission at the spinal level via the activation of rostral ventromedial medulla (RVM) off-cells and inhibition of the region's on-cells (Behbehani et al., 1990; Moreau and Fields, 1986; Foo and Helmstetter, 2000). Ultimately, these actions of MOR agonists result in the reduction of nociceptive transmission from the periphery. Indeed, intra-PAG microinfusion of the MOR antagonist naltrexone blocks the antinociceptive effects of systemic morphine administration in rats, indicating the PAG's critical role in opioid analgesia (Lane et al., 2005). However, opioids also

can directly inhibit the substantia gelatinosa in the dorsal horn and peripheral afferent nociceptive neurons (Pathan and Williams, 2012). While the reward and analgesic pathways are distinct, MOR agonists like morphine can offer therapeutic value through analgesia but come with the unfortunate side effect of high abuse liability, contributing to the current opioid epidemic.

Methadone as an Alternative Opioid Analgesic

In an effort to find solutions to this epidemic, researchers have begun to search for an opioid with lower abuse liability that could replace morphine and other prescription opioids with high addiction potential. One promising candidate is the synthetic opioid methadone, which is effective at decreasing opioid-related cravings in patients with opioid use disorder (Fareed et al., 2011). In patients struggling with opioid use disorder, methadone treatment has been proven to reduce overdose events and opioid-related acute care (Dole and Nyswander, 1965; Wakeman et al., 2020). Animal models show similar results; for example, heroin-dependent rats fail to maintain reward-seeking behaviors when heroin is replaced with methadone (Peng et al., 2010). As a result of this evidence, methadone has been primarily utilized for maintenance therapy because of its slow acting but long-lasting effects (Kreek et al., 2010). These unique characteristics of methadone can be attributed to slower liver metabolism and release into the blood compared to morphine and other opioids (Kreek et al., 1978; Inturrisi et al., 1984). While both methadone and morphine cross the blood-brain barrier via similar transporters like the P-glycoprotein, brain uptake of methadone is significantly higher than morphine (Chaves et al., 2017; Dagenais et al., 2003). Although methadone appears to have potential as a first-line analgesic, its use for patients with severe pain continues to be disputed (Bruera et al., 2004; Moreira de Barros et al., 2021). One issue is that methadone shows higher pharmacokinetic

variability in individuals due to its long half-life when compared to morphine (Eap et al., 2012). Another limitation is that, when used inappropriately for pain control, methadone can cause severe respiratory depression that eventually leads to death (Grissinger, 2011; Ehret et al., 2007). Given these controversies, further investigation into the differential molecular mechanisms and physiological effects of methadone and morphine are warranted.

The Galanin System May Mediate Methadone's Low Abuse Liability

The mechanisms that explain abuse liability differences between methadone and morphine are not yet clear. While the MOR-initiated reward pathway broadly characterizes opioid molecular action, pharmacodynamic analysis indicates that both methadone and morphine have very similar potency and efficacy when interacting with the MOR (Cai et al., 2019). Galanin, a neuropeptide, is widely distributed in the mammalian brain and regulates a diverse set of physiological processes including mood, neuronal survival, pain, food intake, and metabolism (Tatemoto et al., 1983; Perez et al., 2001; Lang et al., 2015; Weinshenker and Holmes, 2016). Importantly for the purposes of this study, accumulating evidence indicates that galanin modulates dopamine transmission and opioid reward. For example, administration of galanin inhibits dopamine release in rat striatal slices (Tsuda et al., 1998). While galanin does not induce preference or aversion on its own, it attenuates morphine-induced conditioned place preference, providing evidence for an antagonistic interaction between galanin and opioids (Zachariou et al., 1999). Galanin knockout mice showed enhanced morphine place preference and increased opioid induced ERK and CREB phosphorylation in NAc and BLA, while administration of galanin agonists attenuate the behavioral and neurochemical changes induced by morphine (Hawes et al., 2007; Zhao et al., 2013). Together, these data suggest that galanin opposes opioid reward, but potential underlying mechanisms were not identified until recently.

Galanin signals through 3 G-protein coupled receptors: GalR1, GalR2, and GalR3. GalR1 is hypothesized to contribute to the different abuse liabilities of morphine and methadone. GalR1 is G_i-coupled and located in brain regions associated with opioid-sensitive behaviors including the VTA, RMTg, substantia nigra, and the nucleus accumbens, implicating the receptor in the dopaminergic activation related to substance abuse (Kerr et al., 2015; Foster et al., 2021; our unpublished data). When GalR1 and MOR are expressed in the same cells, these receptors preferentially form a heteromer with antagonistic interactions that alter the function of the MOR, especially when the ligand for GalR1, galanin, is present. By contrast, GalR2 does not form heteromers with MOR (Moreno et al., 2017; Cai et al., 2019). In a functional assessment of MOR-GalR1 interactions with bioluminescence resonance energy transfer (BRET) in human embryonic kidney cells, both MOR and GalR1 individually promoted activation of G_i proteins with their respective agonists, with all opioids (including morphine and methadone) showing similar potencies in activating the MOR G_i protein. However, when MOR and GalR1 were co-expressed in the same cell, each receptor's agonists promoted activation of G_i proteins, but methadone had a significantly lower potency than other opioids at activating the MOR-GalR1 heteromer G_i protein. To investigate heteromeric effects on downstream signaling, basal and forskolin-induced cAMP formation was studied in cells transfected with GalR1 and MOR. In cells expressing GalR1 or MOR individually, respective receptor agonists did not modify basal levels of cAMP but decreased forskolin-induced cAMP formation, indicating that both receptors individually signal via activation of their G_i-protein. However, in cells co-expressing GalR1 and MOR, GalR1 agonists *increased* basal cAMP formation and did not decrease forskolin-induced cAMP, suggesting that the heteromeric GalR1 switches from signaling via activation of their G_i-protein to G_s-protein. This evidence points to the MOR-GalR1 heteromer mediating differences

between methadone and other opioids via antagonist G_s- G_i interactions between the GalR1 and MOR proteins (De Oliveira et al., 2022).

Using downstream kinase phosphorylation after administration of selective receptor agonists of MOR and GalR1, biochemical markers for heteromeric activation could be identified in rat VTA slices, suggesting that this heteromer exists at critical sites along the mesolimbic reward circuit. To provide evidence for the heteromers as a mediator for opioid reward, these intracellular changes were correlated to dopaminergic changes in the VTA. Most MOR agonists increased dopamine release in the VTA, which was attenuated by co-administration of galanin. Moreover, this inhibitory action of galanin was prevented by the presence of a synthetic peptide that selectively disrupted MOR-GalR1 heteromeric interactions. This study furthers supports the role of GalR1-MOR heteromers in the reward circuit, as the heteromer modulates cell signaling pathways that ultimately cause dopamine release that is critical for the rewarding properties of opioids (Moreno et al., 2017). When directly comparing the ability of morphine and methadone to activate the GalR1-MOR heteromers through BRET assays or dopamine release in freely moving rats, methadone had a significantly lower potency. Critically, the GalR1-MOR interfering peptide increased methadone-induced dopamine release, suggesting that the heteromer mediates the potency differences between morphine and methadone explains why methadone has lower abuse liability than morphine (Cai et al., 2019). To summarize, when MOR is expressed alone, methadone and other MOR agonists have similar effects, but when GalR1 heteromerizes with MOR, methadone loses potency. Because most MORs in the reward circuit but not the pain circuit appear to be in complex with GalR1, methadone may have reduced abuse liability while maintaining robust analgesic properties (Cai et al., 2019).

Signal Transduction and Expression following Opioid Activation

It is important to note that mechanisms beyond the MOR-GalR1 heteromer may explain differences in methadone and morphine abuse liability. Following receptor activation and continued presence of agonists, the MOR is phosphorylated by a G-protein-coupled receptor kinase, preventing further immediate stimulation (Ferguson et al., 1998). Depending on the agonist, the MOR then binds β-arrestin with high affinity and gets trafficked into early endosomes as β-arrestin links phosphorylated receptors to clathrin-coated pits and promotes endocytosis (Zastrow et al., 2003). The MOR depends on phosphatase action to get recycled back to the plasma membrane or trafficked to lysosomes for destruction (Tanowitz and von Zastrow, 2003). Continued agonist action on the MOR can lead to the removal of the receptor from the membrane (He et al., 2002). Indeed, repeated morphine exposure causes tolerance through a downregulation of MOR abundance, as shown by decreases in MOR density in the mouse striatum and downregulation of oPRM1-gene mRNA expression (Petruzzi et al., 1997; Prenus et al., 2012).

Although the MOR mediates the action of both methadone and morphine, these ligands may operate using distinct signal transduction pathways and intracellular signaling proteins in a biased fashion. While methadone promotes significant internalization following phosphorylation, morphine does not show evidence for MOR internalization or translocation from the cell membrane to the cytoplasm (Ma et al., 2020; Arttamangkul et al., 2008; Arden et al., 1995; Keith et al., 1996). When compared to wild-type mice where methadone antinociception does not change over chronic administration, methadone at a 10 mg/kg dose produces tolerance in an animal model where the MOR is mutated to be targeted for degradation following endocytosis, suggesting that MOR is normally recycled back to the plasma membrane following

internalization (Enquist et al., 2012). Methadone treatment also shows no differences in MOR recovery from desensitization in the noradrenergic locus coeruleus (LC), while morphine shows substantially lower recovery from desensitization. These findings were correlated with lower trafficking of MOR back to the plasma membrane following morphine treatment, but not methadone treatment (Quillinan et al., 2011). When compared to methadone, the relative efficacy of morphine to promote MOR internalization is much lower than the efficacies of morphine to activate G-protein signaling or promote rapid desensitization, suggesting that the difference in internalization is a critical driver in mediating differences between methadone and morphine (Borgland et al., 2003). These findings indicate that while morphine induces homeostatic adaptations for MOR regulation, MOR is quickly trafficked back to the membrane following activation by methadone. Morphine's mechanism of action to prevent entrance in the recycling pathway is suggested to be the persistent phosphorylation of carboxy-terminal residue 375, while other MOR-agonists like methadone induce phosphorylation that can quickly be dephosphorylated for MOR recycling (Schulz et al., 2004). After morphine treatment, the MOR may also be phosphorylated with Protein Kinase C (PKC) at Serine-375 or Protein Kinase A and require phosphatases that take much longer to dephosphorylate the receptor for reactivation (Bailey et al., 2004; Schulz et al., 2004; Bernstein and Welch, 1998; Gabra et al., 2007). To a certain extent, all 11 phosphorylation sites on the MOR's C terminus are suggested to play a role in the development of tolerance (Arttamangkul et al., 2019). Despite a lack of clarity regarding its mechanism, internalization and endocytosis may be critical to understanding the differences in the impact of between chronic methadone and morphine treatment.

Some studies suggest that these differences between MOR agonists may be mediated by the binding of β-arrestin. In human embryonic kidney cells that modelled MOR function and β-

arrestin-2 recruitment following activation by its agonists, MORs showed significant internalization and dose-dependent increases in β-arrestin-2 recruitment following activation by methadone, but not morphine (Doi et al., 2016). These findings support methadone's characterization as a β-arrestin-biased MOR agonist, while morphine may preferentially operate through a more traditional G-protein-biased activation pathway. However, this study's finding conflicts with results showing that chronic morphine attenuates analgesic tolerance and enhances conditioned place preference in β-arrestin-2 knockout mice, suggesting that β-arrestin-2 does play a role in morphine analgesic and reward potency (Bohn et al., 2003; Raehal and Bohn, 2011; Quillinan et al., 2011). Studies involving these β-arrestin-2 knockout mice have been limited by concerns of mixed genetic backgrounds as a confounding factor in the experimental design (Kliewer et al., 2020). However, downregulation of β-arrestin-2 expression via antisense RNAs produce similar responses in mice (Bu et al., 2014). Another potential explanation is that the β-arrestin-2 knockout or -depleted mice may potentially recruit compensatory mechanisms to induce G-protein signaling, even without β-arrestin. The β-arrestin-mediated pathway has also been linked to ERK1/2 activation. Drugs of abuse, like cocaine and opioids, are associated with specific patterns of ERK1/2 activation, which have been correlated with activity in reward-related brain regions and rewarding behaviors (Berhow and Nestler, 1996; Valjent et al., 2000, Valjent et al., 2004). In LC neurons, disruption of both the GRK/β-arrestin-2 and ERK1/2 activation is required to abolish desensitization of the MOR following sustained activation by an agonist, while individually these pathways do not completely abolish MOR desensitization (Dang et al., 2009). ERK1/2 activation may simply be a selective downstream effect of GRK/β-arrestin-2 activation, as ERK1/2 activation in striatal neurons requires transfection of arrestin that functions without the presence of GRK (Macey et al., 2007). Across many signal

transduction pathways, ERK1/2 activation leads to downstream phosphorylation events that eventually affects transcription factors (Chalmers et al., 2007). Most importantly, as shown later with GalR1, ERK1/2 activation and MOR gene expression are also regulated in a CREB- and cAMP- dependent manner, suggesting that expression of GalR1 and MOR share similar regulatory mechanisms (Ligeza et al., 2008; Shen et al., 2000; Lee and Lee, 2003). Other local regulation of MOR expression have been suggested to occur at the transcriptional and post-transcriptional levels through agents like miR-103, miR-107, microRNA 339, LncRNA MRAK159688, NFkappaB, and AP-1 (Lu et al., 2014; Wu et al., 2013; Gach et al., 2008; Deng et al., 2022). Epigenetic modulation, like DNA methylation and histone acetylation, may also be a mechanism for the regulation of MOR expression (Oertel et al., 2012; Wagley et al., 2017; Reid et al., 2022; Wei and Loh, 2011). Despite the presence of many regulatory mechanisms, differences of internalization and endocytosis following methadone and morphine treatment may be linked to MOR and GalR1 gene expression.

Studying Abuse Liability via Conditioned Place Preference

One method to understand the abuse liability as a phenotype for opioids is conditioned place preference (CPP). CPP relies on Pavlovian learning to assess the rewarding properties of drugs. The CPP paradigm offers the opportunity to study the brain's formation of drug-context associations in a preclinical model and better understand how drug-associated contexts can evoke drug craving in humans (McKendrick and Graziane, 2020). In both animal and human studies, the association of environmental cues with opioids serves as a powerful mediator of the maintenance and recurrence of drug-seeking behaviors (Davis and Smith, 1976; Childress et al., 1986; Perry et al., 2014). In a clinical fMRI study of cue-elicited cravings in opioid-dependent patients, heroin-related environmental cues were associated with increases in activation of the

VTA, indicating the role of the mesolimbic reward circuit in evoking drug cravings after exposure to opioid-associated cues (Zijlstra et al., 2008). As the association between environmental cues and opioid use requires learning and memory processes, evidence supports the presence of a neuronal circuit between the VTA and hippocampal CA3 region that is involved in the formation of morphine-induced place preference (McNamara et al., 2014; Jiang et al., 2018). Contextual cues can be as behaviorally and biologically powerful as morphine itself, as conditioned drug cues evoked similar locomotion and ERK activity in the VTA and NAc when compared to morphine-induced responses (Crespo et al., 2022). In rodents, activation of the VTA-NAc dopaminergic system is critical for the induction of a CPP by morphine and other opioids (Harris et al., 2004; Soderman and Unterwald, 2008; Moaddab et al., 2009; Narita et al., 2010; Koo et al., 2012; Wu et al., 2021). Transient stimulation or inactivation of the VTA can also modulate morphine-induced place preference in rats (Alaei and Pour, 2021). This suggests that the molecular interactions of MOR agonists in this system have behavioral correlates that could be observed in a CPP paradigm. Morphine-induced place preference has been extensively studied in rodents, with a preference induced at doses as a low as 0.4 mg/kg and as high as 80 mg/kg, indicating the strength of the drug-context associations created by morphine (Mucha and Iversen, 1984; Blander et al., 1984; Mucha et al., 1982; Bardo et al., 1989). However, to our knowledge, methadone-induced CPP has not been rigorously examined.

Studying Analgesia via Antinociception Assays

Methadone and morphine are also suggested to have different sites of activation along the analgesic circuit. For example, microinjections of morphine, but not methadone, into the PAG increases thermal antinociception (Morgan et al., 2014). When administered directly into the brain, naloxone, a competitive opioid receptor antagonist, blocks morphine antinociception but

does not block methadone antinociception. Conversely, systemically administered naloxone blocks methadone antinociception but has little effect on morphine antinociception (He et al., 2009). Together, these findings suggest that methadone antinociception depends on modulating transmission at peripheral opioid receptors, while morphine modulates nociceptive transmission at central opioid receptors on GABAergic neurons that project to regions like the PAG. Interestingly, unlike the brain regions that mediate opioid reward that co-express MOR and GalR1 and thus are capable of hosting heteromers, the MOR-expressing cells that mediate the analgesic properties of opioids do not appear to co-express GalR1, suggesting that methadone may be an effective analgesic while having a reduced ability to trigger addiction (Figure 1). While morphine and methadone modulate nociceptive transmission at different sites, nociception assays can help directly compare the analgesic characteristics of opioids like morphine and methadone to better understand their effect on behavioral responses to painful stimuli (Mogil, 2019). Morphine provides strong dose- and time-dependent analgesic effects (Gades et al., 2000; Pantouli et al., 2020; Minami et al., 2009). Methadone has also shown similar dose-dependent analgesic effects in rodents (Holtman and Wala, 2007). However, the analgesic doses of methadone and morphine have not yet been rigorously compared in reference to their abuse liabilities in healthy naïve mice. Because methadone and morphine are hypothesized to have different effects on the VTA-dopaminergic system, each substance's dose response for their place preferences and analgesic effects should be directly compared and understood at the molecular level.

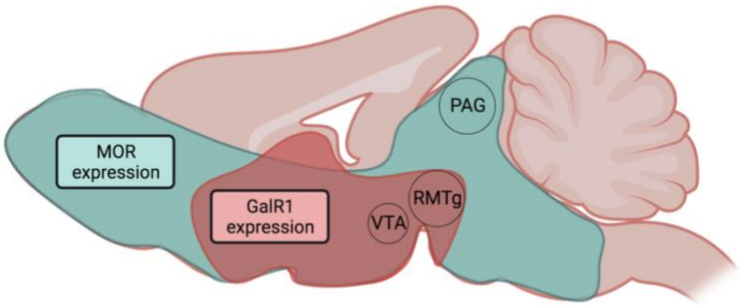


Figure 1: Sites of MOR expression and GalR1 expression in the mouse brain. While MOR alone is expressed in the analgesic circuits, both GalR1 and MOR are co-expressed in the reward circuit, suggesting that the heteromer mediates differences in the abuse liabilities of methadone and morphine but not analgesia.

Study Aims

In this study, we explored whether methadone can be considered an alternative analgesic drug with lower abuse liability when compared to morphine in a preclinical mouse model. We identified the lowest dose at which a conditioned place reference is induced for methadone and morphine to draw conclusions about the relative abuse liabilities of each drug. We also conducted antinociception assays for methadone and morphine to identify the doses where a significant analgesic effect is produced. Finally, to explore potential differences in cellular responses to the two drugs, we investigated the effects of intermittent morphine and methadone administration during CPP testing on the relative co-expression of MOR and GalR1 mRNA in the RMTg. Although the effects of chronic exposure to morphine and methadone on heteromeric expression have not been explored, acute and chronic morphine treatment can alter other opioid receptor levels in the mesocorticolimbic system (Yu and Gong, 2012). Very little is known about the ability of opioids to regulate GalR1 expression in these brain regions. This study will further

clarify methadone's potential use as a first-line analgesic in clinical treatment and add to our understanding of its molecular mechanism.

Prediction

We predicted that morphine would induce a place preference at a lower dose than methadone because of morphine's more potent MOR-GalR1 heteromer binding and dopaminergic activation, while methadone and morphine would have similar analgesic dose-response curves. Because the MOR is easily recycled and endocytosed via recruitment of β-arrestin following methadone administration, morphine may induce greater down-regulation of receptor expression in the brain following intermittent activation of receptors. We expected that MOR and GalR1 co-expression in the RMTg would be lower in the morphine-treated mice, while methadone would not change co-expression.

Methods

Animals

While it would have been ideal to study both male and female mice, we exclusively used female C57BL/6J mice due to time constraints. Sex differences have been identified in the neural mechanisms and behavioral outcomes of opioid reward and addiction (Becker and Chartoff, 2019), and female rodents are more sensitive to opiate reward (Lynch and Carroll, 1999). These findings suggest that stronger differences in the analgesic properties and abuse liability between methadone and morphine are more likely to be observed in female mice than male mice. 30 mice were used for CPP with methadone and morphine (6-8 mice per group), and 45 mice were used for nociception assays with both morphine and methadone (6-8 mice per group).

Conditioned Place Preference (CPP)

CPP experiments were conducted in a three-compartment chamber with one neutral compartment and two conditioning compartments (Figure 2). Each conditioning compartment had unique contextual cues, like a barred floor, light background, a lemongrass scent or a checkered floor, dark background, and an ethanol scent. On the first day, the mice were placed in the neutral compartment and given freedom to explore the entire chamber for 15 min. During this preconditioning day, the time spent in each conditioning compartment was recorded using ANY-maze software to measure the initial side preference. During the next 8 days of conditioning, the mice was administered subcutaneous injections of either drug or saline on alternating days and restricted to the appropriate conditioning chamber for 30 min. The drug was paired to the compartment that was on the less preferred side, and saline was paired with the more preferred compartment. The morphine and methadone doses were 0 mg/kg (saline), 0.3 mg/kg, 1 mg/kg, and 10 mg/kg. On the final test day, the mice were placed into neutral compartment and again given access to the entire chamber for 15 min. The time spent in each conditioning compartment was recorded, and the CPP score was calculated as the difference between the test day preference and initial preference.

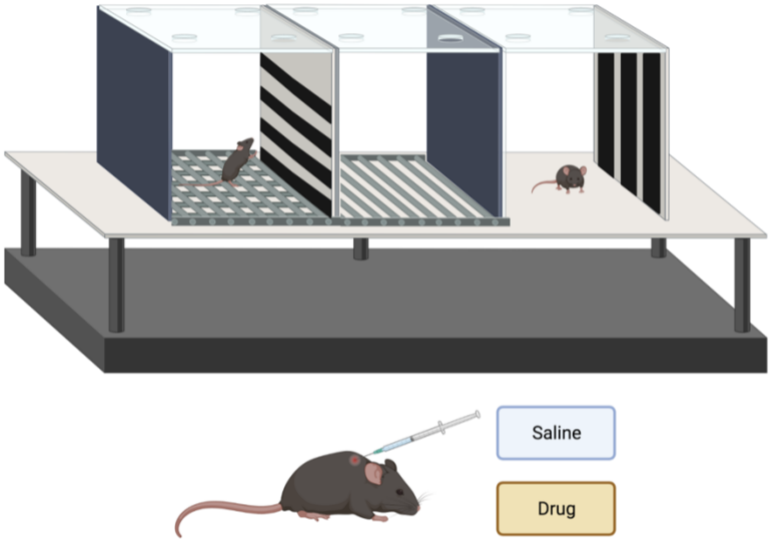


Figure 2: CPP apparatus and chamber cues. Mice were first habituated in an open chamber, subcutaneously administered alternating days of saline and drug, and tested again in an open chamber. The change in preference was recorded.

Nociception Assays

The analgesic characteristics of methadone and morphine were determined using a mechanical nociception assay (Von Frey Filaments Test) and a thermal nociception assay (Hot Plate Test) (Figure 3). These assays were chosen so that different aspects of antinociception could be measured in a preclinical model for methadone and morphine.

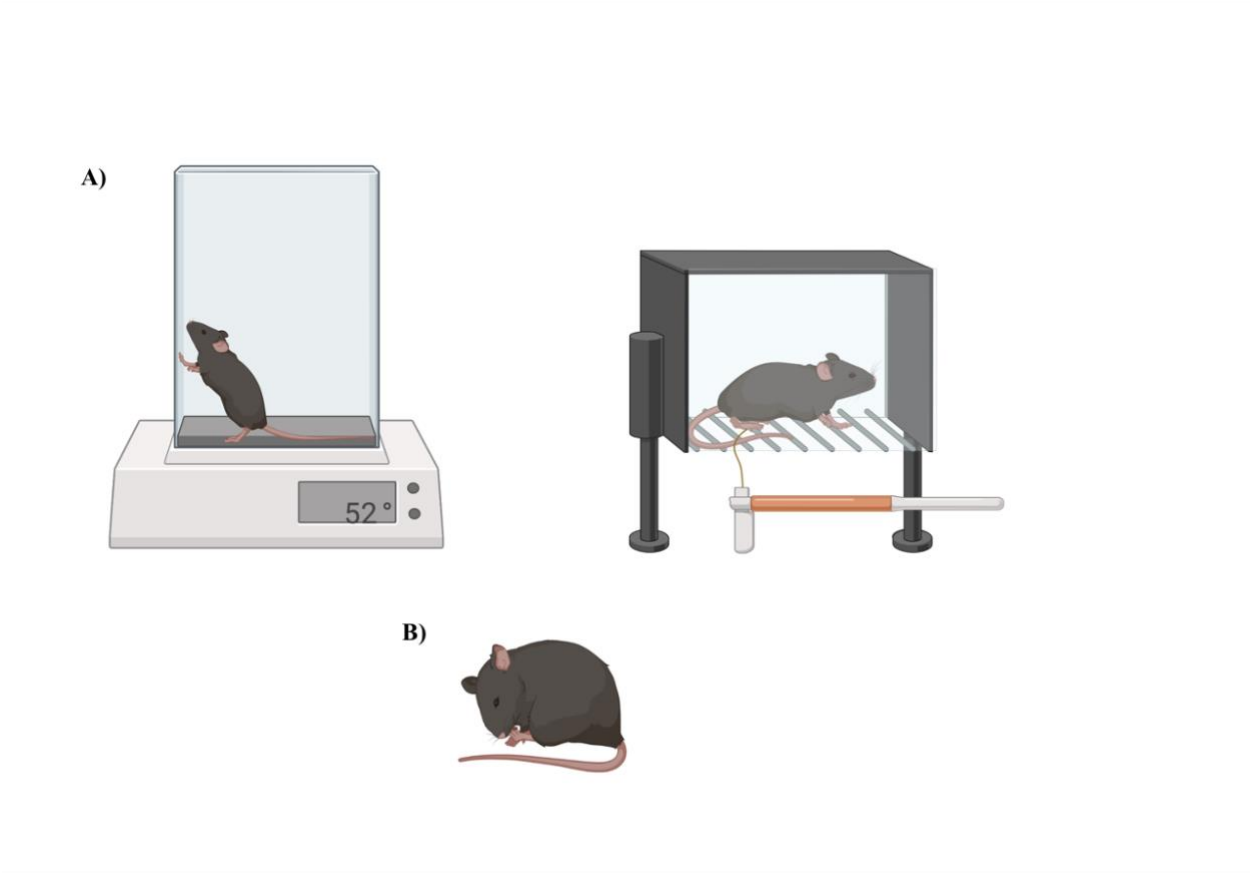


Figure 3: Thermal and Mechanical Antinociception Assays. The hot plate test (A) and von Frey filaments (B) were used in these experiments. A nociceptive reaction (paw-licking) is also shown below.

Von Frey Filaments Test

The Von Frey test is a standard and validated measure of mechanical sensitivity (Deuis et al., 2017). The animals were habituated on a covered wire-mesh platform for 5 min prior to the assay. The mice were given a subcutaneous injection of saline or drug (morphine or methadone 0.3, 1, 3, or 10 mg/kg) and given 30 min to habituate on the platform. Von Frey filaments were applied from the underside of the metal platform to the midplantar surface of the hind paw for up to 3 s to determine if a reaction was elicited. Lifting, licking, or shaking the hindpaw were considered positive reactions. The target force of the filaments ranged from 0.16 grams to 6

grams. The 50% mechanical threshold was determined using the up-down method, described previously (Dixon, 1965; Chaplan et al., 1993). If the animal withdrew the paw, a weaker hair was applied; if the animal did not display a response, the next stronger hair was applied. The maximum number of applications was 9, and the cutoff target force of filament was 6 grams. The surface of the platform was cleaned with 70% ethanol between each test.

Hot Plate Test

Two days after the von Frey test, the mice were given the hot plate test, a standard and validated measure of thermal nociception (Bannon and Malmberg, 2007; Deuis et al., 2017). The mice were first habituated to the hot plate at room temperature for 5 min. The mice were then injected subcutaneously with saline or drug (morphine or methadone 0.3, 1, 3, or 10 mg/kg). 30 min later, mice were placed on a covered hot plate set to 52°C, and the latency to produce a nociceptive reaction that included lifting, licking, or shaking the paws was recorded. The mice were immediately removed after a nociceptive reaction was shown. If no responses were observed after 30 s, mice were removed from the hot plate. The surface of the hot plate was cleaned with 70% ethanol between each test.

Tissue Collection

Mice were anesthetized with isoflurane and quickly decapitated 3 h after the final CPP test. Brains were frozen in an OCT-filled cryomold submerged in isopentane chilled with dry ice and stored at -80°C until sectioning. The brains were sectioned at 16 μm, and sections containing the RMTg were mounted on charged slides and stored at -80°C until the RNAscope assay.

RNAScope Assay of RMTg

Sample pretreatment was performed as instructed using the RNAscope Multiplex Fluorescent Reagent Kit v2 Assay User Manual. Slides were removed from storage and

immediately immersed in 4% PFA for 15 min. After rinsing the slides twice in 1X PBS, the slides were dehydrated using the following ethanol wash series for 5 min each: 50%, 70%, 100%, and 100%. The slides were air-dried for 5 min and then incubated in hydrogen peroxide for 10 min. The slides were washed in distilled water twice and then incubated in Protease IV for 30 min. The slides were washed twice in 1X PBS, and the experimental probe was added to each slide. Experiments analyzing GalR1 and MOR in GABAergic cells in the RMTg used mouse probes for GalR1 (ACD cat. 448821), MOR (ACD cat. 315841), and the GABAergic neuron marker glutamic acid decarbosylase (GAD; ACD cat. 400591). The RMTg was also located in one series of sections using GAD and dopaminergic cell marker tyrosine hydroxylase (TH; ACD cat. 317629). Mouse multiplex positive (ACD cat no. 320881) and multiplex negative (ACD cat no. 320871) control probes were used to validate experimental probe signal. The slides were then placed in a HybEZ Oven at 40°C for 2 h. After hybridization, the slides were washed twice in 1X wash buffer for 2 min. Three subsequent rounds of amplification and two washes with 1X wash buffer for 2 minutes were performed. HRP was added to each slide and incubated in the oven at 40°C for 15 min, and the slides were washed twice in 1X wash buffer for 2 min. Opal 520 was added to each slide, and two washes with 1X wash buffer for 2 min were performed. HRP blocker was added to each slide, and the slides were incubated in the oven at 40°C for 15 min. The slides were washed twice in 1X wash buffer for 2 min. The HRP signal development was repeated with Opal 570 and 690. Slides was then coverslipped using Prolong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA) and stored in the dark at room temperature overnight. All slides were imaged between 12 to 48 h after performing RNAscope.

Image Analysis

For each mouse, 3 RMTg images were analyzed for the number of cells that express MOR alone, GalR1 alone, or MOR + GalR1. Sections were also co-labeled for GAD1 mRNA to characterize MOR-GalR1 co-expression specifically in GABAergic neurons. The outcome measures were the percentage of GABAergic neurons and non-GABAergic neurons that express MOR alone, GalR1 alone, or MOR + GalR1. Values were averaged across all sections from each individual mouse to obtain a single value, which was then used for comparisons between groups.

Statistical Analysis

All statistical analysis and graphs were generated using Prism9 or ggplot (GraphPad, San Diego, CA). A repeated measures two-way ANOVA followed by a priori planned post-hoc Sidak's multiple comparisons tests were conducted to compare the pre-test and post-test preference at each dose for methadone and morphine CPP. A one-way ANOVA and post-hoc Tukey's multiple comparisons tests were used to compare the delta preference at each dose for methadone and morphine. A one-way ANOVA and Dunnet's multiple comparisons tests were conducted at each dose for methadone and morphine thermal antinociception and mechanical nociception scores. Pearson's correlation coefficient and a simple linear regression were conducted to compare the relationship between thermal antinociception and mechanical nociception scores. A two-way ANOVA was used to compare the percentage of GAD+ and GAD- cells that expressed MOR, GalR1, or both MOR and GalR1. Another two-way ANOVA was used to compare the number of cells in bins of MOR and GalR1 puncta individually across treatment groups.

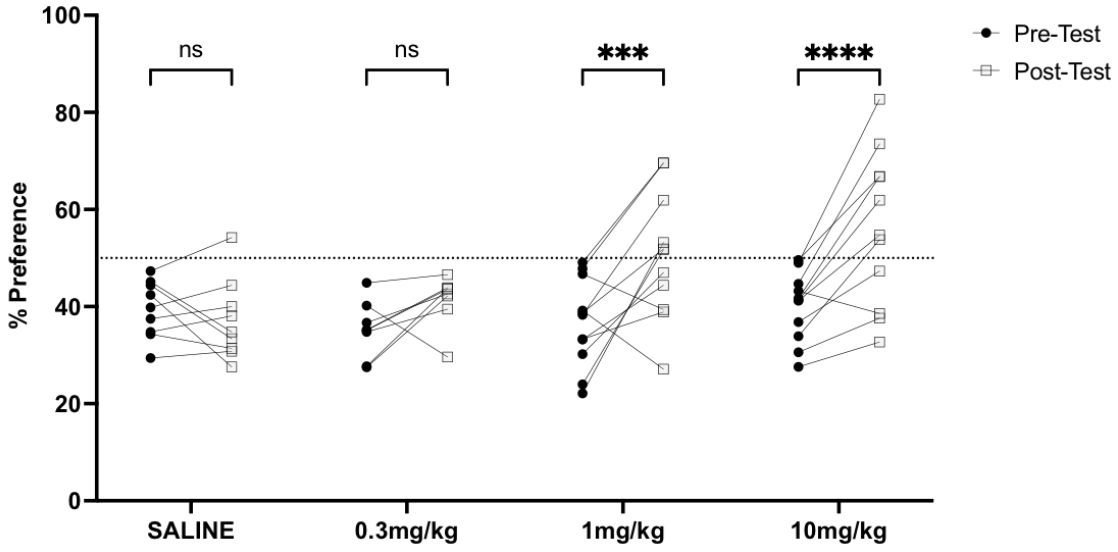
Timeline

This project was conceptualized from September to November. The CPP experiments for methadone were run in 2 cohorts from December 10-20 and January 1-10. After receiving IACUC approval and identifying the appropriate parameters of each antinociception assay in January, the assays were run in February. In early March, the CPP experiments for morphine were run alongside additional nociception assays. In mid-March, the brains of the mice from the CPP experiments were sectioned for RNAscope and run through the procedure. The images from the RNAscope analysis were processed, and the data from the behavioral experiments was analyzed. I defended my thesis on March 31st. On April 10th, my thesis forms and ETD were submitted.

Results and Discussion

We first conducted methadone CPP and identified the lowest dose at which a preference was induced. One mouse in the saline group was excluded as an outlier using the Grubb's Test (Alpha = 0.05). A repeated measures two-way ANOVA was performed to compare the effect of dose and time of test. There were significant main effects of dose ($F(3,35)=3.115$, $p=0.0385$), time $F(1,35)=23.19$, $p<0.0001$), and a significant dose x time interaction (Figure 4A; $F(3,35)=5.664$, $p=0.0029$). Post hoc tests showed that only the 1 mg/kg ($p=0.0007$) and 10 mg/kg ($p<0.0001$) groups differed between pretest and posttest. A one-way ANOVA was also performed to assess the effect of dose on absolute change in preference for the non-preferred side compared to saline, and a statistically significant difference was found (Figure 4B; $F(3,35)=5.664$, $p=0.0029$). Post hoc tests again showed that the methadone-induced preference was only significantly different between the saline and 1 mg/kg ($p=0.0117$) and 10 mg/kg ($p=0.0034$) groups.

A)


Change in Percent Preference for
Non-preferred (Pre-test) / Methadone-paired (Post-test) Chamber
% Preference
100
80
60
40
20
0
ns
ns
***
****
SALINE
0.3mg/kg
1mg/kg
10mg/kg
Pre-Test
Post-Test

B)

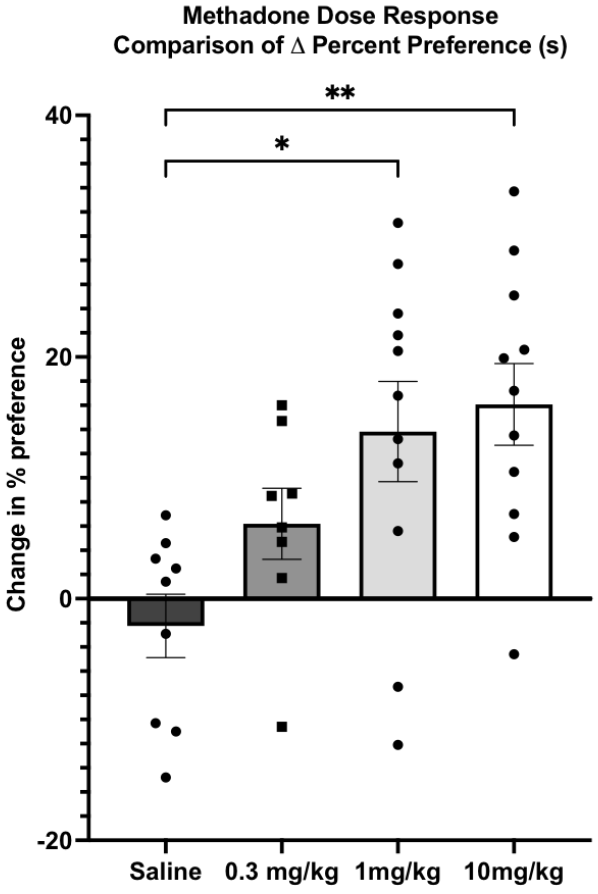

Methadone Dose Response
Comparison of Δ Percent Preference (s)
Change in % preference
40
20
0
-20
**
*
Saline
0.3 mg/kg
1mg/kg
10mg/kg

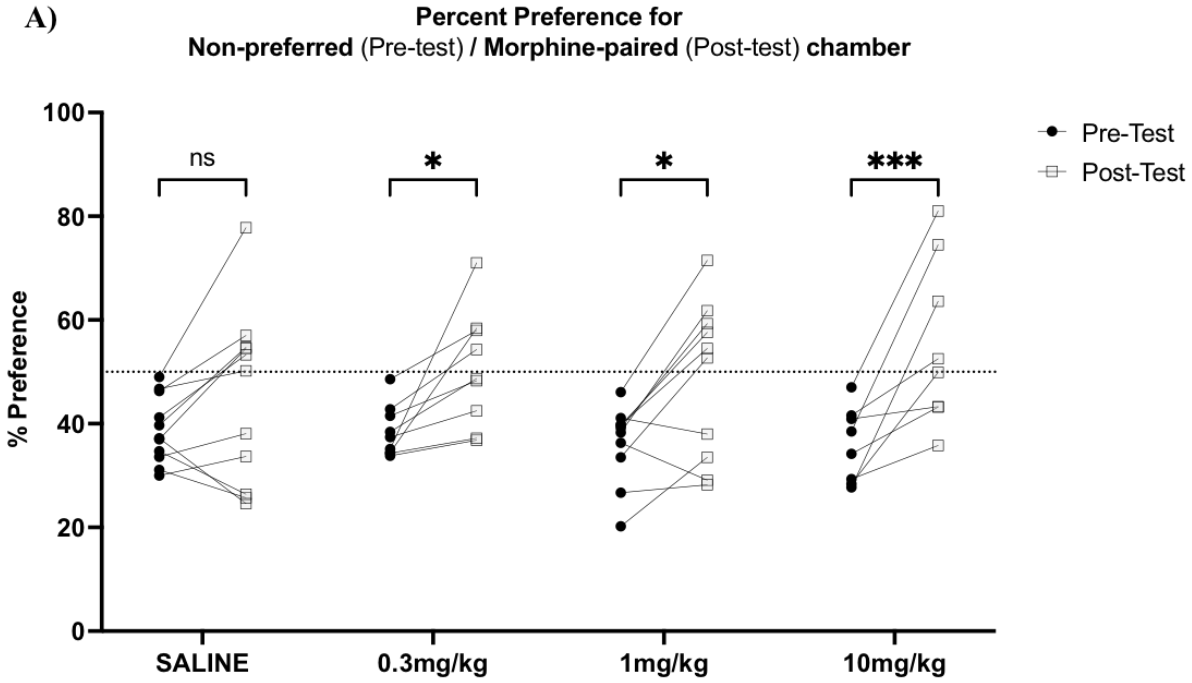
Figure 4. Methadone induces preference in female mice starting at 1 mg/kg. A) Within-subject comparison of time spent in non-preferred side during the pre-test trial and in the methadone-paired side during the post-test trial. ns=not significant; ***P<0.001; ****P<0.0001; repeated measures 2-way ANOVA with Sidak's Multiple Comparisons Test. B) Comparison of absolute change in percent preference between treatment groups. Shown is mean ± SEM. *P<0.05; **P<0.01; 1-way ANOVA with Tukey's multiple comparisons test.

We then conducted morphine CPP to determine the dose at which preference is induced. In a repeated measures two-way ANOVA, the main effect of time was found to be statistically significant (Figure 5A; $F(1,34)=40.35$, $p<0.0001$), while both dose and the interaction term were not significant. Sidak's multiple comparisons test revealed that preference was induced in the 0.3 mg/kg ($p=0.0207$), 1.0 mg/kg ($p=0.0101$), and 10 mg/kg ($p=0.0003$) groups. Preference was not induced in the saline group. A one-way ANOVA was also performed to determine effects of dose on absolute change in preference for the non-preferred side, and no significant differences were found (Figure 5B; $F(3, 34) = 1.827$, $p=0.1607$). However, trends towards a dose response curve could be seen for morphine. The lack of significant differences could be attributed to higher variability in the saline group, when compared to methadone's saline group. Half of our morphine cohort experienced a cage change on the morning of habituation day and were run by a different experimenter, causing them to be much more active and jumpier than expected. CPP is a sensitive assay as it relies on multiple sessions of associative learning, so small differences between cohorts can dramatically impact results. While the comparison of the magnitude of change in preference is another helpful lens through which CPP data can be understood, this plot does not directly address our aim of identifying the doses at which a significant preference is induced, which is better depicted by the analysis for Figure 5A that includes a within-subject

design and is not dependent on effect size. Thus, despite the lack of statistical significance in Figure 5B, we can still conclude that morphine induces a preference at 0.3 mg/kg.

While our data indicate that morphine induced a significant preference in female mice starting at 0.3 mg/kg, this dose of methadone failed to do so – at least 1 mg/kg was required. This result agrees with the initial prediction that a higher dose of methadone would be required to induce a place preference than morphine. The differences in preference-inducing properties of methadone and morphine also align with the previous study of MOR-agonist potency at presumptive MOR-GalR1 heteromers using BRET in cultured cells and dopamine release using microdialysis in freely moving rats (Cai et al., 2019). Very few studies have assessed methadone place preference in mice. After three conditioning sessions, one study found similar results, where a higher dose of methadone (3 mg/kg) was needed to induce preference in male mice when compared to morphine (1 mg/kg) (Holuj et al., 2013). However, an unconventional three-armed CPP apparatus was used in that study, and the effect of the number of conditioning sessions on place preference was also explored, interrupting regular CPP training paradigms prior to testing. Our study is the first to use a traditional CPP paradigm to study methadone's dose response for place preference in female mice.

A)


Percent Preference for
Non-preferred (Pre-test) / Morphine-paired (Post-test) chamber
% Preference
100
80
60
40
20
0
ns
*
*
***
Pre-Test
Post-Test
SALINE
0.3mg/kg
1mg/kg
10mg/kg

B)

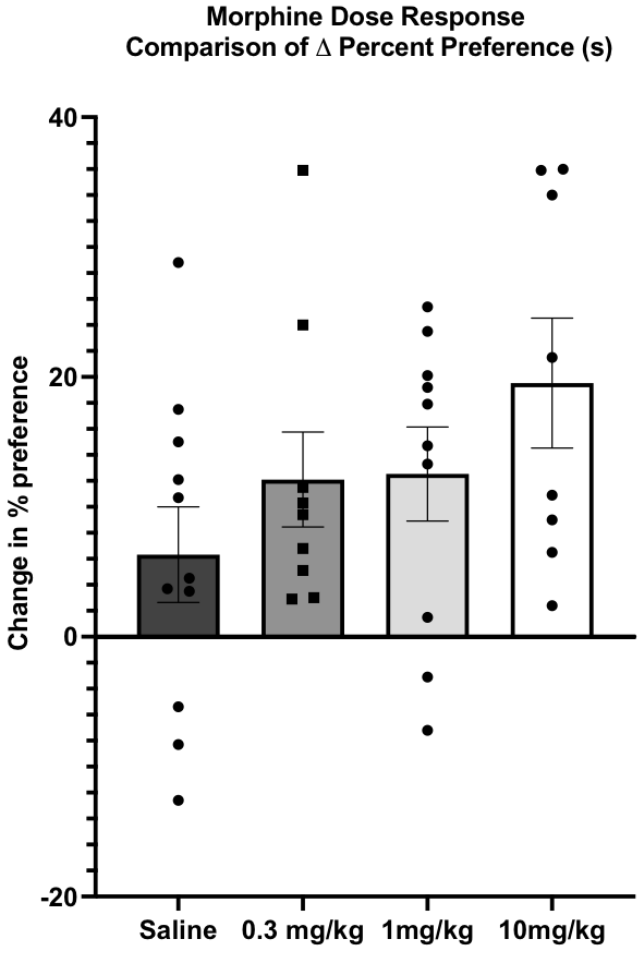

Morphine Dose Response
Comparison of Δ Percent Preference (s)
Change in % preference
40
20
0
-20
Saline
0.3 mg/kg
1mg/kg
10mg/kg

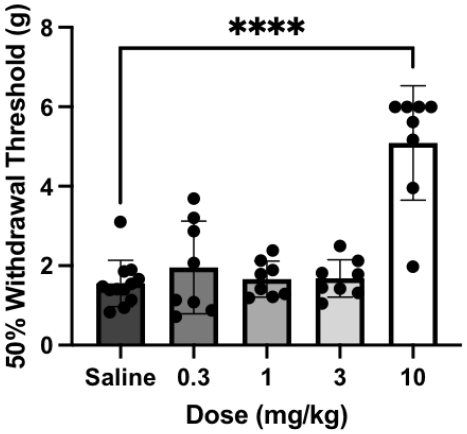
Figure 5. Morphine induces preference in female mice starting at 0.3 mg/kg. A) Within-subject comparison of time spent in non-preferred side during the pre-test trial and in the morphine-paired side during the post-test trial. ns=not significant; *P<0.05; ***P<0.001; repeated measures 2-way ANOVA with Sidak's Multiple Comparisons Test. B) Comparison of absolute change in percent preference between treatment groups. Shown is mean ± SEM. 1-way ANOVA with Tukey's multiple comparisons test.

After identifying differences between methadone and morphine reward, we then compared the analgesic properties of the two drugs using the von Frey test as a measure of mechanical antinociception and the hot plate test as a measure of thermal antinociception. One-way ANOVAs showed dose to be significant for both methadone (Figure 6A; $F(4, 39)=24.12$, $p<0.0001$) and morphine (Figure 6A; $F(4, 39)=20.72$, $p<0.0001$). Dunnett's multiple comparisons tests revealed that mechanical antinociception was only induced in the 10 mg/kg group for both methadone ($p<0.0001$) and morphine ($p<0.0001$) when compared to the saline group. After conducting the hot plate test, we also conducted one-way ANOVAs for thermal antinociception. Again, the test showed dose to be significant for both methadone (Figure 6B; $F(4,37)=56.88$, $p<0.0001$) and morphine (Figure 6B; $F(4,38)=67.33$, $p<0.0001$). Dunnett's multiple comparisons tests showed that thermal antinociception was only induced in the 10 mg/kg group for both methadone ($p<0.0001$) and morphine ($p<0.0001$) when compared to the saline group. Interestingly, the thermal antinociception scores and mechanical antinociception scores showed a strong, positive correlation (Figure 6C; $r =0.78$). As such, a high thermal antinociception score was likely to predict a high mechanical antinociception score, and vice versa, suggesting that individual mice have similar nociceptive and antinociceptive responses to thermal and mechanical pain. The antinociception scores from the morphine and methadone 10

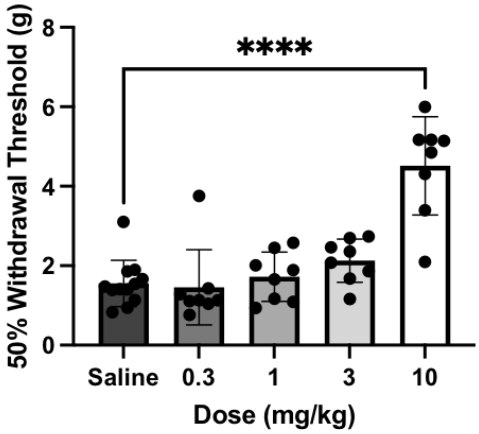
mg/kg treatments appeared to drive this positive correlation, as shown by most of these points in the upper right quadrant of the plot. Together, these findings indicate that, at the doses we tested, methadone and morphine possess similar analgesic potencies. This is consistent with the lack of GalR1 and MOR co-expression/presumptive heteromer formation in analgesic centers of the central nervous system.

Differences in analgesia between methadone and morphine have been well explored in rodents. When directly compared in mice, methadone and morphine have been shown to produce similar analgesia. One study using a standard hot plate test showed that methadone and morphine produced 50% of their maximal antinociceptive effect at similar doses (Fischer et al., 2005). In another study, a dose response effect for both methadone and morphine analgesia was observed in male Swiss-Albino mice using standard hot plate test, showing similar analgesia induced by both drugs at their ED_{80} doses (Lewanowitsch et al., 2006). These finding aligns with our results that thermal analgesia is produced at similar doses for these MOR agonists, and suggest that the similarity in the antinociceptive effect of morphine and methadone might be a general property and not restricted solely to C57BL/6 mice. The comparison of methadone and morphine in rats is not as conclusive. Upon testing the doses of antinociceptive potency for methadone and morphine in Sprague-Dawley rats using the warm-water tail-withdrawal assay, the confidence intervals for methadone and morphine's antinociceptive doses did not overlap, and methadone was more potent than morphine in both males and females (Peckham and Traynor, 2006). However, a comparison of our study to this analysis may be not be appropriate because of the use of different nociceptive assays and species.

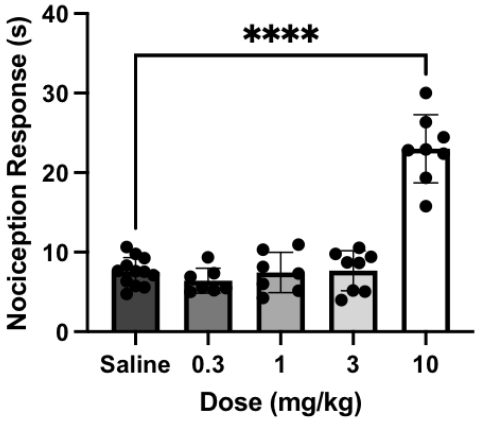
A) Mechanical Nociception Dose Response for Methadone



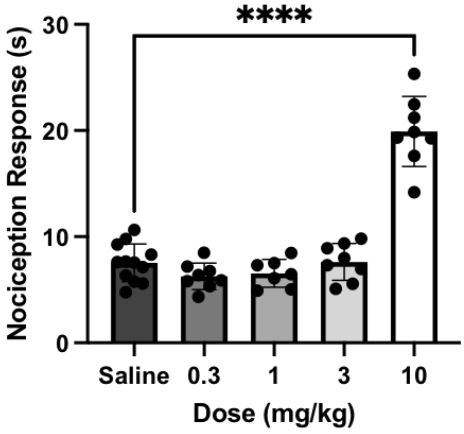
Mechanical Nociception Dose Response for Morphine



B) Thermal Nociception Dose Response for Methadone



Thermal Nociception Dose Response for Morphine



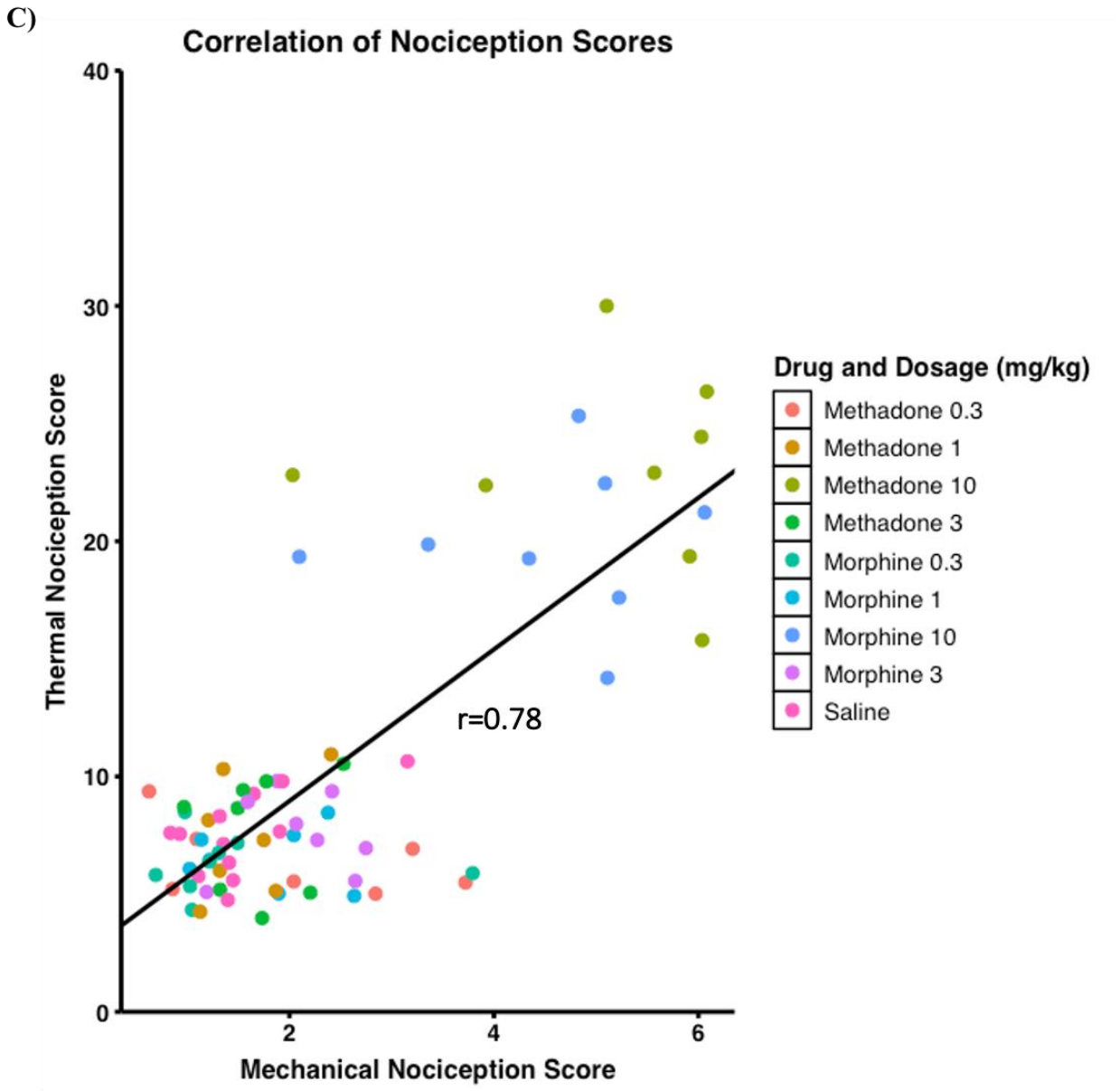
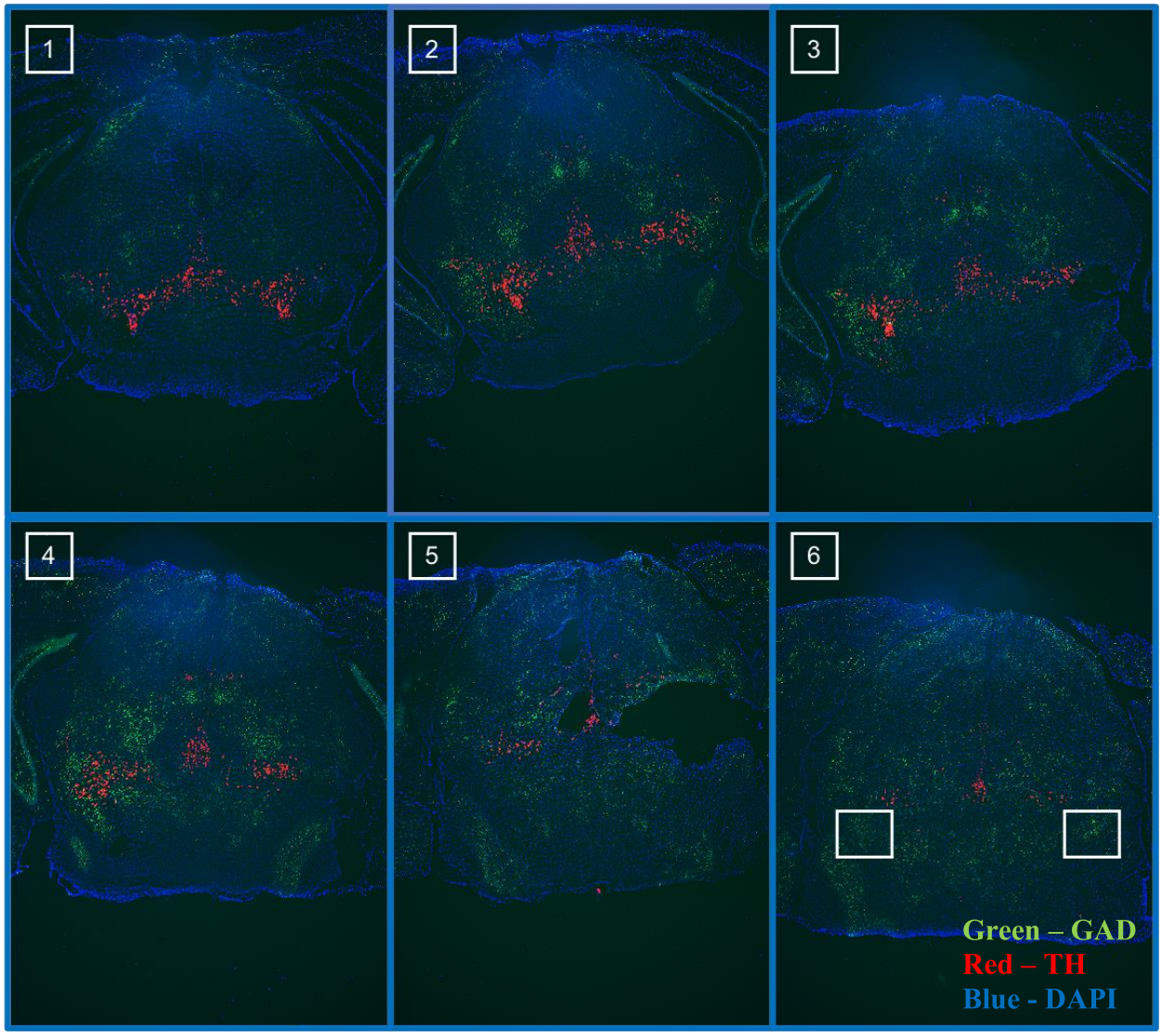


Figure 6. Methadone and morphine induce analgesia in female mice starting at 10 mg/kg. A) Comparison of 50% withdrawal threshold in grams during von Frey test across doses for both methadone and morphine. Shown is mean ± SEM. ****P<0.0001; one-way ANOVA with Dunnett's Multiple Comparisons Test. B) Comparison of time taken for animal to display nociceptive response on hot plate across doses for methadone and morphine. Shown is mean ± SEM. ****P<0.0001; one-way ANOVA with Dunnett's Multiple Comparisons Test. C)

Correlation of mechanical nociception and thermal nociception score across all drugs and doses. Pearson's correlation coefficient (r) and Simple Linear Regression displayed on plot.

After comparing the dose-response curves of the behavioral responses following administration with methadone and morphine, we then sought to understand whether the exposure to intermittent opioids induced changes in MOR and GalR1 mRNA co-expression in the RMTg, the GABAergic tail of the VTA that projects to and modulates the activity of dopaminergic neurons in the VTA. Because the location of this region is not standardized in brain atlases or the literature, we conducted RNAScope to mark dopaminergic neurons with TH and GABAergic neurons with GAD. We stained and imaged slices in series in the rostral to caudal direction to determine where VTA dopaminergic neurons transition to the RMTg GABAergic neurons (Figure 7A). After mapping those regions to a brain atlas, we took tissue from parts of series that matched the second (caudal) atlas image (Figure 7B). Because of this initial staining, we were able to stain sections from the presumptive RMTg and not the VTA proper.

A)



B)

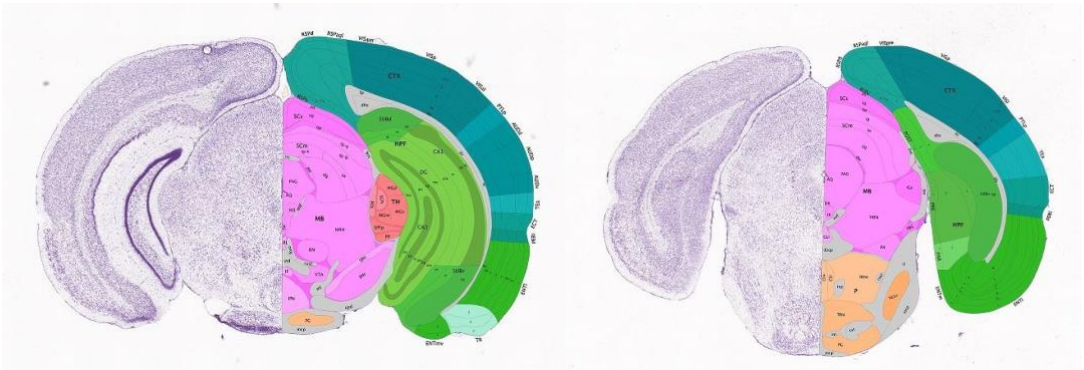
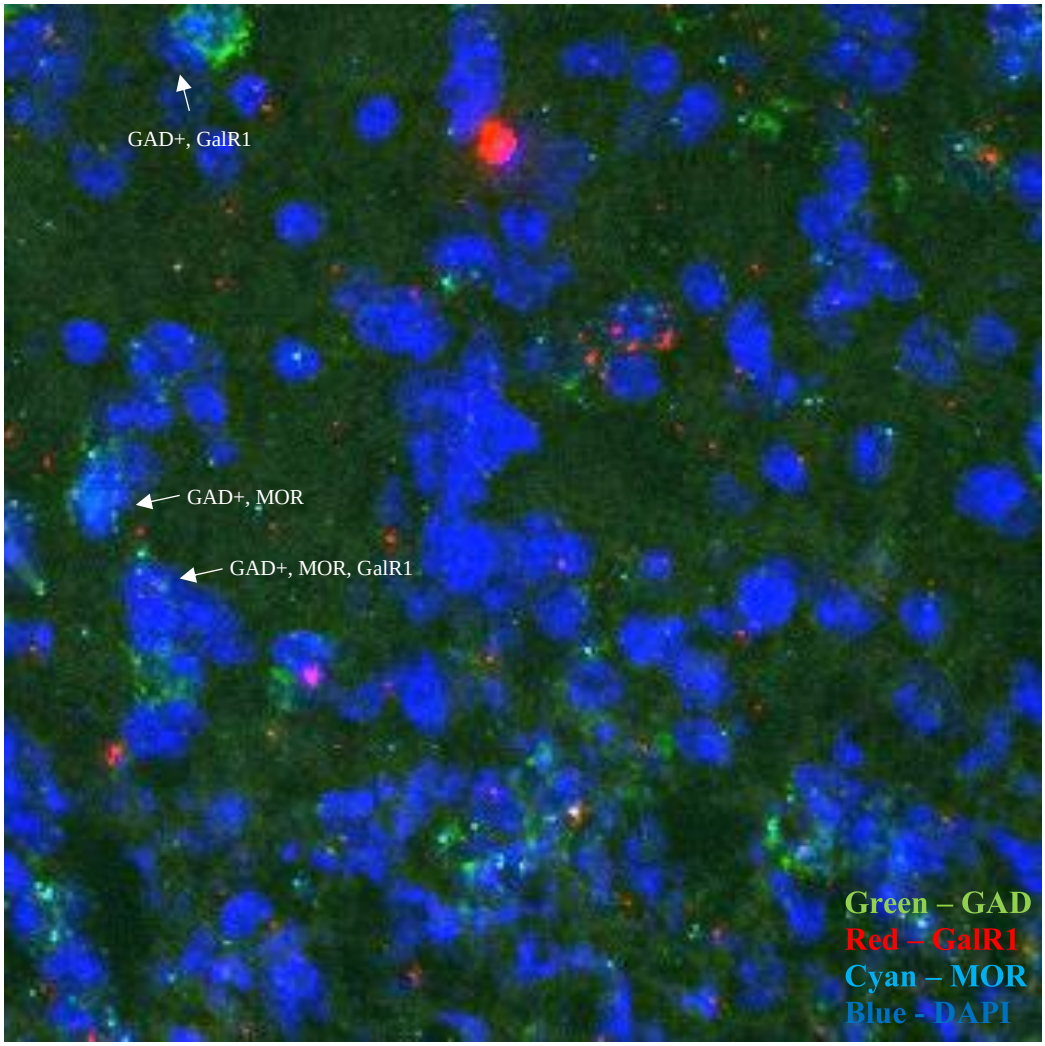


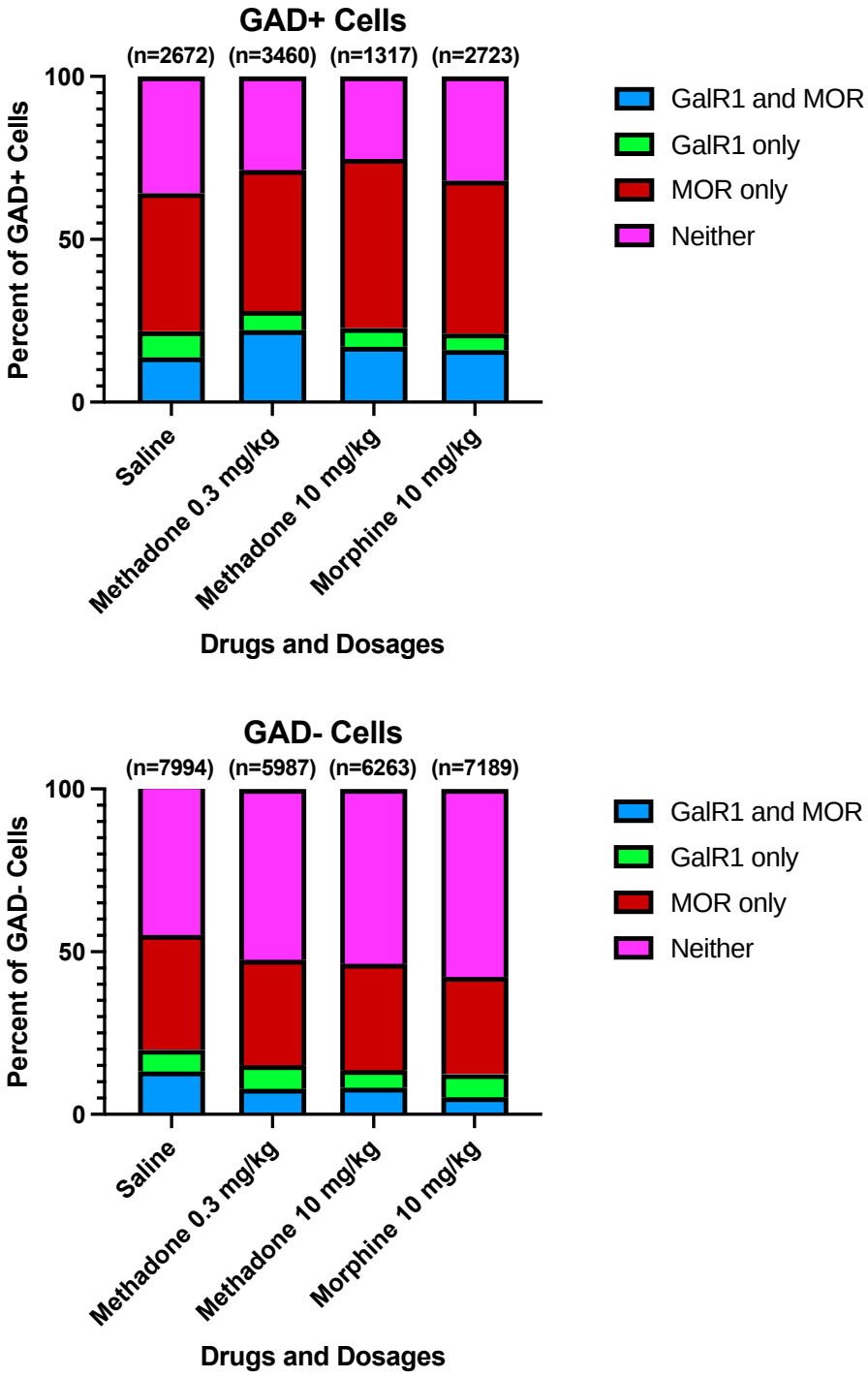
Figure 7. Identifying Location of RMTg using TH and GAD expression. A) 16-micron sections were taken in series. The sections are displayed in ~50-micron rostral to caudal intervals and were stained for TH mRNA (red) as a marker for dopaminergic cells and GAD (green) as a marker for GABAergic cells. This series shows the shift from strong TH (rostral) to GAD (caudal) signal, indicating the presence of the RMTg in images 5 and 6. Representative white boxes were drawn in image 6, where images were taken for RMTg analysis. B) Sections 1 and 6 respectively mapped to images taken from the Allen Brain Atlas (Allen Mouse Brain Atlas).

We then took sections from the RMTg of CPP animals treated with saline, methadone 0.3 mg/kg, methadone 10 mg/kg, and morphine 10 mg/kg and stained for GABAergic neurons with GAD, cells expressing the OPRM gene (MOR), cells expressing GalR1 (GalR1), cells expressing both, and cells expressing neither. Regardless of treatment, ~46% of GAD+ cells expressed MOR alone, ~6% expressed GalR1 alone, ~18% co-expressed MOR and GalR1, and ~30% expressed neither MOR nor GalR1 (Figure 8A). Interestingly, the percentage of cells expressing only GalR1 did not appear to differ between GAD+ cells and GAD- cells (Figure 8B). In GAD- cells, ~33% expressed MOR alone, ~7% expressed GalR1 alone, ~8% co-expressed MOR and GalR1, and ~52% expressed neither MOR nor GalR1 (Figure 5A). No significant differences between treatment groups were found in GAD+ Cells (Figure 8C; $F(3, 44)=1.908e-8$, $p>0.9999$) or GAD- cells (Figure 8C; $F(3, 44)=2.163e-8$, $p>0.9999$). Although we did not statistically compare GAD+ cells to GAD- cells, inspection of the data revealed substantially higher percentages of GAD+ cells expressing MOR alone and co-expressing MOR and GalR1 when compared to GAD- cells.

A)


GAD+, GalR1
GAD+, MOR
GAD+, MOR, GalR1
Green – GAD
Red – GalR1
Cyan – MOR
Blue - DAPI

B)


GAD+ Cells
(n=2672) (n=3460) (n=1317) (n=2723)
Percent of GAD+ Cells
100
50
0
Saline
Methadone 0.3 mg/kg
Methadone 10 mg/kg
Morphine 10 mg/kg
Drugs and Dosages
GalR1 and MOR
GalR1 only
MOR only
Neither
GAD- Cells
(n=7994) (n=5987) (n=6263) (n=7189)
Percent of GAD- Cells
100
50
0
Saline
Methadone 0.3 mg/kg
Methadone 10 mg/kg
Morphine 10 mg/kg
Drugs and Dosages
GalR1 and MOR
GalR1 only
MOR only
Neither

C)

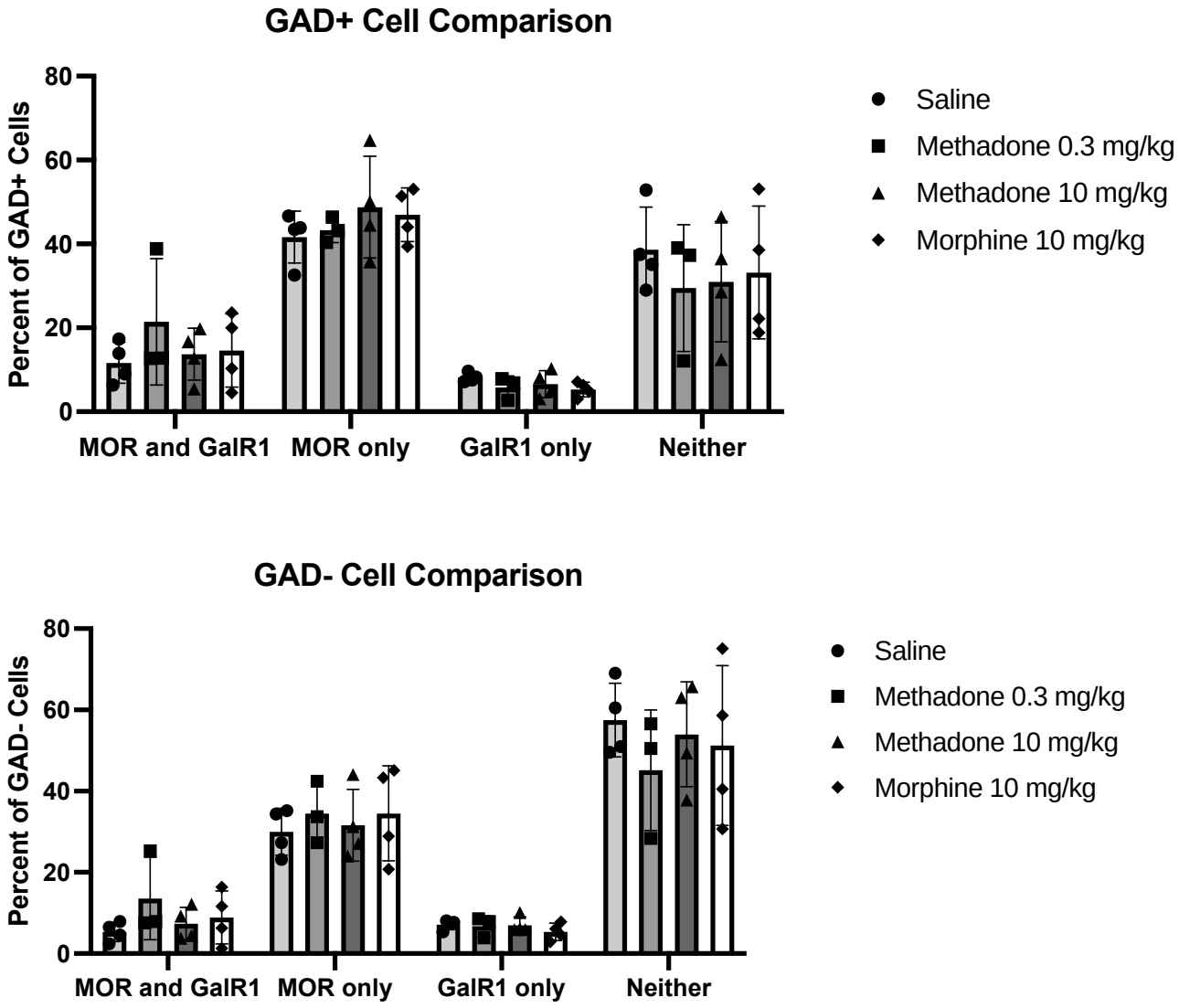


Figure 8. Intermittent morphine or methadone exposure during CPP does not induce changes in GalR1 or MOR mRNA expression. A) Representative image shows staining of GAD (green), GalR1 (red), MOR (cyan), and DAPI (blue). B) Summary stacked bar plots of GAD+ and GAD- cells across all images. C) Percent of GAD+ and GAD- cells expressing MOR and GalR1, MOR only, GalR1 only, and neither receptor was compared across treatment groups.

Shown is mean ± SEM. No significant differences between treatment groups were found; 2-way ANOVA.

We also binned the number of GalR1 and MOR puncta across treatment groups to investigate potential expression changes on a per cell basis. No differences between treatment groups were shown for MOR puncta (Figure 9; $F(3, 55)=1.919e-14$, $p>0.9999$) or GalR1 puncta (Figure 6; $F(3, 55)=6.608e-14$, $p>0.9999$) across all cells. These findings demonstrate that 4 injections of morphine or methadone up to 10 mg/kg over 8 days did not induce changes in mRNA expression for MOR or GalR1 in the RMTg. It is possible that more chronic, higher dose regimens would induce changes in gene expression. In a thesis exploring mRNA expression of MOR and GalR1 following oral fentanyl administration in mice for four weeks, mice administered oral fentanyl showed decreases in GalR1 expression in both GAD+ and GAD- cells in the NAc when compared to mice administered with saccharin, a control sweet compound with intrinsic rewarding properties. No changes were observed in co-expression of MOR and GalR1 or MOR alone (Chen, 2021). Because mRNA abundance does not always predict protein levels, and MOR trafficking affects its function but not total amounts, it is possible that changes are occurring that we cannot detect by RNAScope. Assessing the overlap of MOR and GalR1 protein has been challenging due to the lack of specific antibodies for GalR1. However, the recent development of a GalR1-mCherry knockin mouse has made these experiments more feasible, and we are currently optimizing immunohistochemical techniques to visualize MOR and GalR1 protein co-expression in our laboratory. Methadone's quick recycling back to the membrane via a beta-arrestin-mediated mechanism following activation and morphine's longer phosphorylation and internalization may suggest higher receptor levels following methadone exposure when compared to morphine.

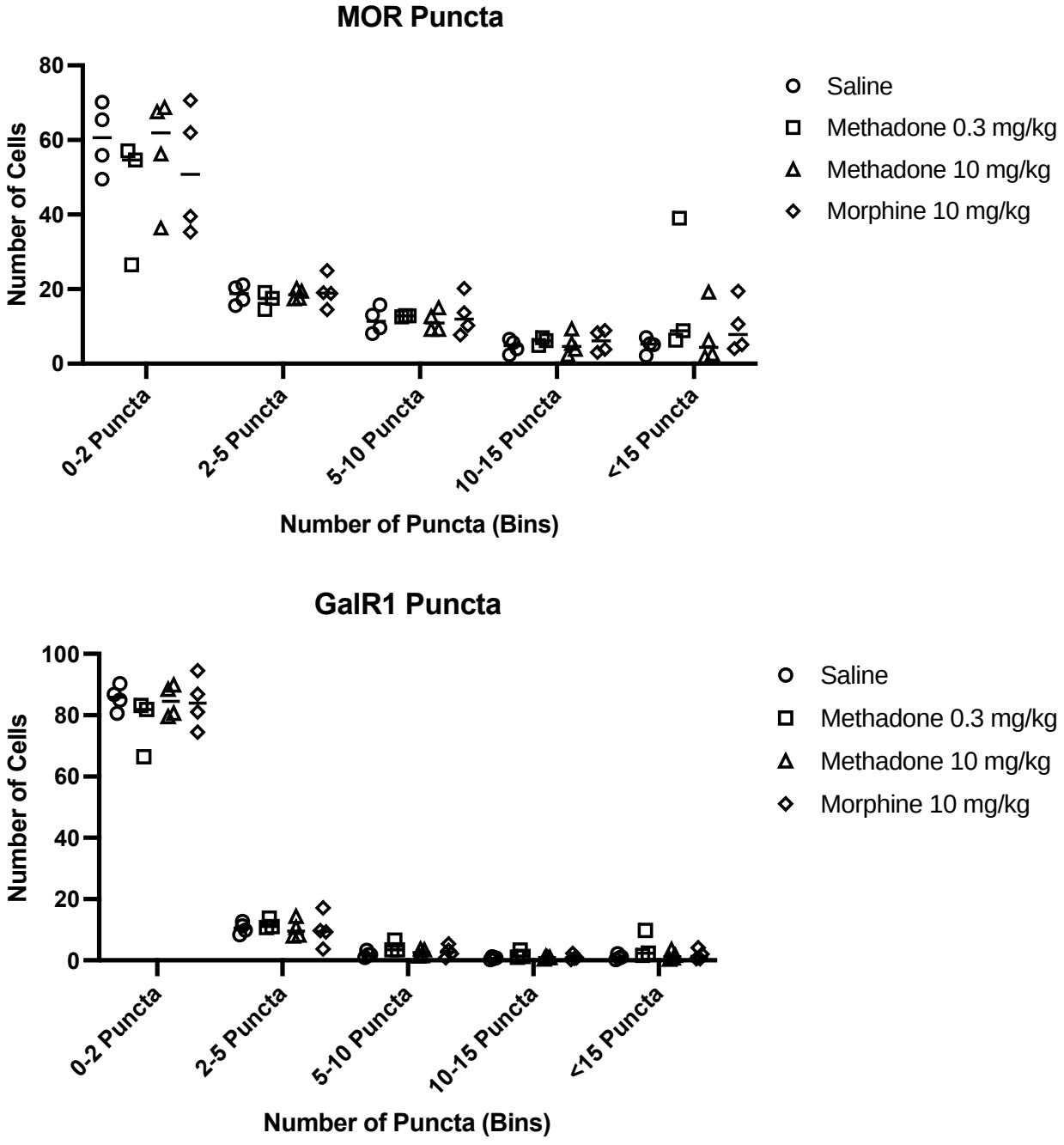


Figure 9. Intermittent morphine or methadone exposure during CPP does not induce changes in GalR1 or MOR puncta that is dependent on the level of expression. No significant differences in the number of MOR or GalR1 puncta per cell were observed between treatment groups; 2-way ANOVA.

This thesis showed that methadone produces similar analgesia and lower reward when compared to morphine without inducing differences in mRNA expression. However, this study has a few limitations. The transcriptomic research presented in this study does not clarify much about differences in signal transduction pathway activation between methadone and morphine. The data simply shows that mRNA expression may not be the site of regulation following intermittent exposure. Future research with immunohistochemistry may be much better for answering questions related to sites of regulation for the heteromer following methadone and morphine activation. The behavioral data was also limited by the potential of observer bias. More stringent studies have blinded the experimenter to doses and treatments given to animals to ensure that qualitative measurements of a nociceptive reaction are not impacted by observer bias (He et al., 2021). Our hot plate videos were scored by both a blind and a non-blind researcher to mitigate this limitation. The antinociception assays also only tested nociceptive responses to acute pain following acute opioid administration, and not chronic pain or following chronic opioid administration. Attenuation of acute pain following acute opioid administration is simply one dimension to understand opioid antinociception. Similarly CPP data is only one measure of abuse liability that does not capture opioid-related reward through an operant-learning lens, like self-administration tasks. Finally, our study could have benefited from testing both male and female mice to examine sex differences. Future research could explore these trends in male mice. Despite these limitations, our study has several strengths. This is the first study to directly compare the rewarding and analgesic properties of methadone and morphine in the same experimental design. As a behavioral assay, CPP represents reward-based associative learning that models the contextual cues that contribute to drug craving and relapse for individuals recovering from drug abuse. Our behavioral data also aligned well with established work

surrounding the relative potencies of morphine and methadone for activating the MOR-GalR1 heteromer. (Cai et al., 2019). Finally, our data also showed the effect of a wide range of doses in abuse liability and analgesia, providing a comprehensive comparison of methadone and morphine in a preclinical mouse model.

Recent pharmacological efforts related to solving the opioid crisis have focused on maximizing antinociception while reducing side-effects like abuse liability and respiratory depression, such as the development of analgesics with G-protein-biased signaling (Azevedo Neto et al., 2020). Cebranopadol, one of these compounds currently in clinical trials, simultaneously activates the nociceptin opioid receptor (NOP) and the mu-opioid receptor (MOR), showing higher therapeutic value and more favorable side-effects (Linz et al., 2014). Advancements in computational pharmacology have allowed researchers to test millions of molecules that bias MOR activation towards G-protein signaling in silico (Manglik et al., 2016). Our behavioral results suggest that MOR-GalR1 heteromer activation should be taken into account as well, using the structure of methadone as a backbone. Further consideration for using methadone itself as a first line analgesic is also warranted. This study provides evidence for methadone as a first-line analgesic as it provides similar analgesia while lowering abuse liability when compared to morphine – the gold standard for first-line analgesia. When compared to other alternative replacement therapies such as buprenorphine and L-α-acetylmethadol, methadone is the most effective in retaining patients and suppressing heroin use (Amato et al., 2005). However, because methadone can only be distributed in opioid treatment programs, which are restricted by inadequate public funding and unfavorable regulations, patients either remain on waitlists for years or travel hundreds of miles to receive methadone treatment (Sigmon, 2013; Rosenblum et al., 2011). An improvement in public opinion surrounding methadone is needed to

unfetter access to methadone maintenance treatment. A better understanding of methadone's abuse liability and analgesia could better inform public opinion on methadone.

Conclusion

Our study directly compared the analgesic and rewarding properties of methadone and morphine in female mice. While methadone and morphine induced similar analgesia at the doses we tested, methadone required a higher dose to induce a CPP when compared to morphine. This difference in preference was not related to changes in mRNA expression for MOR or GalR1. These findings align with previous reports that the MOR-GalR1 heteromer mediates activation by MOR agonists in the reward circuit, while only the MOR mediates activation in the analgesic circuit. Because methadone is less potent at activating the heteromer than morphine but has similar potency for activating MOR alone, methadone and morphine differ in abuse liability but not analgesic properties.

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