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Andy Chen April 10, 2021

Characterization of GalR1 and MOR mRNA Co-Expression in the Nucleus Accumbens

by

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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The neuropeptide galanin is a potential therapeutic target for opioid use disorder and has been shown to be protective against opioid reward and withdrawal symptoms via a potential μ-opioid-galanin receptor (MOR-GalR1) heteromer mechanism. However, the distribution of MOR-GalR1 heteromers within addiction-related circuitry is unknown. Furthermore, it is unclear whether heteromer expression changes in response to chronic opioid exposure, and how these potential changes may affect behavior. The current study utilizes an oral fentanyl self-administration paradigm and RNAscope in situ hybridization to characterize the baseline expression of μ-opioid receptors (MOR) and galanin receptor 1 (GalR1) mRNA in the nucleus accumbens (NAc) and to investigate changes in their abundance following chronic opioid exposure. RNAscope demonstrated successful in situ hybridization of individual galanin receptor 1 and μ-opioid receptor mRNA in the nucleus accumbens and demonstrated the capacity for nucleus accumbens GABAergic neurons to express the MOR-GalR1 heteromer. In addition, RNAscope also demonstrated dynamic expression of galanin receptor 1 and μ-opioid receptor mRNA in GABAergic and non-GABAergic neurons following chronic fentanyl exposure. Together, these results provide evidence for a subpopulation of neurons in the brain to have the capacity to express functional GalR1-MOR heteromers, which may have important implications for the treatment of opioid use disorder.

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Table of Contents

Introduction

The Opioid Epidemic

The use of opioid analgesics for pain management has its utility, but this drug class has a high abuse liability that has contributed to the opioid epidemic in the United States. Since 1999, the rate of drug overdose deaths has more than tripled (Hedegaard, 2020). Importantly, the majority of overdose deaths is attributable to opioids. In 2017 alone, nearly 68% of the approximately 70,000 drug overdose deaths involved an opioid (Colon-Berezin, 2019; Wilson, 2020).

The opioid epidemic has been characterized by three waves of opioid overdose deaths. The first wave, beginning in 1999, was marked by deaths involving prescription opioids that were both overprescribed by physicians and readily available on the black market (Ciccarone, 2019; Dayer et al., 2019). Following increased regulation of these prescription opioids, many prescription opioid users transitioned to heroin. This resulted in the second wave of the opioid epidemic in 2010, which was marked by an increase in opioid mortality involving heroin. Heroin was not only more widely available and potent, but it was also less expensive than prescription opioids on the black market (Jones, 2013). Previous studies indicate that three out of four people who use heroin initially used prescription opioids (Jones, 2013).

The third and most recent wave of the epidemic, which began in 2013, is marked by an increase in overdose deaths involving fentanyl and other illicitly manufactured synthetic opioids. In 2017, fentanyl contributed to 57% of all drug overdose deaths in New York City, and has been the primary driver in deaths involving opioids in the United States in the last decade

(Colon-Berezin, 2019). Interestingly, fentanyl use may be intentional or unintentional. Fentanyl is often mixed with other opioids such as heroin or oxycodone to increase their potency at a low cost (Kuczyńska et al., 2018). However, fentanyl is approximately 50 to 100 times more potent than morphine, and the greatly enhanced potency of fentanyl-laced opioids is therefore much more likely to induce unintentional overdose or death (Kuczyńska et al., 2018).

The everchanging nature of the epidemic continues to pose a challenge for healthcare professionals and policymakers. Despite efforts to broaden treatment, implement educational campaigns, and design abuse-deterrent formulations of opioids, the crisis continues to worsen. With over 11.1 million people aged 12 and older reporting having misused prescription opioids, and 1.7 million admitting to having a prescription opioid use disorder (OUD) in 2017, there are ongoing concerns about the risk of current and future opioid overdose and/or addiction (Substance Abuse and Mental Health Services Administration (US) & Office of the Surgeon General (US), 2018). In addition, the COVID-19 pandemic in 2020 has greatly exacerbated risk factors such as loss of social support, unemployment, and comorbid affective disorders that increase the likelihood of opioid abuse and overdose (Rajkumar, 2020; Webster, 2017). One possible approach to addressing the epidemic would be to intervene earlier in the process of addiction. By addressing opioid misuse early on, the number of individuals who transition from opioid use to addiction could potentially be limited. Specifically, treatments that decrease the addictive properties of opioids might help people misusing opioids curb their intake.

Opioid Signaling

Exogenous opioid drugs mimic the body's endogenous opioid peptides, endorphins and enkephalins, by binding to mu-opioid receptors (MORs) to produce analgesia (Sprouse-Blum et al., 2010). MORs are inhibitory G protein-coupled receptors (GPCRs) that decrease downstream neuronal activity when activated. In pain pathways, MOR activation suppresses nociceptive signaling to reduce pain (Bushnell et al., 2013; Corder et al., 2018). However, MORs are also found in other pathways in the brain. MOR activation in these other brain regions and pathways can produce rewarding effects such as euphoria that can make these drugs addictive.

The Mesolimbic Dopamine System

The mesocorticolimbic system, also known as the reward pathway, is a neural circuit that underlies the motivational and rewarding properties of drugs of abuse, including opioids (Maldonado, 2003). The circuit consists of dopamine (DA) neurons that originate in the ventral tegmental area (VTA) and terminate in the prefrontal cortex (PFC) and limbic structures including the amygdala, hippocampus, and the nucleus accumbens (NAc) (Helbing et al., 2016; Serafini et al., 2020; Söderpalm & Ericson, 2013). When activated by drugs of abuse, these neurons release DA in projection regions, which leads to their rewarding effects (Galaj et al., 2020; Di Chiara & Imperato, 1988; Helbing et al., 2016).

Opioid analgesics are MOR agonists that indirectly increase VTA DA release by binding to MORs expressed on GABAergic projections and disinhibiting VTA DA neurons (Fields & Margolis,

2015; Galaj et al., 2020). These receptors are highly expressed in GABAergic projections to the VTA, including the nucleus accumbens (NAc), ventral pallidum (VP), rostromedial tegmental nucleus (RMTg), and VTA interneurons (Fields & Margolis, 2015; Galaj et al., 2020). Under normal conditions, GABAergic projections from these four regions provide tonic inhibition of VTA DA neurons, which suppresses DA release in the mesolimbic dopamine pathway. However, activation of these MORs by opioids results in an inhibition of downstream GABA release onto VTA DA neurons. The resulting decrease in GABA neurotransmission produces a net excitatory effect that increases DA release from the VTA to mesocorticolimbic structures (Galaj et al., 2020; Williams et al., 2001).

The NAc, a primary site for mediating reward behavior, is one region of interest where targeted therapies could reduce the addictive properties of opioids. A previous intracerebral self-administration study showed that rats learned to self-administer morphine directly into the NAc, but not other brain regions (Olds, 1982). Another study using MOR knockout (MOR-KO) mice found that restoration of MOR expression in D1-receptor expressing medium-spiny neurons (D1-MSNs) in the NAc restored opioid-induced conditioned place preference (CPP) and self-administration (Cui et al., 2014). Interestingly, these D1-MSNs are also known to send GABAergic projections to the substantia nigra and VTA via a monosynaptic pathway (Baimel et al., 2019; Cui et al., 2014; Watabe-Uchida et al., 2012). These converging lines of evidence suggest that modulation of MOR signaling in NAc neurons could reduce the addictive properties of opioids.

Galanin as a Potential Therapeutic Target

A potential therapeutic target for opioid addiction is the neuropeptide galanin. Galanin is widely expressed in the brain, and is co-expressed within classical small molecule neurotransmitters systems in the brain such as the noradrenergic locus coeruleus (LC), the serotonergic dorsal raphe nucleus (DRN), and the cholinergic nucleus basalis (Holets et al., 1988; Lang et al., 2015; Melander et al., 1986). As a neuromodulator, galanin influences neurotransmission in the central and peripheral nervous system through interactions with its three GPCRs (GalR1, GalR2, and GalR3) (Lang et al., 2015). Galanin receptors canonically signal through G_i-protein coupling by inhibiting adenylate cyclase activity and opening potassium channels to suppress downstream neural activity (Fitzgerald et al., 1998; Smith et al., 1998; Wang et al., 1998). In fact, it has been suggested that galanin may confer resilience to depression and anxiety by acting to suppress overexcitation of LC neurons or in other downstream regions (Hökfelt et al., 2018; Tillage et al., 2020). Given its widespread expression and role as a neuromodulator, galanin has been implicated in numerous physiological and pathophysiological processes including feeding behavior, psychiatric disorders, and substance abuse (Lang et al., 2015).

Several lines of evidence suggest that galanin may be protective against opioid effects by reducing opioid reward and withdrawal symptoms. Galanin knockout (GKO) mice show enhanced morphine-induced locomotor activity and conditioned place preference (CPP), indicating that the loss of galanin increases opioid-induced behavioral plasticity and opioid reward (Hawes et al., 2008). Subsequent restoration of galanin receptor signaling with

administration of the non-specific galanin receptor agonist, galnon, normalizes GKO behaviors to wild-type levels (Hawes et al., 2008). Additionally, intracerebroventricular injection of galanin in wild-type (WT) mice reduces morphine CPP (Zachariou et al., 1999). Together, these results suggest that galanin reduces the rewarding effects of morphine. Further evidence implicating galanin as a potential therapeutic target is found in mice lacking galanin receptor 1 (GalR1), which exhibit greater naloxone-precipitated withdrawal symptoms when compared to WT mice (Holmes et al., 2012). In summary, galanin likely attenuates opioid reward and withdrawal symptoms via signaling through GalR1.

A recent study investigating the molecular mechanism underlying galanin's beneficial effects against opioids suggest that heteromerization of MOR and GalR1 is critical (Moreno et al., 2017). *In vitro* experiments using human embryonic kidney (HEK-293T) cells demonstrated selective interaction between MOR and GalR1 to produce MOR-GalR1 heteromers that can interact differentially with ligands (Moreno et al., 2017). The binding of galanin to these MOR-GalR1 heteromers induced a negative cross talk that counteracted normal mitogen-activated protein kinase (MAPK) activation by endomorphin-1, an endogenous MOR agonist (Moreno et al., 2017). Follow-up *in situ* and *in vivo* studies performed in the rat VTA showed that galanin blocks opioid-induced VTA DA release, and that addition of the TM5 interfering peptide that prevents MOR-GalR1 heteromerization attenuates the protective effects of galanin (Moreno et al., 2017). As such, these MOR-GalR1 heteromers may be potential targets for opioid use disorder.

Although MOR-GalR1 heteromers may explain the protective effects of galanin in opioid reward and withdrawal, the distribution of MOR-GalR1 heteromers within addiction-related circuitry is unknown. Furthermore, it is unclear whether heteromer abundance or distribution changes in response to chronic opioid exposure, and how these potential changes may affect behavior. Given the lack of selective antibodies for GalR1, an immunohistochemical approach to detecting the heteromer at the protein level has not been possible (Lu & Bartfai, 2009). An alternative approach is to use *in situ* hybridization to identify cells that co-express both GalR1 and MOR mRNA, and thus have the capacity to express the heteromer.

The present study has two aims: (1) to characterize MOR and GalR1 mRNA co-expression in the NAc, a region that projects to and influences VTA DA activity; and (2) to determine whether chronic opioid consumption alters the expression profiles of MOR and GalR1 mRNA. Characterization of MOR and GalR1 mRNA expression level at baseline and after opioid exposure will help inform our understanding of how the galaninergic and opioid systems interact to influence GABAergic modulation of VTA DA activity.

Materials and Methods

All protocols are consistent with the Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Animals

Eight adult male and eight adult female C57BL/6J mice between three and eight months of age were used for oral self-administration. All animals were individually housed for one week prior to beginning oral self-administration in the home cage and remained singly housed for the duration of the experiment. Food and water were available *ad libitum* during the acclimation period. Water bottles were replaced with 2% saccharin solution or 10 µg/ml of fentanyl in 2% saccharin solution during self-administration. Animals were maintained on a 12h light/dark cycle (7 AM on/7 PM off). Following oral self-administration, two male and two female mice from each treatment group were used for the RNAscope experiments.

Drugs

2% (w/v) saccharin solution was prepared by dissolving saccharin sodium salt dihydrate (Tokyo Chemical Industry, Tokyo, JN) in tap water. 10µg/ml fentanyl citrate (Sigma Aldrich, St. Louis, MO) was prepared by separately dissolving fentanyl citrate in 2% saccharin solution.

Oral Self-Administration

Mice were acclimated to single housing in their home cages for one week. During the last three days of the acclimation period, bottle weights were measured daily at 9:00 AM to assess baseline water intake. At the end of the week, mice were randomly assigned to one of two treatment groups for self-administration and given a single water bottle containing either 2% saccharin solution or 10 μg/ml of fentanyl in 2% saccharin solution. Four male and four female mice were used for each treatment group. Bottles were weighed at approximately 9:00 AM every day for the next four weeks. Daily consumption was calculated by measuring the difference in daily bottle weight and assuming a density of 1g/ml. Mice were weighed every three days to monitor changes in body weight. Saccharin and fentanyl solutions were replaced once a week, consistent with previous studies (Alipio et al., 2021).

Tissue Collection

Following the end of four weeks of oral self-administration, mice were anesthetized with isoflurane and rapidly decapitated. Brains were quickly removed, embedded into a mold with OCT tissue freezing medium, and frozen in isopentane chilled on dry ice. Frozen brains were stored in a -80°C freezer until cryosectioning.

Cryosectioning

Brains were acclimated to -20°C for 1-2 h before sectioning on a cryostat. RNaseZap was used to clean equipment. 10-µm sections of the NAc were collected onto Superfrost Ultra Plus slides (Thermo Fisher Scientific, Waltham, MA). Sections were stored at -80°C.

RNAscope Multiplex Fluorescent Assay

Brain sections were pre-treated and used for RNAscope according to manufacturer's instructions for the RNAscope Fluorescent Multiplex v1 Kit (Advanced Cell Diagnostics, Newark, CA). The following probes were used: *GalR1* (C1, ACD, 448821), *MOR* (Oprm1-C2, ACD, 315841), and *Gad1* (C3, ACD, 400951) for the enzyme glutamate decarboxylase 1 (Gad1), to determine whether cells for GABAergic or non-GABAergic. Probes were combined as *GalR1/MOR/Gad1.* Amp-4-AltA was used for all experiments in amplification step 4. Mouse multiplex positive (ACD, 320881) and multiplex negative (ACD, 320871) controls were also used to ensure the reliability of the assay. DAPI (ACD, 320858) was used for nuclear staining. Slides were coverslipped using Prolong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA) and stored in the dark overnight at room temperature to cure. All slides were imaged the following day.

Confocal Microscopy

Sections were imaged on a Nikon A1R HD25 confocal microscope using NIS Elements software. Images were acquired using a 40x oil-immersion lens. For each image, a 10µm Z-stack (step size 0.95µm,) was acquired. An average of 12 images per mouse was collected for a total of 48 images in each treatment group. Images were collected across both hemispheres over an average of 3 NAc sections per mouse.

Image Analysis

ND2 files acquired from the confocal microscope were converted into grayscale images for each color channel using FIJI software (Schindelin et al., 2012). CellProfiler (McQuin et al., 2018) was then used to process the grayscale images and analyze the expression of GalR1, MOR, and Gad1 mRNA within nuclei. The pipeline first identified all nuclei from the DAPI channel as primary objects. Individual color channels corresponding to GalR1, MOR, and Gad1 mRNA puncta were then filtered to enhance the foci in the image and identify individual puncta. A DAPI mask was then applied to identify puncta within nuclei. Puncta not located within any nuclei were then excluded from the analysis. The final output consisted of counts of GalR1, MOR, and Gad1 puncta in each nucleus. Each nucleus was then classified as either Gad1+ or Gad1-. Nuclei that contained > 3 Gad1 puncta were considered Gad+. Data were sorted to calculate the total number of Gad1+/- cells and the number of cells that expressed GalR1 only, MOR only, GalR1+MOR, or neither. The total number of nuclei were compiled across images for each mouse and then across treatment groups. Proportions of Gad1+/- cells

were calculated by dividing the number of nuclei expressing GalR1 only, MOR only, GalR1+MOR, or neither by the number of Gad1+/- nuclei for each treatment group.

Statistical Analysis

All statistical analysis and graphs were generated using Prism 9 (GraphPad, San Diego, CA). One-way ANOVA was performed to compare differences in the amount of fentanyl doses consumed during self-administration. Two-way ANOVA was performed to compare differences in intake between saccharin and fentanyl mice. Differences in mRNA expression of GalR1, MOR, and GalR1+MOR mRNA expression in Gad1-positive cells across treatment groups were compared by unpaired two-tailed t-tests.

Results

Oral Self-Administration of Water, Saccharin, and Fentanyl Reveal No Differences in Liquid Intake

Baseline daily water intake over the three days prior to beginning oral self-administration was 5.692 ± 0.2755 ml (Figure 1A). Daily consumption of 2% saccharin and 10 µg/ml fentanyl in 2% saccharin solution was 6.062 ± 0.1260 ml and 6.007 ± 0.1091 ml, respectively (Figure 1B). Daily consumption of fluids was stable over time (saccharin, week 1: 6.111 ± 0.287 ml; saccharin, week 4: 5.702 ± 0.386 ml; fentanyl, week 1: 6.136 ± 0.402 ml; fentanyl, week 4: 5.703 ± 0.330 ml) (Figure 1B). A two-way ANOVA (time x treatment) comparing the average daily consumption of saccharin and fentanyl did not reveal any significant differences between mice in their consumption of fentanyl compared with saccharin-only controls. There was no effect of time ($F_{4,69}$ = 1.340, p = 0.2639), treatment ($F_{1,69}$ = 1.179, p = 0.2813), or interaction ($F_{4,69}$ = 1.922, p = 0.1166). Daily dose (mg/kg) by week of fentanyl remained stable over time (week 1: 2.506 ± 0.436; week 4: 2.180 ± 0.452) (Figure 1C). A one-way ANOVA revealed no significant differences in the daily dose consumed each week ($F_{3,28}$ = 1.047, p = 0.3873), indicating that were was no change in fentanyl intake over time. Weekly cumulative doses did not change over time (week 1: 17.539 ± 3.052; week 4: 14.504 ± 3.313).

Characterization of GalR1 and MOR mRNA in Gad1+ and Gad1- Nuclei in Saccharin Mice

To determine the capability of GABAergic and non-GABAergic cells in the NAc to form GalR1-MOR heteromers, we characterized the proportion of cells expressing MOR, GalR1, both, or neither in saccharin controls. Representative images are shown in Fig. 2A and 2B. In the Gad1+ subpopulation, 34.636 ± 2.289% of cells expressed only MOR mRNA; 13.444 ± 0.903% expressed only GalR1 mRNA; 28.079 ± 3.298% expressed both; and 23.849 ± 1.917% expressed neither transcripts (Figure 2C). In the

Gad1- subpopulation, 24.021 ± 0.989% of cells expressed only MOR mRNA; 19.607 ± 1.082% expressed only GalR1 mRNA; 12.920 ± 1.942% expressed both; and 43.450 ± 2.937% expressed neither transcripts (Figure 2D). Multiple unpaired t-tests revealed significant differences in the proportion of nuclei expressing MOR, GalR1, both, or neither between Gad1+ and Gad1- nuclei (Figure 2E). The proportion of nuclei expressing MOR mRNA only was reduced in the Gad1- subpopulation compared with the Gad1+ subpopulation ($t=4.253$, $df=6$, $p<0.01$). The proportion of nuclei expressing GalR1 mRNA only was increased in the Gad1- subpopulation compared with the Gad1+ subpopulation ($t=4.374$, $df=6$, $p<0.01$). The proportion of nuclei expressing both mRNA was reduced in the Gad1- subpopulation compared with the Gad1+ subpopulation ($t=3.960$, $df=6$, $p<0.01$). Lastly, the proportion of nuclei expressing neither mRNA was greater in the Gad1- nuclei compared with Gad1+ nuclei ($t=5.589$, $df=6$, $p<0.01$).

Characterization of GalR1 and MOR mRNA in Gad1+ and Gad1- Nuclei in Fentanyl Mice

Galanin action through GalR1 receptors and MOR-GalR1 heteromers is thought to modulate opioid reward and potentially be a therapeutic target for opioid use disorder. However, changes in the expression of these receptors in response to chronic opioid exposure has not yet been explored. Because GABAergic projections from the NAc to the VTA are known to modulate DA release, we examined the mRNA expression of GalR1, MOR, and GalR1+MOR in Gad1+ and Gad1- cells in mice in the fentanyl group. In the Gad1+ subpopulation, 44.865 ± 4.011% of nuclei expressed only MOR mRNA; 6.660 ± 1.212% expressed only GalR1 mRNA; 16.856 ± 3.522% expressed both; and 31.619 ± 1.283% expressed neither transcripts (Figure 2F). In the Gad1- subpopulation, 27.331 ± 2.343% of nuclei expressed only MOR mRNA; 10.124 ± 2.596% expressed only GalR1 mRNA; 7.870 ± 1.726% expressed both; and 54.675 ± 2.0117% expressed neither transcripts (Figure 2G). Multiple unpaired t-tests revealed significant differences between Gad1+ and Gad1- nuclei in the proportion of nuclei expressing

MOR-only and the proportion expressing neither, but not GalR1 only or both (Figure 2H). The proportion of nuclei expressing MOR mRNA only was reduced in the Gad1- subpopulation compared with the Gad1+ subpopulation ($t=3.774$, $df=6$, $p<0.01$). The proportion of nuclei expressing GalR1 mRNA only in the Gad1- subpopulation compared with the Gad1+ subpopulation was not significantly different ($t=1.209$, $df=6$, $p=0.272$). The proportion of nuclei expressing both mRNA was also similar between subpopulations ($t=2.291$, $df=6$, $p=0.0618$). Lastly, the proportion of nuclei expressing neither mRNA was greater in the Gad1- nuclei compared with Gad1+ nuclei ($t=9.664$, $df=6$, $p<0.001$).

Chronic Exposure to Fentanyl Decreases GalR1 Expression in Gad1+ and Gad1- Nuclei Expressing GalR1 mRNA, but not MOR or GalR1+MOR, in the NAc

To determine whether chronic oral fentanyl self-administration changes the proportion of cells capable of forming GalR1-MOR heteromers, we compared GalR1 and MOR expression alone and in combination between the saccharin and fentanyl groups. Unpaired two-tailed t-test revealed a trend towards an increase in MOR mRNA expression in MOR only Gad1+ nuclei after chronic fentanyl exposure compared with saccharin treatment, but it did not reach significance ($t=2.217$, $df=6$, $p=0.0685$) (Figure 3A). Unpaired two-tailed t-test showed a significant decrease in GalR1 mRNA expression in Gad1+ nuclei following chronic fentanyl exposure ($t=4.489$, $df=6$, $p < 0.01$) (Figure 3B). Unpaired two-tailed t-test showed a trend for decreased MOR+GalR1 mRNA co-expression in Gad1+ nuclei in the fentanyl group, but it was not significant ($t=2.326$, $df=6$, $p=0.0590$) (Figure 3C).

Nearly identical results were observed in Gad1- nuclei. Unpaired two-tailed t-tests revealed a significant decrease in GalR1 mRNA expression in Gad1+ nuclei ($t=3.372$, $df=6$, $p<0.05$), with non-significant trends towards an increase in the proportion of cells expressing MOR only ($t=1.301$, $df=6$,

p=0.2407) and a decrease in those expressing MOR+GalR1 mRNA (t=1.944, df=6, p=0.0999) following chronic fentanyl exposure compared with saccharin (Figure 3D-F).

When all cells expressing GalR1 were considered (GalR1 only and GalR1+MOR), the decrease in GalR1 mRNA remained significant in both Gad1+ (t=3.064, df=6, p=0.0221; Figure 4A) and Gad1- nuclei (t=2.842, df=6, p=0.0295; Figure 4B), while there was no difference in all cells expressing MOR mRNA (MOR only and GalR1+MOR; Figure 4C) in both Gad1+ (t=0.4001, df=6, p=0.7029) and Gad1- nuclei (t=0.6894, df=6, p=0.5164; Figure 4D).

Figures and Graphs

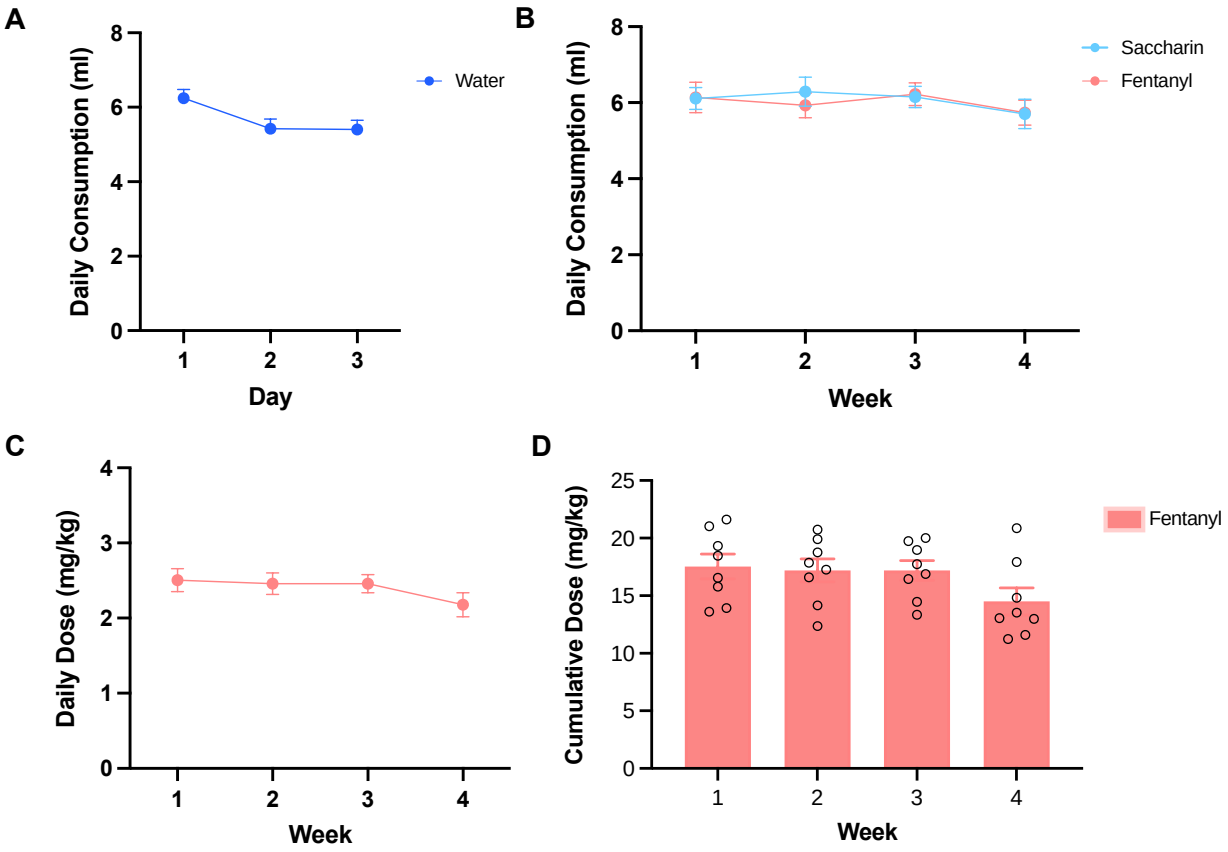
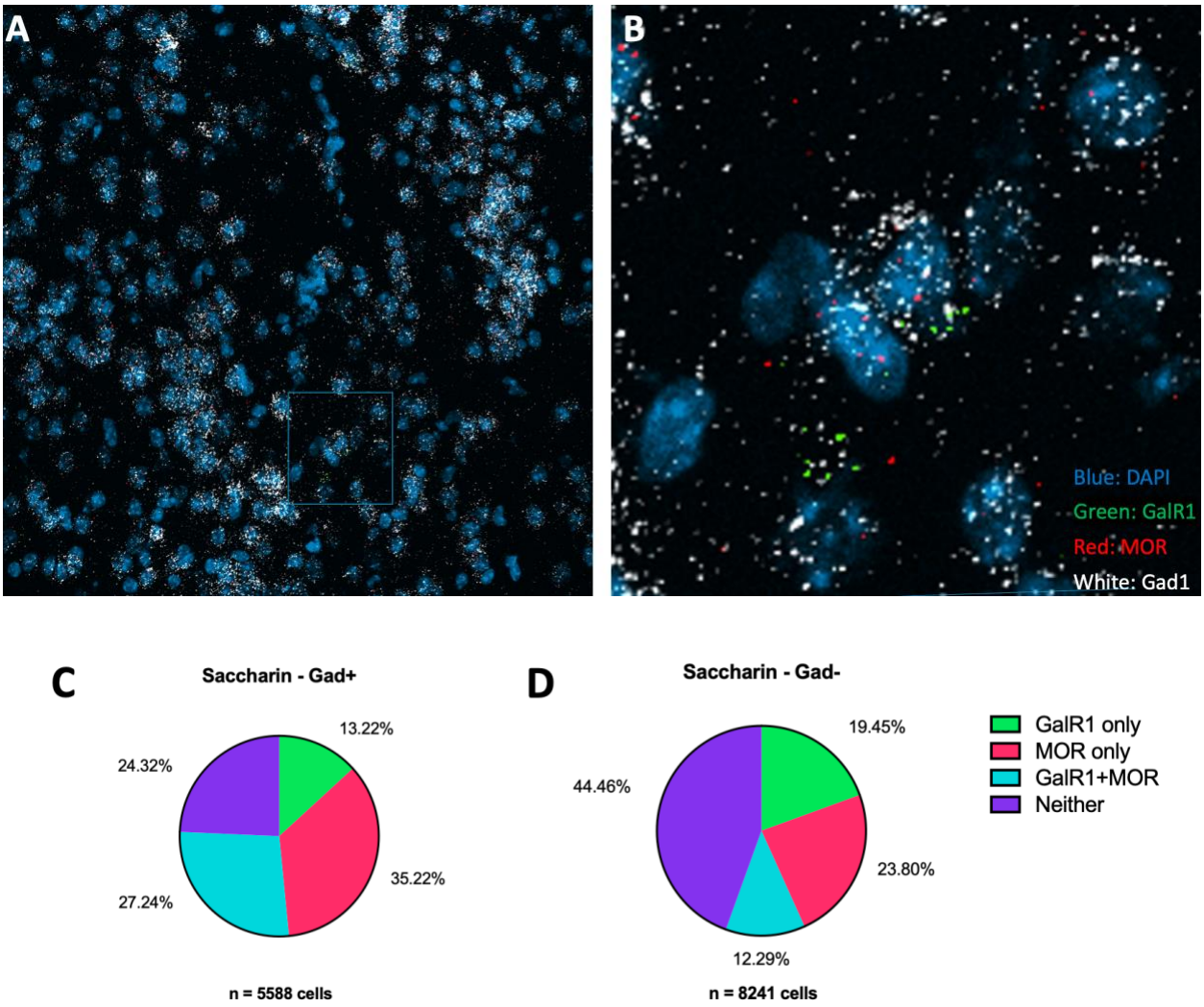


Figure 1. **Oral consumption of saccharin or saccharin + fentanyl is stable over time.**

A) Consumption of water (ml) over three days prior to beginning oral self-administration with 2% saccharin or 10µg/ml fentanyl in 2% saccharin is plotted for baseline comparison. B) Average daily consumption (ml) of saccharin and fentanyl is shown for each week of self-administration. C) Average daily fentanyl dose (mg/kg) does not change over time. D) Cumulative weekly fentanyl dose (mg/kg) with individual data points. All graphs display mean ± SEM. n = 8 mice per treatment group. n.s. non-significant.


A
B
Blue: DAPI
Green: GalR1
Red: MOR
White: Gad1
C
Saccharin - Gad+
13.22%
24.32%
35.22%
27.24%
n = 5588 cells
D
Saccharin - Gad-
19.45%
44.46%
23.80%
12.29%
n = 8241 cells
GalR1 only
MOR only
GalR1+MOR
Neither
E
Expression (%)
60
40
20
0
**
**
**
**
MOR only
GalR1 only
GalR1+MOR
Neither
Gad1+
Gad1-

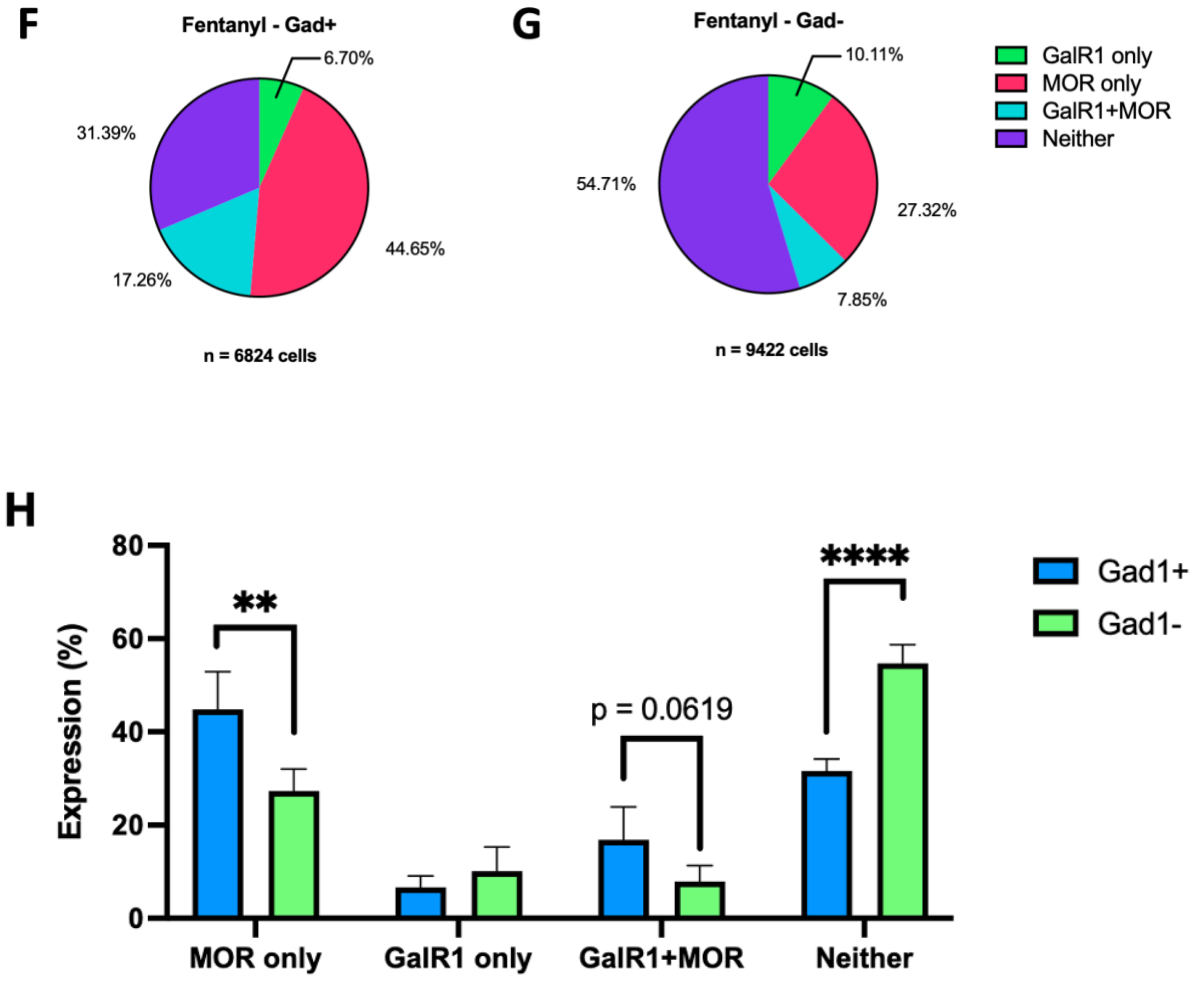


Figure 2. **GalR1 and MOR mRNA expression by cell type and treatment group in the NAc.**

A) Representative RNAscope images show GalR1 (green), MOR (red), Gad1 (white), and nuclear marker, DAPI (blue). B) Co-expression of GalR1 and MOR mRNA can be seen within Gad1-positive nuclei in the zoomed-in image from (A). C, D, F, G) Relative proportions of GalR1 only, MOR only, GalR1+MOR mRNA, or cells containing neither transcript in Gad1+ and Gad- cells by treatment group (saccharin vs saccharin + fentanyl). E) "Baseline" relative proportions of nuclei expressing MOR, GalR1, both, and neither under saccharin condition. H) Relative proportions of nuclei expressing MOR, GalR1, both, and neither under

fentanyl condition. Graph displays mean proportion ± SEM. n = 4 mice per treatment group. **** $p < 0.0001$ ** $p < 0.01$, n.s. not significant

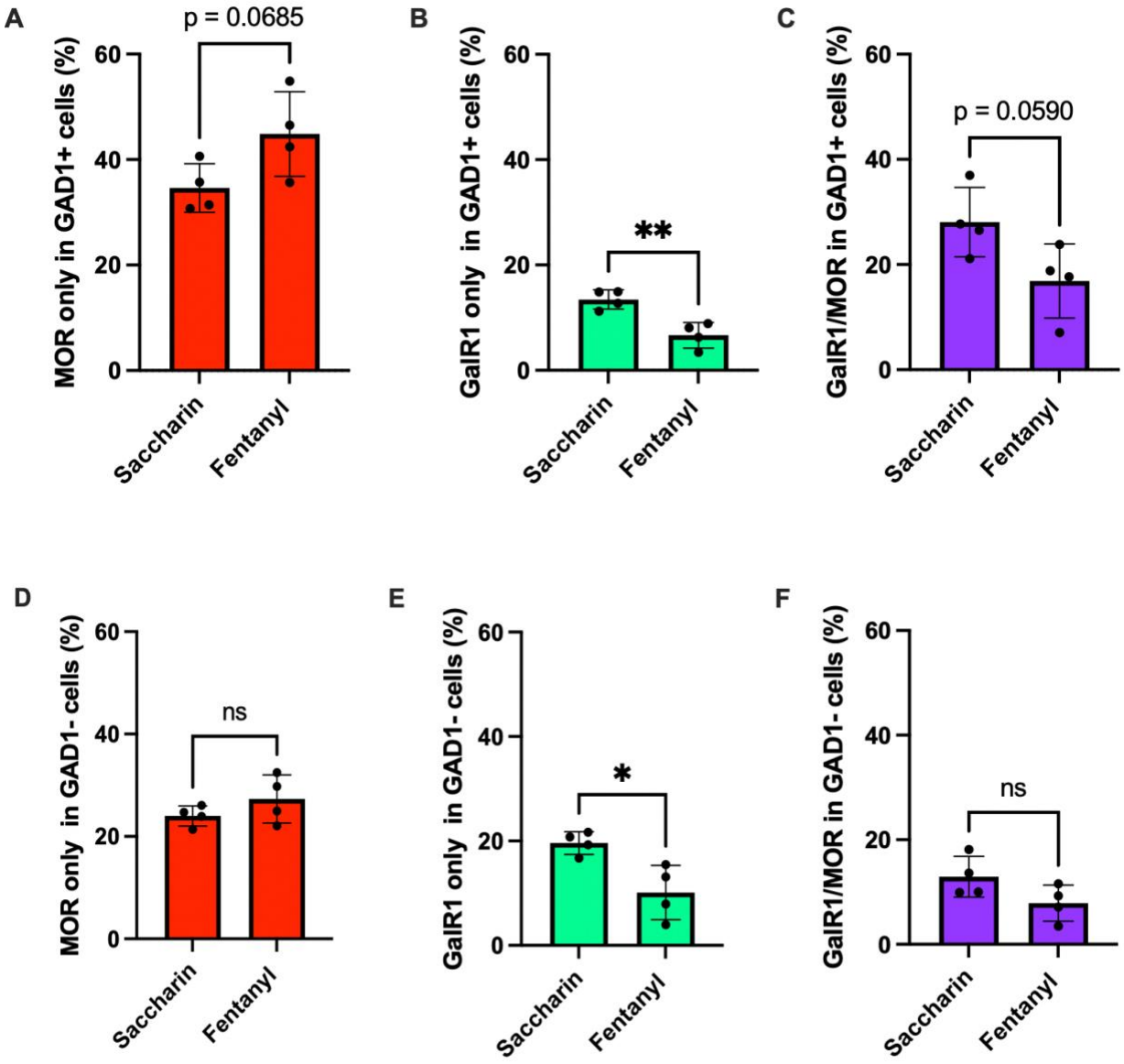


Figure 3. Dynamic expression of GalR1 and MOR mRNA in Gad1+ and Gad1- NAc nuclei following chronic oral fentanyl consumption. A) Percentage of Gad1+ nuclei that express only MOR mRNA tends to increase in response to chronic fentanyl exposure. B) Percentage of Gad1+ nuclei expressing only GalR1 mRNA is significantly decreased in response to chronic fentanyl exposure compared to saccharin controls. C) Percentage of Gad1+ nuclei that co-express GalR1 and MOR mRNA tends to decrease in Gad1+ nuclei after chronic fentanyl exposure. D) Percentage of Gad1- nuclei that express MOR mRNA

only does not change in response to fentanyl treatment compared with saccharin. E) Percentage of Gad1- nuclei that only express GalR1 mRNA is decreased in fentanyl treatment. F) Percentage of Gad1- nuclei that co-express GalR1 and MOR mRNA does not change. All graphs display mean proportion ± SEM. n = 4 mice per treatment group. ** $p < 0.01$, * $p < 0.05$, n.s. not significant.

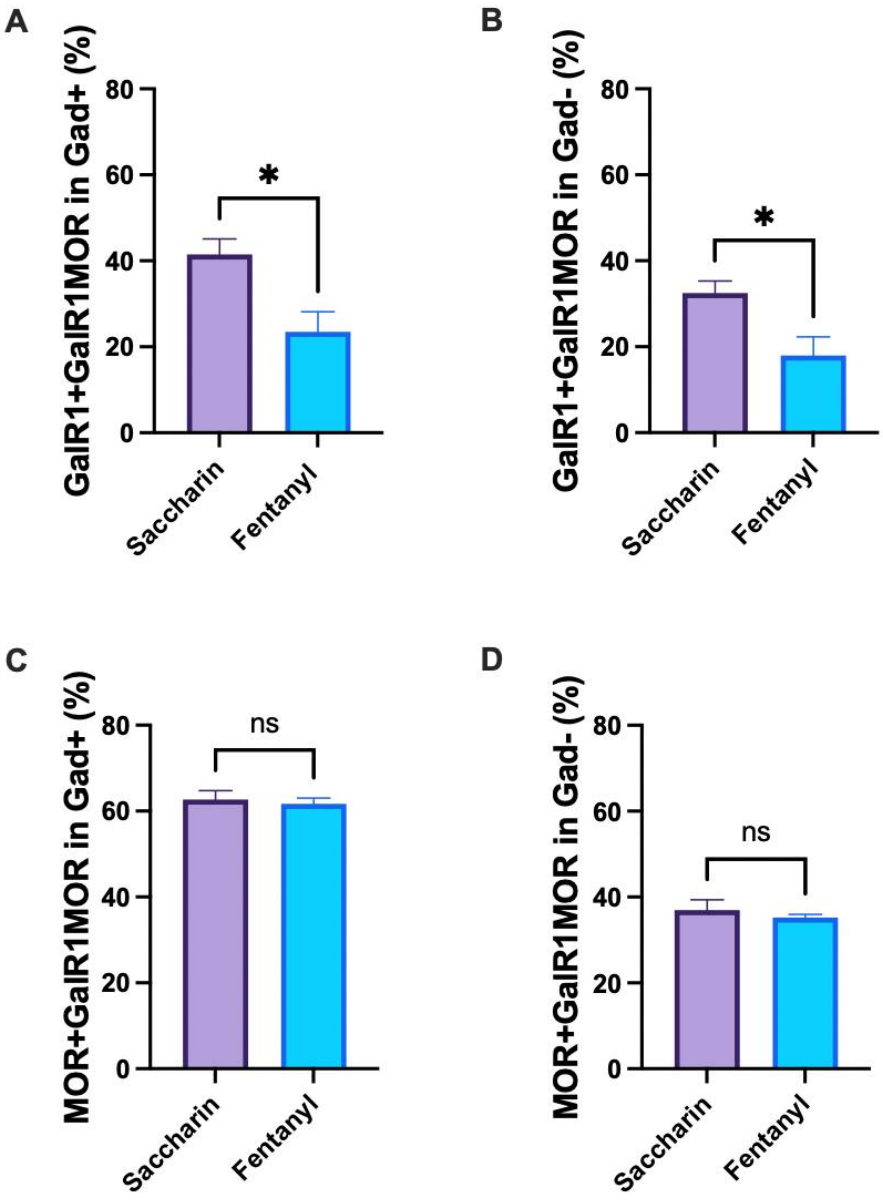


Figure 4. Dynamic expression of all nuclei expressing GalR1 mRNA, but not MOR mRNA, in Gad1+ and Gad1- NAc nuclei following chronic oral fentanyl consumption.

A) Percentage of Gad1+ nuclei that express GalR1 mRNA in GalR1-only and GalR1+MOR-expressing nuclei combined decreases following fentanyl treatment B) Percentage of Gad1+ nuclei that express MOR mRNA in MOR-only expressing nuclei and GalR1+MOR-expressing nuclei combined does not

change C) Percentage of Gad1- nuclei that express GalR1 mRNA in GalR1-only expressing nuclei and GalR1+MOR-expressing nuclei combined also decreases following fentanyl treatment D) Percentage of Gad1- nuclei that express MOR mRNA in MOR-only expressing nuclei and GalR1+MOR-expressing nuclei combined also does not change. All graphs display mean proportion ± SEM. n = 4 mice per treatment group. * $p < 0.05$, n.s. not significant.

Discussion

Previous studies have suggested that galanin released in the VTA may interfere with opioid reinforcement via the GalR1-MOR heteromer to decrease VTA dopamine neuron activity. However, the localization of these heteromers and whether their expression is static or dynamic following opioid exposure is not known. In the present study, we used RNAscope to determine what proportion of cells in the NAc co-express GalR1 and MOR mRNA and are thus capable of containing GalR1-MOR heteromers, and a fentanyl oral self-administration paradigm to investigate potential opioid-induced changes in the co-expression of these receptor mRNAs.

Average baseline water intake in mice prior to beginning oral self-administration was within the previously reported range of 4-6 ml for C57BL/6 mice (Tordoff et al., 2007). Daily consumption of saccharin and fentanyl by week was stable at approximately 6 ml over the four weeks of self-administration. Their average daily dose per week was approximately 2.5mg/kg, which corresponds to a human equivalent dose (HED) of 200µg/kg (Nair and Jacob, 2016). Large doses as high as 100µg/kg is used to critically ill patients and those undergoing intensive surgery to induce and maintain anesthesia (Peng and Sandler, 1999). No difference was observed between treatment groups in their consumption of saccharin or fentanyl, and mice did not demonstrate an escalation in their intake of fentanyl. This is interesting because the escalation of drug intake is often interpreted as a measure of drug dependence, which would validate self-administration paradigms such as the one used in the present study (Zernig et al., 2007). However, previous studies have also reported that mice do not escalate their intake of fentanyl over time, consistent with our results (Grim et al., 2018). In fact, mice displayed no preference for fentanyl at any concentration compared to water, and total fentanyl intake did not differ when tested with saccharin (Grim et al., 2018). However, the absence of escalation does not preclude the validity of oral fentanyl self-administration, and a cursory test of withdrawal behavior following self-

administration indicated that the animals were indeed physically dependent. Specifically, naloxone-precipitated withdrawal in a subset of these mice demonstrated paw tremors, diarrhea, and jumping associated with opioid dependence (Lichtman et al., 2001; Kest et al., 2002). It is important to note that opioids vary in their pharmacokinetic properties and that can affect the ways in which they respond in the body (Mercadante, 2015). Future work can explore the use of other behavioral measures to characterize physical dependence associated with fentanyl use. Another possibility would be to compare how the magnitude of fentanyl precipitated withdrawal following oral self-administration compares with other opioids using the same paradigm.

We successfully detected GalR1, MOR, and Gad1 mRNA in the NAc using RNAscope. Under "baseline" (i.e. saccharin) condition, co-expression of MOR and GalR1 mRNA was observed primarily in GABAergic (Gad1+) neurons in the NAc, although co-expression was also detected to a lesser degree in non-GABAergic neurons. This finding suggests that neurons in the NAc, more prominently GABAergic neurons, have the potential to express MOR-GalR1 heteromers that are hypothesized to protect against opioid reward.

The proportion of Gad1+ nuclei that expressed GalR1 decreased significantly following fentanyl treatment compared with saccharin controls. A previous study using RNA-seq analysis of reward-related genes in the NAc also reported similar changes in the expression of GalR1 following a 14-day oxycodone self-administration paradigm, further implicating the role of galanin receptors in opioid use disorder (Zhang et al., 2018). Our findings demonstrate that the decrease in GalR1 expression is sustained over the four week period of oral fentanyl self-administration. However, future studies should evaluate if gene expression changes occur immediately following oral self-administration and evaluate the impact of these gene expression changes.

Within the Gad1+ subpopulation, the proportion of nuclei expressing MOR but not GalR1 mRNA did not change significantly between saccharin and fentanyl treatment groups. While the data suggested a trend towards an increase in the number of nuclei expressing MOR in the fentanyl group, the variability in the data suggests that the study was underpowered. This was also true of the proportion of Gad1+ nuclei that co-expressed GalR1 and MOR mRNA; there was a trend for a decrease, but the variability in data indicates that a larger number of animals need to be incorporated into the study. Future studies should be fully powered to represent a conclusive finding of the trends. If the present trends were conclusive, our findings would suggest that the decrease in the expression of GalR1 mRNA occurs in both nuclei that express only GalR1 and nuclei that expressed GalR1+MOR mRNA, resulting in an increase in cells that express MOR only.

While galanin has been shown to reduce opioid reward in previous studies, decreased GalR1 expression in the fentanyl group potentially leads to a reduction in the proportion of heteromers and an increase in MOR homomers. If true, this suggests that chronic opioid use might compromise the GalR1 protective mechanism. However, these differences in expression could be region-specific and not indicative of the potential of GalR1-MOR heteromers as therapeutic targets for modulating opioid reward. Importantly, the NAc is only one of four brain regions that provide GABAergic input to VTA DA neurons (Fields & Margolis, 2015). One possibility is that GABAergic projections from the RMTg, VP, or the VTA itself play a larger role in modulating opioid effects in the VTA following chronic opioid exposure.

Future Directions

The use of RNAscope in this study helped address the two aims of the study. First, we demonstrated that co-expression of GalR1 and MOR mRNA occurs in the NAc. Second, we demonstrated dynamic expression of these mRNAs in response to chronic fentanyl exposure. However, it is also important to consider the limitations of using RNAscope in this study. While it is a valuable method for detecting mRNA signal when immunohistochemistry is not possible, it should be noted that mRNA levels do not necessarily correlate with protein levels. Another caveat to the use of RNAscope is that GalR1-MOR mRNA co-expression only indicates the *capacity* to express the heteromer, but provides no evidence of their actual presence. Supplementing the data from this study with methods that investigate the localization of MOR-GalR1 heteromers, possibly using a GalR1-mCherry line and complementary IHC may help address this gap in the literature (Foster et al., 2021; Kerr et al., 2015).

In addition to investigating the localization of the heteromer at the protein level in the NAc, another direction would be to investigate differences in expression between the NAc core and the NAc shell. These two subregions of the NAc preferentially receive input from different brain, which could affect their expression of GalR1, MOR, and MOR-GalR1 receptors (Li et al., 2018). This specificity could also provide additional insights into the role of GalR1 and MOR-GalR1 heteromers in the NAc depending on their projection sites.

Lastly, this study can be extended to include the investigation of GalR1, MOR, and GalR1+MOR mRNA expression in the RMTg, VP, and VTA GABAergic interneurons. In the present study, decreased GalR1 mRNA expression and low GalR1+MOR co-expression suggests that the NAc may contribute only slightly to the galanin system and its role in protecting against opioid effects. One region of specific interest is the RMTg, which projects heavily to VTA dopaminergic neurons, oppose reward, and is powerfully inhibited by opioids (Jhou et al., 2009; Lavezzi & Zahm, 2011; Matsui et al., 2014).

Conclusion

In summary, our study demonstrated that GABAergic neurons in the NAc co-express GalR1 and MOR mRNA, suggesting that a subpopulation of neurons in this brain region have the potential to express functional GalR1-MOR heteromers. In response to chronic fentanyl exposure, we found a significant decrease in GalR1 mRNA expression and trends for increases in MOR and decreases in GalR1+MOR mRNA expression that did not reach significance. Future work should investigate the co-expression of these mRNA transcripts in other brain regions providing GABAergic efferents to VTA dopaminergic neurons, and ideally utilize alternative methods of localizing protein expression such as the GalR1-mCherry line.

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