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Galanin and Opioids: Molecular and Behavioral Interactions in Opioid Use Disorder Circuits

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B.S., University of Maryland – College Park, 2011

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By Stephanie L. Foster

The neuropeptide galanin has been shown to oppose the behavioral effects of opioids, particularly withdrawal and reward. The galaninergic system has therefore been identified as a possible therapeutic target for opioid use disorder (OUD). However, most studies have utilized body- and brain-wide galanin manipulations, leaving system-specific questions about galanin – which could inform targeted therapies for OUD – largely unanswered. Given that the noradrenergic system is implicated in opioid-mediated behaviors and that its major nucleus, the locus coeruleus (LC), strongly expresses galanin, noradrenergic-derived galanin is a prime target for system-specific investigation. Additionally, the mechanisms by which galanin blocks opioid reward and withdrawal remain unclear, but current theories for both implicate galanin receptor 1 (GalR1). Previous work suggests that LC-derived galanin acts on GalR1 within the LC to suppress withdrawal symptoms. In the ventral tegmental area (VTA), GalR1 forms heteromers with the mu opioid receptor (MOR), potentially allowing galanin to directly interfere with opioid signaling and attenuate reward. Therefore, the goal of this dissertation was to examine 1) whether noradrenergic galanin modulates opioid withdrawal and reward behaviors, and 2) to evaluate whether the pattern of GalR1 expression in the LC and in reward circuits provides clues about its contribution to these two aspects of OUD. We demonstrate here that manipulating noradrenergic galanin does not affect naloxone-precipitated withdrawal symptoms. We also show, for the first time, that the distribution of GalR1 in the LC is inconsistent with previous mechanistic theories of galanin-mediated suppression of withdrawal. Regarding opioid reward and reinforcement, we find that altered noradrenergic galanin levels do not affect by acute morphine-induced locomotion, morphine conditioned place preference, or intravenous remifentanil self-administration. Characterization of GalR1 and MOR mRNA co-expression indicates that the rostromedial tegmental nucleus and the VTA, two populations that exert GABAergic control over VTA dopamine neurons, both exhibit GalR1-MOR co-expression in a quarter of GABAergic neurons. Together, these findings constitute the first system-specific investigation of galanin effects on OUD-related behaviors. This work indicates that while noradrenergic galanin does not modulate opioid withdrawal or reward behaviors, enhancing GalR1-MOR heteromeric activity in the VTA may be an important area of focus for future studies.

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This work is dedicated to my fellow underrepresented PhD students. We bear visible (and invisible) burdens that many may not acknowledge or understand. To the PhD students reading this while working a second job, caring for children or sick family members, or feeling culturally isolated – you are not alone. Your presence in graduate school will help ensure that our struggles do not become those of the next generation. You can do it, and you can do it well.

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CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.1 OPIOID PHARMACOLOGY

1.1.1. The Endogenous Opioid System

The endogenous opioid system is comprised of opioid peptides and their cognate G protein-coupled receptors. Beginning with the initial discovery of the opioid peptides Leu- and Met-enkephalin in the 1970's (Hughes et al., 1975), more than 20 opioid peptides have since been identified (Fricker et al., 2020). A trait shared by all known opioid peptides is that they are cleavage products of the precursor molecules prodynorphin, proopiomelanocortin, and proenkephalin, the products of which are dynorphins, beta-endorphins, and enkephalins, respectively (Fricker et al., 2020). Despite their common origin, differential proteolytic processing of opioid precursors confers an array of biologic functions and binding profiles to this group of peptides (Fricker et al., 2020).

Opioid peptides are the endogenous ligands for opioid receptors, which are seven transmembrane G protein-coupled receptors that are divided into three subtypes: mu (MOR), delta (DOR), and kappa (KOR) (Al-Hasani & Bruchas, 2011). While opioid peptides can signal through more than one opioid receptor, the field has classically associated each peptide with a "preferred" receptor subtype. It was previously thought that endorphins preferentially bind MOR, enkephalins bind DOR, and dynorphins bind KOR (Höllt, 1986; Trescot et al., 2008). However, more recent work revealed that the majority of opioid peptides bind with high affinity to all three opioid receptor subtypes, greatly increasing the potential repertoire of endogenous opioid signaling (Fricker et al., 2020; Gomes et al., 2020).

1.1.2. Cellular and Circuit-Level Opioid Signaling

Opioid receptors are G protein-coupled receptors that, under most conditions, couple to $G\alpha_i$ subunits (Al-Hasani & Bruchas, 2011; Chakrabarti, Regec, & Gintzler, 2005; H. Y. Wang et

al., 2005). The net result of opioid receptor binding is neuronal hyperpolarization, which occurs through distinct actions of the Gα and Gβγ subunits (**Fig. 1.1.**). Upon dissociation, Gα inhibits adenylate cyclase and suppresses the formation of cyclic adenosine monophosphate. The Gα subunit also activates G protein-coupled inwardly rectifying potassium channels to hyperpolarize the cell (Al-Hasani & Bruchas, 2011). Meanwhile, the Gβγ subunit binds to voltage-gated calcium channels and inhibits calcium conductance, ultimately preventing calcium-dependent fusion of vesicles at the axon terminal and suppressing neurotransmission (Al-Hasani & Bruchas, 2011; Corder et al., 2018). The collective effect of opioid receptor signaling is therefore a decrease in cellular activity.

The primary and most studied function of the endogenous opioid system is its modulation of pain through its actions in both the peripheral and central nervous system (Friedman & Nabong, 2020). When noxious stimuli activate peripheral nociceptors, this somatosensory signal is transmitted via primary afferent neurons to the dorsal horn of the spinal cord. From there, neurons in ascending nociceptive pathways transmit pain signals to the thalamus and reticular formation, and finally to the cerebral cortex (Julius & Basbaum, 2001). Nociceptive signals are then modulated by descending pain pathways, which can exert inhibitory or facilitatory effects on nociception (Heinricher et al., 2009).

The analgesic effects mediated by endogenous or exogenous opioids occur through signaling at MORs located at multiple nodes of nociception circuitry. When noxious stimuli activate nociceptors, they also elicit the release of endorphins (Bach, 1997), which can bind MORs in the dorsal horn to directly block pain transmission at the level of the spinal cord (Friedman & Nabong, 2020). Opioids can also bind MORs in the brain regions that influence descending pain control, especially the periaqueductal gray (PAG), locus coeruleus (LC), dorsal reticular nucleus,

and ventrolateral medulla (Al-Hasani & Bruchas, 2011; Heinricher et al., 2009). While this mechanism is not yet entirely understood, MOR activation is thought to disinhibit neurons capable of inhibiting nociceptive transmission in the spinal cord, ultimately producing analgesia (Al-Hasani & Bruchas, 2011; Heinricher et al., 2009). Importantly, the analgesic effects of opioids are ablated in MOR knockout mice (Kitanaka et al., 1998; Matthes et al., 1996), indicating that MOR signaling critically mediates the analgesia-producing effects of opioids. However, the importance of the MOR ligand, beta-endorphin, is less clear. Although beta-endorphin-deficient mice exhibit some analgesic deficits as would be expected, there is evidence that these mice exhibit compensatory analgesic mechanisms that obscure the impact of beta-endorphin deletion on analgesia (Rubinstein et al., 1996). Future studies can better assess the role of beta-endorphin without the confound of developmental changes by employing inducible knockout approaches.

1.1.3. Clinical Utility

In a clinical context, the endogenous opioid system can be leveraged to treat pain by giving patients drugs that stimulate MORs. These drugs, also called opioids, are MOR agonists that can be sub-classified into three groups based on their means of production: naturally occurring, semi-synthetic, and synthetic (Nafziger & Barkin, 2018). Naturally occurring opioids (also called opiates) are compounds derived from the opium poppy plant, which include drugs like morphine and codeine (Friedman & Nabong, 2020). Semi-synthetic opioids, such as oxycodone and heroin, are chemically modified versions of natural opioids. These compounds broadly exhibit increased potency and, in some cases, a faster onset of action than their natural counterparts (Trescot et al., 2008). Synthetic opioids, as the name implies, are entirely laboratory-made. This class includes the therapeutic partial MOR agonist methadone, as well as the highly potent MOR agonist fentanyl and its various analogs (Friedman & Nabong, 2020; Trescot et al., 2008).

Opioids have historically been a first-line therapy for the treatment of pain (Friedman & Nabong, 2020). All three classes of opioids can be used for the treatment of moderate to severe pain, with the selection of a particular opioid depending on a variety of factors including the patient's medical history, the desired duration of effect, and the type and intensity of pain (Ahlbeck, 2011). Synthetic opioids are frequently used in surgical and intensive care settings and have various clinical uses that depend on their dose and mode of administration. For example, in surgical settings, synthetic opioids are used during the intraoperative period to reduce nociception and supplement anesthesia. In addition, opioids suppress the sympathetic system and enhance hemodynamic stability during surgery (Lavand'homme & Estebe, 2018). In critical care settings, opioids can similarly be used to provide a combination of analgesic and sedative effects (Scholz, Steinfath, & Schulz, 1996).

While opioids have unparalleled efficacy regarding pain relief, their benefits are tempered by a number of side effects associated with their use. The use of high potency or high dose opioids, which is common in surgical settings, is known to contribute to paradoxical hyperalgesia (Corder et al., 2018), a phenomenon characterized by increased sensitivity to nociception following opioid use. Chronic opioid use also facilitates neural adaptations leading to both tolerance (decreased responsiveness to opioid analgesia) and withdrawal (physical dependence) (Rivat & Ballantyne, 2016). Moreover, the wide central and peripheral distribution of MORs, combined with systemic modes of administration, give rise to numerous adverse effects. Some side effects like constipation, urinary retention, and dizziness are manageable, but others are far more serious, and include delirium, respiratory suppression/overdose, and risk for developing opioid use disorder (Ahlbeck, 2011). In particular, the highly addictive properties of opioids necessitate careful clinical oversight to ensure adequate pain management while minimizing liability for abuse.

1.1.4. Endogenous Opioid Reward

Opioid drugs are frequently abused because apart from their ability to suppress pain, they induce powerful euphoric sensations. In the same way that exogenous opioids are used to treat pain by mimicking the effects of endogenous opioids on nociceptive circuits, they also modulate hedonic states by acting on reward circuitry. The euphoric effects caused by opioid drugs reflect pharmacological activation of natural opioid reward processes typically mediated by the endogenous opioid beta-endorphin.

Beta-endorphin is primarily synthesized in the pituitary gland, hypothalamus, and nucleus of the solitary tract (NTS). It is released in response to physical and environmental stress (Nikolarakis, Almeida, & Herz, 1986; Xue et al., 2020) within multiple brain regions including those that mediate reward processes, such as the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Roth-Deri, Green-Sadan, & Yadid, 2008). Central administration of beta-endorphin is rewarding and reinforcing to rats (Roth-Deri, Green-Sadan, & Yadid, 2008), and stimulates increased dopamine (DA) release into the NAc (De Vries & Shippenberg, 2002) similar to drugs of abuse. These findings indicate that beta-endorphin is an endogenous opioid capable of inducing reward. Apart from being rewarding itself, endogenous opioid signaling enhances the rewarding effects mediated by other endogenous peptides and exogenous drugs. For example, pharmacological blockade of MORs in the NAc blocks endocannabinoid- and benzodiazepine-induced euphoria (Mitchell, Berridge, & Mahler, 2018; Richardson et al., 2005). These results indicate that endogenous opioids produce rewarding effects through MORs, and explains why opioid drugs, which mimic this effect, can be highly addictive.

1.2. OPIOID USE DISORDER

1.2.1. The Opioid Epidemic

Rates of opioid use disorder (OUD) and overdose deaths have reaches crisis levels in the U.S. over the last several decades (Murthy, 2016), leading many to refer to this issue as an epidemic. The opioid epidemic began in the 1990's due to a confluence of factors, including a newfound pressure for healthcare providers to treat pain, pervasive and misleading marketing of powerful, semi-synthetic opioids, and rampant licit and elicit over prescribing of opioids (Bernard et al., 2018; Oesterle et al., 2019). Reports indicate that by 2010, enough opioids had been sold in the U.S. to medicate every adult every four hours with 5 mg/kg hydrocodone for a month ("Vital signs: overdoses of prescription opioid pain relievers---United States, 1999--2008," 2011). The population's unprecedented exposure and access to opioids ultimately culminated in a public health crisis that is still unfolding today.

The progression of the opioid epidemic is currently recognized as having three phases differentiated by the major causes of opioid overdose deaths. The initial phase, beginning around 1999, was characterized by overdoses of natural and semi-synthethic opioids, particularly oxycodone and hydrocodone (Ossiander, 2014; "Vital signs: overdoses of prescription opioid pain relievers---United States, 1999--2008," 2011). While these deaths spurred stricter regulation of prescription opioids and changes in prescribing practices, a second wave of overdose deaths occurred in 2010. This time, deaths were primarily attributable to heroin, as former prescription opioid users turned to heroin when prescription opioids became harder to access (Rudd et al., 2014). A third wave of opioid overdose deaths, unparalleled in scale, then began around 2013 with the spread of potent illicit synthetic opioids, particularly fentanyl (J. K. O'Donnell et al., 2017). Recent national statistics indicate that while death rates including all opioids decreased two percent during 2017 – 2018, the rates for synthetic opioids increased ten percent, and this class accounted for two-thirds of all opioid deaths (Wilson et al., 2020). These findings show that the issue of

opioid overdose is a persistent and evolving public health threat, which necessitates discussion about OUD and associated treatment.

1.2.2. Diagnostic Criteria

To receive a diagnosis of OUD, the DSM-5 indicates that a patient must exhibit two or more of 11 criteria consistent with problematic use occurring within a 12-month period (Coffa & Snyder, 2019). The criteria include escalating use, failure to control or reduce intake, continued use despite harms, and an inability to fulfill obligations at work or home due to opioid use, among other behaviors. Tolerance and withdrawal are also critical OUD criteria. Tolerance is defined as the need to take increasing doses of a drug to achieve the same effect over time, while withdrawal is an aversive reaction induced by cessation of drug intake in a physically dependent individual (Listos et al., 2019). During prolonged opioid use, the body adapts to the presence of the drug and becomes dependent upon its presence for normal neural function. Opioid withdrawal is triggered when intake stops, and is characterized by somatic symptoms including muscle spasms, tremors, insomnia, lacrimation, rhinorrhea, and gastrointestinal issues including nausea and diarrhea (Kosten & Baxter, 2019). See section 1.3.2. for more on the neural processes underlying withdrawal.

It is important to note that while tolerance and dependence occur in OUD, these symptoms alone are not indicative of OUD. Appropriate, medically-approved use of opioids can also result in tolerance and dependence if the treatment regimen is long-term (J. V. Pergolizzi, Jr., Raffa, & Rosenblatt, 2020). For this reason, tolerance and/or dependence must be present along with other signs of aberrant behavior before OUD can be diagnosed.

1.2.3. Treatment

OUD requires both short- and long-term pharmacological management, and FDA-approved medications exist for each purpose. Naloxone is a competitive antagonist of the MOR that is administered as a reversal agent to patients experiencing acute overdose. It can be administered intranasally or via injection, and if given quickly enough, displaces the offending opioid at MORs to relieve patients of opioid-induced respiratory suppression (Peprah & Severn, 2019). Additionally, the alpha-2 adrenergic receptor agonist lofexidine is FDA-approved for acute management of withdrawal symptoms. This medication agonizes alpha-2 autoreceptors, which reduces the central and peripheral norepinephrine (NE) release associated with somatic withdrawal symptoms (Joseph V. Pergolizzi, Jr. et al., 2019).

There are three FDA-approved medications for long-term OUD treatment, all of which act at the MOR: methadone (a full agonist), buprenorphine (partial agonist), and naltrexone (opioid antagonist). Each medication differs slightly regarding accessibility, pharmacokinetics, abuse liability, and potential side effects. As a result, the selection of a particular treatment must be based on individual patient characteristics and desired treatment outcomes (Oesterle et al., 2019). Combined pharmacological and behavioral treatment of OUD is clinically referred to as medication-assisted treatment (MAT), which has been shown to significantly improve OUD patient outcomes (Korthuis et al., 2017).

1.2.4. Current Challenges

Recent national statistics on opioid overdose deaths highlight the need to connect patients with MAT, but patients face many barriers to treatment (Wilson et al., 2020). While methadone and buprenorphine are efficacious, FDA-approved treatments for OUD, they are still opioid agonists with abuse liability, and are therefore subject to federal restrictions as controlled substances ("Medication Assisted Treatment for Opioid Use Disorders. Final rule," 2016; Oesterle

et al., 2019). These restrictions require patients to receive treatment at a federally approved opioid treatment program, or through a limited number of approved physicians. The difficulty of finding willing and qualified providers severely limits the ability of patients to receive treatment (C. M. Jones et al., 2015). Given the persistence of legal barriers to MAT, the development of effective non-opioid therapies that do not require such control could markedly facilitate access to treatment. This may be achieved by developing medications that are not opioids themselves, but rather modulate neural circuits that contribute to opioid reward and withdrawal.

Apart from managing the existing burden of OUD in the population, another challenge is preventing future cases of OUD through early-stage intervention. Substance use disorders (SUDs) are thought to develop over time (Everitt & Robbins, 2016), such that the initial voluntary or recreational misuse of a drug eventually transitions into behaviors consistent with SUD. Approximately 21 – 29 percent of people prescribed an opioid will misuse it (Vowles et al., 2015), and prescription opioid misuse strongly increases the odds of escalating to more dangerous opioids such as heroin (Banerjee et al., 2016; Palamar & Shearston, 2018). These findings indicate that opioid misuse is a critical window for early intervention; however, such intervention would require giving patients a treatment that reduces the rewarding or addictive effects of opioids to curb further misuse. Current OUD medications manage withdrawal and craving in patients who have already progressed to a state of physical dependence, and might not be appropriate for patients in earlier stages of misuse. New treatments must therefore be developed to specifically attenuate opioid reward, as this could facilitate early-stage opioid cessation.

1.3. NEURAL CIRCUITRY OF SUBSTANCE USE DISORDERS

SUDs are complex, chronic disease states in which drugs of abuse interact with an individual's biology and psychosocial experience to alter brain function. However, despite diverse

etiology, these disorders typically manifest as a set of stereotypical, maladaptive behaviors, which have been conceptualized as a three-stage cycle of drug addiction (Koob & Volkow, 2010). The first phase, binge/intoxication, refers to the acute euphoric and rewarding effects associated with drug intake that promote future use. After chronic use of the drug, its absence elicits the withdrawal/negative affect phase, which encompasses acute somatic withdrawal symptoms and the emergence of longer lasting negative emotionality (e.g. irritability and dysphoria) (Koob, 2013). Last, the preoccupation/anticipation stage is associated with intense craving and motivation for the drug (Koob & Volkow, 2010). This stage is also associated with relapse, which occurs when an individual resumes drug use after a period of abstinence. Importantly, this cycle changes over time. While early drug use is motivated by the desire for appetitive effects, sustained drug use is driven by a need to stave off increasingly aversive withdrawal effects, which is referred to as opponent process theory (Koob et al., 1989; Solomon, 1980).

The following sections will describe the neural circuits underlying the three "stages" of drug addiction, which are largely conserved across SUDs. Opioid-specific differences will be discussed where appropriate. Each section will also review the assays used to study different behavioral components of the addiction cycle in animals. As this dissertation modeled OUD in mice using assays of reward/reinforcement, withdrawal, and relapse, these are the specific behaviors that will be discussed.

1.3.1. Reward and Reinforcement

Circuitry

The binge/intoxication stage of SUDs can be understood by examining the circuits that confer the rewarding and reinforcing effects of drugs. The foundational work on reward circuitry was conducted in the 1950's by Olds and Milner, who showed that rats would lever press for

electrical stimulation of specific brain areas, particularly the VTA (Olds & Milner, 1954). The fact that intracranial stimulation of the VTA strongly supported lever pressing behavior indicated that the subjective effects induced by VTA stimulation were likely appetitive in nature. While not known at the time, these experiments were stimulating the mesolimbic DA system, which consists of VTA DAergic neurons that project to the NAc (Moore & Bloom, 1978). The discovery that nearly all drugs of abuse elicit DA release from the VTA into NAc (Di Chiara & Imperato, 1988) cemented the importance of the mesolimbic DA system in the addiction neuroscience field. Many studies have since shown that VTA DA release in NAc is a critical mediator of both natural (i.e. food) and drug reward (Bergamini et al., 2016; Solinas et al., 2019; Wise, 1989).

It is important to note, however, that while VTA DA release is clearly triggered by food and drugs, exactly what this neural phenomenon represents is still under debate. The studies discussed above imply that VTA DA confers the hedonic or, in other words "rewarding," properties of a stimulus. However, other studies show that DA signals unexpected events (Horvitz, 2002), reward prediction error (Schultz, Dayan, & Montague, 1997), and facilitates goal-directed behaviors (Cannon & Palmiter, 2003). Additionally, in the context of addiction, it is thought that DA release does not confer a drug's hedonic value (inherent "liking") per se, but rather mediates a process of incentive salience (learning to "want" a drug) (Robinson & Berridge, 2000). Ultimately, it appears that these functions need not be mutually exclusive, and that heterogenous subpopulations of VTA DA neurons could explain the various, and even conflicting, functions ascribed to VTA DA (Lammel, Lim, & Malenka, 2014; Morales & Margolis, 2017).

While drugs of abuse similarly induce VTA DA release in NAc, this effect is achieved through mechanisms that differ between and even within drug classes. Psychostimulants exert direct effects on dopamine neurons by reducing DA reuptake, impairing vesicular packaging,

and/or inducing release (Anderson & Pierce, 2005; Hedges et al., 2018). With opioids however, the primary site of action is not the VTA DA neurons themselves, but rather the gamma-aminobutyric acid (GABA) neurons that synapse onto VTA DA neurons (**Fig. 1.2.**). Normally, VTA DA release is tightly controlled by GABAergic neurons within the VTA itself (i.e. interneurons), as well as from other areas including the ventral pallidum (VO), rostromedial tegmental nucleus (RMTg), and NAc (Fields & Margolis, 2015; Hjelmstad et al., 2013; Matsui et al., 2014). When opioids are present, they act through Gi-coupled MORs to suppress GABAergic inputs to VTA and thereby disinhibit VTA DA neurons. Indeed, MOR agonists decrease the firing rate of VTA GABA neurons that exhibit plasmalemmal MOR immunoreactivity (Steffensen et al., 2006). Slice electrophysiology studies show that opioids and MOR agonists reduce GABA inhibitory postsynaptic currents on VTA DA neurons (Matsui et al., 2014). Finally, microdialysis studies show that administering MOR agonists into the VTA reduces GABA release while concurrently increasing somatodendritic DA release in awake behaving animals (Chefer et al., 2009). Opioids therefore exert an indirect, disinhibitory effect on VTA DA neurons by altering GABAergic tone.

In addition to cellular studies, behavioral data indicate that opioids induce rewarding effects, particularly through actions in the VTA. Morphine conditioned place preference (CPP) is ablated in MOR knockout mice (Matthes et al., 1996), indicating a critical function of this receptor in facilitating rewarding effects of opioids. Intra-VTA infusion of morphine produces a CPP that is readily blocked when naloxone is infused into the same region (Olmstead & Franklin, 1997). Likewise, heroin CPP and locomotor activation are blocked in mice that received intra-VTA infusion of an siRNA targeting the MOR (Y. Zhang et al., 2009). Moreover, MOR agonists are readily self-administered into the VTA (Bozarth & Wise, 1981; Zangen et al., 2002). These studies

collectively demonstrate that opioid actions in the VTA are sufficient to produce the rewarding effects of opioids, and that these effects necessitate intact MOR signaling. Importantly, aside from a small effect in PAG, site-specific infusion of morphine into various other brain regions does not alter morphine CPP, underscoring the importance of the VTA (Olmstead & Franklin, 1997).

Apart from differences in mechanism of action, another important distinction between psychostimulant and opioid reward circuitry is that while psychostimulants require the VTA DA system, growing evidence suggests that opioid actions do not. High-doses of the non-selective DA receptor antagonist alpha-flupenthixol abolished operant self-administration of cocaine in rats, but did not have an equally suppressive effect on heroin self-administration (Ettenberg et al., 1982). In addition, destruction of DA terminals in NAc with 6-hydroxydopamine attenuated cocaine but not heroin self-administration (Pettit et al., 1984). Moreover, DA-deficient mice can still exhibit robust morphine reward (Hnasko, Sotak, & Palmiter, 2005). Such findings indicate that opioids likely elicit rewarding effects through both DA-dependent and DA-independent mechanisms. However, the specific mechanism(s) underlying DA-independent reward are still under investigation. Current theories include opioid reward mediated by the pedunculopontine tegmental nucleus or by $GABA_A$ receptor-expressing neurons in the VTA, and even reward resulting from direct opioid inhibition of a subset of VTA dopamine neurons that encode noxious stimuli (Fields & Margolis, 2015; Fujita, Ide, & Ikeda, 2019).

Animal Models

Several behavioral assays can be used to measure drug "reward," which, in this context, refers to the appetitive effects of a substance allowing it to function as a positive reinforcer (Fields & Margolis, 2015; Roberts, Corcoran, & Fibiger, 1977). In animal models, the rewarding effects

of drugs are not explicit, and must instead be inferred by the ability of a drug to promote a particular behavior.

Conditioned place preference (CPP) is a behavioral assay of reward that relies on an animal's ability to associate an experimenter-administered, non-contingent treatment with a particular context through Pavlovian conditioning (Charbogne, Kieffer, & Befort, 2014; Huston et al., 2013). The animal learns to associate contextual cues with certain effects of a treatment, and over time, those cues develop their own salience, such that the animal "seeks" them out (Berridge, 2018). In morphine CPP, a mouse learns to associate the interoceptive effects of either morphine or a control treatment (saline) with a unique context that is paired with that treatment. Morphine CPP involves exposing a mouse to a novel apparatus that contains at least two compartments, each of which are contextually distinct (e.g. different floor texture, wall pattern, scent). The task starts with a pre-test, in which the mouse can freely explore the apparatus and its chambers. Ideally, mice exhibit no strong preference for either chamber at the pre-test, since both contexts are novel. The next several exposures to the chamber (which vary in number by protocol) are conditioning sessions, in which the mouse is injected with saline and confined to one chamber, typically between 30 minutes to one hour. The mouse is removed from the chamber for a period of time, and then injected with morphine and confined to the opposite chamber. After repeated conditioning sessions, the animals learn to associate the effects of saline or morphine with its respective context. On the last day of the paradigm, the mice undergo a post-test in which they have unrestricted access to either chamber in a drug-free state, just as in the pre-test. The time spent on the drug-paired side at pre-test is then subtracted from the post-test value. A positive value indicates that the mouse spent more time in the morphine-paired chamber at post-test, which is indicative of place preference formation. Conversely, a negative value indicates that the effects of the drug

reduced the time spent in its associated context, meaning it induced a place aversion. Overall, CPP is favored for measuring drug reward due to its simplicity and its ability to measure both appetitive and aversion treatments.

Acute drug-induced locomotion is another behavioral assay that can be used to characterize animal responses to drugs of abuse. Locomotor activity is enhanced by a host of drugs including morphine, cocaine, heroin, and methamphetamine (J.-J. Zhang & Kong, 2017), which are known to trigger VTA DA release (Di Chiara & Imperato, 1988). Critically, destruction of dopaminergic terminals in rat NAc abolishes psychostimulant-induced locomotion (Kelly & Iversen, 1976), indicating that drug-induced changes in locomotion are indeed mediated by actions on mesolimbic DA neurons. While this assay is not a model for drug reward per se, drug-enhanced locomotion functions as an observable readout of the mesolimbic DA activation that underlies reward-related processes (Kalivas & Duffy, 1987; Kelly & Iversen, 1976).

This simple assay involves administering a drug to an animal and placing it into a familiar apparatus where locomotion can be measured over time. Typically, this apparatus will incorporate infrared beams arranged in a grid pattern, and beam breaks made by the moving animal are recorded (Manvich et al., 2019). Conducting the assay in a familiar/habituated environment is important for measuring drug-specific effects, because both drugs of abuse and novelty enhance locomotion (Fraser et al., 2010). Locomotor studies can be performed once after a single drug dose to measure the acute effects of a drug on locomotion, or they can be performed repeatedly to study behavioral sensitization, a phenomenon in which repeated exposure to a drug amplifies certain behaviors for extended periods of time (Kuhn, Kalivas, & Bobadilla, 2019). Chronic drug exposure induces persistent neurochemical changes within the mesocorticolimbic system (Robinson et al., 1988; Wolf, 1998; Wolf et al., 1995), and sensitization studies provide an

observable readout of underlying drug-induced plasticity. While drug-induced locomotor activation may not have high face validity, it is nevertheless a useful initial test to assess the impact of genetic, behavioral, or pharmacological manipulations on mesolimbic DA activity.

Reinforcement is another behavioral aspect of the binge/intoxication phase of addiction that can be modeled in animals. As discussed above, in operant conditioning tasks, an animal learns to associate an action with an outcome, and a particular outcome can reinforce performance of said action (Panlilio & Goldberg, 2007). Operant self-administration, the gold-standard animal model of drug addiction, utilizes reinforcement in order to study how animals facilitate their own drug consumption (Thomsen & Caine, 2005).

In operant drug self-administration procedures, animals are placed in operant conditioning chambers for a set period of time, during which they control their own drug intake. Drug access is contingent upon the animal performing an action (e.g. nose-poking, lever-pressing, or screen touching). Drugs can be delivered via inhalation (Moussawi et al., 2020), oral consumption of a drug solution (Phillips et al., 2020), or intravenously by an infusion through an indwelling catheter (Thomsen & Caine, 2005). The self-administered aspect of the task is superior to other assays of assays of SUD-like behavior because the animal voluntarily initiates, and therefore controls, its own drug intake, as opposed to other models in which drugs are delivered to the animal by the experimenter (Panlilio & Goldberg, 2007).

Operant conditioning chambers typically contain at least two nose poke apertures or levers, one of which is “active” and delivers the drug when acted upon, and the other which is “inactive” and does not deliver drug. The animal must learn to differentiate the active and inactive sides, and preferentially choose the active side, demonstrating “selectivity.” Percent selectivity is therefore a useful indicator of whether the animal has learned the task and is allocating more effort to the

active side as expected. When an animal is not being reinforced as expected (as seen in extinction sessions), decreases in selectivity can arise as the animal attempts alternative strategies to obtain reinforcement, such as working on the inactive side. Impaired selectivity can also be indicative of learning or memory impairments, which are important to monitor, as these issues can confound operant self-administration studies.

There are numerous ways in which operant self-administration behaviors can be evaluated. The time it takes animals to "acquire," or learn the operant task, can be viewed as a proxy for the initiation of drug use. The number of drug deliveries per session during the stable "maintenance" phase can indicate the reinforcing value of a drug to an animal. Moreover, dose response curves can identify the maximally reinforcing dose of a drug, and shifts in the dose response curve can show whether a particular manipulation (genetic, pharmacological, optogenetic, etc.) alters the reinforcing value of a drug (Thomsen & Caine, 2005). Furthermore, the complexity of operant self-administration procedures can vary greatly depending on the schedule of reinforcement and the duration of access to the drug (Kuhn, Kalivas, & Bobadilla, 2019). The simplest studies implement a fixed ratio schedule of reinforcement, meaning that a set number of responses will reliably result in drug delivery. However, introducing unpredictability to these paradigms, either through variable schedules of reinforcement or through intermittent access, is frequently used for its ability to induce escalation of intake, which can be viewed as a measure of craving (Kuhn, Kalivas, & Bobadilla, 2019).

Motivation is another aspect of drug reward that can be modeled using operant self-administration assays. In a progressive ratio paradigm, there is a linear or exponential increase in the schedule of reinforcement following each drug delivery. This paradigm requires the animal to work increasingly harder to earn each subsequent reward. The maximum number of responses the

animal makes to earn a reward is referred to as the "break point," which can be compared between animals to assess willingness to work for a reward (Kuhn, Kalivas, & Bobadilla, 2019). Animals with higher break points are considered to be more motivated for the drug, which is indicative of the strength of the drug's reinforcing properties.

1.3.2. Withdrawal

Circuitry

As discussed previously, withdrawal refers to the distressing symptoms that arise in physically dependent individuals when circulating levels of the abused drug dissipate. While withdrawal can occur with other drugs of abuse, this phenomenon is most pronounced with opioids. Opioid withdrawal can occur spontaneously when an opioid-dependent animal or individual undergoes a period of abstinence, or it can be precipitated pharmacologically by administration of a MOR antagonist, such as naloxone.

Somatic withdrawal symptoms are "physical" and largely attributed to neuronal hyperactivity that emerges when neurons, which have adapted to the suppressive effects of opioids, become disinhibited once opioids are no longer present. This phenomenon is especially, although not exclusively, prominent within the noradrenergic system and its major nucleus, the locus coeruleus (LC) (Maldonado et al., 1992). As such, the LC has long been a region of intense focus when studying the cellular and functional changes that mediate withdrawal (Maldonado, 1997). While acute opioid exposure suppresses LC activity, chronic exposure induces upregulation of the cyclic AMP signaling pathway and allows the LC to maintain a normal firing rate (Aghajanian, 1978; Cao et al., 2010). This compensatory response normalizes LC function in the presence of opioids, but when opioid intake ceases, the unopposed LC becomes hyperactive and withdrawal symptoms arise (Aghajanian, 1978; Mazei-Robison & Nestler, 2012). Indeed, injection of the

MOR antagonist naloxone into the LC, but not surrounding structures, of opioid-dependent rats is sufficient to induce a withdrawal-related increase in firing rate (Aghajanian, 1978). Conversely, intra-LC infusion of the alpha-2-adrenergic receptor agonist clonidine is sufficient to reduce somatic signs of withdrawal and levels of NE metabolites (J. R. Taylor et al., 1988), and electrolytic lesions of the LC likewise attenuate somatic withdrawal signs (Maldonado & Koob, 1993). While these findings indicate a role for the LC in withdrawal, the inability of LC-specific manipulations to completely abolish somatic withdrawal indicates that the LC is not the sole brain region mediating this phenomenon.

Notably, the role of other noradrenergic nuclei in withdrawal is supported by evidence that A1 and A2 are also activated by withdrawal and influence withdrawal-induced aversion, while the LC is not (Delfs et al., 2000). A1 and A2 also project to regions involved in stress and drug responses, like the bed nucleus of the stria terminalis (BNST) (Delfs et al., 2000). Microinjections of beta-adrenergic receptor antagonists into the BNST markedly reduce withdrawal-conditioned place aversion and somatic signs of withdrawal (Delfs et al., 2000), indicating that noradrenergic signaling in this region modulates both the affective and somatic aspects of withdrawal. These findings expand upon LC-specific withdrawal studies and show that noradrenergic neurotransmission in the brain is broadly impacted by opioids.

It is important to acknowledge that early work may have overestimated the importance of the LC in opioid withdrawal. In fact, the causal role of the LC in withdrawal has been strongly contested (Christie et al., 1997), mainly because qualities observed in the LC – such as withdrawal-induced hyperactivity and the association between regional hyperactivity and symptom severity – are not exclusive to the LC. Several other brain regions, particularly the PAG, are hyperactive during withdrawal and can modulate somatic symptom severity, indicating that this attribute is not

unique to the LC (Bozarth, 1994). Beyond that, several studies directly contradict the notion that the LC is necessary for withdrawal. One group reported that destruction of noradrenergic LC terminals in morphine-dependent rats using the neurotoxin N-(2-chloroethyl)-N-ethyl-bromo-benzylamine (DSP4) failed to reduce naloxone-precipitated withdrawal symptoms (Chieng & Christie, 1995). Additionally, a second study showed that chemically lesioning the LC with 6-hydroxydopamine did not alter naloxone-precipitated nor spontaneous opioid withdrawal symptoms (Caillé et al., 1999). Altogether, these studies are difficult to reconcile with previous ones demonstrating a clear role for the LC and imply a more complex mechanism underlying opioid withdrawal, which likely encompasses a network of brain regions including the LC. One potential explanation for the disparate results is that ablation of the LC reduces not only release of NE but all LC co-transmitters, while acute pharmacological approaches more subtly and specifically modulate NE transmission. There is therefore a need to assess the role of LC co-transmitters in the development and expression of opioid withdrawal.

Withdrawal does not only manifest through somatic symptoms, but also through affective impairments. Importantly, somatic and affective withdrawal symptoms show appear to be temporally distinct, with somatic symptoms occurring more acutely and affective symptoms occurring over days to weeks following opioid abstinence. These protracted withdrawal effects are mediated in part by NE, but also by neuropeptides such as corticotropin releasing factor and the endogenous opioid dynorphin (Koob & Volkow, 2016). Additionally, brain regions that encode negatively valenced stimuli, such as the lateral habenula, become more active during protracted withdrawal. Increased activity in this region is significant, as the lateral habenula strongly drives GABAergic neurons in the rostromedial tegmentum to suppress VTA DA neuron activity (Brown et al., 2017). Withdrawal-associated increases in stress neuropeptides and the activity of circuits

encoding negative valence ultimately coalesce to induce an emotional state characterized by anhedonia, irritability, and malaise (Koob & Volkow, 2016). Affective symptoms are therefore an equally important part of the withdrawal phenomenon that invites further study.

Animal Models

The physical and behavioral manifestation of somatic withdrawal symptoms in rodents are fairly similar to those experienced by humans, and can be readily observed by the experimenter (Kest et al., 2002). In order to induce withdrawal, animals first need to become physically dependent on opioids. Dependence can be induced passively via repeated experimenter-administered injections of an opioid, or by implantation of a subcutaneous drug-imbued pellet or minipump to achieve chronic opioid delivery. Alternatively, the animal can facilitate their own dependence through opioid self-administration, either through non-contingent access in the home cage or via contingent access in an operant conditioning chamber. After dependence is established, withdrawal studies can be performed using spontaneous or precipitated withdrawal protocols. With spontaneous withdrawal, animals undergo forced opioid abstinence, which induces somatic withdrawal symptoms that arise within hours and can persist for several days (Bobzean et al., 2019). In precipitated withdrawal studies, animals are administered a MOR antagonist such as naloxone or naltrexone, which rapidly induces a briefer but more intense episode of withdrawal accompanied by somatic symptoms (Welsch et al., 2020). Somatic withdrawal signs in rodents have strong face validity because the symptoms – which include diarrhea and neuromuscular effects resulting in jumps, tremors, and shakes – closely resemble the symptoms experienced by humans during opioid detoxification (Kosten & Baxter, 2019).

However, more recent work shows that another component of withdrawal, affective symptoms, can model the negative emotional state that emerges days to weeks after withdrawal in

humans (Welsch et al., 2020). This "protracted" withdrawal state is associated with increased anxiety-like behaviors, impairments in social interaction, and decreased reinforcement by natural rewards like food (Welsch et al., 2020). Negative emotionality is also reflected in increased intracranial self-stimulation reward thresholds among animals undergoing withdrawal (Koob et al., 2014). Protracted withdrawal studies can capture this affective component of withdrawal by examining whether animals demonstrate impairments in social interaction or increased despair- and anxiety-like behavior as measured by tail suspension and open field tests (Goeldner et al., 2011; Lutz et al., 2013). Withdrawal assays are therefore the most direct way to model this component of OUD; depending on how withdrawal is induced and the time course over which behaviors are monitored, it can be useful for examining both somatic and affective symptoms.

1.3.3. Craving and Relapse

Circuitry

Craving and relapse have traditionally been studied in the context of cocaine use disorder; however, the brain regions implicated in these studies appear to largely overlap with those identified in opioid-specific studies (Rogers, Ghee, & See, 2008). Converging evidence in humans and animals indicates that craving and relapse involve the prefrontal cortex (PFC), a brain region that critically impacts decision making and inhibitory control (Koob & Volkow, 2010). fMRI studies have found that people with SUD exhibit stronger PFC responses to drug-related cues than healthy controls, which suggests that decision making may be influenced by the heightened salience of drugs (Goldstein & Volkow, 2011). Other imaging studies found that cue-induced craving among people who use drugs is associated with increased DA release in the PFC, as well as in the striatum and amygdala (Koob & Volkow, 2016). Indeed, these PFC-related findings are supported by circuit-specific studies in rodents, which implicate the medial PFC in supporting

drug- and cue-induced opioid- and psychostimulant-seeking behavior (Reiner et al., 2019; Steketee & Kalivas, 2011).

Beyond the PFC, animal studies show that relapse-like behavior encompasses an extensive limbic-cortical-striatal circuit that is engaged by both heroin and cocaine (Rogers, Ghee, & See, 2008). Regional inactivation experiments demonstrate that impairment of the dorsomedial PFC, NAc core, VP, amygdala, dorsolateral striatum, or VTA can similarly suppress cue- and/or drug-induced seeking of either cocaine or heroin (Kalivas & McFarland, 2003). But in contrast to cocaine-seeking circuitry, heroin-seeking circuitry appears to be modulated by an even broader network, which includes the ventromedial PFC, NAc shell, central amygdala, and BNST (De Vries & Shippenberg, 2002; Rogers, Ghee, & See, 2008). These findings reveal that opioids and psychostimulants promote relapse-like behavior through overlapping but not identical circuits.

Noradrenergic dysregulation also plays a role in relapse, specifically with respect to stress. Of the patients with OUD who can access MAT, many are unable to adhere to treatment, and relapse rates are high (Broers et al., 2000; Gossop et al., 1989). Stressful life events have been shown to increase relapse rates across SUD involving cannabis, alcohol, and prescription opioids (McCabe et al., 2018). The role of the noradrenergic system in stress-induced relapse is evident from rodent studies showing that pharmacological blockade of NE release or signaling attenuates stress-induced cocaine- and opioid-seeking behaviors (Schmidt et al., 2017; Schroeder et al., 2013; Shaham et al., 2000). Similar noradrenergic-suppressing pharmacological studies in humans have likewise reported decreases in stress-induced craving (Jobes et al., 2011; Sinha et al., 2007). In addition, studies in rats indicate that chronic morphine administration sensitizes LC neurons to become hyperactive in response to even minor stressors (G. P. Xu et al., 2004). Collectively, these findings demonstrate that noradrenergic activity can promote relapse-like behavior, and that

opioid-induced sensitization of the noradrenergic system to stressors may contribute to the high relapse rates observed with OUD.

Animal Models

Relapse-like behavior can be modeled in animals using reinstatement assays. These assays can be performed in a range of behavioral contexts such as CPP or self-administration. Typically, animals that have been repeatedly exposed to a drug first undergo an extinction period to extinguish the drug-conditioned contextual association or behavioral response, followed by a reinstatement test. The abstinence phase can be a passive process if it is forced by the experimenter, and an active process if it is voluntary or mediated by extinction (Reiner et al., 2019). With forced abstinence, animals are confined to the home cage, and do not have access to the drug until reinstatement. Alternatively, voluntary abstinence protocols introduce measures that either punish responding for drug or provide an alternative non-drug reward such as food, allowing the animal to naturally cease drug-seeking. Extinction involves exposing the animal to the operant chamber but changing the paradigm so that active responses have no consequence. The animal will initially make many active responses, but over time will taper off as the original contingency is degraded. Once temporal and/or behavioral criteria for abstinence/extinction are met, animals can undergo reinstatement.

Reinstatement paradigms resemble the human experience of relapse in that drug-seeking behavior can be elicited in an abstinent rodent by exposing them to stimuli known to induce relapse in humans (Reiner et al., 2019). Reinstatement is typically primed by non-contingent presentation of one of three stimuli: the drug itself, cues associated with prior drug intake (such as tones or lights), or stress, which can be induced by physical stress (e.g. foot shock, forced swim), social defeat stress, or pharmacological agents like yohimbine (Shaham et al., 2003). These triggers will

then invigorate the previously extinguished response, such as spending more time in a drug-paired context in the case of CPP, or responding on a formerly active manipulandum in the case of self-administration. In reinstatement procedures, active responses have no consequences and do not result in drug administration, so the magnitude of the response is considered to only reflect the level of drug-seeking behavior. Importantly, the different means of inducing reinstatement can be influenced by distinct receptors and circuits (Reiner et al., 2019). For example, in the case of adrenergic receptor signaling and reinstatement, alpha-1 receptors mediate drug-primed reinstatement, while alpha-2 and beta receptors mediate stress-induced reinstatement, and both alpha-1 and beta receptors can influence cue-induced reinstatement (Schmidt et al., 2017; Schmidt & Weinshenker, 2014). Therefore, characterization of relapse-like behavior can be better defined by testing more than one reinstatement modality.

1.3.4. Neuropeptide Modulation of Addiction-Related Circuits

Upon reviewing how drugs of abuse mediate addictive behaviors, a natural conclusion is that the most effective treatments for substance use disorders would be those that block or modulate mesolimbic DA transmission or signaling. However, years of research on this topic have consistently failed to identify successful DA-based treatments for a variety of reasons (Mariani & Levin, 2012; Schmidt & Weinshenker, 2014). Identifying treatments specifically for OUD is even more challenging because opioids can exert rewarding effects through DA-dependent or DA-independent mechanisms (Fields & Margolis, 2015). For these reasons, much research now focuses on ways to indirectly modulate the mesolimbic DA system.

In recent years, efforts to better understand how drugs of abuse alter brain function has led to a renewed focus on neuropeptides as key neuromodulators of addiction-related brain circuitry. Indeed, a review of the literature illustrates that motivational and stress circuits, which are

dysregulated in addiction, are critically influenced by the actions of neuropeptides (Castro & Bruchas, 2019; Collins, Wolff, & Saunders, 2019; M. H. James et al., 2017; King, Gano, & Becker, 2020; Schank et al., 2012). In addition, disruption of specific neuropeptides, particularly those in the hypothalamus, can perturb consummatory drive and result in maladaptive binge-eating behavior that resembles compulsive drug-taking (Ferré, 2017; Novelle & Diéguez, 2018). It is therefore important to study not just the classical neurotransmitters released within these addiction-related circuits, but also the neuropeptides that can be co-released along with them. In addition, while many studies have demonstrated that peptidergic manipulations impact behavior, there is a lack of mechanistic data underlying these observations, largely due to the technical difficulty of measuring real-time neuropeptide release (Al-Hasani et al., 2018; Kash et al., 2015). However, recent work indicates that the effects of certain neuropeptides on addiction-like behaviors can be mechanistically linked to heteromerization of neuropeptide receptors (Ferré, 2017), which will be discussed in more detail later in this chapter. Further study of neuropeptides, especially their receptor-level effects on neural activity, will not only help elucidate their complex role in shaping neurotransmission, but could also facilitate development of therapeutic approaches to diseases such as OUD.

1.5. GALANIN

1.5.1. Discovery and Function

The neuropeptide galanin was discovered in the 1983 from porcine intestinal extracts (Tatemoto et al., 1983). At the time, it was found to facilitate smooth muscle contraction and cause hyperglycemia. Since then, galanin has been shown to modulate numerous physiologic states including pain, nerve injury, inflammation, osmotic regulation, and neuroendocrine function (Lang et al., 2015). Galanin also mediates numerous innate behaviors, ranging from feeding (Abramov

et al., 2004) to parenting (Wu et al., 2014). Additionally, galanin modulates behavior in animal models of anxiety, depression, and drug addiction (Genders, Scheller, & Djouma, 2020; Hokfelt et al., 2018; Picciotto, 2008; R. P. Tillage, N. R. Sciolino, et al., 2020), suggesting that the galaninergic system may be an effective target for neuropsychiatric therapies. Due to the myriad functions of galanin, this neuropeptide has been investigated in the context of many human diseases including Alzheimer's disease, epilepsy, stroke, and mental illnesses such as depression, anxiety disorders, and substance use disorders (Lang et al., 2015; Weinshenker & Holmes, 2016).

1.5.2. Galanin Signaling

Similar to opioid peptides, galanin is derived from a large precursor protein, called preprogalanin, which undergoes subsequent cleavage to yield bioactive peptides including galanin (Mains et al., 1987). Galanin is then stored in large dense core vesicles, which differs from classical neurotransmitters that can be stored in either large dense core or clear synaptic vesicles (Lang et al., 2015). Due to the specific storage conditions of neuropeptides, they are thought to be preferentially released under conditions of high frequency burst firing, as this state can sufficiently mobilize large dense core vesicles to release their contents (Lang et al., 2015).

Galanin signals through three receptor subtypes: GalR1, GalR2, and GalR3 (**Fig. 1.3.**). GalR1 and GalR3 are G_i-coupled, and activation of these receptors inhibits adenylate cyclase, reducing cyclic AMP levels and decreasing neuronal activity (Hokfelt et al., 2018). GalR2, however, is unique in that it can flexibly signal via G_i or G_q pathways. As such, GalR2 activation can also reduce neuronal function, or it can activate phospholipase C to increase neural activity (Weinshenker & Holmes, 2016). Given that all three receptor subtypes can signal via G_i mechanisms, neuromodulatory galanin is typically viewed as a negative regulator of neural activity

(Lang et al., 2015), while GalR2 G_q signaling is thought to mediate the long-term trophic effects of the neuropeptide.

1.5.3. Central Expression of Galanin and its Receptors

Galanin is widely expressed throughout the brain of several species, and is co-expressed with a variety of classic small molecule neurotransmitters including DA, GABA, acetylcholine, NE, and serotonin (Lang et al., 2015). Rats and mice exhibit fairly similar central galanin expression, with galanin and its precursor peptide being abundant in the forebrain, hypothalamus (especially the periventricular, preoptic, and dorsomedial nuclei) the medial and lateral amygdala, the BNST, and noradrenergic regions including the nucleus of the solitary tract and the LC (Cheung et al., 2001; Ryan & Gundlach, 1996). However, only mice express galanin in the inferior olive, and rats, but not mice, express galanin in the cerebellum and serotonergic dorsal raphe nucleus (Cheung et al., 2001; Larm, Shen, & Gundlach, 2003; Ryan & Gundlach, 1996; Skofitsch & Jacobowitz, 1986). Humans do not express galanin in the dorsal raphe nucleus either, making this region more similar to mice in that respect (Le Maitre et al., 2013). Importantly, one brain region that is consistent across species is the LC, which exhibits robust galanin expression in mice, rats, and humans (Cheung et al., 2001; Le Maitre et al., 2013; Perez et al., 2001; Skofitsch & Jacobowitz, 1986).

Galanin receptor expression has been thoroughly examined in rats, but characterization in mice remains incomplete. Autoradiography studies, which used radiolabeled galanin to identify regions of galanin binding (i.e. putative receptors), revealed similar, widespread binding patterns throughout the rat and mouse brain (Jungnickel & Gundlach, 2005; Melander et al., 1986; Skofitsch, Sills, & Jacobowitz, 1986). But while these studies provided broad insight regarding possible receptor expression, autoradiography cannot indicate which receptors are present at

galanin binding sites unless subtype-specific compounds are used. Furthermore, immunohistochemical (IHC) approaches to examine receptor protein are not possible because there are no reliable antibodies for galanin receptors (Hawes & Picciotto, 2004; Lu & Bartfai, 2009). Therefore, RNA-based techniques, such as *in situ* hybridization (ISH) or quantitative, real-time polymerase chain reaction (RT-PCR) have been used to assess galanin receptor expression.

Currently, GalR1 is the most well-characterized receptor in rats and mice. It is highly expressed across the rostral-caudal axis of both rat and mouse brain, often in areas that co-express galanin (Lang et al., 2015). In fact, the visible similarities between ISH for GalR1 and previous autoradiography studies suggested that the majority of galanin binding might be occurring though GalR1 (Gustafson et al., 1996). Indeed, this is further supported by an autoradiography study in GalR1 knockout mice, which noted a complete lack of galanin binding in the absence of the receptor (Jungnickel & Gundlach, 2005), although sensitivity issues are a potential caveat to this result.

ISH studies in rat showed GalR1 expression to be highest in the lateral olfactory tract, ventral hippocampus, and lateral parabrachial nucleus (Gustafson et al., 1996). Moderate signal was also reported in the piriform cortex, lateral septum, NAc shell, VP, amygdala, and certain thalamic and hypothalamic nuclei, with weak signal in the LC (Gustafson et al., 1996). Mouse studies that characterized GalR1 expression using either a fluorescent GalR1 reporter line or ISH reported similar expression patterns, with particularly high levels of GalR1 in the thalamus, hypothalamus, and amygdala (Hohmann et al., 2003; Kerr et al., 2015). These complementary results indicate that GalR1 expression patterns are similar between mice and rats, and that levels of GalR1 mRNA expression may be a reliable indicator of GalR1 protein expression. The reporter study also identified GalR1 protein signal in the mouse dorsal pons, in a region anatomically

consistent with the LC (Kerr et al., 2015). LC GalR1 protein expression has not been similarly examined in the rat, but ISH studies report an interesting pattern of GalR1 mRNA expression in the LC, as well as in adjacent areas such as Barrington's nucleus (Z. Q. Xu, Shi, & Hokfelt, 1998). It is still unclear whether this pattern of expression is unique to the rat, or if it is also present in mice.

In the rat brain, GalR2 is also expressed in many regions, but with a slightly more restricted distribution compared to GalR1. GalR2 mRNA is highest in the hippocampus (particularly the dentate gyrus), cerebellar cortex, and hypothalamus, with moderate expression in the olfactory bulb and tubercle and substantia nigra pars compacta (D. O'Donnell et al., 1999; Z. Q. Xu, Shi, & Hokfelt, 1998). Weak expression is seen the PAG and several pontine and medullary nuclei, including the LC, lateral parabrachial nucleus, and the motor nucleus of the trigeminal nerve (Burazin et al., 2000; D. O'Donnell et al., 1999). In contrast to the rat, GalR2 expression in mouse appears very low. GalR2 could not be detected by ISH or IHC in wild-type C57BL/6N mice (Le Maître et al., 2011). Only when GalR2 was genetically overexpressed could it be observed in the subiculum, cingulate cortex, and subregions of the prefrontal cortex (Le Maître et al., 2011), suggesting that baseline expression was below the limit of detection by ISH and IHC. Interestingly, this study did not detect GalR2 in the LC of either wild-type or GalR2 overexpressor mice, even though GalR2 was previously identified in the LC by brain-wide gene mapping (Lein et al., 2007). Therefore, further investigation is needed to characterize GalR2 expression in the mouse brain.

GalR3 is the most poorly characterized of the three receptor subtypes, and appears to be more abundant in the periphery than in the brain (Lang et al., 2015). In the rat, GalR3 mRNA is expressed in the hypothalamus (paraventricular, ventromedial, and dorsomedial nuclei), diagonal band of Broca, periaqueductal grey, and LC (Mennicken et al., 2002; Waters & Krause, 2000).

Human studies also report GalR3 expression in the LC, but at much higher levels than observed in rat (Lang et al., 2015; Le Maitre et al., 2013). In mouse, GalR3 has been identified by qPCR in the olfactory bulb, medial septum/diagonal band of Broca, amygdala, hippocampus, and piriform and frontal cortices (He et al., 2005).

1.6. GALANINERGIC MODULATION OF OPIOID EFFECTS

1.6.1. Therapeutic Effects of Galanin in Rodents

The galaninergic system has been proposed as therapeutic target for OUD because of its ability oppose the behavioral effects of opioids. Central administration of galanin attenuates morphine reward as measured by CPP (Zachariou, Parikh, & Picciotto, 1999), and enhancing galanin signaling by genetic overexpression or administration of the galanin receptor agonist, galnon, is reported to attenuate withdrawal symptoms (F. E. Holmes et al., 2012; Zachariou et al., 2003). These findings are particularly intriguing because they indicate that galanin-based therapies could potentially attenuate behaviors associated with multiple components of the addiction cycle. The development of such a multimodal therapy could be used to treat a spectrum of patients, from those experiencing problematic misuse to those seeking treatment for OUD.

Prior studies also indicate that impairments in galanin signaling increase susceptibility to opioid effects. Galanin knockout mice show increased morphine-induced locomotor activity, morphine CPP, and precipitated withdrawal symptoms, all of which can be corrected by restoring galanin signaling with administration of galnon (Hawes et al., 2008; Zachariou et al., 2003; Zachariou, Parikh, & Picciotto, 1999). Receptor-specific manipulations, like genetic deletion of GalR1, also exacerbate withdrawal symptoms (F. E. Holmes et al., 2012). Considering that human studies have identified polymorphisms in the galanin gene which are associated with increased susceptibility for opioid addiction (Beer et al., 2013; Levran et al., 2008), functional impairments

in the galaninergic system may influence opioid-related phenomena in humans. In addition, galanin promotes antinociception in several animal models of acute and inflammatory pain (M. L. Zhang, Fu, & Yu, 2017; X. Y. Zhang et al., 2015; Y. Zhang et al., 2019). This finding further increases the value of galaninergic therapies for OUD, because it suggests that galaninergic signaling might block the addictive properties of opioids while simultaneously preserving, or even enhancing, analgesic properties. Collectively, the literature indicates that broad (brain- or body-wide) alterations in galanin signaling influence susceptibility to the behavioral effects of opioids, and also suggests that galanin-enhancing treatments could help combat OUD-associated behaviors.

1.6.2. Discovery of GalR1-MOR Heteromers

While the ability of galanin to block opioid-mediated behaviors is well-documented, the mechanism underlying this phenomenon has remained unclear. But the recent discovery of GalR1-MOR heteromers in the VTA (Moreno et al., 2017) revealed a direct interaction between the galanin and opioid systems that explains the ability of this neuropeptide to modulate opioid effects. This study found that GalR1, but not GalR2, forms heteromers with MOR in a mammalian transfected cell line (Moreno et al., 2017). It also revealed that the GalR1-MOR heteromer exhibits biochemical properties that differ from isolated GalR1 or MOR functions. Endomorphin-1 is a MOR-selective endogenous opioid that typically elicits ERK1/2 phosphorylation. Interestingly, combined addition of endomorphin-1 and galanin to the heteromer-expressing cell line produced pERK1/2 levels lower than endomorphin-1 alone, indicating that the activated GalR1 protomer inhibited MOR function (Moreno et al., 2017) (**Fig. 1.4**.). Complementary *in vivo* microdialysis experiments then demonstrated that while intra-VTA endomorphin-1 elicited dendritic DA release, intra-VTA galanin completely blocked this effect. Furthermore, infusion of an interfering peptide

that disrupted the GalR1-MOR heteromer counteracted the suppressive effect of galanin on endomorphin-1-mediated DA release (Moreno et al., 2017)., indicating that the heteromer identified through *in vitro* approaches is functional *in vivo*. These data provided the first evidence that GalR1-MOR heteromers are present in the rat VTA, and that galanin signaling through this heteromer can block opioid-mediated signaling through MORs. This mechanistic insight is critically important for developing galanin-based therapies for OUD, and therefore further characterization of the GalR1-MOR heteromer is needed.

1.7. CRITICAL QUESTIONS IN THE FIELD

1.7.1. Effects of Noradrenergic Galanin on Opioid-Mediated Behaviors

Collectively, previous findings indicate that the galaninergic system could be leveraged to reduce OUD-associated behaviors. However, the overwhelming majority of these studies examined galanin effects using body- or brain-wide manipulations (Hawes et al., 2008; F. E. Holmes et al., 2012; Zachariou et al., 2003; Zachariou, Parikh, & Picciotto, 1999). Given that galanin and its receptors are expressed widely in the brain and within multiple neurotransmitter systems (Lang et al., 2015), system-specific effects of galanin will need to be characterized in order to inform targeted therapeutic approaches. Currently, there is a dearth of information indicating whether or how different sources of galanin contribute to the protective effects of this neuropeptide against opioids. Therefore, the relative importance of specific sources of galanin must be evaluated.

When considering which sources of galanin may contribute most to suppressing opioid-related behaviors, noradrenergic galanin is a prime candidate. Noradrenergic neurons provide the major source of galanin to the pons, hippocampus, and prefrontal cortex – brain regions implicated in withdrawal, craving, and relapse, respectively (Hokfelt et al., 1998; Koob & Volkow, 2016; R.

P. Tillage, N. R. Sciolino, et al., 2020). Interestingly, the majority of noradrenergic galanin appears to originate from the LC. Examination of galanin expression in several brainstem noradrenergic nuclei, including the LC, A1, A2 and A5, only found galanin co-expressed within noradrenergic neurons of the LC (R. P. Tillage, N. R. Sciolino, et al., 2020). It is therefore important to note that investigation of endogenous noradrenergic galanin functionally constitutes examination of LC-derived galanin. Indeed, characterization studies of galanin mRNA expression and immunoreactivity show that the LC co-expresses galanin in the overwhelming majority of its neurons (Cheung et al., 2001; Perez et al., 2001). Moreover, while galanin exhibits some regional heterogeneity across the brains of different species, it is highly expressed in the LC of mice, rats, and humans (Le Maitre et al., 2013; Perez et al., 2001; Skofitsch & Jacobowitz, 1986). The conservation of noradrenergic (i.e. LC-derived) galanin suggests an evolutionary importance of this source of the neuropeptide, and further underscores its translational relevance to human therapeutic approaches.

As of yet, only one study has examined the role of noradrenergic galanin on OUD-related behaviors. Genetic overexpression of galanin under the control of a noradrenergic-specific promoter was reported to attenuate precipitated withdrawal symptoms compared to wild-type controls (Zachariou et al., 2003). However, the critical question, namely whether selective *depletion* of noradrenergic galanin exacerbates withdrawal, has not been explored. Furthermore, noradrenergic galanin has not been evaluated in other opioid-related behaviors that central galanin has been shown to modulate, such as reward and reinforcement (Zachariou, Parikh, & Picciotto, 1999). Given that the noradrenergic system is a key component of OUD-related circuitry and is a prominent source of central galanin, it is possible that manipulation of noradrenergic galanin alone would be sufficient to modulate opioid reward, reinforcement, and withdrawal behaviors.

Determining the impact of noradrenergic galanin on these three behaviors will help characterize the role of this particular source of galanin, and will also inform the design of possible galanin-based therapies for OUD.

1.7.2. Cell-Type Specific Expression of Galanin and its Receptors

While galanin and its receptors have been characterized at the mRNA level, previous studies do not provide insight regarding cell-type specificity. These ISH studies used single probes for either galanin or a single galanin receptor subtype, which revealed anatomically distinct patterns in mRNA expression, but not indicate which cell-types were expressing the mRNA (Cheung et al., 2001; D. O'Donnell et al., 1999; Zachariou et al., 2000). The lack of cell-type specific information regarding the galaninergic system means that current assumptions in the field – for example, that the LC expresses GalR1 – remain speculative. However, this knowledge gap can be addressed by using new and improved RNA detection methods. Current ISH approaches allow for multiplexing of probes, such that target mRNAs and cell-type markers can be labeled in tandem. Additionally, these methods yield enhanced resolution of single mRNA transcripts, enabling direct quantification of mRNA transcripts (Buxbaum et al., 2015). Evaluating the cell-type specific expression of galanin and its receptors, particularly GalR1, will provide anatomical insights about the galaninergic system and help refine current theories on galanin mediated-actions on opioid reward and withdrawal.

1.7.3. Characterization of Central GalR1-MOR Co-Expression

New insights regarding the existence of GalR1-MOR heteromers in the VTA (Moreno et al., 2017) provide a mechanistic explanation for galanin-mediated attenuation of opioid reward. But while these data demonstrate a functional impact of GalR1-MOR heteromers within the VTA, they do not reveal which cell type the heteromer is modulating. It will be critical to determine

where the GalR1-MOR heteromer is acting in order to fully elucidate this protective mechanism. Given that 1) VTA DA neurons do not express MOR mRNA (Galaj et al., 2020), 2) MORs inhibit GABAergic neurons to induce VTA DA release (Fields & Margolis, 2015), and 3) the biochemical effects of the heteromer are consistent with modulating GABAergic outflow (Moreno et al., 2017), it is most likely that heteromer is present on GABAergic neurons.

Anatomically, the VTA receives GABAergic inputs from several brain regions, and also contains its own population of GABAergic interneurons (Fields & Margolis, 2015; Hjelmstad et al., 2013; Matsui et al., 2014). The GalR1-MOR heteromer could feasibly act on any one, or all, of these sources to modulate VTA DA release. As such, it will be important to characterize which GABAergic inputs could express the GalR1-MOR heteromer. However, identifying where the GalR1-MOR heteromers exist is technically challenging. GPCR heteromers cannot be readily visualized *in vivo* (González-Maeso, 2014), and the lack of GalR1 antibodies (Hawes & Picciotto, 2004; Lu & Bartfai, 2009) precludes an IHC-based approach to visualize GalR1-MOR protein co-localization. An alternative approach then, is to use ISH to examine GalR1 and MOR mRNA co-expression. While this approach does not localize the heteromer itself, it can be used indicate which GABAergic regions that project to the VTA would be capable of forming the GalR1-MOR heteromer. Comparing the relative proportions of GalR1 and MOR co-expression across GABAergic regions of interest could also indicate which areas are more likely to exhibit heteromeric activity.

1.8. DISSERTATION AIMS

The overall goal of this dissertation is to address the critical questions described in the previous section by determining how cell-type specific actions of galanin and its receptors affect opioid-related behaviors and circuitry. We ultimately aimed to expand our understanding of

galaninergic interactions with opioids by studying the most pressing questions related to the peptide itself, as well as its receptor, GalR1. We therefore sought to 1) test whether noradrenergic-derived galanin modulates opioid withdrawal, reward, or reinforcement and 2) characterize GalR1 and MOR mRNA co-expression in GABAergic projections to VTA to indicate which areas might exhibit GalR1-MOR heteromeric activity.

In chapter two of this dissertation, we evaluated whether noradrenergic galanin alters somatic withdrawal symptoms via a mechanism involving LC-derived galanin and GalR1 in LC neurons. We used RNAscope to characterize GalR1 expression in the dorsal pons, and performed naloxone-precipitated withdrawal in mouse lines containing altered noradrenergic galanin levels to determine whether these manipulations impacted somatic symptom severity. We also performed systemic pharmacological experiments to compare the ability of the galanin receptor agonist, galnon, to attenuate withdrawal versus the alpha-2 adrenergic receptor agonist clonidine. In chapter three, we again utilized mouse lines with altered noradrenergic galanin levels to evaluate the impact of this source of galanin on opioid reward and reinforcement as measured by morphine-induced locomotion, morphine CPP, and intravenous remifentanil self-administration. In chapter four, we used RNAscope and advanced image analysis approaches to quantify GalR1-MOR mRNA co-localization in GABAergic regions that project to VTA to identify potential areas of GalR1-MOR heteromeric activity.

Collectively, these results provide important behavioral, molecular, and neuroanatomical insights on the complexity of the galaninergic system and its interactions with the opioid system. These results have important implications for the development of OUD-related therapies, and suggest that continued system-specific approaches to studying galanin will help reveal how this system can be leveraged for therapeutic benefit.

1.9. FIGURES

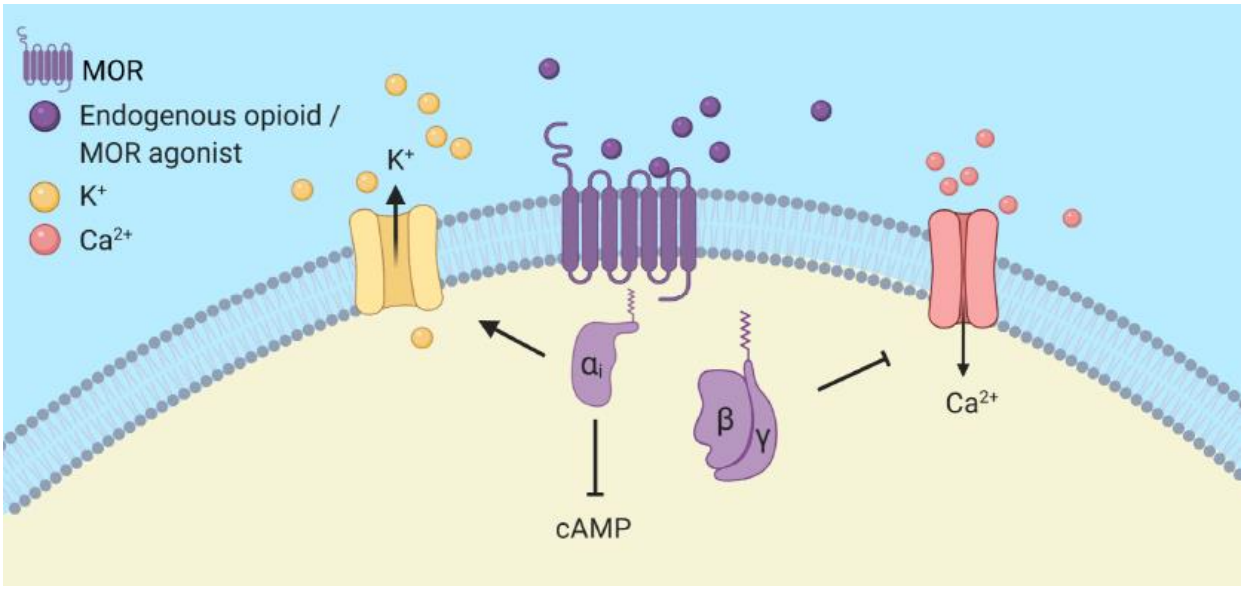

Figure 1.1. Cellular effects of mu opioid receptor activation. The mu opioid receptor (MOR) is a seven-transmembrane domain G protein-coupled receptor. Activation of the MOR by endogenous opioids or MOR agonists induces signaling via its associated inhibitory Gα subunit. After dissociation of the Gα and Gβγ subunits, Gα inhibits adenylate cyclase to suppresses cAMP production, and hyperpolarizes the cell by activating G protein-gated inwardly rectifying potassium (K^+) channels. The Gβγ subunit also induces neuronal hyperpolarization by binding to calcium (Ca^{2+}) channels and suppressing their activity. Arrows indicate effects enhanced by MOR activation; ⊤ indicates processes that are inhibited. Figure adapted from Al-Hasani and Bruchas 2011.

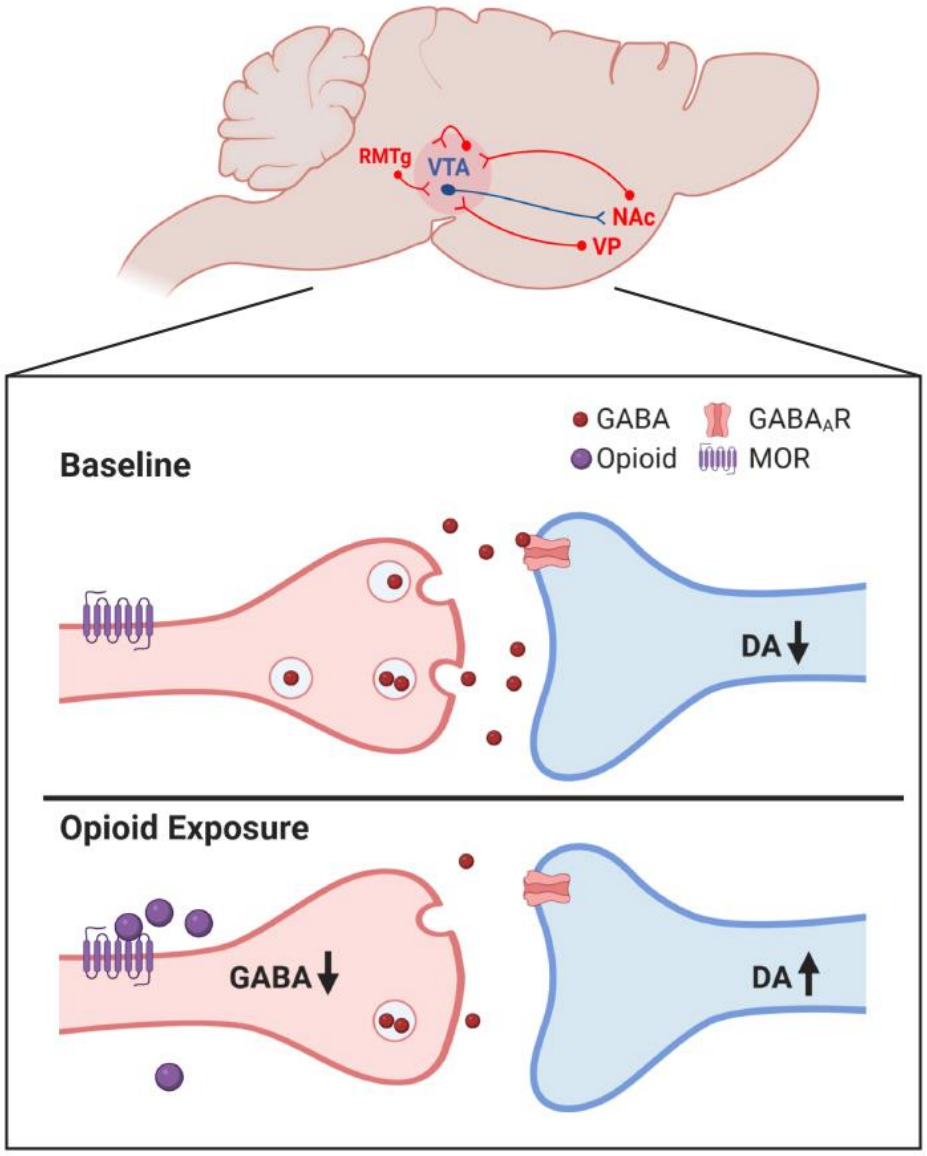

Figure 1.2. Opioid signaling in the ventral tegmental area. A diagram of the mouse brain highlighting GABAergic inputs (red) to the ventral tegmental area (VTA), which arise from within the VTA itself (interneurons), the rostromedial tegmental nucleus (RMTg), the ventral pallidum (VP), and the nucleus accumbens (NAc). Activity of these GABAergic afferents affects the activity of dopaminergic VTA neurons (blue) projecting to NAc, which modulates opioid reward. The inset shows how under baseline conditions, GABAergic neurons (red) provide inhibitory tone to VTA dopamine (DA) neurons (blue). During opioid exposure, activation of mu opioid receptors (MOR)

located on GABAergic terminals in the VTA suppresses GABA outflow and disinhibits DA neurons, resulting in increased DA release.

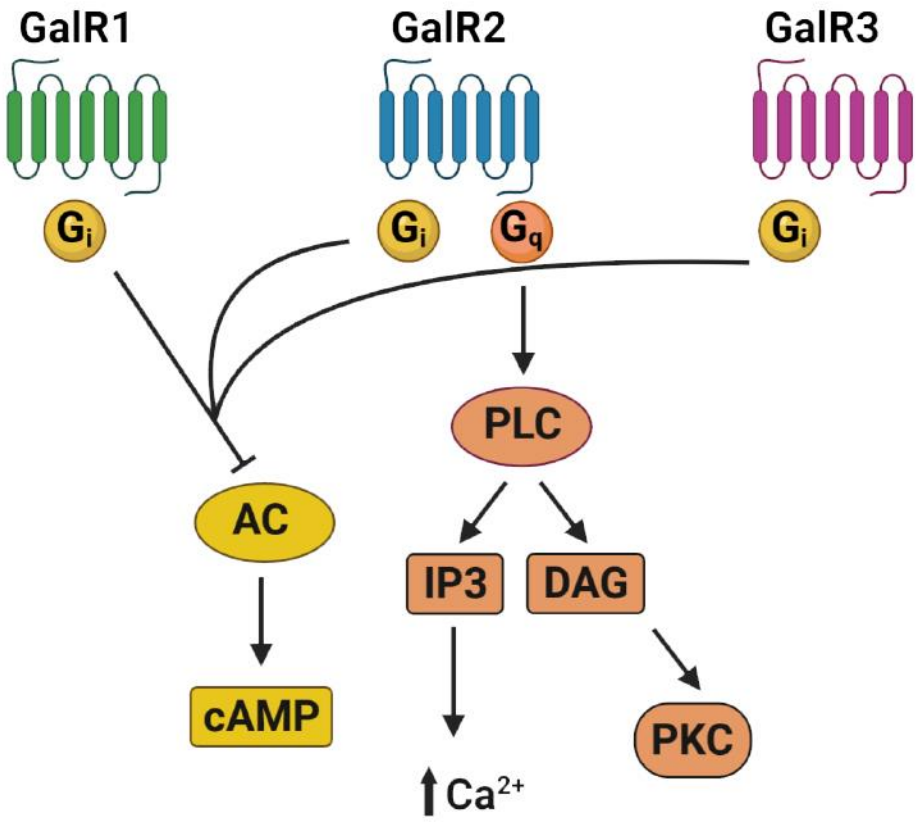

Figure 1.3. Signaling through galanin receptor subtypes. Galanin signals through three receptor subtypes: GalR1, GalR2, and GalR3. All three subtypes can signal through G_i to inactivate adenylate cyclase (AC), suppress cyclic adenosine monophosphate (cAMP) formation, and ultimately decrease cellular activity. GalR2 can also flexibly signal through G_q to activate phospholipase C (PLC) and produce inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 actions on the endoplasmic reticulum increase cytosolic calcium levels, and DAG-mediated activation of protein kinase C (PKC) triggers downstream signaling cascades leading to increased cellular activity.

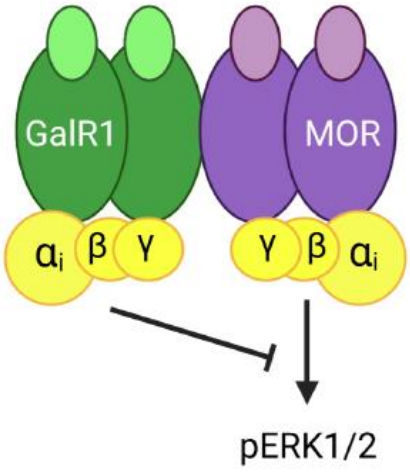

Figure 1.4. Galanin blocks opioid signaling through GalR1-MOR heteromers. Galanin receptor 1 (GalR1) and the mu opioid receptor (MOR) can form a heterotetramer comprised of GalR1 and MOR homodimers. While ligand binding to MOR alone elicits a secondary messenger cascade resulting in phosphorylation of ERK1/2 (pERK1/2), concurrent ligand binding to the GalR1 and MOR protomers results in cross-antagonism of MOR signaling by GalR1. Adapted from Ferré 2017.

CHAPTER 2: CELL-TYPE SPECIFIC EXPRESSION AND BEHAVIORAL IMPACT OF GALANIN AND GALR1 IN THE LOCUS COERULEUS DURING OPIOID WITHDRAWAL

Portions of this chapter were used verbatim, with permission, from the following publication: Foster SL, Galaj E, Karne SL, Ferré S, Weinshenker D. Cell-type specific expression and behavioral impact of galanin and GalR1 in the locus coeruleus during opioid withdrawal. Addict Biol. 2021 Mar 25:e13037. PMID: 33768673.

2.1. ABSTRACT

The neuropeptide galanin is reported to attenuate opioid withdrawal symptoms, potentially by reducing neuronal hyperactivity in the noradrenergic locus coeruleus (LC) via galanin receptor 1 (GalR1). We evaluated this mechanism by using RNAscope *in situ* hybridization to characterize GalR1 mRNA distribution in the dorsal pons and to compare galanin and GalR1 mRNA expression in tyrosine hydroxylase-positive (TH+) LC cells at baseline and following chronic morphine or precipitated withdrawal. We then used genetically altered mouse lines and pharmacology to test whether noradrenergic galanin (NE-Gal) modulates withdrawal symptoms. RNAscope revealed that, while GalR1 signal was abundant in the dorsal pons, 80.7% of the signal was attributable to TH- neurons outside the LC. Galanin and TH mRNA were abundant in LC cells at baseline and were further increased by withdrawal, whereas low basal GalR1 mRNA expression was unaltered by chronic morphine or withdrawal. Naloxone-precipitated withdrawal symptoms in mice lacking NE-Gal ($Gal^{cKO\text{-}Dbh}$) were largely similar to WT littermates, indicating that loss of NE-Gal does not exacerbate withdrawal. Complimentary experiments using NE-Gal overexpressor mice (NE-Gal OX) and systemic administration of the galanin receptor agonist galnon revealed that increasing galanin signaling also failed to alter behavioral withdrawal, while suppressing noradrenergic transmission with the alpha-2 adrenergic receptor agonist clonidine attenuated multiple symptoms. These results indicate that galanin does not acutely attenuate precipitated opioid withdrawal via an LC-specific mechanism, which has important implications for the general role of galanin in regulation of somatic and affective opioid responses and LC activity.

2.2. INTRODUCTION

Opioid withdrawal is characterized by somatic symptoms resulting from neuronal hyperactivity in multiple brain regions (Kosten & Baxter, 2019; Rasmussen et al., 1990). The neuropeptide galanin is a negative regulator of neural activity (Lang et al., 2015), and genetic deletion of either galanin or one of its receptors, galanin receptor 1 (GalR1), exacerbates withdrawal symptoms (F. E. Holmes et al., 2012; Zachariou et al., 2003). Conversely, genetic or pharmacological enhancement of galanin signaling attenuates withdrawal symptoms (F. E. Holmes et al., 2012; Zachariou et al., 2003). These studies employing whole-body manipulations to galanin have prompted interest in defining the specific neuroanatomical substrates underlying the protective effects of galanin-GalR1 transmission in the brain.

Among the brain regions thought to contribute to opioid withdrawal is the noradrenergic locus coeruleus (LC) (Nestler, 2004), which strongly expresses galanin (Cheung et al., 2001; Perez et al., 2001; Skofitsch & Jacobowitz, 1985; R. P. Tillage, N. R. Sciolino, et al., 2020). Attenuation of opioid withdrawal by galanin is posited to involve a negative feedback loop in the LC that is maintained by galanin and GalR1 (Picciotto et al., 2005; Zachariou et al., 2003; Zachariou et al., 2000). During states of LC hyperactivity such as opioid withdrawal, somatodendritic galanin release may engage Gi-coupled GalR1 autoreceptors on LC neurons, suppressing LC firing and restoring normal activity (Hokfelt et al., 2018; Vila-Porcile et al., 2009). Indeed, electron microscopy studies indicate that the LC is capable of dendritic galanin release (Vila-Porcile et al., 2009), and galanin is known to induce a potent GalR1-mediated hyperpolarization of LC neurons in slice preparations (Bai et al., 2018; Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989; Sevcik, Finta, & Illes, 1993). In addition, galanin and GalR1 are reported to be dynamically regulated in the LC by opioids, with microarray, *in situ* hybridization (ISH), and galanin reporter

mouse results collectively indicating that their expression is increased by chronic morphine and precipitated withdrawal (F. E. Holmes et al., 2012; McClung, Nestler, & Zachariou, 2005; Zachariou et al., 2000). These findings support the hypothesis that galanin and GalR1 could assuage LC hyperactive states and suppress opioid withdrawal symptoms via a local negative feedback system.

Importantly, the LC-galanin negative feedback model is predicated on the presence of both galanin and GalR1 in noradrenergic LC neurons. Galanin expression is robust in the rat, mouse, and human LC (Cheung et al., 2001; Le Maitre et al., 2013; Perez et al., 2001; Skofitsch & Jacobowitz, 1985; R. P. Tillage, N. R. Sciolino, et al., 2020), but GalR1 expression has been difficult to characterize. Because there are no reliable antibodies for detecting GalR1 protein (Hawes & Picciotto, 2004; Lu & Bartfai, 2009), ISH has been relied upon to examine GalR1 mRNA expression (F. E. Holmes et al., 2012; D. O'Donnell et al., 1999; Z. Q. Xu, Shi, & Hokfelt, 1998; Zachariou et al., 2000). Though these studies revealed the presence of GalR1 mRNA in a neuroanatomical location consistent with the LC, they lacked double labeling to confirm cellular identity and sufficiently high resolution to definitively attribute GalR1 mRNA signal to noradrenergic LC neurons. Such limitations also apply to previous studies that identified upregulation of galanin and GalR1 in the LC after precipitated withdrawal (F. E. Holmes et al., 2012; Zachariou et al., 2000). Therefore, the distribution of GalR1 mRNA in the LC remains speculative, as does potential regulation of galanin and GalR1 expression by opioids.

The second tenet of the LC-galanin negative feedback model is that the LC, specifically, is the source of galanin that attenuates withdrawal severity. However, little is known about the effects of LC-derived galanin in the context of opioid withdrawal. While one study reported attenuated withdrawal symptoms in mice overexpressing galanin under the control of a noradrenergic

promoter (Zachariou et al., 2003), a notable caveat is that this transgenic line exhibits ectopic galanin expression in noradrenergic neurons that do not normally contain the neuropeptide, in addition to some non-noradrenergic cells (Steiner et al., 2001). Moreover, no study has selectively depleted noradrenergic galanin (NE-Gal) to determine whether its absence in the LC exacerbates withdrawal severity.

In this report, we sought to address remaining gaps in knowledge regarding 1) basal and opioid-induced changes in galanin and GalR1 mRNA expression in noradrenergic LC neurons, and 2) the specific role of NE-Gal in opioid withdrawal behaviors. We first employed RNAscope ISH to visualize GalR1 expression in the mouse and rat dorsal pons. We then compared GalR1 mRNA and protein expression in the mouse LC to that of adjacent non-noradrenergic neurons in dorsal pons using RNAscope and a fluorescently-tagged GalR1 transgenic mouse line, respectively. We also generated the first high-resolution, cell-type specific characterization of galanin and GalR1 mRNA expression in noradrenergic LC neurons of mice, and tested whether chronic morphine or naloxone-precipitated withdrawal altered galanin or GalR1 mRNA expression. To test whether loss of NE-Gal exacerbates withdrawal symptoms, we performed naloxone-precipitated withdrawal in genetically modified mice that lack NE-Gal ($Gal^{cKO\text{-}Dbh}$). We also performed complimentary tests using NE-Gal overexpressing mice (NE-Gal OX) and WT mice treated with the galanin receptor agonist, galnon, to assess whether withdrawal symptoms could be attenuated by enhanced noradrenergic-derived or central galanin signaling, respectively.

2.3. MATERIALS AND METHODS

Animals

The following studies used 3-6 month old mice (both sexes) on a C57 BL/6J background unless otherwise specified. Mice were group housed on static racks with food and water available

ad libitum in a temperature-controlled room with a 12:12 light/dark cycle unless otherwise stated. All procedures were performed during the light phase. Procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee.

RNAscope was performed using C57 BL/6J mice and Long-Evans rats (3 months old). Immunohistochemistry was performed using GalR1-mCherry knock-in mice, which express an mCherry tag at the C-terminus of GalR1 (Kerr et al., 2015). This strain was on a mixed 129P2/OlaHsd background and was back-crossed with C57 BL/6J mice. Withdrawal studies used previously characterized $Gal^{cKO\text{-}Dbh}$ and NE-Gal OX mice with respective wild-type littermates serving as controls (Steiner et al., 2001; R. P. Tillage, N. R. Sciolino, et al., 2020). $Gal^{cKO\text{-}Dbh}$ mice were generated by crossing a line expressing *cre* recombinase under the noradrenergic-specific dopamine β-hydroxylase (*Dbh*) promoter with a floxed galanin line (Gal^{cKO}) (JAX stock no. 034319). $Dbh^{cre/+}$;Gal^{cKO} homozygotes were crossed with Gal^{cKO} homozygotes to generate $Gal^{cKO\text{-}Dbh}$ progeny as previously described (R. P. Tillage, N. R. Sciolino, et al., 2020). NE-Gal OX mice contain a transgene in which galanin expression is driven by the *Dbh* promoter, resulting in a five-fold increase in galanin mRNA in the LC and increased galanin immunoreactivity in LC projection regions (JAX stock no. 004996) (Steiner et al., 2001).

Drugs

Morphine sulfate (NIDA Drug Supply Program), naloxone hydrochloride (0.4 mg/ml stock) (Hospira, Lake Forest, IL), and clonidine hydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved or diluted in normal sterile saline. Galnon trifluoroacetate salt (Bachem, Torrance, CA) was dissolved in a vehicle of 1% DMSO in normal sterile saline. All solutions were administered using an injection volume of 10 ml/kg.

In Situ Hybridization

Tissue Collection: Animals were deeply anesthetized with isoflurane and quickly decapitated. For RNAscope studies in mice that received saline, chronic morphine, or withdrawal, mice were sacrificed 3 h after the final injection, as previously described (Zachariou et al., 2000). Brains were immediately frozen in an OCT-filled cryomold that was submerged in isopentane chilled with dry ice. OCT blocks were stored at -80°C until sectioning. Brains were sectioned at 16 μm increments onto charged slides and stored at -80°C until used for RNAscope.

RNAscope Assay: Sample pretreatment was performed as instructed using the RNAscope Sample Preparation and Pretreatment Guide for Fresh Frozen Tissue. Briefly, slides were removed from the -80°C freezer and immediately fixed in pre-chilled 10% NBF for 15 min. Slides were then dehydrated using the following ethanol wash series in 5-min increments: 50%, 70%, 100%, 100%. Slides were air dried, a hydrophobic barrier was drawn around the tissue, and slides were incubated with Pretreat IV at room temperature for 30 min. RNAscope for mouse and rat LC sections was then performed using the RNAscope Fluorescent Multiplex Assay v1 kit according to manufacturer's instructions for fresh frozen tissue (Advanced Cell Diagnostics, Newark, CA). Slides were washed 2x in PBS, experimental probe was added to each section, and slides were incubated in the HybEZ oven at 40°C for 2 h.

For qualitative images of GalR1 mRNA in mouse LC, mouse probes for GalR1 (ACD cat no. 448821), and tyrosine hydroxylase (TH, a marker of noradrenergic cells) (ACD cat no. 317621) were used. Mouse multiplex positive (ACD cat no. 320881) and multiplex negative (ACD cat no. 320871) control probes were used to validate experimental probe signal. For qualitative images of GalR1 signal in rat LC, rat probes for GalR1 (ACD cat no. 439791) and TH (ACD cat no. 314651) were used. Experiments analyzing GalR1 in TH+ and TH- neurons within the LC field of view

used probes for GalR1, TH, and the neuronal marker Synaptosome Associated Protein 25 (SNAP25) (ACD cat no. 516471-C3). For the LC-specific analysis of GalR1 mRNA expression at baseline and after chronic morphine or withdrawal, mouse probes for GalR1, galanin (ACD cat no. 400961), and TH were used.

After hybridization, slides were washed 2x 2 min with wash buffer. Four subsequent rounds of amplification and 2x 2 min washes with wash buffer were performed as instructed. In amplification step 4, color module Alt A-FL was chosen to assign the following fluorophores to each channel: C1 Alexa 488, C2 Atto 550, C3 Atto 647. Slides were 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 24 to 48 hours after performing RNAscope.

Imaging: Slides were imaged using a Nikon A1R HD25 confocal microscope with NIS Elements Software. Representative images of GalR1 signal in the LC and surrounding areas were acquired with a 20x objective lens. For quantitative RNAscope studies, the LC was centered in the field of view, and a Z-stack (~14 μm thickness with 0.95 μm steps) was taken at a resolution of 1024 x 1024 pixels using a 40x objective oil immersion lens. Gain settings were chosen to maximize probe signal without oversaturation and validated with positive and negative control probe slides.

Immunohistochemistry

GalR1-mCherry knock-in mice (Kerr et al., 2015) were deeply anesthetized with isoflurane and transcardially perfused with 0.1M KPBS followed by 4% PFA in 0.1 M KPBS. Brains were post-fixed in 4% PFA overnight and transferred to 30% sucrose in PBS for ~48 h. Brains were frozen in chilled isopentane and sectioned on a cryostat at 40 μm increments. Sections from the

LC and paraventricular nucleus of the thalamus (PVT), which strongly expresses GalR1 (Kerr et al., 2015), were stained using an adapted protocol for mCherry detection in mu opioid receptor-mCherry tagged mice (Gardon et al., 2014).

Sections were washed in PBS 3x for 10 min each and incubated in blocking solution (PBS-Triton (0.3%), 2% normal goat serum, 1% BSA) for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C. A rabbit anti-DsRed polyclonal antibody (Takara Bio, Mountain View, CA, cat no. 632406, dilution 1:1000) was used for mCherry detection, and a chicken anti-TH polyclonal antibody (Abcam, Cambridge, MA, cat no. ab76442, dilution 1:1000) was used to define noradrenergic LC neurons. The next day, sections were washed in PBS 3x for 10 min each. Then sections were incubated in goat anti-rabbit 488 conjugated (Invitrogen Cat no. A11008, dilution 1:2000) and goat anti-chicken 633 conjugated (Invitrogen A21103, dilution 1:600) secondary antibodies for 2 h at room temperature, washed in PBS 3x for 10 min each, mounted onto charged slides, and dried overnight. Slides were coverslipped using Fluoromount-G with DAPI (Thermo Fisher Scientific, Waltham, MA, cat no. 00-4959-52) the following day.

Slides were imaged using a Nikon A1R HD25 confocal microscope with NIS Elements Software. Z-stack images with 4 μm steps were acquired for LC and PVT sections after confirming lack of signal in secondary-only slides.

Image Analysis

RNAscope image analysis was performed using Imaris software (Bitplane Inc., Concord, MA). All RNAscope experiments contained 6 mice per group. For each mouse, 3 LC images were analyzed. Values were averaged across images for each mouse, and then across mice for each group.

GalR1 mRNA expression in LC versus LC-adjacent neurons: To identify individual neurons in each image, we capitalized on the expression pattern of SNAP25, which fills the cell soma (Jolly et al., 2019). The image channel corresponding to the SNAP25 probe was isolated, and a Surface layer was generated in Imaris to identify and segment individual SNAP25+ cells in 3-D. The channel corresponding to the GalR1 probe was then used to generate a Spots layer identifying individual GalR1 puncta. The GalR1 puncta were then filtered to select only for puncta contained within the surfaces of the SNAP25+ cells identified in the Surface layer. Then, the TH channel was overlaid and used to label each SNAP25+ cell as either TH+ or TH-. GalR1 puncta per cell counts were generated for each cell, which was also classified as TH+ or TH-. Distribution of GalR1 puncta by cell type was determined for each image by dividing the total puncta within a cell population (TH+ or TH-) by the total puncta within SNAP25-defined cells. To account for differences in the number of TH+ and TH- cells observed per image, GalR1 density was also calculated by cell type. The total 2-D surface area for each cell population was calculated by summing the surface areas of the individual cells for that population within each image. Then, the total GalR1 puncta contained within a cell population was divided by the estimated total 2-D surface area occupied by that population in the image.

LC galanin and GalR1 mRNA regulation: LC sections from mice that received saline, chronic morphine, or underwent withdrawal were run with RNAscope probes for galanin, TH, and GalR1. Due to high fluorescent signal, galanin and TH mRNA expression were determined by acquiring respective fluorescence intensity values from each LC cell and calculating the average intensity per image. For GalR1, the same segmentation process was used as in the GalR1 SNAP25 analysis, except that the TH channel was used to segment noradrenergic LC cells and associated GalR1 puncta.

Naloxone-Precipitated Withdrawal

Behavior: Naloxone-precipitated withdrawal was conducted as previously described (Contet et al., 2008). Mice received five consecutive days of intraperitoneal (i.p.) morphine injections at 08:00 and 18:00. Morphine doses escalated by 20 mg/kg each day (i.e. 20, 40, 60, 80, 100 mg/kg). Body weights were recorded prior to each injection to detect differences in morphine-induced weight loss (Koek, 2014). On day six, mice were transported to a separate room, weighed, and given 100 mg/kg of morphine at 08:00. Two hours later, mice were removed from their home cages, injected with naloxone (1 mg/kg, s.c.), and placed into a transparent polycarbonate observation chamber. For pharmacology experiments, mice were pre-treated with galnon (2 mg/kg, i.p.) or vehicle 15 min before receiving naloxone, and clonidine (0.3 mg/kg i.p.) or saline vehicle 30 min before naloxone, consistent with previous studies (Hosseinzadeh & Jahanian, 2010; Zachariou et al., 2003; G. Zhang et al., 2016). Withdrawal behaviors were video recorded for 30 min following naloxone injection, after which mice were weighed and returned to home cages. Fecal boli were counted at the end of the session; all other behaviors were scored during review of withdrawal videos.

Video Scoring: Withdrawal behaviors were scored by a blinded observer using Behavioral Observation Research Interactive Software (Friard & Gamba, 2016). For every mouse, each occurrence of the following behaviors during the 30-min observation period was marked as a point event: rearing, jumping, wet dog shakes, paw tremor, backwards steps, and sniffing. The criteria below were used to score each behavior:

Rearing: Mouse supporting itself on extended hind legs.

Jumping: All four paws leaving the ground at the same time.

Wet dog shake: Brief, rapid shake involving both the head and body of the mouse.

Backwards steps: Mouse jumping or shuffling backwards, with visible movement of hind paws.

Paw tremor: Brief, rapid shaking of one or both front paws or hind paws. During instances where this occurred rapidly, each "bout" of tremor was counted. Instances where mice exhibited hind paw tremor, often prior to a jump or during rearing, were also counted as paw tremor.

Sniffing: Movements of the nose and whiskers (distinct from chewing), often accompanying rearing or head-scanning behavior prior to and while walking.

Galnon Feeding Test

Galnon is a non-selective galanin receptor agonist that crosses the blood-brain barrier (Saar et al., 2002), and i.p. administration of galnon attenuates feeding in mice and rats through actions on central galanin receptors (Abramov et al., 2004). To validate the use of i.p. administered galnon in our withdrawal study, we measured whether galnon increased the latency of food-deprived mice to bite a food pellet or reduced their food consumption over a 30-min interval compared to vehicle-treated mice.

C57 Bl/6J mice were single-housed one week prior to testing. Mice had food removed 24 h before behavioral testing to increase motivation to eat. The next day, 15 min prior to the start of testing, mice were given an i.p. injection of either galnon (2 mg/kg) or vehicle. A pre-weighed pellet of standard mouse chow was introduced into the home cage at the end opposite to the mouse's location, and the latency to eat the food was timed. The test ended when the mouse bit the pellet and started consuming the food, or once 5 min elapsed, whichever occurred first. Thirty min after the start of each feeding test, the food pellet was re-weighed, and the amount consumed was calculated.

Statistical Analysis

Statistical analyses and graphs were generated in GraphPad Prism Version 8 (GraphPad Software, San Diego, CA). GalR1 SNAP25 data were compared by unpaired one-tailed t-test given prior observations that GalR1 was overwhelmingly higher in TH- cells. LC-specific data comparing expression at baseline, after chronic morphine, or after withdrawal, were analyzed by one-way or two-way ANOVA with Tukey's multiple comparisons test as appropriate. For behavioral studies, weight loss during induction of morphine dependence was compared by two-way repeated measures ANOVA (time x genotype). Withdrawal data were assessed for equality of variance using a Brown-Forsythe test. Behaviors demonstrating equal variance across groups were compared using one-way ANOVA with Tukey's multiple comparisons test; behaviors lacking equal variance were compared using a Kruskal-Wallis test with Dunn's multiple comparisons test. Feeding test data were compared by unpaired one-tailed t-test, since effect direction (decreased feeding) was predetermined from the literature.

2.4. RESULTS

The LC exhibits low GalR1 mRNA expression

As no study has yet demonstrated GalR1 expression in verified noradrenergic LC neurons, we used RNAscope to visualize GalR1 mRNA expression in TH+ LC neurons in mice. Images revealed low LC GalR1 mRNA expression in a small number of cells, with comparatively higher expression in many cells adjacent (medial, lateral, and dorsal) to the LC (**Fig 2.1A**). The few cells that did co-express GalR1 and TH were located on the LC periphery, as opposed to the LC core (**Fig. 2.1B**). GalR1 signal in hypothalamus, a positive control region (Kerr et al., 2015), showed robust signal as expected (**Fig. 2.1C**).

Previous work suggests that galanin attenuates withdrawal in mice but not rats (P. V. Holmes et al., 1994; Zachariou et al., 2003). To assess whether this species difference is also

reflected in GalR1 distribution, we performed RNAscope for GalR1 in rat LC sections. Similar to observations in the mouse, GalR1 expression was low in TH+ LC cells, yet abundant just outside this nucleus (**Fig. 2.1D**), demonstrating that LC GalR1 mRNA expression patterns are consistent between mouse and rat.

A potential caveat is that mRNA levels do not necessarily correlate with protein abundance. We therefore used a knock-in mouse line that expresses mCherry-tagged GalR1 protein (Kerr et al., 2015) and found that immunohistochemistry for mCherry broadly recapitulated RNAscope results. GalR1-mCherry immunoreactivity in TH+ LC neurons was negligible, but strong signal was detected just outside the LC (**Fig. 2.1E**), as well as in a positive control region, the paraventricular nucleus of the thalamus (PVT) (Kerr et al., 2015) (**Fig. 2.1F**).

The majority of dorsal pontine GalR1 mRNA expression is outside of the LC

To characterize GalR1 expression in dorsal pons, we performed RNAscope using probes for GalR1, TH, and the neuronal marker SNAP25 (**Fig. 2.2**) (Jolly et al., 2019). The proportion of GalR1 signal was then compared between SNAP25+/TH+ LC cells and SNAP25+/TH- cells surrounding the LC. Only 19.3% of GalR1 puncta were located in TH+ cells, while 80.7% of puncta were located in TH- cells (**Fig. 2.2G**). An unpaired t-test of GalR1 density by cell type also indicated significantly higher GalR1 signal in the TH- cell population ($t_{10} = 8.408$, $p < 0.0001$) (**Fig. 2.2H**). These results indicate that the majority of GalR1 mRNA signal in the dorsal pons emanates from non-noradrenergic, LC-adjacent cells, rather than the LC itself.

Morphine withdrawal increases TH and galanin mRNA, but not GalR1 mRNA, in the LC

Galanin and GalR1 are reported to be dynamically regulated in the LC by opioid exposure (F. E. Holmes et al., 2012; McClung, Nestler, & Zachariou, 2005; Zachariou et al., 2000). However, this has not been demonstrated with cell-type specificity. We therefore compared

galanin and GalR1 mRNA expression in TH+ LC neurons of mice that received saline, chronic morphine, or underwent naloxone-precipitated withdrawal following chronic morphine (**Fig. 2.3**). TH mRNA was quantified as a positive control because its expression in LC is consistently enhanced by opioid exposure and withdrawal (Jalali Mashayekhi et al., 2018; McClung, Nestler, & Zachariou, 2005; Nestler, 2004). For TH, a one-way ANOVA indicated a significant effect of treatment on intensity value ($F_{2,15} = 6.739$, $p = 0.0010$); intensity was significantly increased after chronic morphine ($p = 0.0177$) and withdrawal ($p = 0.0008$) compared to saline (**Fig. 2.3A-C; Fig. 2.4A**). Because TH intensity was higher in opioid-exposed groups, and TH signal was used to identify cells for LC GalR1 quantification, we wanted to ensure that these treatment groups were not biased to detect more TH+ cells than the saline group. A one-way ANOVA showed no effect of treatment group on TH+ cells detected per LC image ($F_{2, 15} = 2.490$, $p = 0.1165$), indicating that GalR1 puncta were quantified in approximately the same number of TH+ LC cells per image across treatments (saline: 102.3 ± 3.62, chronic morphine: 92.67 ± 3.64, withdrawal: 101 ± 2.55) (**Fig. 2.4B**). For galanin, a one-way ANOVA also showed a significant effect of treatment on intensity value ($F_{2,15} = 6.739$, $p = 0.0082$); withdrawal was significantly higher than saline ($p = 0.0069$), and there was a trend for chronic morphine ($p = 0.0708$) (saline: 200.2 ± 62.44, chronic morphine: 374.8 ± 29.74; withdrawal: 461.2 ± 55.55) (**Fig. 2.3 D-F; Fig. 2.4C**).

Due to comparatively low expression levels, GalR1 expression was analyzed by binning TH+ cells in each LC image by the number of GalR1 puncta per cell. A two-way ANOVA (bin x treatment) showed a main effect of bin ($F_{4,75} = 929.1$, $p < 0.0001$), but not treatment ($F_{2,75} = 2.440$, $p = 0.0941$), and no interaction ($F_{8,75} = 0.5410$, $p = 0.8220$), indicating that treatment did not influence the relative proportions of GalR1 expression (**Fig. 2.3G-I; Fig. 2.4D**). The majority of TH+ cells did not exhibit any GalR1 puncta (saline: 65.39 ± 4, chronic morphine: 63.06 ± 1.98,

withdrawal: 65.72 ± 1.59). Approximately one third of cells contained between one and three GalR1 puncta (saline: 29.78 ± 2.31, chronic morphine: 24.17 ± 1.85, withdrawal: 27.72 ± 1.75), with the remaining small proportion containing four or more puncta. To examine GalR1 among the cells that expressed the transcript, we performed a second analysis restricted to cells containing one or more GalR1 puncta. Again, one-way ANOVA indicated no effect of treatment group on the average number of GalR1 puncta per cell ($F_{2,15} = 0.5221$, $p = 0.6037$), with similar values across treatments (saline: 2.47 ± 0.19, chronic morphine: 2.44 ± 0.14, withdrawal: 2.72 ± 0.17) (**Fig. 2.4E**). These results indicate that while TH and galanin mRNA increase in the LC following chronic morphine and/or withdrawal, GalR1 expression does not change from low baseline levels.

Noradrenergic-derived galanin does not modulate precipitated withdrawal symptoms

To determine whether selective depletion of NE-Gal exacerbates withdrawal symptoms, naloxone-precipitated withdrawal was performed in $Gal^{cKO\text{-}Dbh}$ mice and WT littermates alongside a cohort of NE-Gal OX mice and their WT littermates as a control for NE-Gal modulation of withdrawal symptoms (Zachariou et al., 2003). Two-way repeated measures ANOVAs showed that during the morphine dosing period, there were no differences in weight loss between $Gal^{cKO\text{-}Dbh}$ or NE-Gal OX mice and their respective WT littermates (**Fig. 2.25A, B**). For both $Gal^{cKO\text{-}Dbh}$ and NE-Gal OX analyses, there was a main effect of time ($Gal^{cKO\text{-}Dbh}$: $F_{3.223,61.23} = 73.72$, $p < 0.0001$; NE-Gal OX: $F_{2.437,46.29} = 97.46$, $p < 0.0001$) but not genotype ($Gal^{cKO\text{-}Dbh}$: $F_{1,19} = 1.320$, $p = 0.2649$; NE-Gal OX: $F_{1,19} = 0.05025$, $p = 0.8250$), and no interaction ($Gal^{cKO\text{-}Dbh}$: $F_{10,190} = 0.8423$, $p = 0.5885$; NE-Gal OX: $F_{10,190} = 0.4376$, $p = 0.9266$). One-way ANOVAs for withdrawal behaviors also revealed no genotype differences for withdrawal-induced weight loss ($F_{2,39} = 0.3357$, $p = 0.7169$), jumps ($F_{2,39} = 0.2570$, $p = 0.7746$), sniffing ($F_{2,39} = 2.119$, $p = 0.1338$), paw tremor ($F_{2,39} = 1.628$, $p = 0.2093$), rearing ($F_{2,39} = 1.028$, $p = 0.3672$), wet dog shakes ($F_{2,39} = 1.116$,

$p = 0.3379$), or fecal boli ($F_{2,39} = 0.7528$, $p = 0.4778$) (**Fig. 2.5C-G, I, J**). Only backwards steps were significantly different ($F_{2,39} = 4.603$, $p = 0.0160$), in which $Gal^{cKO\text{-}Dbh}$ mice were lower than WT ($p = 0.0124$) (**Fig. 2.5H**). Collectively, these results indicate that neither depletion nor overexpression of NE-Gal substantially alters withdrawal behavioral profiles.

Activation of central galanin receptors does not reduce precipitated withdrawal symptoms

To confirm that peripherally administered galnon sufficiently activates central galanin receptors, we reproduced the finding that i.p. galnon reduces feeding, which requires activation of hypothalamic galanin receptors (Abramov et al., 2004). An unpaired one-tailed t-test showed that galnon significantly reduced the amount of food consumed ($t_{12} = 1.849$, $p = 0.0446$), and increased latency to eat (vehicle 86.14 ± 38.4; galnon 179.40 ± 43.58), although this measure did not reach significance ($t_{12} = 1.606$, $p = 0.0671$) (**Fig. 2.6A, B**).

We then evaluated withdrawal behaviors in mice pre-treated with vehicle, galnon, or the anti-adrenergic drug clonidine (positive control). The vehicle group included mice treated with 1% DMSO in saline (vehicle for galnon) or saline alone (vehicle for clonidine). Significant differences were detected for weight loss ($F_{2,36} = 8.508$, $p = 0.0009$), sniffing ($F_{2,35} = 27.29$, $p < 0.0001$), paw tremor ($H_2 = 21.55$, $p < 0.0001$), rearing ($F_{2,36} = 32.05$, $p < 0.0001$), backward steps ($H_2 = 15.70$, $p = 0.0004$), and fecal boli ($H_2 = 18.42$, $p < 0.0001$). However, all differences were attributable to clonidine, as galnon did not reduce any symptoms (**Fig. 2.6C, E-H, J)**. Post-hoc tests revealed that clonidine significantly reduced symptoms compared to both vehicle (sniffing $p < 0.0001$, paw tremor $p < 0.0001$, rearing $p < 0.0001$, backward steps $p < 0.0015$, fecal boli $p < 0.0001$) and galnon (sniffing $p < 0.0001$, paw tremor $p < 0.0019$, rearing $p < 0.0001$, backward steps $p < 0.0012$, fecal boli $p < 0.0111$). Unexpectedly, clonidine significantly increased weight loss compared to galnon ($p = 0.0006$), but not vehicle ($p = 0.0538$) (**Fig. 2.6C**). Jumps ($F_{2,36} = 0.2856$, $p = 0.7532$)

and wet dog shakes ($F_{2,36} = 1.850$, $p = 0.1719$) were unaffected by either galnon or clonidine (**Fig. 2.6D, I**). Overall, these data imply that even broad activation of central galanin receptors fails to reduce withdrawal symptoms.

2.5. DISCUSSION

The neuropeptide galanin has been shown to modulate opioid withdrawal symptoms, which is speculated to involve an autoinhibitory feedback loop in the LC in which galanin is locally released under hyperactive conditions, and binds Gi-coupled GalR1 receptors located on LC neurons to suppress excessive activity (Hokfelt et al., 2018; Picciotto et al., 2005; Zachariou et al., 2003; Zachariou et al., 2000). Surprisingly, we found that that noradrenergic LC neurons express little GalR1 mRNA, and that the majority of GalR1 mRNA signal in the dorsal pons emanates from LC-adjacent regions, rather than the LC itself. GalR1 RNAscope findings in the mouse dorsal pons were also recapitulated in rat. While precipitated withdrawal enhanced galanin mRNA expression in the LC, neither chronic morphine nor withdrawal altered GalR1 expression. Furthermore, neither decreasing nor increasing NE-Gal levels affected withdrawal symptoms. Pharmacological activation of central galanin receptors also failed to reduce withdrawal symptoms, contradicting previous reports. Our molecular and behavioral findings therefore do not support an LC-centric mechanism for galaninergic modulation of withdrawal symptoms, consistent with previous evidence that the LC is just one of several brain regions contributing to the development and expression of withdrawal (Christie et al., 1997; Maldonado, 1997).

We found that most of the GalR1 signal in the dorsal pons, which has historically been attributed to the LC, was actually located in TH-, LC-adjacent regions including those neuroanatomically consistent with Barrington's nucleus, the parabrachial nucleus, the pontine central gray, and the mesencephalic nucleus of the trigeminal nerve. Similar observations had been

previously reported in the rat (Pieribone et al., 1995; Z. Q. Xu, Shi, & Hokfelt, 1998), but our RNAscope data now substantiate these findings with cell-type specificity, and also indicate that the pattern of GalR1 expression in the dorsal pons is conserved between rats and mice. While our study revealed basal GalR1 expression outside the LC to be higher than previously appreciated, we simultaneously found that GalR1 expression within the LC is quite low. Most unexpected was the finding that the majority of noradrenergic LC neurons do not express any GalR1, and of those cells that do, most exhibited only one to three GalR1 puncta.

These results have broader implications for the suppression of LC activity by galanin, and invite renewed discussion of previous slice electrophysiology studies. Specifically, how does galanin potently inhibit LC firing if these neurons contain so little GalR1? The first possibility is that LC GalR1 mRNA levels may not correlate with GalR1 protein; however, our data from GalR1-mCherry mice suggest that GalR1 protein is also quite low in the LC. Another explanation is that inhibitory effects of galanin are due to alternative galanin receptor subtypes, either GalR2 or GalR3. While both GalR2 and GalR3 have been identified in the rat LC (Mennicken et al., 2002; D. O'Donnell et al., 1999), pharmacological and siRNA experiments do not support a GalR2-based mechanism (Bai et al., 2018; Ma et al., 2001), and our preliminary RNAscope results suggest that GalR3 is also sparse in TH+ LC cells of the rat. Because the published slice electrophysiology experiments were all performed in rat, it remains unclear whether these observations pertain to mice. Alternatively, galanin may modulate LC activity through an indirect mechanism involving adjacent GalR1-rich regions that in turn affect LC firing. Previous slice electrophysiology experiments bath-applied galanin while recording from LC neurons and attributed resulting inhibition to GalR1 in the LC (Bai et al., 2018; Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989; Sevcik, Finta, & Illes, 1993). However, our data showing that pontine GalR1 is largely

outside the LC suggest that the inhibition could reflect summed actions across multiple GalR1-expressing regions, complicating interpretation of past results. The high sensitivity of RNAscope, combined with the consistent pattern of signal with our GalR1-mCherry immunohistochemistry approach, further support this possibility. Finally, it is possible that a small number of receptors may be sufficient to transduce the powerful inhibitory effect of galanin on LC firing, or perhaps many LC neurons recorded from in slice experiments happened to be those few that contained appreciable amounts of GalR1. Future studies should utilize optogenetics, cell-type specific galanin receptor knockouts, and targeted pharmacological approaches to determine whether galanin affects LC activity through a direct or indirect mechanism.

Our findings on opioid regulation of LC galanin and GalR1 expression also challenge previous work. We reproduced the finding that withdrawal enhances LC galanin mRNA expression (F. E. Holmes et al., 2012; McClung, Nestler, & Zachariou, 2005), but saw no change in LC GalR1 mRNA, in contrast to a report that withdrawal increases its expression (Zachariou et al., 2000). Given that the previous ISH study lacked double-labeling, it is possible that the increased GalR1 signal included upregulation of this transcript outside the LC. However, even total GalR1 signal in our images did not differ between saline and withdrawal groups (data not shown). This disparity may be attributable to experimental differences, chiefly that we used naloxone to precipitate withdrawal, whereas the previous study used naltrexone. Additionally, we only evaluated GalR1 expression at the one significant time point used in the previous study, so there may be temporal dynamics in GalR1 expression that were not captured.

Because previous research used conventional knockout mice to argue that loss of galanin exacerbates morphine withdrawal symptoms, our data from noradrenergic-specific $Gal^{cKO\text{-}Dbh}$ mice do not directly refute this finding. However, the lack of altered withdrawal in $Gal^{cKO\text{-}Dbh}$ mice

and in NE-Gal OX mice, which were reported to exhibit attenuated withdrawal symptoms (Zachariou et al., 2003), suggest that neither depletion nor enhancement of NE-Gal modulates symptom severity. Moreover, our noradrenergic-specific results are superseded by pharmacological data showing that even brain-wide activation of galanin receptors with galnon is insufficient to attenuate withdrawal, again in contrast to prior work (Zachariou et al., 2003). A caveat of our study is that galnon and clonidine were delivered systemically, so possible peripheral actions cannot be discounted. Even so, feeding test data indicate that central galanin receptors were sufficiently activated by galnon during the time frame of withdrawal evaluation, and the ability of systemic clonidine to suppress central noradrenergic transmission and opioid withdrawal is well-established (Dehpour et al., 2001; Grant & Redmond, 1981; Ozdoğan, Lähdesmäki, & Scheinin, 2003; Svensson, Bunney, & Aghajanian, 1975). Our behavioral data therefore suggest that manipulations to noradrenergic and even widespread central galanin signaling do not affect withdrawal symptoms. Our finding is consistent with a report in rats in which intraventricular infusion of galanin was sufficient to modulate feeding but failed to alter naloxone-precipitated withdrawal symptoms (P. V. Holmes et al., 1994). The lack of galanin effect reported by Holmes and colleagues was originally attributed to possible species-specific differences, or methodological limitations relating to peptide diffusion and/or proteolysis (Zachariou et al., 2003). Yet our study also failed to detect galanin effects using the same species and similar genetic and pharmacological approaches as previous mouse studies, providing an important counterpoint to the existing literature.

Although we designed our studies to align with prior work, methodological differences should be noted. Many precipitated withdrawal protocols exist, each capable of engendering different levels of symptom severity that vary widely by mouse strain (Kest et al., 2002). We did

not use the exact protocol of Zachariou and colleagues (Zachariou et al., 2003), but both studies employed chronic, escalating morphine doses resulting in a high cumulative dose (500 versus 700 mg/kg), and the same naloxone dose (1 mg/kg). Though we evaluated withdrawal in the same NE-Gal OX mice and used C57 BL6/J mice, genetic drift can influence phenotypic differences (Zeldovich, 2017). We also used a broader and slightly older age range for our studies (3-6 months versus 6-12 weeks). Additionally, many withdrawal studies do not include descriptions of behaviors scored, contributing to potential variation in scoring that complicates direct comparison. To that end, we provided our behavioral scoring criteria as a resource. It is possible that these collective differences impaired our ability to detect effects of galanin on opioid withdrawal; if that is the case, it would suggest that galaninergic effects are modest and require narrow experimental parameters.

One prior result we reproduced was upregulation of galanin mRNA in the LC following withdrawal (F. E. Holmes et al., 2012; McClung, Nestler, & Zachariou, 2005), which implies a possible role for this source of the neuropeptide in the context of opioid use disorder, even if not detected in our withdrawal studies. Notably, the present and previous studies focused on the acute effects of galanin on somatic symptoms using precipitated withdrawal models, which are translationally similar to acute opioid detoxification in humans (Welsch et al., 2020). But given the contribution of galanin to stress responses and depression- and anxiety-like behaviors (Kozlovsky et al., 2009; Lang et al., 2015; R. P. Tillage, G. E. Wilson, et al., 2020), future work should explore whether galanin modulates affective withdrawal symptoms that arise over extended periods of time, and can be examined in spontaneous and protracted withdrawal models (Bravo et al., 2020; Welsch et al., 2020). This approach may be crucial for detecting neuropeptide effects, which develop over a longer time scale than classical fast neurotransmitters (Hokfelt et al., 2018).

Spontaneous withdrawal models may also more accurately reflect the human experience, in which withdrawal symptoms emerge over time due to prolonged opioid abstinence (Aronowitz & Laurent, 2016).

In summary, we found that in contrast to galanin mRNA, GalR1 mRNA expression is low in the LC, and is not modulated by chronic morphine or withdrawal. Our results regarding LC GalR1 expression, in combination with behavioral data suggesting NE-Gal does not modulate withdrawal, argue against a mechanism by which acute galaninergic actions in the LC attenuate precipitated somatic withdrawal symptoms. Future work should utilize the molecular findings identified here to probe alternative mechanisms underlying GalR1 effects on LC function, as well as other behavioral aspects of opioid withdrawal.

2.6. FIGURES

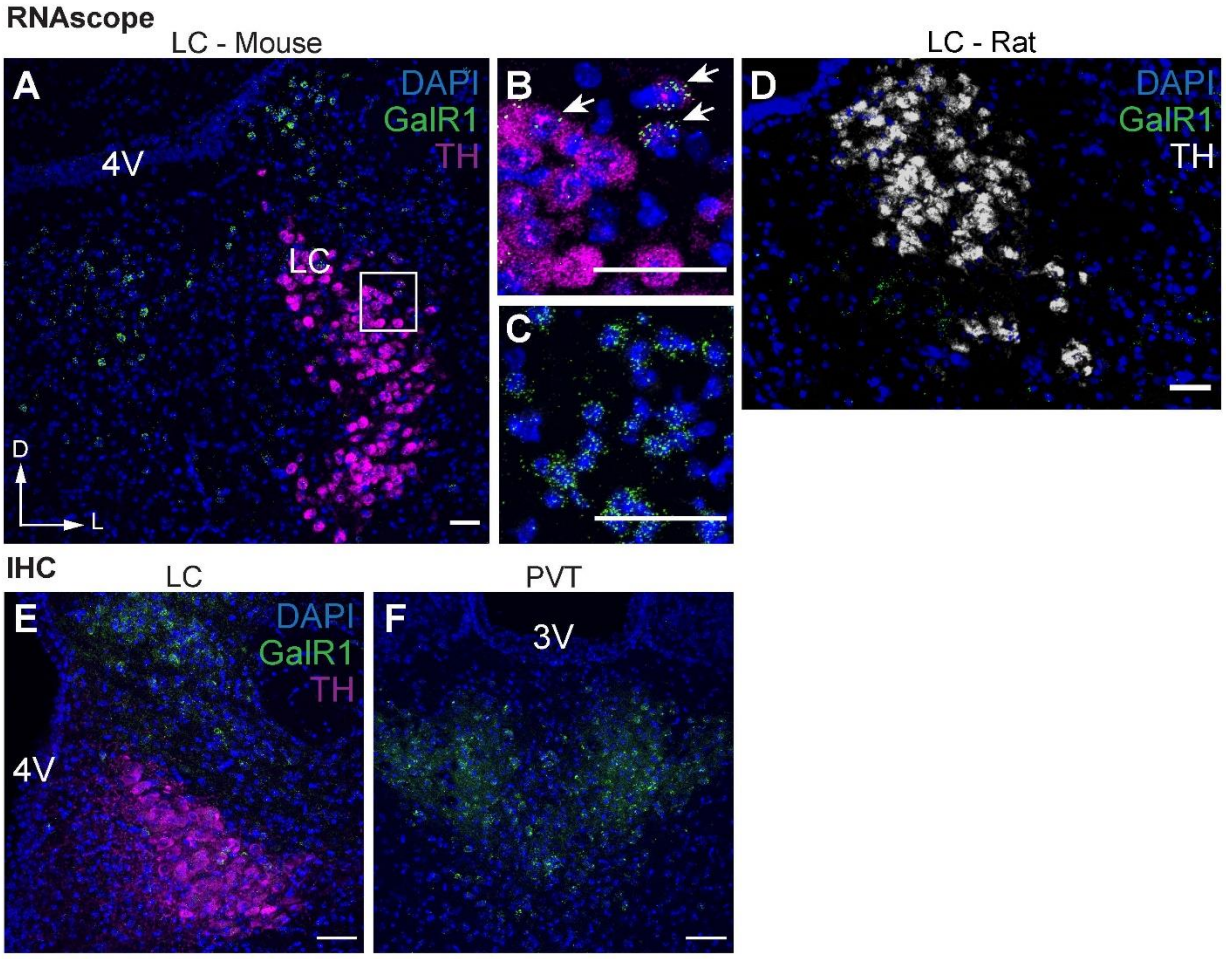

Figure 2.1. GalR1 expression is low in noradrenergic neurons of the LC. RNAscope was performed to identify GalR1 mRNA (GalR1, green) and noradrenergic neurons of the LC defined by tyrosine hydroxylase mRNA (TH, magenta) along with DAPI nuclear stain (blue). A representative image of a coronal mouse brain section shows strong GalR1 expression around, and little within, the LC (**A**). The few TH+ cells expressing GalR1 are observed in the LC periphery (white arrows) in comparison to the LC core (**B**). GalR1 mRNA was also readily observed in control sections of hypothalamus, a region previously shown to strongly express GalR1 mRNA (**C**). RNAscope for GalR1 mRNA (green) in the rat LC (TH, white) shows a similar pattern with

GalR1 primarily outside the LC border (**D**). IHC for mCherry in a GalR1-mCherry mouse line reveals a pattern of GalR1 protein consistent with mRNA findings (**E**), and robust signal as expected in positive control sections containing the paraventricular nucleus of the thalamus (PVT, **F**). All scale bars are 50 µm. Abbreviations: V4 (fourth ventricle); LC (locus coeruleus); D (dorsal); L (lateral); V3 (third ventricle).

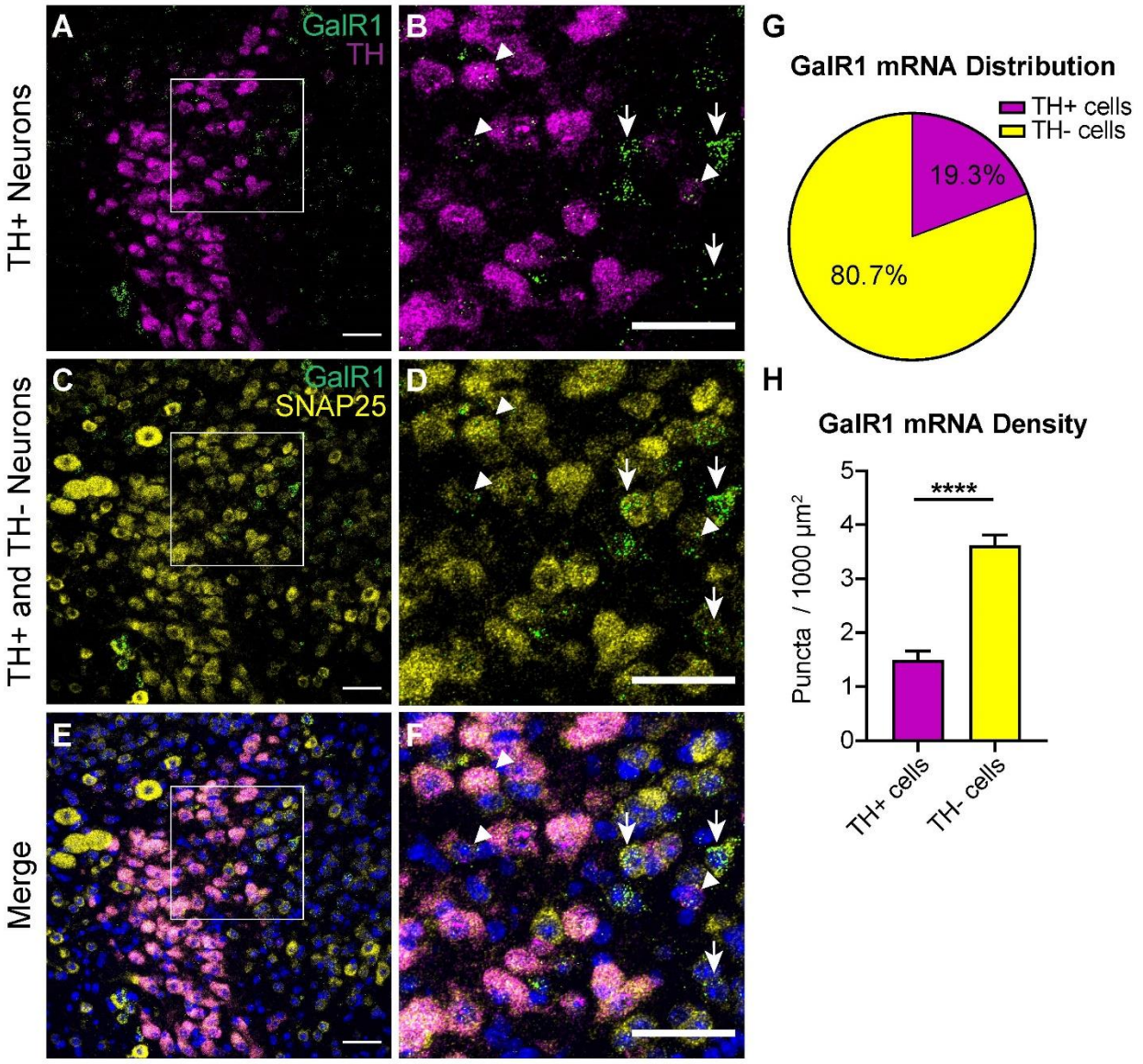

Figure 2.2. Pontine GalR1 mRNA expression is higher in LC-adjacent regions than the LC itself. RNAscope was performed for GalR1 (green) and TH (magenta) in addition to the neuronal marker SNAP25 (yellow) to evaluate GalR1 mRNA expression in TH+ and TH- populations. SNAP25 labels both TH+ LC neurons and TH- neurons in the surrounding field of view (**A,C**). Enlarged images show low GalR1 signal in LC neurons that are both TH+ (**B**, arrowheads) and SNAP25+ (**D**, arrowheads). Higher GalR1 signal can be seen in neurons that are TH- (B, arrows) but SNAP25+ (**D**, arrows). Merged images with DAPI nuclear stain (blue) highlight robust GalR1

signal in SNAP25+ neurons outside the LC (**E,F**). The majority of GalR1 signal in each LC image is contained within TH-, rather than TH+ cells (**G**). Analysis of GalR1 puncta density by cell type shows that within the LC field of view, TH- cells express significantly more GalR1 than TH+ cells (H). n = 6 mice, 3 LC images per mouse. All scale bars are 50 μm. Bar graphs display mean ± SEM. **** $p < 0.0001$.

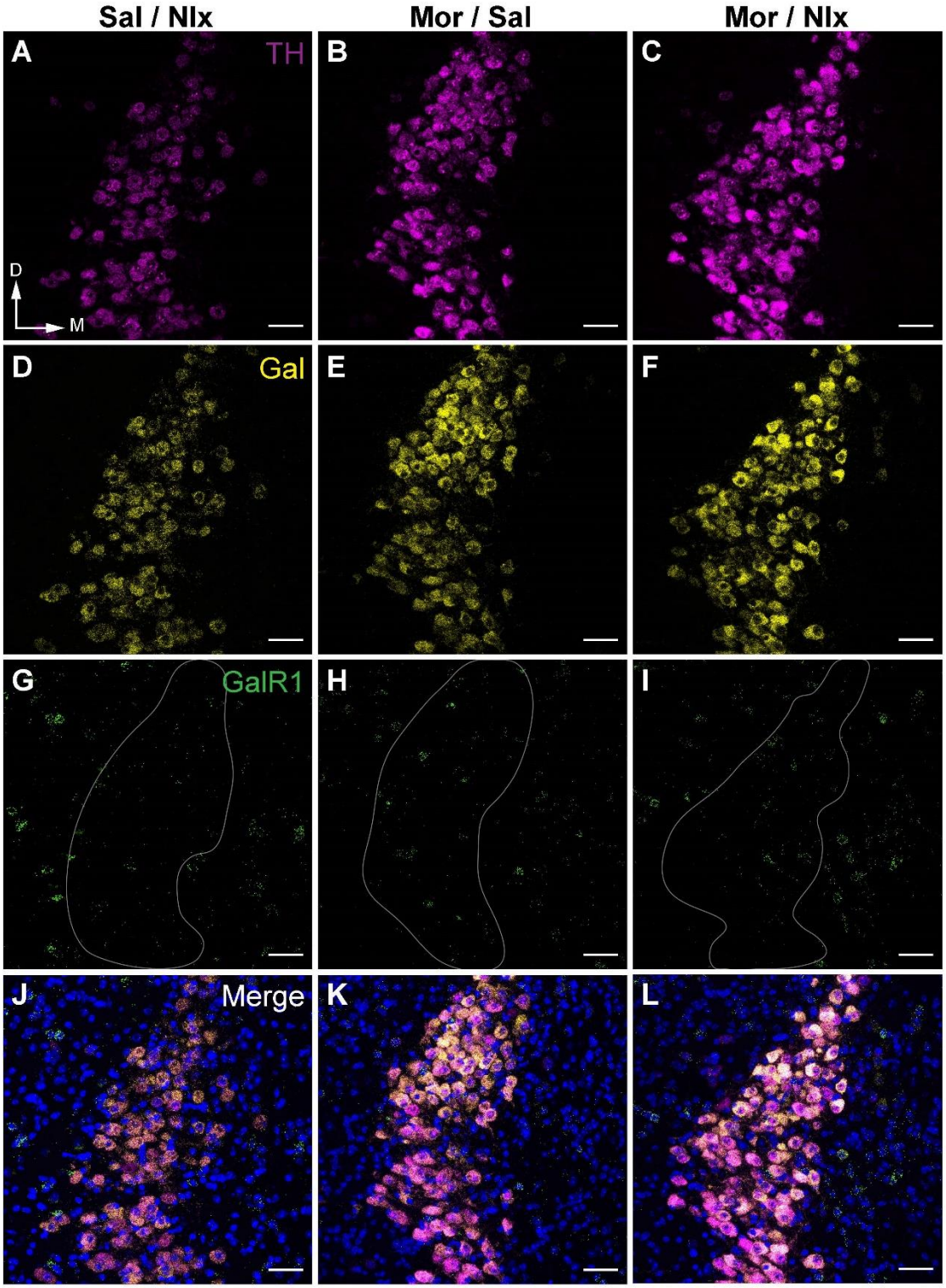

Figure 2.3. Morphine withdrawal increases LC expression of TH and galanin, but not GalR1 mRNA. Representative 40x LC images from mice that received saline injections (Sal / Nlx),

chronic morphine injections (Mor / Sal), or underwent naloxone-precipitated withdrawal following induction of morphine dependence (Mor / Nlx). RNAscope was performed with probes for TH (magenta), galanin (Gal, yellow), and GalR1 (green). Compared to saline treatment, chronic morphine and withdrawal increased TH expression, indicated by elevated fluorescent signal intensity (**A-C**). Withdrawal also increased galanin expression compared to saline treatment (**D-F**). Baseline GalR1 expression was markedly lower than either TH or Gal, and expression was unaffected by chronic morphine or withdrawal (**G-I**, LC outlined in gray). Merged images display all probes with DAPI nuclear stain (blue) (**J-L**). All scale bars are 50 µm. Abbreviations: D (dorsal); M (medial).

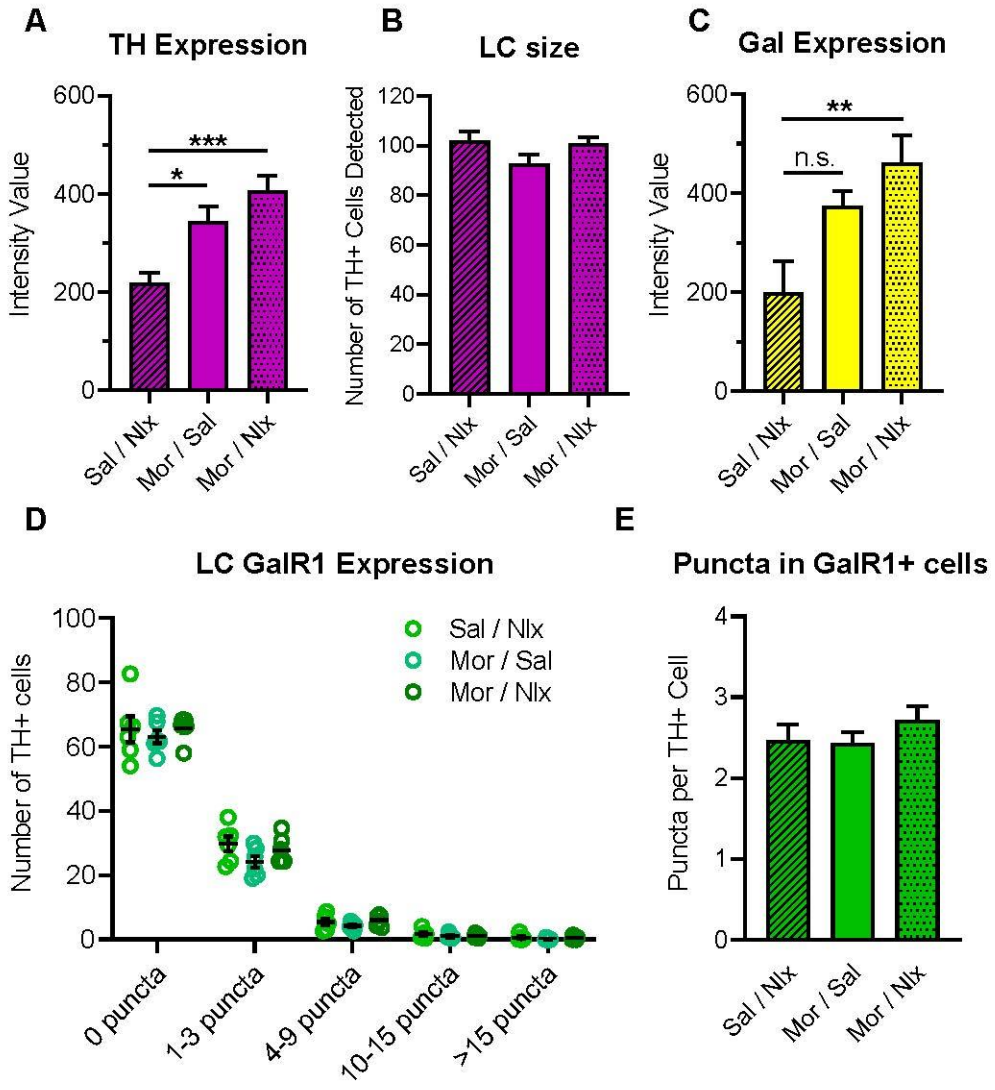

Figure 2.4. Low GalR1 mRNA expression in the LC is unaltered by chronic morphine or withdrawal. Quantification of TH, Gal, and GalR1 mRNA signal in 40x LC images from mice that received saline injections (Sal / Nlx), chronic morphine injections (Mor / Sal), or underwent withdrawal (Mor / Nlx). Withdrawal and chronic morphine increased TH mRNA expression as measured by fluorescence intensity (**A**). There were no differences in the number of TH+ LC cells detected per treatment group, indicating that changes in TH intensity did not affect LC quantification for GalR1 analysis (**B**). Withdrawal increased galanin mRNA expression (**C**).

GalR1 is not expressed in the majority of TH+ LC neurons, and relative proportions of GalR1 expression are not altered by chronic morphine or withdrawal (**D**). Among cells that expressed any GalR1 puncta, treatment group did not affect the average number of GalR1 puncta per cell (**E**). *n* = 6 mice per group, 3 LC images per mouse. All graphs display mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

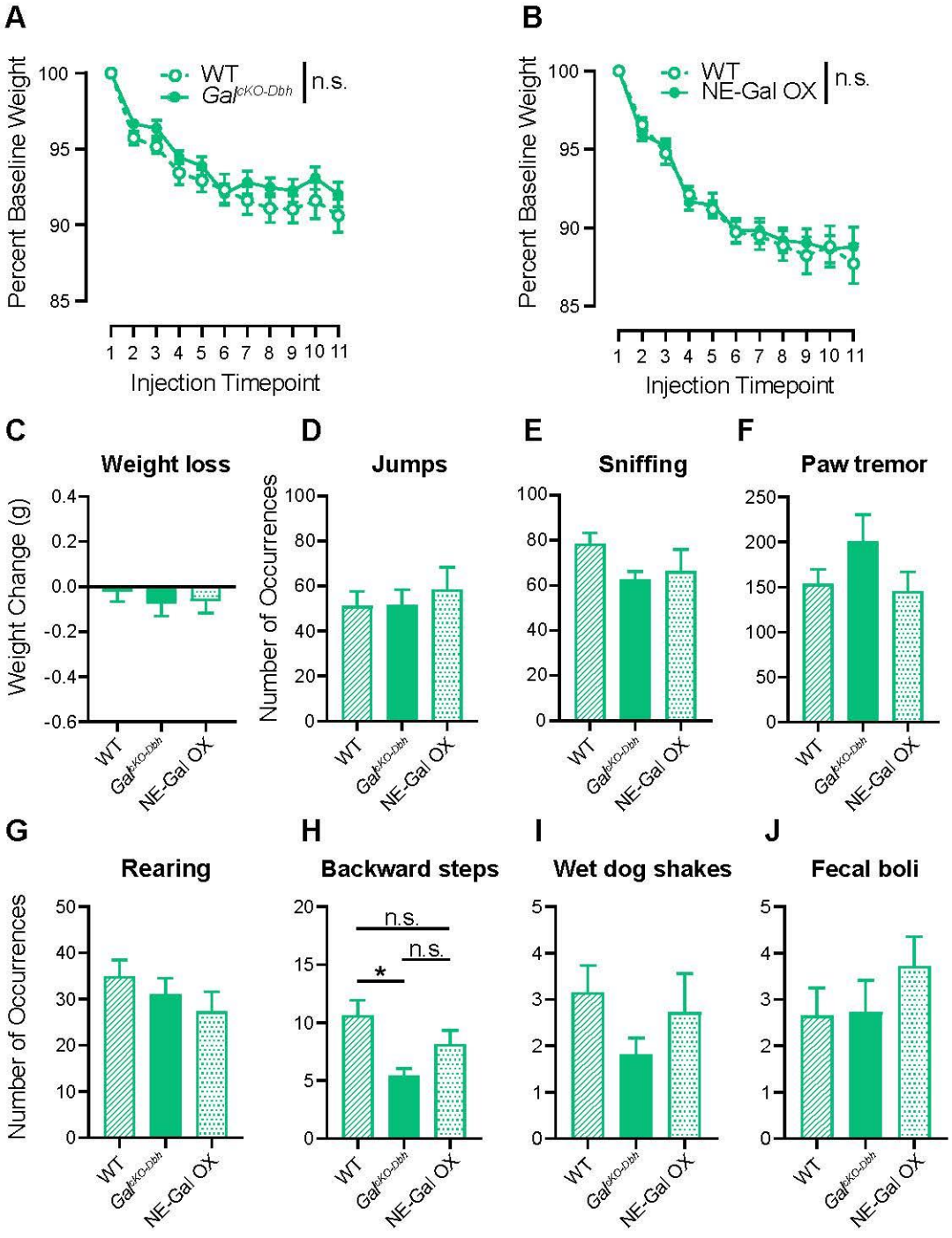

Figure 2.5. Noradrenergic galanin does not modulate precipitated withdrawal symptoms in mice. Noradrenergic-specific galanin knockout mice (*Gal*$^{cKO\text{-}Dbh}$), noradrenergic galanin overexpressor mice (NE-Gal OX), and wild-type littermates (WT) received escalating doses of

morphine twice daily for 5 days (20, 40, 60, 80, 100 mg/kg, i.p.) to induce dependence. On day 6, mice received a final dose of 100 mg/kg morphine and 2 h later underwent naloxone-precipitated withdrawal (1 mg/kg, s.c.). Neither *Gal*$^{cKO\text{-}Dbh}$ nor NE-Gal OX differed from WT littermates in weight lost during the morphine dosing period (**A, B**). *Gal*$^{cKO\text{-}Dbh}$, NE-Gal OX, and WT littermates exhibited similar occurrences of most withdrawal symptoms (**C-G, I, J**). Only backwards steps were significantly different between groups, in which *Gal*$^{cKO\text{-}Dbh}$ exhibited fewer occurrences than WT mice (**H**). n = 10-11 for *Gal*$^{cKO\text{-}Dbh}$ and NE-Gal OX; n = 20 for WT. All graphs display mean ± SEM. * $p < 0$ .05, n.s. = not significant.

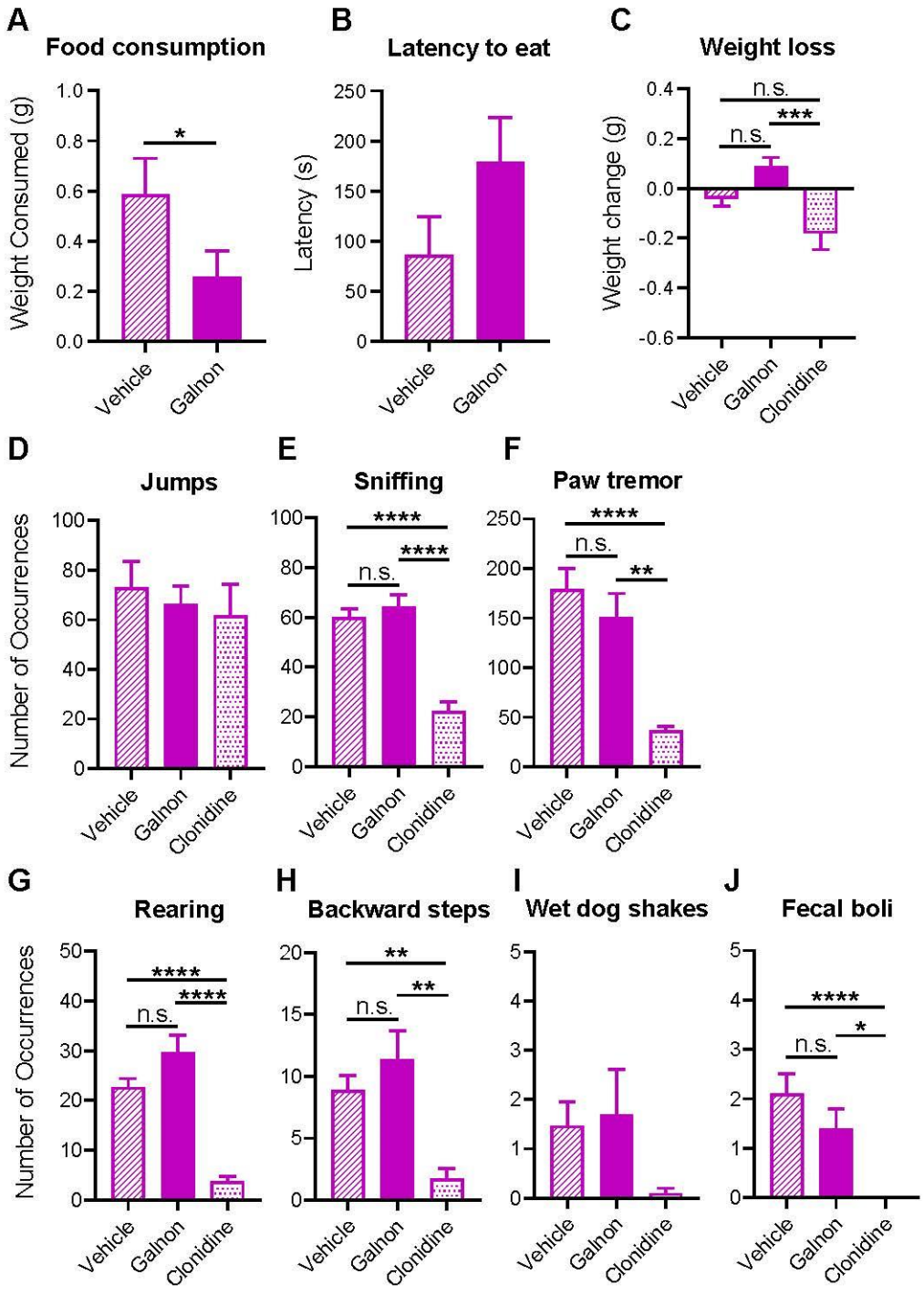

Figure 2.6. Activation of central galanin receptors does not alter withdrawal symptoms. Food-deprived mice were pre-treated with either vehicle or galnon (2 mg/kg, i.p.) prior to a feeding test. Galnon-treated mice consumed less food than vehicle treated mice (**A**), and latency to eat was

greater than vehicle but not significantly different (**B**), indicating that systemic galnon was sufficient to exert central effects on feeding. $n = 7$ per group. A separate cohort was pre-treated with galnon, clonidine (0.3 mg/kg, i.p.), or respective vehicle before naloxone-precipitated withdrawal. Galnon did not affect any symptoms compared to vehicle, while clonidine significantly reduced multiple symptoms compared to either vehicle or galnon treatment (**E-H, J**). Clonidine significantly increased weight loss compared to galnon, but not vehicle (**C**). Jumps and wet dog shakes did not differ from vehicle for either treatment (**D, I**). $n = 10\text{-}11$ for galnon and clonidine groups; $n = 19$ for vehicle. All graphs display mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. = not significant.

CHAPTER 3: NORADRENERGIC GALANIN DOES NOT MODULATE OPIOID REWARD OR REINFORCEMENT

3.1. ABSTRACT

Galanin is a neuropeptide that has been shown to oppose opioid reward and withdrawal, and may be a viable biological target for attenuating the addictive properties of opioids. However, galanin is widely expressed throughout the brain, and it is unclear which source(s) of galanin are responsible for its protective effects. Because the locus coeruleus (LC), the major noradrenergic (NE) nucleus of the brain, strongly expresses galanin and is implicated in opioid use disorder, the noradrenergic galanin may be capable of modulating the rewarding effects of opioids. Here, we sought to examine the role of NE-galanin on opioid reward and reinforcement. Mice that either lack ($Gal^{cKO\text{-}Dbh}$) or overexpress (NE-Gal OX) NE-galanin and their wild-type (WT) littermates were compared using acute morphine-induced locomotion and conditioned place preference (CPP) assays. Intravenous self-administration of the synthetic opioid remifentanil was also examined in $Gal^{cKO\text{-}Dbh}$ and WT mice. We found that neither increasing nor decreasing levels of NE-galanin altered locomotor or CPP behaviors elicited by morphine compared to controls. Additionally, loss of NE-galanin did not alter remifentanil self-administration behaviors. These findings suggest that NE-galanin does not robustly contribute to opioid reward or reinforcement.

3.2. INTRODUCTION

Opioid use disorder (OUD) is a complex neurological disease that typically develops over time as initial opioid misuse escalates to abuse (Bonar et al., 2020). As with many other progressive conditions, early intervention can improve long-term outcomes, and curbing problematic opioid use early on can reduce the likelihood of developing OUD (Substance, Mental Health Services, & Office of the Surgeon, 2016). Therefore, there is a critical need to identify neurobiological systems that can be targeted to suppress the rewarding and addictive effects of opioids. The development of therapies that target these systems could greatly reduce the number of people misusing opioids, as well as relapse rates among those with OUD.

The neuropeptide galanin has been proposed as therapeutic target for OUD because it is part of an endogenous system that opposes the behavioral effects of opioids in rodent models (Lang et al., 2015; Picciotto, 2008). Intraventricular administration of galanin attenuates morphine conditioned place preference (CPP) (Zachariou, Parikh, & Picciotto, 1999). Conversely, reducing galanin signaling increases susceptibility to opioid reward. Galanin knockout mice show exaggerated morphine-induced locomotor activity and CPP, and importantly, locomotion can be normalized with acute administration of the galanin receptor agonist, galnon (Hawes et al., 2008). Human studies have identified gene variants of galanin and its receptor, GalR1, that are associated with increased susceptibility for opioid and nicotine use disorders (Beer et al., 2013; Gold et al., 2012; Levran et al., 2008; Lori et al., 2011), implicating the galaninergic system in addiction. However, galanin is co-expressed in several neurotransmitter systems (Cheung et al., 2001; Skofitsch & Jacobowitz, 1985), and it is unclear whether the protective effects of galanin against opioids can be attributed to a particular source of this neuropeptide. The development of galanin-based therapies will require an understanding of the neuroanatomical and neurochemical substrates

that mediate the protective effects of galanin. Therefore, it is critical to identify the specific sources of galanin that oppose opioid-induced behaviors.

The noradrenergic (NE) system, specifically its major nucleus, the locus coeruleus (LC), is potentially a major source of protective galanin. The neural circuitry underlying drug reward and reinforcement has classically implicated the mesolimbic system, which consists of dopaminergic neurons of the ventral tegmental area (VTA) and their projections to the nucleus accumbens (Di Chiara & Imperato, 1988). Interestingly, the VTA receives projections from the LC, which is involved in substance use disorders and strongly expresses galanin (Alhadeff, Rupprecht, & Hayes, 2012; Cheung et al., 2001; Mazei-Robison & Nestler, 2012; R. P. Tillage, N. R. Sciolino, et al., 2020; Weinshenker & Holmes, 2016). Therefore, it is possible that the NE system provides a functionally important source of galanin to the VTA, capable of suppressing opioid reward. Indeed, the link between galanin, the LC, and opioids motivated a previous study examining the role of NE-galanin on withdrawal (Zachariou et al., 2003). However, no study has yet examined the specific role of NE-galanin on opioid reward.

In addition to a lack of system-specific studies on galanin and opioid reward, there is also shockingly little information regarding the effects of galanin on opioid self-administration. In contrast to methods like CPP, which employ experimenter-administered drugs, self-administration approaches involve voluntary drug intake by the animals themselves, serving as a more translationally relevant model of human drug use (Spanagel, 2017). So far, only one study has examined the role of galaninergic system in opioid reinforcement using operant self-administration (Scheller et al., 2017), and it is not yet clear how specific sources of galanin might modulate opioid self-administration patterns. Additionally, virtually all studies examining galanin have used the natural opioid, morphine. Given that synthetic opioids are the leading cause of opioid overdose

deaths in the U.S. (Wilson et al., 2020), we sought to study the effects of NE-galanin on intravenous self-administration (IVSA) behaviors using the synthetic opioid remifentanil.

In this report, we examined how manipulation of NE-galanin influenced performance in behavioral assays where galanin has previously been shown to modulate the effects of opioids. We used genetically altered mouse lines that either lack ($Gal^{cKO\text{-}Dbh}$) or overexpress (NE-Gal OX) NE-galanin, and compared their behavioral responses to wild-type littermates (WT) in acute morphine-induced locomotion and morphine CPP. We also examined whether NE-galanin modulates opioid reinforcement by performing IVSA studies in both mouse lines using the synthetic opioid, remifentanil.

3.3. MATERIALS AND METHODS

Animals

The following experiments utilized two genetically manipulated mouse lines, $Gal^{cKO\text{-}Dbh}$ and NE-Gal OX, both of which are on a C57BL/6 background and have been previously published (Steiner et al., 2001; R. P. Tillage, N. R. Sciolino, et al., 2020). All experimental cohorts were comprised of male and female mice between 3-8 months old. Mice were group housed in a temperature-controlled room with a 12h light/dark cycle (07:00 on / 19:00 off) with food and water available *ad libitum* for locomotor and CPP studies. For IVSA, mice were under a reverse-light cycle (07:00 off / 19:00 on) and were housed individually, with food access dependent upon the experiment (see below). Locomotor and CPP assays were performed during the light cycle, while self-administration was performed during the dark cycle. All procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee.

Drugs

Morphine sulfate and remifentanil hydrochloride were obtained from the NIDA Drug Supply Program. Morphine was dissolved in normal saline and was delivered by intraperitoneal injection in a volume of 10 ml/kg. For IVSA studies, stock solutions of remifentanil (1920 μg/ml) were prepared by dissolving the drug in normal saline and freezing aliquots at -20 C. Aliquots were thawed and serially diluted with normal saline each day according to the dose being administered in that day's session.

Acute Morphine-Induced Locomotion

Dose response curves of acute morphine-induced locomotor activity were generated for $Gal^{cKO\text{-}Dbh}$, NE-Gal OX, and respective WT littermate controls over five weeks of testing. The locomotor apparatus consisted of a polycarbonate chamber (22 x 43 x 22 cm) placed inside a grid of infrared beams, where two consecutive beam breaks by the mouse were recorded in the computer software as one ambulation (San Diego Instruments, San Diego, CA). Ambulations were recorded in 5-min bins throughout each test session. On each test day, mice were individually habituated to chambers for 30 min, then given an intraperitoneal injection of either saline or 5, 10, 20, or 40 mg/kg morphine. Ambulations were monitored for an additional 120 min following injection, after which mice were removed from the chambers and returned to their home cages. Mice were given a week between test days to prevent sensitization, and the order of morphine doses was counterbalanced across sex and genotype.

Morphine Conditioned Place Preference

An unbiased CPP procedure was conducted using an 8-day paradigm as others have previously described (Gaspari et al., 2017). On the first day, a 20-min pre-test was performed in which the mouse could move freely throughout a three-chambered apparatus consisting of two contextually distinct side chambers and a neutral middle section. Animals exhibiting a strong side

bias at pre-test (defined as a difference in time spent between the two sides exceeding 150 s) were excluded from the study. Each mouse was then assigned a "drug-paired" side and a "saline-paired" side of the chamber, and assignments were counterbalanced across groups. Mice were also assigned to a saline or morphine treatment group; saline mice received saline injections on both the saline- and drug-paired side as a control condition, whereas morphine mice received saline on the saline-paired side, and morphine on the drug-paired side. For the next six days, mice received one 45-minute conditioning session per day in which they were alternately confined to the drug-paired side or saline-paired side after receiving an intraperitoneal injection of morphine (5, 10, or 20 mg/kg depending on the experiment) or saline. On the last day, a 20-min post-test was administered as on the pre-test day. Preference scores were determined by calculating the difference in time spent on the drug-paired side minus the saline-paired side for each test day. Changes in preference were determined by subtracting preference score at pre-test from the post-test value.

The morphine doses used for CPP experiments with Gal$^{cKO\text{-}Dbh}$ mice and NE-Gal OX mice were chosen based on anticipated genotype effects. Based on the literature demonstrating that galanin opposes opioid reward (Zachariou, Parikh, & Picciotto, 1999), Gal$^{cKO\text{-}Dbh}$ mice were predicted to be more sensitive to opioid reward than WT, while NE-Gal OX were predicted to be comparatively resistant. Gal$^{cKO\text{-}Dbh}$ mice were therefore tested at a dose of morphine that did not elicit a CPP in pilot studies with WT mice (5 mg/kg) in order to detect a possible increase in preference. A high dose of morphine (20 mg/kg) was also tested as a point of comparison, as this dose induces a robust CPP and elicited the most activity in our locomotor studies. NE-Gal OX mice were predicted to exhibit an attenuated CPP response, so a moderate dose of 10 mg/kg morphine was selected in order to detect a potential decrease in CPP in comparison to WT.

Jugular Vein Catheterization Surgery

For self-administration studies, mice underwent surgical catheterization of the right jugular vein. Gal$^{cKO\text{-}Dbh}$, NE-Gal OX, and WT mice were anesthetized via intraperitoneal injection of a ketamine : dexmedetomidine mixture (80 mg/kg : 0.5 mg/kg, Patterson Veterinary, Greeley, CO), and meloxicam (5 mg/kg, Patterson Veterinary, Greeley, CO) was administered subcutaneously to provide analgesic relief. Once mice reached the surgical plane, the dorsal subscapular region and anterior aspect of the right-side of the neck were cleaned. A horizontal incision was made low on the back, and a biopsy punch was used to make an opening superior to the incision. A catheter made in-house was inserted through the horizontal incision, and the cannula guide was externalized through the superior opening. The mouse was placed on its side, and a 1 cm incision was made on the right side of the neck, superficial to the jugular pulse. Catheter tubing was routed subcutaneously from the dorsum over the scapula and through neck incision. The jugular vein was isolated by dissecting surrounding tissue, and forceps were placed under the vein to keep it taut and isolated. Using microscissors, a small hole was cut in the jugular vein, and 1 cm of the catheter tubing was gently inserted into the vein up to a silicone bead. The catheter tubing was sutured to the vein, and the neck incision was sutured closed. The mouse was then placed in a prone position and the horizontal incision on the dorsum was sutured. Atipamezole (1 mg/kg, Patterson Veterinary, Greeley, CO) was administered subcutaneously to reverse anesthetic effects, and animals were returned to clean, individual cages on a heating pad to recover.

Mice remained single-housed for the remainder of the study and received daily meloxicam (5 mg/kg s.c.) for the first 3 days post-surgery. Mice began IVSA studies approximately one week post-surgery. Catheters were maintained by flushing daily with 0.03 - 0.05 ml each of gentamicin (4 mg/ml, Patterson Veterinary, Greeley, CO) and heparinized saline (30 USP, Patterson

Veterinary, Greeley, CO). Catheter patency was assessed by flushing 0.03 ml of ketamine (15 mg/ml, Patterson Veterinary, Greeley, CO) and observing a corresponding loss of muscle tone. Patency tests were performed prior to the start of self-administration, periodically throughout the study (no sooner than 2 h following a session), at the end of the study, and on an individual basis if session responses seemed aberrant.

Intravenous Self-Administration of Remifentanil

To comprehensively evaluate the potential impact of noradrenergic manipulations on opioid-mediated behaviors, we compared behavioral responses of the Gal$^{cKO\text{-}Dbh}$ and NE-Gal OX mice against WT mice in an IVSA assay using the rapidly acting and highly potent synthetic opioid, remifentanil (A. S. James et al., 2013; Porter-Stransky, Bentzley, & Aston-Jones, 2017). In order to determine the best dose to examine remifentanil IVSA acquisition in Gal$^{cKO\text{-}Dbh}$ and NE-Gal OX mice, we first had to characterize the remifentanil dose response curve in WT C57BL/6J mice.

Experiment 1: Characterization of remifentanil dose response curve.

Prior to surgery, freely-fed mice were trained to nose poke for palatable 20 mg chocolate-flavored food pellets (Bio-Serv, Flemington, NJ) in operant conditioning chambers containing two nose poke apertures and a food delivery magazine (Med Associates, Fairfax, VT). Mice were first exposed to the pellets in the home cage to limit neophobic responses. Mice then underwent 4 consecutive days of fixed-ratio (FR) 1 food training, in which responses on the active nose poke resulted in delivery of one pellet, followed by a 5 s timeout during which the house light was extinguished and a light in the active nose poke was illuminated. Responses on the inactive nose poke were also recorded but had no consequence. The active nose poke side (right or left aperture)

was counterbalanced across subjects. Sessions terminated after 30 reinforcers were earned or 3 h elapsed, whichever occurred first.

After recovering from surgery, mice began IVSA. Mice were re-introduced to operant conditioning chambers and the externalized guide of the catheter was connected to polyethylene tubing attached to a syringe containing remifentanil. Responses on the active nose poke now triggered a syringe pump to administer an intravenous infusion of remifentanil (~10 µl per infusion, depending on body weight). Sessions were run at FR1 with 10 s timeouts, during which the house light was extinguished and the active nose poke illuminated. Sessions were terminated after 100 infusions were earned or 1 h elapsed, whichever occurred first. Mice first acquired IVSA at a dose of 64 µg/kg/inf, and were transitioned into the dose response component of the experiment after meeting the following criteria: 1) self-administering remifentanil $\geq$ 5 days, 2) selectivity for active nose poke was $\geq$ 75 percent for the past 3 days, and 3) variability in response rate $\geq$ 30 percent for the past 3 days. Five remifentanil doses were then tested in a pseudorandomized order: 6.4, 32, 64, 320, and 640 µg/kg/inf. To ensure that operant behavioral responses were reliable at each dose, mice were not transitioned to a new dose until response rates for the current dose varied by less than 30 percent from the previous day.

Experiment 2: Effect of NE-galanin on intravenous remifentanil self-administration. The role of NE-galanin on opioid reinforcement was assessed by comparing acquisition of remifentanil IVSA in Gal$^{cKO\text{-}Dbh}$, NE-Gal OX, and WT littermates at a dose of 320 µg/kg, the peak of the WT dose-response curve. After recovering to pre-surgical body weights, mice were food-restricted to ~ 90% of their free-feeding weight. Mice then began intravenous self-administration in operant chambers containing two nose poke apertures. Responses on the active nose poke resulted in delivery of an intravenous infusion of remifentanil (320 µg/kg) accompanied by a 10 s timeout,

extinction of the house light, and illumination of the active nose poke; responses on the inactive nose poke were recorded but had no programmed consequences. Mice underwent daily FR1 sessions which terminated at 1 h or after delivery of 100 infusions, whichever occurred first. Mice were allowed to self-administer remifentanil for 13 days.

A subset of Gal$^{cKO\text{-}Dbh}$ and WT mice also underwent a progressive ratio (PR) test followed by extinction and cue-induced reinstatement. For the PR test, the session started on a schedule of FR1 and increased with each successive infusion: 1, 3, 6, 9, 12, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. (Suto et al., 2002). Each infusion earned was accompanied by the same 10 s timeout as in acquisition, and sessions terminated after 3.5 h had elapsed in total or more than 30 min had elapsed since the last infusion was earned. Breakpoints were recorded as the highest FR schedule attained before session termination. Following the PR test, mice underwent daily 1 h extinction sessions where responding on the active nose poke had no programmed consequences. Mice were required to meet the following extinction criteria before undergoing reinstatement: 1) $\geq$ 5 extinction sessions and 2) at least 3 consecutive days where active responses were < 50 percent of the average active response over the last 3 days of acquisition. Cue-induced reinstatement, 1 h in duration, was performed by presenting the mouse with a 5 s visual cue previously paired with remifentanil delivery (illumination of the active nose poke), after which nose poke responses were recorded but had no programmed consequence.

Statistical Analysis

Morphine-induced locomotor dose response curves between the experimental strain (Gal$^{cKO\text{-}Dbh}$ or NE-Gal OX) and respective WT controls were compared by repeated measures two-way ANOVA (dose x genotype). Morphine CPP data were analyzed by two-way ANOVA (dose x genotype), followed by Tukey's post-hoc tests where appropriate. To account for data points lost

from catheter occlusions, IVSA data were analyzed using a mixed-effects model with Tukey's post-hoc test where appropriate.

3.4. RESULTS

Acute morphine-induced locomotion is not altered by noradrenergic galanin

A repeated measures two-way ANOVA (dose x genotype) of dose response curves for *Gal*$^{cKO\text{-}Dbh}$ mice and WT littermates revealed a significant effect of dose ($F_{2.4,\ 53.4} = 14.79$, $p < 0.0001$) but not genotype ($F_{1,\ 22} = 0.002250$, $p = 0.9626$) or a dose x genotype interaction ($F_{4,\ 88} = 0.5612$, $p = 0.6914$) (**Fig. 3.1. C**). Likewise, the same analysis for NE-Gal OX mice and their WT littermates showed a significant effect of dose ($F_{2.6,\ 54.6} = 27.26$, $p < 0.0001$) but not genotype ($F_{1,\ 21} = 2.007$, $p = 0.1712$) or interaction ($F_{4,\ 84} = 0.9338$, $p = 0.4485$) (**Fig. 3.2. C**). These results indicate that noradrenergic galanin levels to not impact the acute locomotor-activating effects of morphine at the doses tested.

Noradrenergic galanin does not modulate opioid reward

Previous work indicates that the locomotor-activating and reward-producing effects of drugs of abuse occur through partially overlapping but distinct circuits (Runegaard et al., 2018). Therefore, we also sought to compare these two mouse lines using morphine CPP, an assay thought to reflect opioid reward. A two-way ANOVA (dose x genotype) of morphine CPP data for *Gal*$^{cKO\text{-}Dbh}$ mice and their WT littermates showed a main effect of dose ($F_{2,\ 58} = 10.78$, $p = 0.0001$), but not genotype ($F_{1,\ 58} = 0.1248$, $p = 0.7252$) or dose x genotype interaction ($F_{2,58} = 0.0574$, $p = 0.9442$) (**Fig. 3.3. A)**. Tukey's multiple comparisons test showed that the 20 mg/kg morphine dose elicited a CPP compared to saline (0 mg/kg) for each genotype (WT $p = 0.0098$; *Gal*$^{cKO\text{-}Dbh}$ p = 0.0450). However, comparison of the 5 mg/kg and 0 mg/kg doses did not reveal a significant difference between doses in either genotype (WT $p = 0.6959$; *Gal*$^{cKO\text{-}Dbh}$ $p = 0.9254$). Therefore,

20 mg/kg morphine induced a significant morphine CPP in both genotypes as expected, and the sub-threshold dose, 5 mg/kg, did not induce a CPP in either genotype. These data indicate that loss of NE-galanin does not increase sensitivity to opioid reward at the doses tested.

NE-Gal OX mice were also evaluated by CPP using a moderate dose of 10 mg/kg morphine. A two-way ANOVA (dose x genotype) showed a main effect of dose ($F_{1, 46} = 6.498$, $p = 0.0142$), but no effect of genotype ($F_{1, 46} = 0.1656$, $p = 0.6859$) or interaction ($F_{1, 46} = 0.0828$, $p = 0.7748$) (**Fig. 3.3. B**). Post-hoc comparisons between saline (0 mg/kg) and 10 mg/kg morphine were not significant by Tukey's multiple comparisons test. Therefore, 10 mg/kg morphine did not induce a statistically significant CPP in either genotype. Overall, CPP findings indicate that bi-directional manipulation of NE-galanin levels do not appreciably impact opioid reward.

Noradrenergic galanin levels do not influence intravenous self-administration of the opioid remifentanil

Prior to examining the effects of NE-galanin on self-administration behavior, the remifentanil IVSA dose response curve needed to be evaluated in WT mice. Because the background strain of the $Gal^{cKO\text{-}Dbh}$ and NE-Gal OX lines was C57BL/6, this strain was chosen for dose-response studies. Analysis of active/inactive responses using a mixed-effects model demonstrated main effects of response type ($F_{1, 18} = 231.2$, $p < 0.0001$), time ($F_{1.6, 27.65} = 7.83$, $p = 0.0035$), and a response type x time interaction ($F_{3, 52} = 8.879$, $p < 0.0001$), with active and inactive responses being significantly different at all time points by Tukey's multiple comparisons test. (**Fig. 3.4. B**). These data indicate that mice readily learned to nose poke for a reinforcer as early as the first training session. Following catheterization, IVSA acquisition responses demonstrated the potent and highly reinforcing effects of remifentanil. A mixed-effects analysis of active/inactive responses showed a main effect of response type ($F_{1, 18} = 54.47$, $p < 0.0001$), but

not time ($F_{2.52, 39.44}$ = 1.677, p = 0.1939) or interaction ($F_{6, 94}$ = 1.944, p = 0.0816). Tukey's post-hoc tests indicated that active responses were significantly higher than inactive responses at all time points. In addition, mice readily acquired remifentanil IVSA at 64 μg/kg, with the cohort averaging 33.4 ± 7.5 infusions during the first IVSA session and consistently showing high nose poke selectivity. (**Fig. 3.4. C,D**). The ability of remifentanil to support behavior at many doses was also reflected in the dose response curve, which was fairly flat across the 100-fold difference in the lowest and highest doses tested (**Fig. 3.4. E**). A slight peak in infusion number was observed at the 320 ug/kg dose (52.7 ± 6.5 inf/session) and a modest reduction in infusion number was observed at the 640 ug/kg dose (37 ± 6.2) compared to the other three doses (6.4 ug/kg: 43.2 ± 8.7; 32 ug/kg: 47.3 ± 8; 64 ug/kg: 46.2 ± 6.1). Therefore, the 320 ug/kg dose was considered maximally reinforcing and was selected for the subsequent studies with Gal$^{cKO\text{-}Dbh}$ and NE-Gal OX mice.

A separate cohort of Gal$^{cKO\text{-}Dbh}$, NE-Gal OX, and respective WT littermates self-administered remifentanil (320 ug/kg/inf) over a 13-day period. A mixed-effects analysis of infusions per session for Gal$^{cKO\text{-}Dbh}$ and WT mice indicated a main effect of time ($F_{3.2, 25.6}$ = 23.60, p < 0.0001), but not genotype ($F_{1, 8}$ = 0.03736, p = 0.8516) or interaction ($F_{12, 95}$ = 0.4333, p = 0.9462) (**Fig. 3.5. A**). Similarly, infusion data from NE-Gal OX mice also showed an effect of time ($F_{2.6, 19.8}$ = 18.08, p < 0.0001), but not genotype ($F_{1, 10}$ = 0.9707, p = 0.3477) or interaction ($F_{12, 90}$ = 1.732, p = 0.0728) (**Fig. 3.5. B**). Individual traces of IVSA activity demonstrate that there is appreciable variability within and between genotypes, and overall no obvious differences in remifentanil intake (**Fig. 3.5. C,D**). A mixed effects analysis of active and inactive response data showed main effects of time ($F_{3.93, 62.2}$ = 13.39, p < 0.0001), and response type ($F_{3, 16}$ = 38.88, p < 0.0001), and a time x response type interaction ($F_{36, 190}$ = 6.167, p < 0.0001). However, Tukey's multiple comparisons test did not reveal any differences between genotypes among active or

inactive responses at any time point (**Fig. 3.5. E**). The corresponding analysis of NE-Gal OX responses also indicated main effects of time ($F_{2.31, 36.54} = 6.098$, $p = 0.0037$), and response type ($F_{3, 20} = 22.38$, $p < 0.0001$), and a time x response type interaction ($F_{36, 190} = 2.587$, $p < 0.0001$). (**Fig. 3.5. F**). Again, Tukey's post-hoc comparison did not identify any time points where active and inactive responses differed by genotype. Together, these data indicate that NE-galanin levels do not impact the acquisition of remifentanil IVSA at the 320 ug/kg dose.

Preliminary progressive ratio, extinction, and cue-induced reinstatement data were also collected in Gal$^{cKO\text{-}Dbh}$ and WT mice, but due to small sample sizes, were not sufficiently powered for statistical analysis (**Fig. 3.6.**). Qualitative overview of these data suggest that Gal$^{cKO\text{-}Dbh}$ mice might exhibit a higher breakpoint ratio for remifentanil, as well as higher initial responding during extinction sessions which normalized over time. Cue-induced reinstatement responses appeared similar between genotypes, but ultimately future studies will be needed to more fully assess the impact of NE-galanin on these more complex self-administration behaviors.

3.5. DISCUSSION

Several studies have examined how brain- or body-wide manipulations of galanin affect opioid reward, but no study has yet investigated the role of specific sources of galanin. This study assessed the impact of NE-galanin on both opioid reward and reinforcement. Using genetically engineered mouse lines to deplete or overexpress noradrenergic galanin, we found that manipulation of NE-galanin levels did not alter acute morphine-induced locomotion or morphine CPP compared to WT controls, nor did it affect the reinforcing properties of the synthetic opioid remifentanil in an IVSA model. To our knowledge, this is the first study to directly assess the role of a specific source of galanin in opioid reward and reinforcement.

Previous studies using conventional galanin knockout mice reported enhanced morphine-induced locomotion compared to WT mice at 5, 10, and 20 mg/kg doses (Hawes et al., 2008). We found no increase in activity of Gal$^{cKO\text{-}Dbh}$ mice at any of these doses. It is important to note that the conventional galanin knockout mice were on a 129OlaHsd background, whereas our mice were on a C57 BL/6 background. Given that these two strains show different sensitivity to the effects of opioids (Kest et al., 2002), strain differences likely influenced our findings. Nevertheless, the range of morphine doses tested included a sub-threshold dose (5 mg/kg) and a highly activating dose (40 mg/kg), and at no point did locomotion differ significantly between Gal$^{cKO\text{-}Dbh}$ mice and WT littermates. Therefore, it appears that while complete loss of galanin increases sensitivity to the locomotor activating effects opioids, the partial loss of galanin signaling, induced by our NE-specific depletion, is not sufficient to do so. We also evaluated this behavior in NE-Gal OX mice, and similarly observed no difference in locomotor activity compared to WT, suggesting that neither increasing or decreasing NE-galanin levels affects acute locomotor responses to morphine.

To better characterize the effects of NE-galanin on opioid reward, we also performed morphine CPP. Morphine dose is important to consider when interpreting these data. Previous work by our lab and others showed that 5 mg/kg is the lowest dose of morphine that can elicit a CPP in C57BL/6 mice (Porter-Stransky et al., 2020; Zachariou, Parikh, & Picciotto, 1999). However, pilot studies for these experiments indicated that 5 mg/kg morphine was a "sub-threshold" dose that could not consistently elicit a place preference, and additionally did not increase locomotion. Given that Gal$^{cKO\text{-}Dbh}$ mice were expected to be more sensitive to the effects of opioids, we predicted that we would detect a CPP in the Gal$^{cKO\text{-}Dbh}$, but not WT, at 5 mg/kg morphine. However, we observed no genotype differences. Notably, the previous study that examined morphine CPP in conventional galanin knockout mice only showed a modest phenotype

at a very low morphine dose, 0.25 mg/kg (Hawes et al., 2008). Again, these contrasting findings may reflect strain differences between 129OlaHsd and C57Bl/6 mice. Given that the Gal$^{cKO\text{-}Dbh}$ mice exhibit a less substantial loss of galanin compared to the full knockout mice, they may have enough central galanin to maintain a morphine CPP response similar to that of WT mice. In the case of the NE-Gal OX mice, we anticipated that they would exhibit an attenuated response to morphine and would therefore fail to develop a CPP to 10 mg/kg morphine, while WT mice would. Interestingly, neither group developed a CPP with this dose, due to apparently variability between subjects. While our data suggest that overexpression of NE-galanin is insufficient to modulate opioid reward, future studies will need to confirm this finding at a higher dose of morphine that reliably produces a CPP in WT mice. If there is still no genotype difference, this result could reflect that galanin receptors are already maximally engaged to the point that peptide overexpression provides no added benefit, or that increased galanin expression might not translate into increased galanin release. At present, it appears that bidirectional manipulation of NE-galanin levels does not critically influence opioid reward.

We also sought to determine how NE-galanin modulates opioid reinforcement, and again did not observe any difference in acquisition or maintenance of remifentanil IVSA between Gal$^{cKO\text{-}Dbh}$ or NE-Gal OX mice and their WT controls. Based on our findings, we would conclude that manipulation of NE-galanin is not sufficient to alter opioid self-administration behaviors. However, it should be noted that these studies used a single, maximally reinforcing dose of remifentanil (320 μg/kg), as determined by our remifentanil dose response study performed in C57BL/6J mice. While technically demanding, future studies could further characterize genotype differences in remifentanil reinforcement by performing the same dose response studies in the Gal$^{cKO\text{-}Dbh}$ or NE-Gal OX mice. It is possible that such an approach might capture shifts in the dose

response curve that could not be detected with our single-dose approach. To date, only one other paper has examined how the galaninergic system affects opioid IVSA. Scheller and colleagues found that systemic pre-treatment with the GalR3 antagonist SNAP 37889 acutely reduced FR1 responding for morphine (Scheller et al., 2017). Interestingly, it is the first study to suggests that blocking, rather than activating, galanin receptor signaling opposes the behavioral effects of opioids. SNAP 37889 shows high selectivity for GalR3 over GalR1 or GalR2, and little cross-reactivity with other GPCRs (over 100-fold difference) (Swanson et al., 2005). It should be noted though, that these findings are difficult to interpret due to our limited understanding of GalR3. Expression patterns of this receptor in the brain and periphery are not well characterized (Lang et al., 2015), and as the antagonist was administered systemically, it is unclear whether the observed effects were due to blockade of GalR3 in the brain or periphery. These findings highlight the complexity of the galaninergic system, and specifically the ability of galanin to induce divergent effects on behavior through its receptor subtypes. This finding also reaffirms that galaninergic actions need to be assessed on a smaller scale using targeted manipulations of the peptide, as in this report, or by performing site specific infusions of galanin receptor agonists/antagonists, in order to differentiate the effects of galanin receptor subtypes.

While preliminary, our progressive ratio and reinstatement data suggest that Gal$^{cKO\text{-}Dbh}$ mice may exhibit increased motivation for remifentanil, but future studies will be needed before conclusions about genotype differences can be made. In some cases, genotype or treatment differences that are not apparent under an FR1 schedule of reinforcement can emerge when animals are subjected to more demanding and complex tasks like progressive ratio (Thomsen & Caine, 2007), underscoring the value of this IVSA task. Furthermore, future reinstatement studies may yet reveal an important role for galanin. Indeed, previous work has shown that galanin receptor

agonist, galnon, attenuates cocaine-primed reinstatement (Ogbonmwan et al., 2015), but similar work has not been performed with opioids. While the current study found no apparent differences between Gal$^{cKO\text{-}Dbh}$ and WT mice in cue-induced reinstatement, future experiments should focus on stress-induced reinstatement. Recent findings on the role of NE-galanin in modulating stress responses (R. P. Tillage, N. R. Sciolino, et al., 2020; R. P. Tillage, G. E. Wilson, et al., 2020; Weinshenker & Holmes, 2016) suggest that this particular modality of relapse-like behavior is most likely to be influenced by galanin.

An important consideration is whether the null effects observed here are reflective of the role of noradrenergic galanin, or whether they are attributable to compensatory mechanisms resulting from genetic alteration of noradrenergic galanin levels in the Gal$^{cKO\text{-}Dbh}$ and NE-Gal OX mice. It is possible that galanin receptor density is altered in the VTA or other brain regions of these mouse lines, masking a functional consequence of altered noradrenergic galanin levels. Future studies will also need to employ *in situ* hybridization or reporter line approaches to determine whether chronic, system-specific changes in galanin expression are associated with compensatory changes in galanin receptor density.

The noradrenergic nuclei A1 and A2 provide the primary noradrenergic innervation to the VTA (Mejías-Aponte, Drouin, & Aston-Jones, 2009), but interestingly, lack galanin expression in NE neurons (R. P. Tillage, N. R. Sciolino, et al., 2020). Given that the LC projects to VTA (B. E. Jones & Moore, 1977; Simon et al., 1979) and strongly expresses galanin (Cheung et al., 2001), there were anatomical reasons to suspect that LC-derived galanin would alter opioid reward. The findings presented here overwhelmingly show little impact of this source of galanin on opioid reward, which is surprising given the significant effects of galanin reported in previous studies (F. E. Holmes et al., 2012; Zachariou et al., 2003; Zachariou, Parikh, & Picciotto, 1999). However, a

recent characterization study of the Gal$^{cKO\text{-}Dbh}$ mice found that levels of galanin in the midbrain, as well as galanin fiber density in the VTA, are comparable to that of WT mice (R. P. Tillage, N. R. Sciolino, et al., 2020). Therefore, the LC does not provide a major source of galanin to the VTA. While the proportion of VTA galanin provided by the noradrenergic system does not necessarily correspond to its functional impact, it is nevertheless an important consideration for the results observed here. Galaninergic inputs to VTA have not yet been systematically mapped, so it remains unclear which brain regions, if not the LC, act as a source of galanin to the VTA. Retrograde mapping of inputs to VTA (Soden et al., 2020) in the context of previous galanin ISH studies (Cheung et al., 2001) suggest that the lateral septum, BNST, medial amygdala, and several hypothalamic nuclei (medial and lateral preoptic areas, lateral hypothalamus, posterior hypothalamic area, and dorsomedial hypothalamic nucleus) could be possible sources of VTA galanin.

Perhaps more important than galanin peptide itself is the distribution of galanin receptors in addiction-related circuitry. The discovery of functional GalR1- mu opioid receptor (MOR) heteromers in the VTA (Moreno et al., 2017) indicates that galanin may indeed affect opioid reward, but through receptor-level interactions. Currently, little is known about galanin receptor density in the VTA, especially with respect to which cell-types and neuronal elements might express galanin receptors. Studies that previously characterized GalR1 expression in the mouse brain did not examine VTA, resulting in a lack of knowledge about galanin receptors in this important region. Future studies will need to investigate GalR1 and MOR co-expression in the VTA itself, as well as in GABAergic inputs to VTA. Understanding the relative distribution of GalR1-MOR co-expression within this network could help indicate which projections might express the heteromer.

In summary, we find that neither genetic depletion nor overexpression of NE- galanin alters acute morphine-induced locomotion, morphine CPP, or remifentanil IVSA. These behavioral findings, in combination with prior work, suggest that NE-galanin does not critically modulate the rewarding effects of opioids, likely because other brain regions provide alternative sources of galanin to the VTA. The effects of galanin on opioid reward should be further investigated by evaluating the role of GalR1 and MOR specifically in the VTA, as this region is a key component of opioid reward circuitry.

3.6. FIGURES

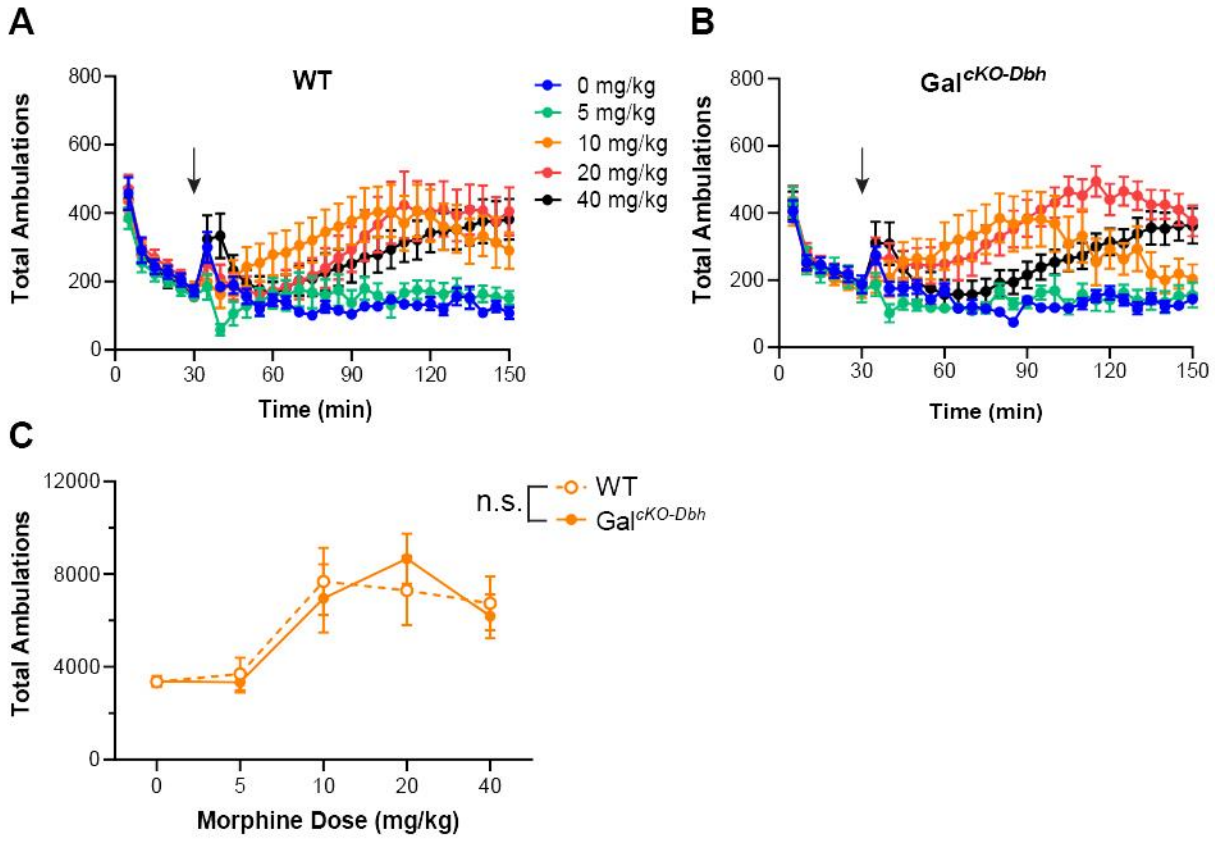

Figure 3.1. Genetic depletion of noradrenergic galanin does not alter acute morphine-induced locomotor activity. Testing session traces of morphine-induced locomotion in wild-type (WT) littermate controls (**A**) and NE-galanin knockout mice (Gal$^{cKO\text{-}Dbh}$) (**B**). For each session, mice were habituated to locomotor chambers for 30 min, injected (arrow) with either 0 (saline), 5, 10, 20, or 40 mg/kg morphine, and monitored for another 120 min. Dose response curves of morphine-induced locomotion did not differ by genotype (**C**). Data are displayed as mean ± SEM; n = 12 mice per genotype. n.s. = not significant.

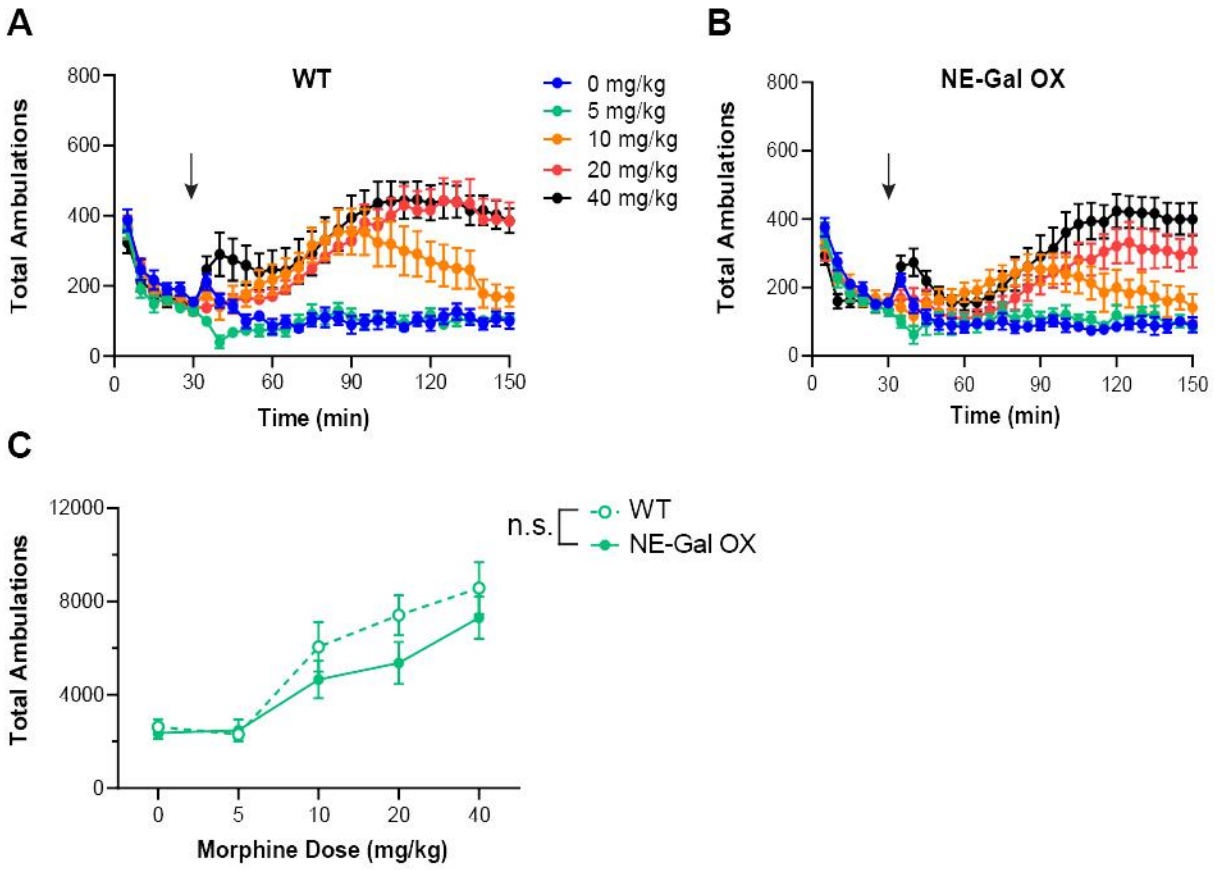

Figure 3.2. Genetic overexpression of noradrenergic galanin does not alter the locomotor activating effects of morphine. Testing session traces of morphine-induced locomotion in wild-type (WT) littermate controls (**A**) and noradrenergic galanin overexpressor mice (NE-Gal OX) (**B**). For each session, mice were habituated to locomotor chambers for 30 min, injected (arrow) with either 0 (saline), 5, 10, 20, or 40 mg/kg morphine, and monitored for another 120 min. There was no effect of genotype on dose response curves of morphine-induced locomotion (**C**). Data are displayed as mean ± SEM; n = 11-12 mice per genotype. n.s. = not significant.

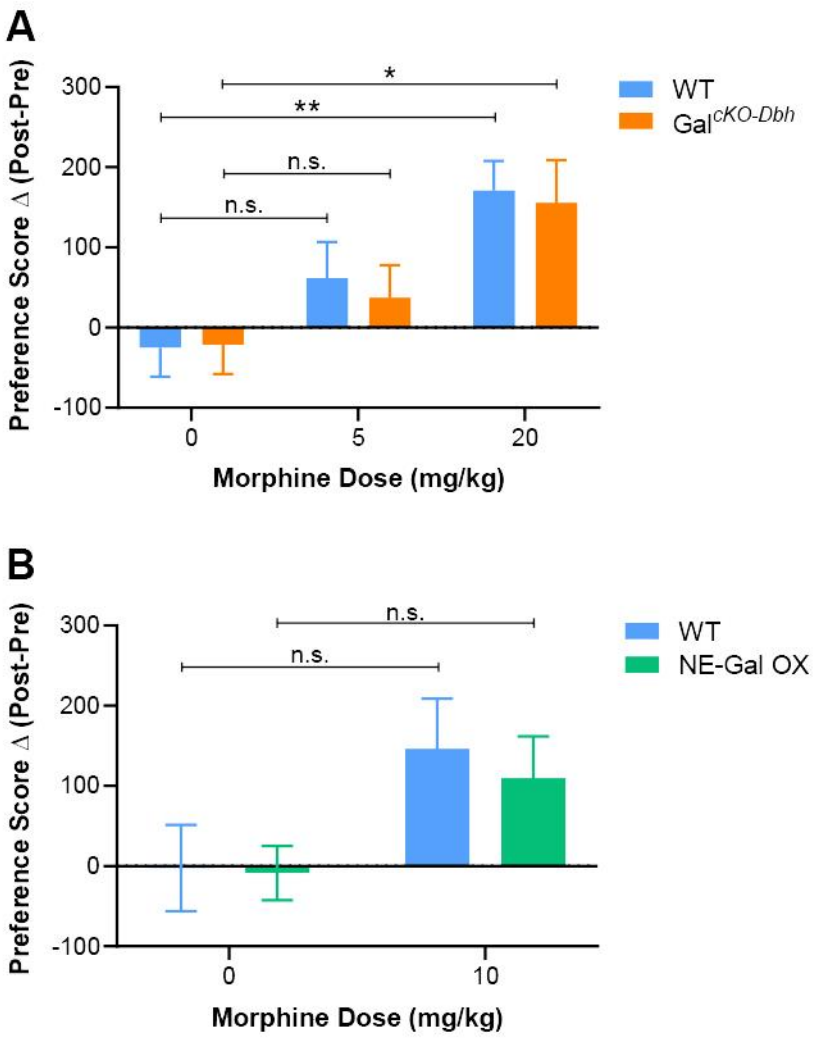

Figure 3.3. Morphine conditioned place preference is not modulated by noradrenergic galanin levels. Morphine conditioned place preference (CPP) was performed in Gal$^{cKO\text{-}Dbh}$ mice and WT littermates with 0 (saline), 5, or 20 mg/kg morphine. Both WT and Gal$^{cKO\text{-}Dbh}$ mice developed a CPP to 20 mg/kg, but not 5 mg/kg morphine (**A**). NE-Gal OX and WT mice underwent CPP with 0 and 10 mg/kg morphine, but neither genotype developed a CPP (**B**). Data represented as mean ± SEM; n = 10-11 mice per genotype. * $p < 0.05$, ** $p < 0.01$, n.s. = not significant.

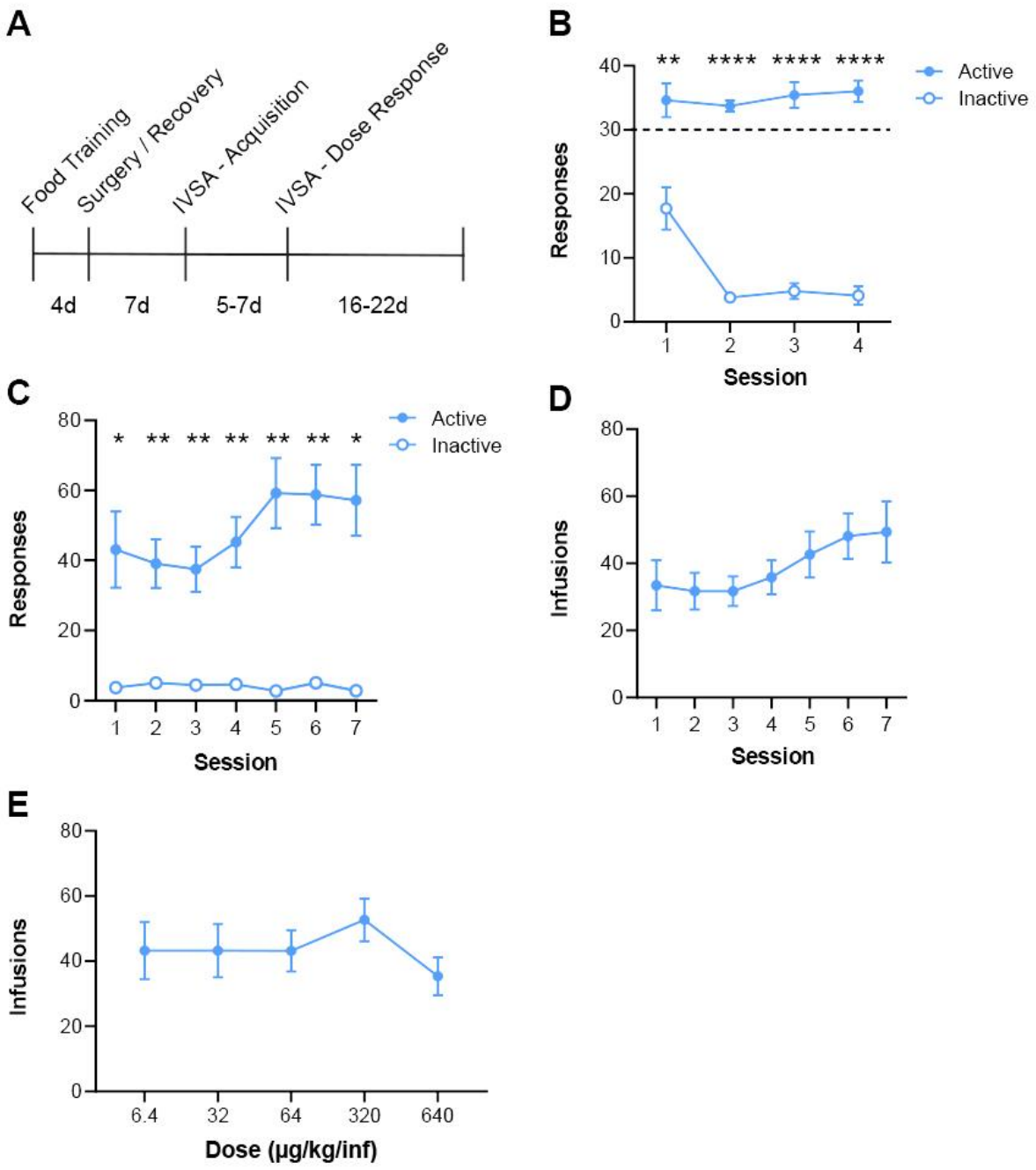

Figure 3.4. Characterization of the dose response curve for intravenously self-administered remifentanil in C57BL/6 mice. Experimental timeline indicates the different components of the remifentanil intravenous self-administration (IVSA) experiment (**A**). Food training data prior to catheter surgery indicate that C57BL/6 mice readily allocate responses to the active (filled circles)

rather than inactive (empty circles) nose poke to reach 30 maximum reinforcers (dashed line) as early as the first training session (**B**). When transitioned from food to intravenous remifentanil reinforcers (64 μg/kg/inf), mice maintain nose poke selectivity (**C**) and gradually escalate the number of infusions earned per session (**D**). Infusions earned per FR1 session for the following doses of remifentanil: 6.4, 32, 64, 320, and 640 μg/kg (**E**). All doses tested supported operant behavior, but the dose response curve peaked slightly at 320 μg/kg/inf. Data displayed as mean ± SEM; n = 9-11 mice per dose. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

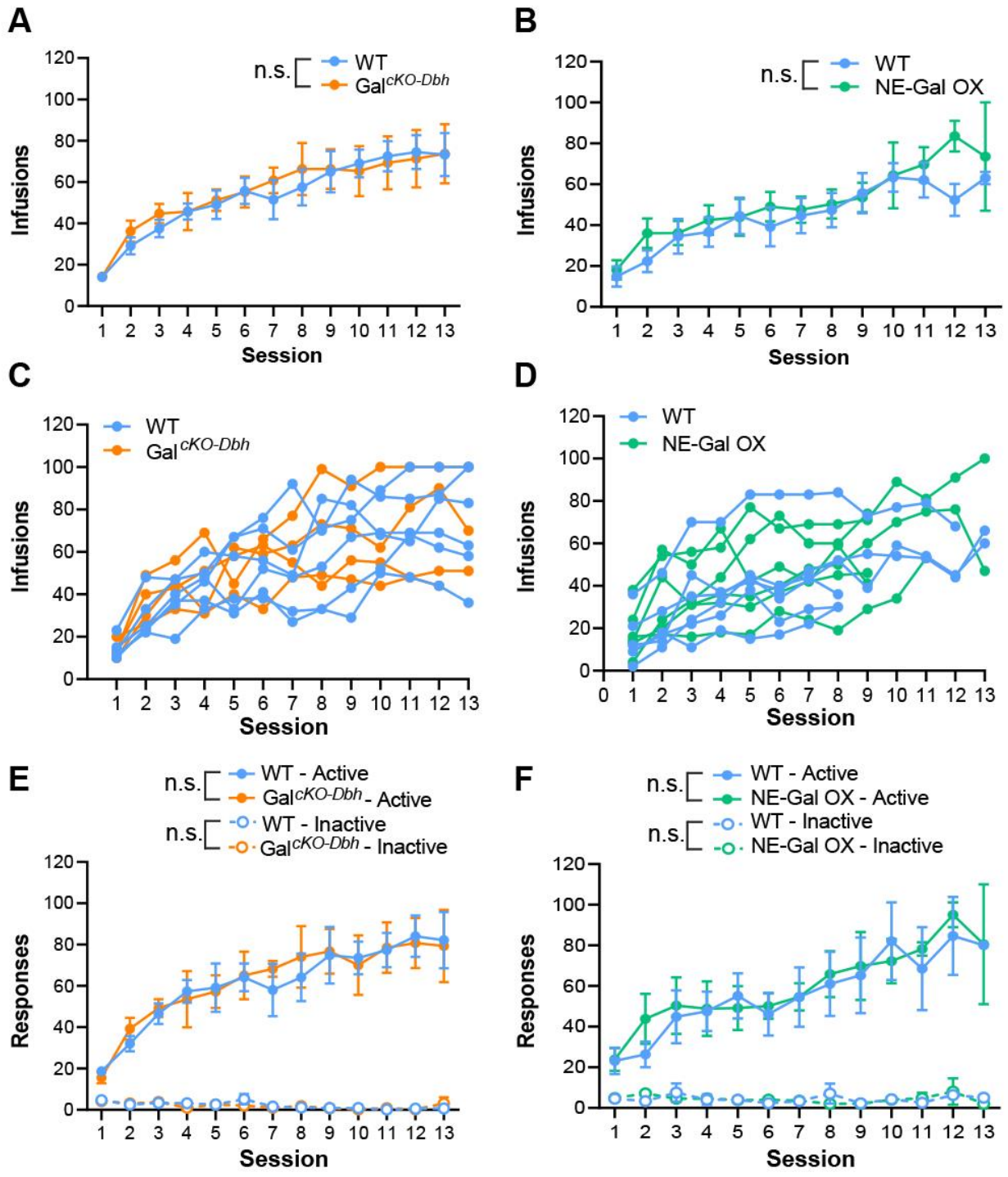

Figure 3.5. Noradrenergic galanin does not modulate acquisition of intravenously self-administered remifentanil. Gal$^{cKO\text{-}Dbh}$, NE-Gal OX, and their respective WT littermates self-administered the synthetic opioid remifentanil (320 μg/kg/inf) on an FR1 schedule for

approximately 13 days. The mean number of infusions earned per session escalated over time, but did not differ between Gal$^{cKO\text{-}Dbh}$ or NE-Gal OX mice and WT littermates (**A, B**). Traces of infusions earned by each animal over the 13-d acquisition period demonstrate individual differences in remifentanil intake across both experiments, and a similar range of responses across genotypes (**C, D**). Mice demonstrated strong behavioral selectivity for the active nose poke, but neither active nor inactive responses differed by genotype (**E, F**). Data displayed as mean ± SEM; n = 4-6 mice per genotype. n.s. = not significant.

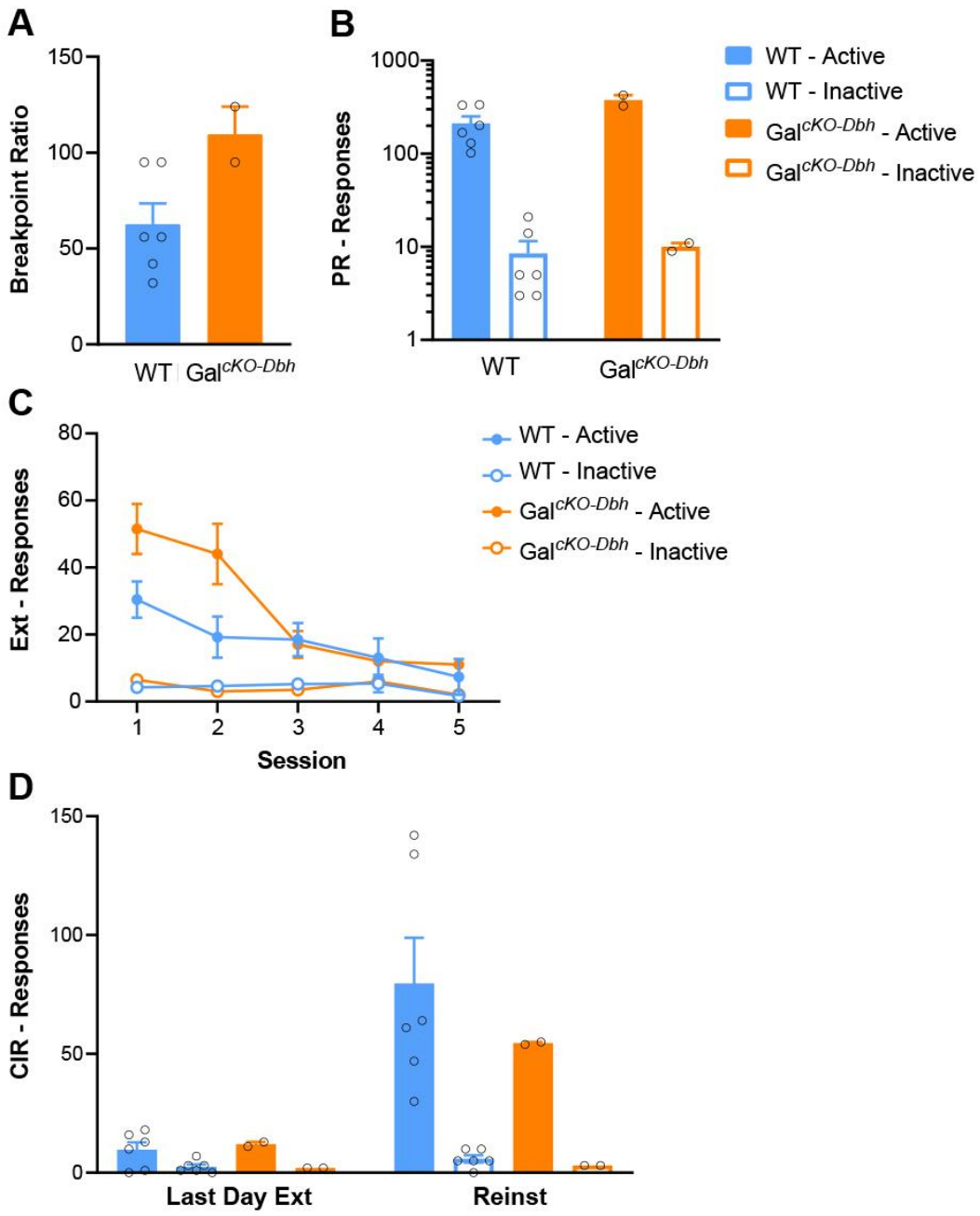

Figure 3.6. Effect of noradrenergic galanin depletion on motivation and craving for remifentanil. Preliminary progressive ratio (PR) data suggest that Gal$^{cKO\text{-}Dbh}$ mice may exhibit enhanced motivation for remifentanil (320 µg/kg/inf), as demonstrated by a higher breakpoint ratio (**A**) and greater active responses than WT (**B**). Extinction (Ext) of remifentanil reinforcement shows that Gal$^{cKO\text{-}Dbh}$ mice initially demonstrated more active responses, but eventually matched

WT responses (**C**). Cue-induced reinstatement (CIR) invigorated previously extinguished remifentanil-seeking behavior in both genotypes (**D**). Data displayed as mean ± SEM; n = 2-6 mice per genotype.

CHAPTER 4: CHARACTERIZATION OF GALR1 AND MOR MRNA IN OPIOID REWARD CIRCUITRY

4.1. ABSTRACT

Opioids exert their rewarding effects by binding to mu opioid receptors (MORs) on GABAergic neurons and inhibiting GABA release on ventral tegmental area (VTA) dopamine (DA) neurons. The neuropeptide galanin is reported to attenuate the cellular and behavioral effects of opioids, but the mechanism underlying these effects is unknown. Recent work indicates that galanin suppresses MOR signaling via galanin receptor 1 (GalR1) – MOR heteromers in the VTA. It is therefore critical understand where GalR1-MOR heteromers are expressed, but visualizing G protein complexes *in vivo* is technically challenging. In this report, we sought to characterize GalR1 and MOR RNA co-expression as an indicator of which brain regions and neuronal subtypes might be capable of assembling the heteromer. We used fluorescent *in situ* hybridization (ISH) to characterize GalR1 and MOR RNA expression in the rostromedial tegmental nucleus (RMTg) and VTA, both of which exert GABAergic control over VTA DA neurons, but exhibit different sensitivities to opioids. We found that GalR1 expression is similar in RMTg and VTA GABA neurons (RMTg: 31.54% ± 5.2; VTA: 33.80% ± 4.7), while expression of MOR is higher in RMTg GABA neurons (RMTg: 73.29% ± 2.8; VTA: 54.62% ± 4.5). Surprisingly, GalR1-MOR co-expression was similar between brain regions (RMTg: 26.51% ± 4.9; VTA: 21.08% ± 3.5). Low levels of GalR1, MOR, or co-expressed GalR1 and MOR were also observed in small populations of non-GABAergic neurons in each region. These findings provide the first cell-type specific characterization of GalR1 and MOR expression in the RMTg and VTA. Understanding the distribution of GalR1 and MOR in GABAergic inputs to VTA DA neurons will inform future studies attempting to target GalR1-MOR heteromers.

4.2. INTRODUCTION

The rewarding effects of drugs of abuse are linked to their ability to enhance dopamine (DA) release from ventral tegmental area (VTA) DA neurons into the nucleus accumbens (NAc) (Di Chiara & Imperato, 1988). While psychostimulants act directly on VTA DA neurons to enhance DA release, opioids primarily induce DA release via indirect actions on GABAergic inputs to VTA DA neurons. Specifically, opioids bind to Gi-coupled mu opioid receptors (MORs) on GABAergic neurons, which reduces GABA neurotransmission and disinhibits VTA DA neurons, facilitating DA release (Fields & Margolis, 2015). Importantly, VTA DA activity is influenced by local VTA interneurons, as well as GABAergic projections from other brain regions, including the rostromedial tegmental nucleus (RMTg), nucleus accumbens, and ventral pallidum (Bouarab, Thompson, & Polter, 2019; Matsui et al., 2014). GABAergic neurons therefore represent a neuronal subtype that critically shapes DA-dependent opioid reward processes.

Galanin is a neuropeptide that is reported to oppose the rewarding effects of opioids (Hawes et al., 2008; F. E. Holmes et al., 2012; Zachariou et al., 2003; Zachariou, Parikh, & Picciotto, 1999), yet the mechanism underlying these effects is poorly understood. Recent work revealed that the galanin receptor subtype, GalR1, can form functional heteromers with MOR, and that galanin binding to GalR1 within this heteromer blocks the downstream signaling effects of its activated MOR protomer (Ferré, 2017; Moreno et al., 2017). Furthermore, *in vivo* disruption of the GalR1-MOR heteromer in the VTA abolishes the ability of galanin to suppress opioid-induced DA release in the VTA (Moreno et al., 2017), indicating that heteromeric function is necessary for galanin-mediated effects.

Given that the presence of GalR1-MOR heteromers in a neuron could drastically alter its response to opioids, further characterization of the heteromer is needed. However, it is unclear

which VTA elements express the heteromer. As opioids are known to act on GABAergic inputs to VTA, and VTA DA neurons do not express MOR (Galaj et al., 2020), the GalR1-MOR heteromers are most likely to be found in GABAergic neurons. Indeed, GABAergic inputs to VTA exhibit different sensitivities to the effects of opioids (Matsui et al., 2014). It is possible that these differences reflect regional variation in GalR1-MOR heteromer density, such that GABAergic regions with more GalR1-MOR heteromerization are less sensitive to opioid suppression. Characterization of GalR1-MOR heteromer density across GABAergic inputs to VTA could help reveal if this is the case. A technical drawback though, is that GPCR heteromers cannot be readily visualized in *vivo* (Erbs et al., 2015). Additionally, immunohistochemical approaches for GPCR protein detection can be challenging due to low expression levels and frequent issues with non-specific antibodies (Marchalant et al., 2014). In the specific context of detecting the GalR1-MOR heteromer, reliable antibodies exist for MOR, but not for GalR1 (Hawes & Picciotto, 2004; Lu & Bartfai, 2009; Rodgers et al., 2019; D. Wang et al., 2018). We therefore sought to use *in situ* hybridization (ISH) to characterize the co-localization of GalR1 and MOR mRNA as a proxy for which cells could putatively contain the GalR1-MOR heteromer. In this study, high-resolution, cell-type specific ISH was used to characterize GalR1 and MOR mRNA co-expression in GABAergic and non-GABAergic neurons of the VTA and RMTg, two areas that modulate VTA DA transmission and exhibit differential sensitivity to the effects of opioids (Matsui et al., 2014).

4.3. MATERIALS AND METHODS

Animals

The following studies used 3-5 month old C57 BL/6J mice (both sexes) (JAX stock no. 000664). Mice were group housed on static racks with food and water available *ad libitum* in a temperature-controlled room with a 12:12 light/dark cycle. Procedures were conducted in

accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee.

Fluorescent In Situ Hybridization

RNAscope: With the exception of the types of probes used, tissue collection and RNAscope ISH was performed on fresh frozen mouse brain sections as described in Chapter 2, Section 2.3. For each region of interest (ROI), RNAscope was performed on 2 brain sections per mouse across 4 C57 BL/6J mice. GalR1 and MOR RNA were detected using GalR1 (ACD cat no. 448821) and OPRM1 (ACD cat no. 315841) probes. Cells were also identified as GABAergic or non-GABAergic with the use of a glutamate decarboxylase 1 (GAD1) probe (ACD cat no. 400951).

Imaging: Slides were imaged using a Nikon A1R HD25 confocal microscope with NIS Elements Software. A 10x tile of DAPI and GAD1 signal was acquired to visualize the entire brain section and delineate GABAergic regions. A high-magnification, tiled image that encompassed the ROI across both hemispheres was subsequently acquired (RMTg: ~100 fields of view (FOV) per section; VTA: ~200 FOV per section). For each FOV, a Z-stack (12 µm thickness with 1 µm steps) was acquired at a resolution of 1024 x 1024 pixels using a 20x lens with a 4x digital zoom. Gain settings were chosen to maximize probe signal without oversaturation and were validated with positive and negative control probe slides.

Image Analysis

ROI validation: Each photomerged, high-magnification tile was atlas-matched in order to delineate the boundaries of the ROI in the image. Then individual FOVs were chosen that were within the ROI boundaries and distributed evenly throughout the ROI. Approximately 10-20

FOVs, encompassing both hemispheres, were selected for analysis within each brain section. This process was completed for 2 brain sections per mouse, and 4 mice per ROI.

Image processing: Each nd2 file was separated into grayscale tifs for each color channel using FIJI image processing software. Then, images were processed using Cell Profiler software (McQuin et al., 2018). Images were analyzed using a speckle counting pipeline that identifies objects and foci and then uses a relationship module to perform per-object quantification of foci. We modified this pipeline to enable a nuclear-based analysis of RNAscope puncta. Specifically, nuclei were identified as objects and RNAscope puncta were identified as foci, which generated puncta-per-cell counts for each RNAscope probe within each cell nucleus.

Quantification: An R script was created to sort and compile the data generated by Cell Profiler. For each image, a nucleus was designated as GAD1+ or GAD1- based on the presence of $\geq$ 3 GAD1 puncta. Within each of these categories, cells were further sorted as GalR1 only, MOR only, GalR1 + MOR, or Neither, based on the presence of GalR1 and/or MOR puncta in the nucleus. Due to the comparatively low expression of these two RNA targets and the high specificity of RNAscope, the presence of 1 or more GalR1 or MOR puncta in a nucleus was considered positive for that target.

Statistics

Statistical analyses and graphs were generated in GraphPad Prism Version 8 (GraphPad Software, San Diego, CA). Pie charts display the summed expression findings across all mice for each ROI. GalR1 and MOR expression in GABAergic cell populations between ROIs were compared by unpaired t-test using average values per mouse.

4.4. RESULTS

GalR1 expression is similar in VTA and RMTg, and is consistent across cell types

RNAscope images indicated that GalR1 was expressed in both the RMTg and VTA (**Fig. 4.1.**). In RMTg, GalR1-only cells comprised 5.2% and 6.5% of GABA and non-GABA neurons, respectively (**Fig. 4.2.**). The VTA contained more GalR1-only cells, with 13.3% and 13.9% in GABA and non-GABA neurons (**Fig. 4.2.**). Total GalR1 expression (combined proportions of GalR1-only and GalR1-MOR co-expressing cells) was also evaluated. In RMTg, 31.9% of GABA cells and 11.9% of non-GABA cells expressed GalR1 with or without MOR. The same measurement in VTA encompassed 35.7% of GABA cells and 19% of non-GABA cells. An unpaired t-test indicated that average GalR1 expression in GABAergic neurons was not significantly different between regions (t_6 = 0.3233, p = 0.7575) (**Fig. 4.3. A**). Therefore, expression of GalR1 alone is similar between RMTg and VTA, and expression within each region is consistent among GABA and non-GABA populations.

MOR expression is prominent in GABAergic RMTg neurons

Within the RMTg, MOR-only cells accounted for 47.8% of GABAergic neurons and 15.0% of non-GABA neurons. In VTA, MOR-only cells comprised 26.4% and 13.1% of GABA and non-GABA neurons, respectively (**Fig. 4.2.**). Total MOR expression (combined proportion of MOR-only and GalR1-MOR co-expressing cells) was 74.5% in RMTg GABA cells and 19.4% in RMTg non-GABA cells. In the VTA, 48.8% of GABA neurons and 18.2% of non-GABA neurons expressed MOR with or without GalR1. An unpaired t-test indicated that average MOR expression in GABA neurons was significantly higher in RMTg (73.29% ± 2.8) compared to VTA (54.62 ± 4.5) (t_6 = 3.492 , p = 0.0129) (**Fig. 4.3. B**). Therefore, MOR was preferentially expressed in GABAergic neurons, with notably higher levels of expression in the RMTg compared to VTA.

RMTg and VTA exhibit similar GalR1-MOR co-expression

RNAscope images demonstrated GalR1-MOR co-expression in both GABAergic and non-GABAergic populations of the RMTg and VTA (**Fig. 4.1.**). Among GABAergic neurons, 26.7% of RMTg neurons and 22.4% of VTA neurons exhibited GalR1-MOR co-expression (**Fig. 4.2.**). An unpaired t-test indicated no significant difference in co-expression between brain regions (t_6 = 0.9104 , p = 0.3977) (**Fig. 4.3. C**). GalR1-MOR co-expression was also highly similar in non-GABAergic neurons, encompassing 5.4% of RMTg neurons and 5.1% of VTA neurons. Overall, these data indicate that GalR1-MOR co-expression occurs in roughly one-quarter of GABA neurons in either RMTg or VTA, with a much smaller fraction of non-GABA neurons containing both transcripts in these two regions.

4.5. DISCUSSION

The discovery of GalR1-MOR heteromers (Moreno et al., 2017) presents a potential mechanism by which galanin can attenuate the effects of opioids. However, GPCR heteromers cannot be readily visualized *in vivo* (Erbs et al., 2015). By understanding where the individual components of the GalR1-MOR heteromer are expressed, we can gain an alternative insight into which regions and neuronal subtypes are most likely to contain the heteromer itself. In this report, characterized the expression of GalR1 and MOR RNA in RMTg and VTA, two regions whose GABAergic neurons project to and influence VTA DA function (Matsui et al., 2014). We found that while MOR expression in GABA neurons was significantly higher in RMTg than VTA, neither GalR1 nor GalR1 and MOR co-expression differed between RMTg and VTA. GalR1 and MOR were preferentially co-expressed in GABAergic cells in both regions.

Although previous work has employed ISH and reporter line approaches to detect GalR1 in the mouse brain, neither study examined the RMTg or VTA (Hohmann et al., 2003; Kerr et al., 2015). In addition, while opioid-induced changes in VTA galanin receptors have been investigated

using radioligand assays (Zachariou et al., 2000), those data are not specific for GalR1, nor do they allow for direct measurement of receptor expression. Our study therefore represents the first cellular quantification of GalR1 RNA expression in the RMTg and VTA. We report that GalR1 expression is present in roughly one third of GABAergic cells in both the RMTg and VTA. Furthermore, the proportion of GalR1-only cells is similar between GABAergic and non-GABAergic cell populations within each region.

The RMTg contains a high proportion of GABAergic neurons, and exhibits robust MOR expression (Galaj et al., 2020; Jhou et al., 2009). Galaj and colleagues found that approximately 72% of GABA RMTg neurons express MOR in the rat (Galaj et al., 2020). Our finding that 74.5% of mouse RMTg GABA neurons express MOR is consistent with this previous observation. We also found MOR expression in 19.4% of non-GABA neurons.

MOR expression in the VTA has yielded more variable results across studies. While MOR expression in the VTA was previously reported to be dense (Erbs et al., 2015), a recent cell-type specific analysis in rat reported MOR in only 28% of GABAergic neurons (Galaj et al., 2020). In contrast, our cell-type specific analysis found MOR in 48.8% of mouse GABA neurons. Our increased detection of MOR may reflect procedural differences in sampling or possible species differences. While the prior study examined 450 - 1500 cells in VTA, our analysis examined over 3,700 cells. As the VTA is known to be regionally heterogenous (Lammel, Lim, & Malenka, 2014; Morales & Margolis, 2017), our larger sampling strategy may have been less impacted by regional variability in MOR expression. In addition, we cannot discount possible differences in MOR expression between rats and mice, which has not yet been directly compared.

Interestingly, we also found slight differences in MOR expression among non-GABAergic cells. While MOR was previously reported to be absent in VTA DA neurons, we found that 18.2%

of non-GABAergic neurons expressed MORs. Because we did not perform experiments with DA-specific markers, this MOR+, non-GABAergic population could encompass putative DAergic and/or glutamatergic cells, the other major cell types found in the VTA (S. R. Taylor et al., 2014). Given that the VTA is comprised of roughly 65% DAergic neurons, 30% GABAergic neurons, and a small proportion of glutamatergic neurons (Bouarab, Thompson, & Polter, 2019), it is likely that at least some of the non-GABAergic, MOR+ cells were DAergic. However, future work will need to confirm the neurochemical identity of this cell population.

The most novel aspect of this characterization study was our examination of GalR1-MOR co-expression in GABAergic cells as a proxy for GalR1-MOR heteromer formation. Given that galanin signaling through the heteromer blocks MOR signaling (Moreno et al., 2017), it would be assumed that less opioid-sensitive areas might exhibit increase heteromer density. And yet, GalR1-MOR co-expression was present in approximately a quarter of GABAergic neurons in both RMTg and VTA. This result was unexpected, given that these regions exhibit divergent responses to opioids, with RMTg and VTA GABA neurons being strongly and weakly suppressed by opioids, respectively (Matsui et al., 2014). Future experiments will need to assess GalR1-MOR co-expression in other GABAergic projections to VTA, such as nucleus accumbens and ventral pallidum (Hjelmstad et al., 2013; Matsui et al., 2014). It will be interesting to see whether or not GalR1-MOR co-expression recapitulates the findings reported here. If GalR1-MOR RNA co-expression is indeed reflective of heteromer density, this finding could suggest that opioid sensitivity is independent of heteromer prevalence.

However, previous work indicates that the addictive properties of opioids and modulation of VTA DA *is* specifically mediated by the heteromer (Moreno et al., 2017). Opioids such as morphine and fentanyl exhibit high potency for the GalR1-MOR heteromer and induce a subjective

"high," whereas methadone exhibits low potency at the heteromer and is far less likely to induce a "high" (Cai et al., 2019). This finding indicates that the ability of an opioid to readily bind the GalR1-MOR heteromer predicts its abuse liability. In light of these findings, if RNA co-expression does in fact correlate with heteromer density, the similar levels in RMTg and VTA could suggest that local galanin transmission might in fact influence when and where the heteromer will be activated.

Follow-up studies can expand on our initial findings, particularly at the protein level. A GalR1-mCherry reporter mouse line has been generated (Kerr et al., 2015), which allows for detection of GalR1 by performing IHC against the mCherry tag. Triple-IHC for GalR1, MOR, and a cell-type marker in tissue from these mice could readily define brain regions where there is high GalR1-MOR co-expression at the protein level. Again, co-expression does not equate with heteromerization, but this approach would be one step closer to elucidating GalR1-MOR co-expression at the protein level within the same neuron or neuronal element.

Future studies should also use optogenetics and slice electrophysiology to activate or inhibit specific GABAergic terminals in the VTA under varied conditions of opioid and/or galanin exposure. Specifically, GalR1-mCherry/Vgat-Cre mice could receive site-specific infusions of a Cre-dependent channelrhodopsin virus in GABAergic regions of interest. This strategy would enable optogenetic control of specific GABAergic populations while also labeling GalR1. Slice electrophysiology studies could then be performed where specific GABAergic terminals in the VTA are optogenetically activated in the presence of an opioid, galanin, or both, and the resulting IPSCs on VTA DA neurons measured. Combined use of two-photon approaches for these studies could also enable visualization of mCherry-tagged GalR1 on the GABAergic terminals that are being stimulated. In doing so, VTA DA neuron responses to be categorized based on whether the

stimulated GABAergic terminals contained GalR1. Ultimately, these experiments will help us better understand the way in which the galaninergic and opioid systems interact to control VTA DA neuron activity, and the specific role played by GalR1 in this circuitry.

Overall, we have confirmed previous work showing that the RMTg exhibits high MOR mRNA expression in its GABAergic cells, and additionally report that the RMTg and VTA exhibit similar levels of GalR1-MOR co-expression in approximately a quarter of GABAergic neurons. Future work should evaluate GalR1-MOR co-expression in additional GABAergic projections to VTA to fully assess GalR1-MOR co-expression in this circuit. Characterization of GalR1-MOR density may be helpful in determining which areas to test new therapeutic ligands specific for the GalR1-MOR heteromer.

4.6. FIGURES

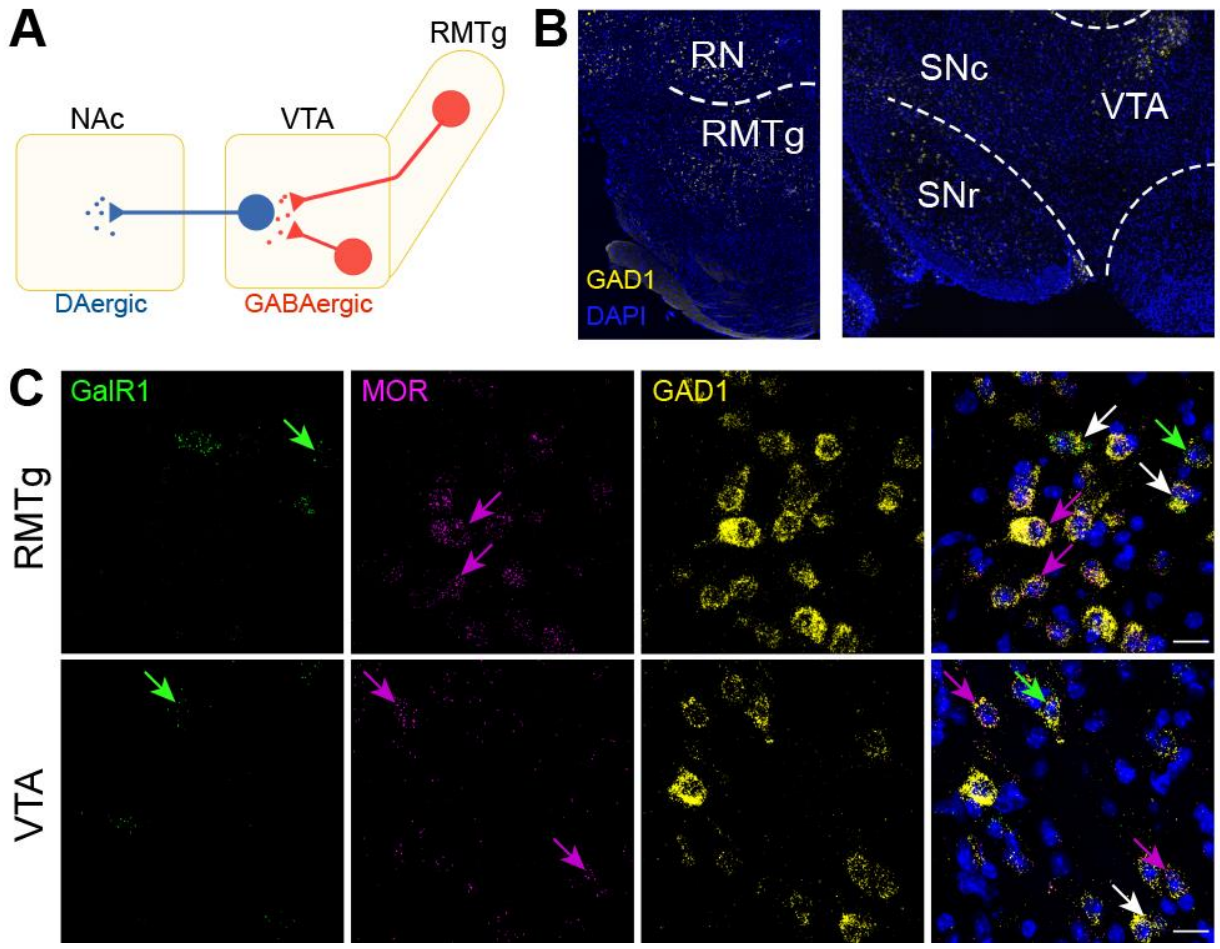

Figure 4.1. RMTg and VTA GABA neurons express GalR1 and MOR. Diagram depicting how ventral tegmental area (VTA) dopamine neurons that project to the nucleus accumbens (NAc) are modulated by GABAergic neurons (**A**). Sources of VTA GABA include afferents from the rostromedial tegmental nucleus (RMTg) and local VTA interneurons. Low magnification (10x) images of the RMTg and VTA with DAPI nuclear staining and RNAscope signal for GABAergic neurons using glutamate decarboxylase 1 (GAD1) (**B**). The RMTg is located ventral to the red nucleus (RN), and the VTA is medial to the substantia nigra pars reticulata (SNr) and compacta (SNc). High-magnification (20x with 4x digital zoom) images of GalR1 (green) and mu opioid receptor (MOR; magenta) RNA expression in GABAergic neurons (GAD1; yellow) of the RMTg

and VTA (**C**). Green arrows = GalR1 expression; magenta arrows = MOR expression; white arrows = GalR1 and MOR co-expression. Scale bar = 20 μm. Diagram adapted from Barrot et al. 2012.

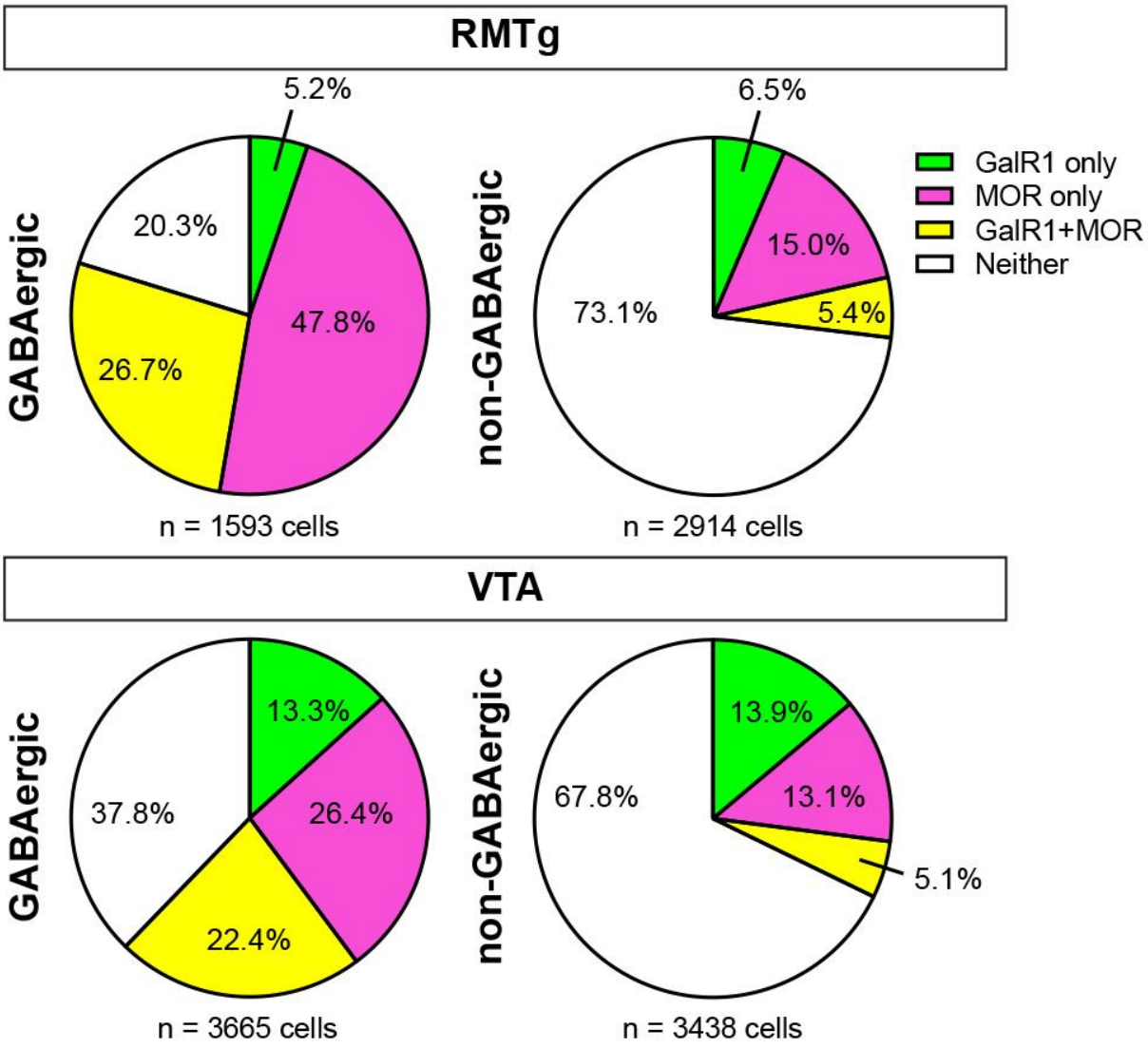

Figure 4.2. The RMTg and VTA display similar GalR1-MOR co-expression. Among GABAergic neurons, GalR1 expression is similar between the RMTg (**left, top**) and VTA (**left, bottom**). MOR expression is higher in the RMTg. Both regions exhibit co-expression of GalR1 and MOR in approximately a quarter of cells. The majority of non-GABAergic neurons express neither GalR1 nor MOR in the RMTg (**right, top**) or VTA (**right, bottom**). A small proportion of cells express similar amounts of GalR1, MOR, and GalR1-MOR co-expression between the RMTg and VTA.

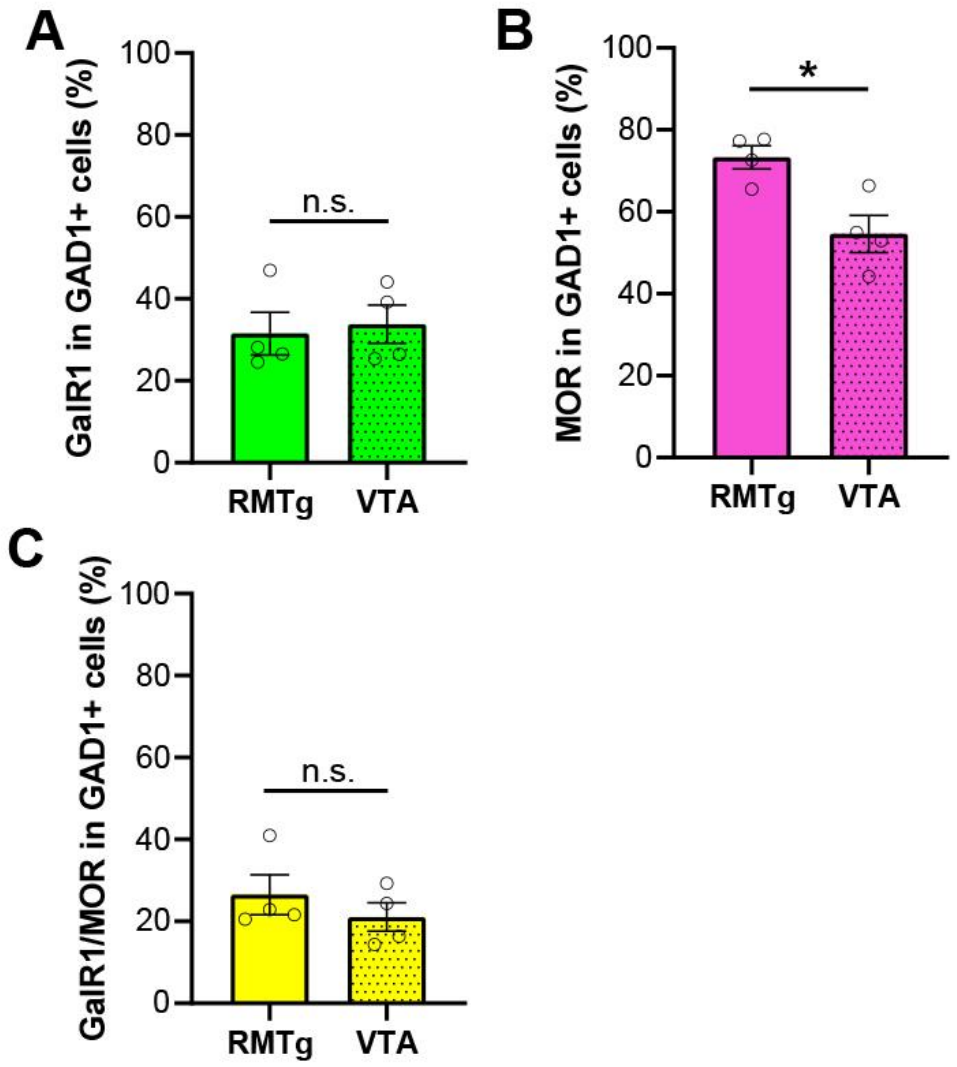

Figure 4.3. MOR expression, but not GalR1 or GalR1-MOR co-expression, differs between RMTg and VTA GABA neurons. RMTg and VTA GABA neurons express similar levels of GalR1 (**A**). The RMTg exhibits significantly higher MOR expression in GABA neurons compared to VTA (**B**). GalR1-MOR co-expression also did not differ between brain regions in GABA neurons (**C**). Data displayed as average ± SEM. n = 4 mice per region. * $p < 0.05$, n.s. = not significant.

CHAPTER 5: DISCUSSION

5.1. SUMMARY

This dissertation sought to characterize the specific role of noradrenergic (NE) galanin in opioid withdrawal and reward behaviors. To complement these behavioral studies, we also used molecular approaches to examine the expression of galanin receptor 1 (GalR1) in opioid withdrawal and reward circuitry. We demonstrated that NE-galanin does not modulate behavioral measures of opioid withdrawal, reward, or reinforcement. Using advanced *in situ* hybridization approaches, we also revealed that GalR1 expression is primarily outside, rather than within, the mouse LC. This molecular/anatomical finding may therefore explain why NE-galanin does not affect opioid withdrawal behaviors, and strongly challenges the dominating mechanistic theory regarding galanin effects on withdrawal. In an effort to identify molecular evidence for the presence of the GalR1-MOR heteromer in reward circuitry, we characterized GalR1 and MOR co-expression in the VTA and RMTg. We found GalR1-MOR co-expression in both the RMTg and VTA in similar proportions of GABA neurons. Overall, these results show that NE-galanin does not critically affect opioid-mediated behaviors, and suggests that investigating galanin receptor-level interactions with the opioid system may be as informative as studies examining galanin itself.

5.2. CONCLUSIONS AND FUTURE DIRECTIONS

5.2.1. Noradrenergic Galanin and Opioid Withdrawal

Of the effects that galanin is reported to exert on opioid-mediated behaviors, its ability to suppress withdrawal appeared to be the most potent (Zachariou et al., 2003). Given that the LC is mechanistically involved in the cellular development and physical expression of opioid withdrawal (Mazei-Robison & Nestler, 2012), we and others suspected that galaninergic actions specifically within the LC were responsible for modulating withdrawal symptom severity (Hokfelt et al., 2018;

F. E. Holmes et al., 2012; P. V. Holmes et al., 1994; Zachariou et al., 2003; Zachariou et al., 2000). Previous work suggested that galanin and GalR1 formed an autoinhibitory negative feedback loop within the LC, which could suppress withdrawal (Hokfelt et al., 2018). However, this potential mechanism had never been formally tested. In chapter two, we determined that neither NE-galanin, nor central galanin receptor signaling, is sufficient to modulate precipitated withdrawal symptoms. We also generated molecular and anatomical evidence showing that, in contrast to prior reports (Zachariou et al., 2000), GalR1 expression is low in the LC and is not altered by withdrawal. Our collective findings that 1) NE-galanin levels do not alter withdrawal symptom severity and 2) GalR1 expression is enriched outside, but not within the LC, argue against the long-standing mechanistic theory that an autoinhibitory GalR1 negative-feedback loop in the LC regulates somatic opioid withdrawal symptoms.

Perhaps the most impactful finding of our withdrawal studies is the cell-type specific distribution of GalR1 mRNA in the dorsal pons. As discussed in Section 1.7.2., no study had evaluated GalR1 mRNA expression in confirmed LC neurons before. Our study is the first to use an ISH approach with multiplexed probes to label both TH+ LC neurons and GalR1 mRNA, providing the first quantitative, cell-type specific examination of GalR1 mRNA in the dorsal pons. An obvious drawback of this approach could be that mRNA levels do not necessarily reflect protein levels. However, similar immunohistochemical results in GalR1-mCherry mice indicate that our GalR1 mRNA expression patterns accurately reflect GalR1 protein levels and distribution. At the very least, the low LC GalR1 expression means that galanin suppression of LC activity, which is quite profound and has been reported by multiple groups (Bai et al., 2018; Ma et al., 2001; Seutin et al., 1989; Sevcik, Finta, & Illes, 1993), does not occur directly via GalR1-containing LC neurons. The natural conclusion of this finding would be that GalR1 in LC-adjacent regions might

instead respond to local galanin release and indirectly modulate the LC to suppress withdrawal. However, if this were the case, then depletion or overexpression of LC-derived galanin, the presumed critical source of galanin for these GalR1-rich areas, should affect withdrawal severity. And yet, our *Gal*$^{cKO\text{-}Dbh}$ and NE-Gal OX mice exhibited little to no change in withdrawal symptoms. The combined lack of GalR1 in the LC and the inability of LC-derived galanin to affect withdrawal indicate that galaninergic actions in the LC do not modulate somatic withdrawal symptoms, contrary to prior mechanistic theories (Hokfelt et al., 2018; Zachariou et al., 2003).

Our follow-up experiments using galnon raise even more questions about how or whether central galanin can suppress withdrawal at all. Even if the LC is not the critical locus for galaninergic suppression of withdrawal, broad activation of galanin receptors should have been sufficient to suppress symptoms as reported previously (Zachariou et al., 2003). While we were able to suppress feeding behavior with galnon, indicating its pharmacological effectiveness, we still could not detect changes in withdrawal. It therefore appears that somatic withdrawal symptoms cannot be modulated by either NE-galanin nor central galanin signaling. From these studies, we conclude that *if* galanin modulates withdrawal, the effect is not LC-dependent, and may be particularly sensitive to slight variations in experimental conditions. Altogether, this work suggests that galanin is not an ideal target for modulating somatic signs of precipitated opioid withdrawal.

Although NE-galanin does not alter acute somatic symptoms in a precipitated withdrawal model, future studies would do well to explore whether phenotypes in the *Gal*$^{cKO\text{-}Dbh}$ and NE-Gal OX mice emerge in protracted withdrawal models. This phase of withdrawal, which follows the acute somatic phase, is defined by the emergence of a negative affective state that manifests as increased anxiety-like behavior and impaired social interaction in rodents several weeks after

opioid cessation or naloxone administration (Bravo et al., 2020; Welsch et al., 2020). Interestingly, both *Gal*$^{cKO\text{-}Dbh}$ and NE-Gal OX mice exhibit phenotypes relevant to protracted withdrawal; *Gal*$^{cKO\text{-}Dbh}$ mice display more active coping in a stressful context (R. P. Tillage, N. R. Sciolino, et al., 2020), while NE-Gal OX mice are resistant to the anxiogenic effects of footshock stress (R. P. Tillage, G. E. Wilson, et al., 2020). Therefore, the protracted withdrawal state could be a more appropriate withdrawal model in which to evaluate how NE-galanin shapes stress-and anxiety-like withdrawal responses. Furthermore, the longer time period of behavioral assessment in protracted withdrawal studies may more reliably capture neuropeptide effects, which are known to act over longer timescales than classical neurotransmitters (Hokfelt et al., 2018; Lang et al., 2015; Rachel P. Tillage et al., 2020).

It is important to note that while many studies have demonstrated that bath-applied galanin suppresses LC pacemaker activity and hyperpolarizes LC neurons in brain slices from naïve animals (Bai et al., 2018; Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989; Sevcik, Finta, & Illes, 1993), no study has evaluated the effects of galanin on LC activity in opioid-dependent animals. Therefore, the effects of galanin on the opioid-dependent LC, particularly during withdrawal, are still unknown. Future studies should address this major gap in the literature, particularly because the LC undergoes opioid-induced cellular changes (Mazei-Robison & Nestler, 2012) that alter baseline LC functionality. It will be critical to understand whether the effects of galanin on LC neurons are conserved or altered between the drug-naïve and opioid-dependent LC.

Independent of this study's implications regarding the LC, galanin, and withdrawal, our findings on GalR1 distribution strongly suggest that future studies should explore how galanin more generally attenuates LC activity. While many studies have demonstrated that bath-applied galanin suppresses LC pacemaker activity and hyperpolarizes LC neurons in slice preparations

(Bai et al., 2018; Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989; Sevcik, Finta, & Illes, 1993), this effect was assumed to be the result of direct actions through GalR1 in the LC. We now know that these effects likely encompass galanin binding to GalR1 located primarily outside of the LC, which suggests indirect modulation of LC activity. A possible indirect mechanism could be tested in LC slice electrophysiology experiments using mice that selectively lack GalR1 in LC neurons. If bath-applied galanin is sufficient to induce hyperpolarization in LC neurons in these mice, this result would definitively demonstrate that galanin signaling via GalR1 located *outside* the LC is responsible for the previously observed suppressive effect of galanin on LC neurons.

One particular brain region that should be explored as a modulator of LC activity is Barrington's nucleus. This brain region contains plentiful GalR1 mRNA according to our study, and is known to send corticotrophin releasing hormone- (CRH) containing afferents to the LC (Lechner & Valentino, 1999). Because CRH increases LC firing (Valentino & Van Bockstaele, 2001), Barrington's nucleus can therefore exert an excitatory influence on LC function. It is possible that galanin activating GalR1 receptors on CRH+ neurons in Barrington's nucleus suppresses this excitatory input, resulting in a concomitant decrease in LC activity.

Slice electrophysiology studies could first test this potential mechanism by examining whether bath-applied galanin suppresses neuronal activity in Barrington's nucleus. Then, Cre-Lox technology and viral approaches could be used to induce expression of an inhibitory opsin in CRH+ neurons of Barrington's nucleus. LC slices could be collected from these mice, and CRH+ cells in Barrington's nucleus could be optogenetically inhibited during simultaneous recording from LC neurons to see if this action recapitulates inhibitory effects of bath-applied galanin on LC activity. Similar strategies could then be used to selectively inactivate other GalR1-expressing candidate regions that project to the LC. Clarifying how galanin exerts its effects on LC function

will provide critical insight on how this neuropeptide modulates NE function. Of course, this approach would then beg the question: what is the source of galanin to the brain regions that indirectly modulate LC activity? This could be tested by infusing a retrograde tracer into the region of interest and performing IHC for galanin on the resulting sections from those mice. A strategy like this would readily indicate which brain regions send galaninergic inputs to the region of interest.

5.2.2. Noradrenergic Galanin and Opioid Reward

Previous studies had suggested that enhanced galaninergic activity was protective against opioid reward, and that decreased activity enhanced sensitivity to opioid reward (Hawes et al., 2008; Zachariou, Parikh, & Picciotto, 1999). However, these studies employed relatively non-specific manipulations to galanin. In chapter three, we attempted to build on prior work by specifically examining the consequence of NE-galanin depletion or overexpression on morphine-induced locomotion, CPP, and remifentanil IVSA. While we predicted that our NE-specific manipulations would be sufficient to largely reproduce the effects of central/systemic galanin manipulations, we instead observed no effect on behavior. Therefore, it appears that NE-galanin is not a critically important source of galanin for opioid reward.

When considering the extensive nature of the galanin system, it is in some ways unsurprising that NE-galanin depletion does not drastically impact behavior. After all, the galaninergic system mediates many fundamental aspects of behavior (parenting, feeding, stress) as well as neuronal activity (Abramov et al., 2004; Lang et al., 2015; Wu et al., 2014). A complex neuromodulatory system such as this likely has redundant or collateral sources of galanin to key brain regions in order to minimize the impact of galaninergic disruption. Indeed, recent characterization of the $Gal^{cKO\text{-}Dbh}$ line by our lab indicate that these mice exhibit normal levels of

galanin in the midbrain (R. P. Tillage, N. R. Sciolino, et al., 2020). Additionally, galaninergic fibers in the VTA of these mice are comparable to that observed in WT mice. Therefore, loss of noradrenergic galanin does not drastically alter galanin levels or galaninergic inputs to VTA. As the VTA is the critical brain region underlying DA-dependent mechanisms of opioid reward and reinforcement, it is possible that the nominal change in midbrain galanin levels correlated with a lack of behavioral effects.

The locomotor findings in this chapter indicate that NE-galanin levels do not impact this behavior. While the CPP findings are reasonably conclusive for the *Gal*$^{cKO\text{-}Dbh}$ mice, future work should test NE-Gal OX mice and WT littermates with a higher dose of morphine that elicits a CPP before definitively concluding that NE-galanin does not alter opioid reward. The greatest remaining potential for the role of NE-galanin in opioid-mediated behaviors involves IVSA. We did not observe differences in acquisition of FR1 remifentanil IVSA, but our progressive ratio and reinstatement work was preliminary and could not definitively assess the impact of NE-galanin on drug motivation or drug-seeking behavior. If there are any effects of NE-galanin on IVSA, they will likely be detected during reinstatement assays for two reasons. First, the *Gal*$^{cKO\text{-}Dbh}$ mice show significantly lower levels of galanin and fewer galaninergic fibers in the PFC compared to WT mice. As both the PFC and the NE-system are critically involved in reinstatement (Koob & Volkow, 2016; Schmidt et al., 2017), decreased PFC galanin could affect relapse circuitry and related behaviors. Second, as mentioned in the previous section, the *Gal*$^{cKO\text{-}Dbh}$ mice show phenotypes in atypical stress- and anxiety-related behaviors that could be engaged by stress-induced reinstatement procedures. Given that these mice demonstrate increased active coping behaviors (R. P. Tillage, N. R. Sciolino, et al., 2020), it is possible that *Gal*$^{cKO\text{-}Dbh}$ mice would display intensified drug-seeking behavior after a stressor as a form of active coping.

As this was the first system-specific investigation of galanin and opioid reward, our studies have inherent value. Within the context of prior work, these findings suggest that global, but not targeted, changes in galanin alter opioid reward. It will be interesting to see whether analogous studies examining serotonergic or cholinergic galanin yield significant effects on behavior. If not, this would indicate that major changes in galanin levels are required to affect opioid-related behaviors. However, such a widespread manipulation to galanin would likely affect a host of other essential behaviors, and would therefore suggest that the galaninergic system might not be an ideal biological target for suppressing opioid-mediated behaviors.

There are essential future studies that could guide further inquiry into the role of galanin and opioid reward/reinforcement. Above all, there is a critical need for combined retrograde tracer/IHC studies that would show which brain regions send galaninergic afferents to addiction-related regions, such as the VTA. Surprisingly few studies have attempted this, and the few that have done so yielded unexpected results. For example, the lateral hypothalamus (LH) contains a population of GABAergic neurons that strongly innervate the VTA (Nieh et al., 2015), and the LH also expresses galanin (Cheung et al., 2001). Therefore, it has been assumed that the LH is a source of VTA galanin. However, recent anatomical work revealed that while many GABAergic LH neurons project to the VTA, those that co-express galanin do not (Qualls-Creekmore et al., 2017). Findings such as these underscore the need for a systematic evaluation of galaninergic inputs to the VTA. Understanding which regions send galaninergic projections to the VTA would help inform future experiments in which galanin could be selectively depleted to assess the effect of particular sources of galanin on behavior. Alternatively, future studies could focus more on galanin receptors, as opposed to sources of galanin itself. Given compelling evidence that galanin may

modulate GABAergic input to the VTA via GalR1-MOR heteromers, this is an important line of investigation that is discussed in greater detail in Section 5.2.3.

One of the greatest challenges with models such as our *Gal*$^{cKO\text{-}Dbh}$ and NE-Gal OX mice is that technical limitations that hinder our ability to confirm whether genetic changes in galanin expression translate to changes in galanin release. While ELISAs can be used to generally gauge peptide levels in tissue punches (R. P. Tillage, N. R. Sciolino, et al., 2020), this approach has poor spatial resolution and cannot be used to measure neuropeptide release in a living animal. The most significant advance in measuring *in vivo* peptide release has been the development of tandem microdialysis and nanoflow liquid chromatography-mass spectrometry (Al-Hasani et al., 2018). However, this approach is highly technically challenging and therefore inaccessible for most labs. Moreover, this technique yields poor temporal resolution (Al-Hasani et al., 2018), a quality that is essential for evaluating changes in peptide release. The most viable *ex vivo* approach for measuring neuropeptide release, which has both high spatial and temporal resolution (Zaelzer et al., 2018), is the use of "sniffer" cells. Sniffers are heterologous cells that typically express a Gq-coupled receptor in conjunction with a fluorescent calcium indicator (Piñol et al., 2014; Zaelzer et al., 2018). Sniffer cells can be topically applied to brain slices, where they emit a fluorescent signal when a neuropeptide ligand binds the receptor and triggers intracellular calcium release.

Future studies could measure real-time galanin release by generating sniffer cells that capitalize on the Gq coupling of the GalR2 receptor. Using GalR2 sniffer cells, electrically or optically evoked galanin release could be visualized with spatial and temporal specificity for the first time in brain slices. Moreover, pairing this approach with electrophysiology could, for the first time, reveal how galanin release affects cellular activity in various regions of interest. This

approach would be particularly useful for evaluating how galaninergic afferents to VTA affect VTA DA neuron activity.

Ultimately, the most informative studies on galanin neurotransmission will need to be performed *in vivo*, which could be accomplished with the use of genetically-encoded fluorescent biosensors. To develop these sensors, a permutated fluorophore is inserted into a GPCR of interest such that binding by its ligand triggers a conformational change resulting in a concomitant increase in fluorescence intensity (Patriarchi et al., 2018). The change in fluorescence, which has high spatial and temporal specificity, can be used to measure neurotransmission of various ligands. This technology has already been successfully applied in the creation of sensors for DA and NE (Feng et al., 2019; Patriarchi et al., 2018), and could also be applied to neuropeptides such as galanin. The development of a galanin biosensor would be a major contribution to the field, as it could be combined with fiber photometry to measure real-time, *in vivo* galanin release for the first time. Learning about the temporal and spatial dynamics of galanin release would significantly impact and inform our understanding of neuropeptide transmission dynamics.

5.2.3. Characterization of GalR1-MOR Co-Expression

In light of our generally negative findings regarding the effects of altered galanin levels on opioid-mediated behaviors, it is important to consider whether the galanin receptors, rather than galanin itself, should be the focus of future research. The discovery that the VTA contains GalR1-MOR heteromers (Moreno et al., 2017) indicates that the galaninergic system directly influences, and interferes with, the opioid system at the receptor level. The GalR1-MOR heteromer therefore has major therapeutic potential, in that agonists specific to the GalR1 component of the heteromer, or small molecules that enhance heteromer formation/stability, might be useful for suppressing opioid effects. Therefore, in chapter four, we chose to characterize GalR1 and MOR mRNA co-

expression in the VTA and RMTg, two areas that have GABAergic neurons capable of altering VTA DA activity. We report that despite the different sensitivities of these two populations to opioids, GalR1-MOR mRNA expression was highly similar.

While the RNAscope experiments in this dissertation provide an important first look at GalR1-MOR co-expression in addiction-related circuitry, future studies will need to expand upon these initial findings. First, anatomical studies are needed to determine specifically which GABAergic projections to VTA express GalR1. This could be achieved by crossing the GalR1-mCherry line with a Vgat-Cre line. Then, a Cre-dependent retrograde virus could be infused into the VTA. IHC could be performed for mCherry and the viral fluorophore, and co-expression would indicate which GABAergic projections to VTA also express GalR1. This experiment could also be performed in conjunction with IHC for MOR to further clarify which GABAergic projections to VTA express both GalR1 *and* MOR.

Additional experiments should also be performed to assess the importance of GalR1-MOR function on not only cellular, but also behavioral effects of opioids. Although disrupting the GalR1-MOR heteromer with an interfering peptide ablates galanin's ability to suppress opioid-induced VTA DA release (Moreno et al., 2017), it is unclear whether the magnitude of this effect is sufficient to modulate behavior. Presumably, interference with GalR1-MOR heteromer function would confer susceptibility to the rewarding effects of opioids. Future studies should therefore test how GalR1-MOR disruption with the interfering peptide affects behaviors such as morphine CPP and opioid IVSA. If GalR1-MOR heteromer disruption increases morphine CPP or self-administration behaviors, we will know that this heteromer normally suppresses opioid reward and reinforcement through its actions in the VTA.

5.3. CONTRIBUTIONS TO THE FIELD

5.3.1. RNA-Based Cell Soma Markers

In chapter two, our RNAscope approach necessitated that we define cell somas in order to quantify GalR1 in neurons of the dorsal pons and LC. Typically, this would be accomplished by running IHC for various cytoplasmic neuronal markers such as beta-III tubulin, neuronal nuclear protein, or enolase 2, among others (D'Andrea, Howanski, & Saller, 2017; Grabinski et al., 2015). However, the tissue digestion required for RNAscope can degrade many cytoskeletal-based cell soma markers, resulting in weak IHC signal when RNAscope and IHC are combined. Therefore, we sought to identify non-specific neuronal markers that exhibited robust, cell-filling RNA expression patterns. This would allow us to employ an RNAscope-only approach in which an additional probe could be used in lieu of an antibody to define cell somas.

After an extensive literature search, we determined that many cell type markers typically used for IHC exhibited RNA expression patterns that were not sufficiently high to define neuronal somas. We did however identify synaptosomal-associated protein 25 (SNAP25) as a non-specific neuronal marker that appeared to exhibit a strong, cell-filling pattern of RNA expression (Jolly et al., 2019; Tafoya et al., 2006). As demonstrated in chapter two, we successfully used SNAP25 RNA expression to broadly define neurons within the dorsal pons. This project therefore revealed that certain RNA targets can be used as an alternative means of defining cell somas when IHC cannot be successfully combined with RNAscope. This approach could be readily adapted for many other types of experiments in which combined RNAscope and IHC is not feasible.

5.3.2. RNAscope Image Analysis

While RNAscope provides an unparalleled ability to resolve single RNA transcripts, analyzing RNAscope images can be technically challenging. Publicly available pipelines to

analyze RNAscope images are difficult to apply to different datasets, and more importantly, may oversimplify analyses by using 2-D approaches to quantify 3-D data (i.e. Z-stack images) (Maynard et al., 2020). We therefore sought to develop a new method for analyzing RNAscope images that would enable us to quantify GalR1 within the borders of cells defined in 3-D.

Using Imaris Software, we were able to generate a method by which RNA signal from cell soma markers, such as SNAP25 or TH, were used to demarcate the 3-D surfaces of cells. Then, GalR1 puncta that fell within each cell's surface could be quantified. This approach to RNAscope analysis is superior to alternative methods because the Z-dimensions of each image can be used to determine whether puncta are truly located within a cell, as opposed to being located in a similar plane and erroneously attributed to being "inside" the cell, which can occur in 2-D analyses. This new analytical approach can be applied in the future when highly specific information is needed about the spatial distribution of RNA transcripts.

5.3.3. Remifentanil IVSA in Mice

This remifentanil IVSA component of this project also provides several important contributions to the field. First, the majority of remifentanil IVSA studies have been performed in rats (Blair et al., 2020; Hofford et al., 2017; Levin et al., 2020; Porter-Stransky, Bentzley, & Aston-Jones, 2017; Thorpe, Lacy, & Strickland, 2020; Venniro & Shaham, 2020), with only a few examining mice (Bornebusch et al., 2019; A. S. James et al., 2013; Severino et al., 2020). The findings in this dissertation provide key insights about the ability of remifentanil to powerfully support IVSA in mice, with or without prior food training. It also characterizes the dose response curve for intravenous remifentanil. Furthermore, these data reveal that remifentanil responding can be extinguished very quickly, which indicates that this particular opioid is excellent choice for reinstatement studies. Rodents can take up to several weeks of extinction training to adequately

extinguish conditioned behavioral responses (Manvich et al., 2016), but using remifentanil may decrease the number of extinction sessions needed, significantly shortening the overall length of these experiments.

5.4. CONCLUDING REMARKS

Overall, this dissertation clarifies the role of the galaninergic system in opioid withdrawal and reward behaviors. Examination of the specific impact of NE-galanin levels on these behaviors revealed that NE- galanin does not modulate opioid withdrawal or reward, which may be explained by specific expression patterns of GalR1 in respective circuits. The findings in this dissertation suggest that mechanistic actions of galanin via GalR1 are more complex than previously appreciated, and indicate that future work should focus on receptor-level interactions between the galanin and opioid systems, in particular the GalR1-MOR heteromer. The future development of small molecules that specifically target the GalR1-MOR heteromer may yield a major advance in galanin-based therapeutic approaches for treating OUD.

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