Regulation of Mu-Opioid Receptors in Thalamic Pathways of Motivation and Stress

By

Omar Koita

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Omar Koita

has been approved

John T. Williams, Ph.D., Advisor

Skyler Jackman, Ph.D., Chair

Susan Ingram, Ph.D., Member

Laurance Trussell, Ph.D., Member

Mario Penzo, Ph.D., Member

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Acknowledgments

I often think of a cartoon comic strip that used to live on the threshold of Gary Westbrook's and Eric Schnell's labs. It starts with a patron standing on the outside of a glass case, likely housing sweet pastries. An employee arrives and asks, "*H*ave you been helped yet?" The patron replies "By so many people my entire life." As cheesy as it was, I found myself gleaming with love at the reminder every time I walked by it. Getting to where I am today has taken the support of more peopl*e* than I'm p*r*obably allowed to list here, so briefly, if you find yourself reading this, no matter how short our interaction may have been, thank you for believing in me and supporting me. And if you didn't... thanks for being my hater, I hope every soda you drink comes pr*e*-shaken up.

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I will probably add the really sappy stuff once my committee has ok's my thesis and save it for whoever is willing to find my thesis in the future.

Preface

“Lately, I get my pills from a doctor” explains poet and musician Jeffery Lamar Williams on the ballad titled Metro Spider. Today Mr. Williams stands trial against the state of Georgia where he is accused of co-founding and overseeing gang activity that terrorized Fulton County with drug sales, armed robberies, shootings, and at least three murders. If we are to believe Mr. Williams's artistry, it is not implausible that he is guilty of what he is accused of. However, we are not here to cast our verdict on this case but to question, what does it mean when an individual with unprecedented access to illicit drugs finds it easier, safer and more reliable to procure opiates from a doctor.

Hip-hop/rap remains an excellent medium to assess the condition of a community. Indeed, the harrowing tales told over tectonic tones remain synonymous with the condition to which they reflect. This is no better illustrated than by the advent of chopped and screwed music in the early 90s. Named after the late DJ that created the sound, Houston native, DJ Screw,chopped and screwed music slows an original song to a slower BPM (~60 bpm) and adds reverb to mimic the effects of the cardiovascular depressant that inspired it; Lean. Lean: Sprite, 3-4 Jolly Ranchers candies, and codeine-promethazine cough syrup. “Lean,” however, wasn’t new to the scene, it had been a part of Houston's music scene since the early 60s when jazz musicians would mix Robitussin with beer to produce a similar effect. The codeine substitution came on the heels of the pharmaceutical industry adding naloxone to pentazocine/tripelennamine in an attempt to curtail the potential for abuse, a goal we are still struggling to get ahead of. Perhaps we are doomed to repeat these mistakes in perpetuity.

While the overdoses associated with this microclimate are often overlooked to use this as an opportunity to condemn rap music for “popularizing” the drug, many lives were lost to this opioid abuse, including DJ Screw. His death is not uncommon among chronic opioid users. A simple miscalculation in measuring these drugs in an attempt to titrate desired effects can often be lethal. Additionally, there is a force manipulating the goalpost called tolerance or a decreased efficacy of drug potency following extended exposure that makes it all the harder to standardize one's dosage.

My heart goes out to anyone struggling with addiction. It is a luxury that I've been allowed to think about this problem. Please be patient with us, I will never stop fighting for you.

Abstract

The development of tolerance/withdrawal is the defining feature that drives opioid addiction or opioid use disorder (OUD). Tolerance reduces receptor-effector coupling potentially driving an individual to take more drugs to get the same response. Withdrawal, or the adaptations seen when chronic opioid signaling is ceased, give rise to a litany of symptoms that potentially bias an animal toward seeking more exogenous opioids. The goal of this dissertation is to characterize the adaptations that lead to tolerance and withdrawal in a circuit relevant to motivated behavior.

The work presented in this dissertation was collected with whole-cell patch clamp recordings from the paraventricular nucleus of the thalamus (PVT) neurons in acute horizontal rat brain slices. Chapter 3 examines the heterogeneity of the PVT and aims to assess if the PVT is a viable model for studying opioid regulation with electrophysiology. Chapter 3 shows that while both the anterior and posterior PVT (aPVT and pPVT, respectively) exhibit similar electrophysiological properties, the aPVT has current responses larger than the pPVT. This makes the aPVT more suitable to study tolerance with the tools we have access to. Chapter 4 examines the chronic actions of opioids on the aPVT and highlights further heterogeneity in both their acute response to opioids but also the adaptations seen following chronic opioid exposure. Chapter 5 details preliminary data aimed at understanding how rapidly GPCRs regulate their neighboring GPCRs and the presynaptic adaptions seen in the aPVT following chronic morphine treatment. By elucidating the interplay between thalamic circuits and opioid modulation, this study advances our understanding of addiction neurobiology.

Chapter 1: Introduction

Statue of Aphrodite holding an opioid poppy in her right hand

The Opioid Crisis: A Historical and Contemporary Overview

The term "opioid crisis" emerged in the 1990s to describe the alarming increase in opioid overdose and addiction in the United States (Schiff, 2002). However, humanity's struggle with opioid addiction spans millennia, tracing back to ancient civilizations that recognized both the medicinal and addictive properties of opium. The earliest references to opioids date back to 3400 BCE in lower Mesopotamia, where the Sumerians cultivated opium poppies and referred to them as *Hul Gil*, the "joy plant" (Bandyopadhyay, 2019; Schiff, 2002). This early use underscores a profound and enduring human fascination with the psychoactive effects of opium.

In ancient Greece, Hippocrates, often called the "father of modern medicine," referred to the poppy as μήκων (*Mḗkōn*), meaning "poppy" (Rosemary, 2023), today it refers to a newborn's first poop for its likeness. Hippocrates acknowledged opium's usefulness as a narcotic, documenting its analgesic properties and contributing to the classification of different poppy species based on their physical characteristics (Fields & Wiegand, 2014). The duality of opium—as both a healer and a potential source of harm—is reflected in Greek mythology, where gods associated with sleep, night, death, and the underworld, such as Hypnos, Nyx, Thanatos, and Hades, are depicted adorned with poppies (Schiff, 2002). Similarly, Aphrodite, the goddess of love and desire, is often shown with poppies, symbolizing the plant's capacity to elicit pleasure and its potential for addiction (Lin & Ko, 2011).

The addictive potential of opium has become a persistent canker sore on humanity no better exemplified by the Opium Wars of the 19th century. These conflicts between China and Western powers were rooted in trade disputes exacerbated by widespread opium addiction in China, which the Qing Dynasty sought to curb by banning opium imports (Dikötter et al., 2004; Nakayama, 2024). The wars highlighted the complex interplay between economics, politics, and public health—a theme that resonates in today's opioid crisis.

In the contemporary context, the opioid crisis has escalated due to the over-prescription of opioid analgesics, aggressive pharmaceutical marketing, and the proliferation of potent synthetic opioids like fentanyl (Van Zee, 2009; Volkow et al., 2014). According to the Centers for Disease Control and Prevention (CDC), opioid-involved overdose deaths have continued to rise, reaching unprecedented levels (CDC, 2021). These modern epidemic underscores the need for a comprehensive understanding of opioids' pharmacology, their impact on neural circuits, and the socio-economic factors contributing to their misuse.

Opioids as Ligands: Endogenous and Exogenous Compounds

Opioids exert their physiological and pharmacological effects by binding to specific opioid receptors in the nervous system. These ligands can be broadly classified into endogenous opioids, which are naturally produced within the body, and exogenous opioids, which are introduced from external sources. A detailed examination of both types is essential for appreciating the complexity of opioid signaling and its implications for health and disease.

Endogenous Opioids

Endogenous opioids are peptides synthesized in the body that modulate pain, stress responses, immune function, and emotional states. They are derived from larger precursor proteins encoded by specific genes and include enkephalins, endorphins, dynorphins, and nociceptin/orphanin FQ (Hughes et al., 1975; Akil et al., 1984).

Enkephalins

Discovered by Hughes and Kosterlitz in 1975, enkephalins were the first endogenous opioid peptides identified (Hughes et al., 1975). Derived from the precursor proenkephalin A, they include [Met^5]-enkephalin and [Leu^5]-enkephalin. These pentapeptides preferentially bind the mu-opioid receptor

(MOR) and delta-opioid receptors (DORs) and play critical roles in modulating nociception, emotional behavior, and neuroendocrine functions (Przewłocki & Przewłocka, 2001; Williams et al., 2011).

- **[Met^5]-enkephalin (ME)**: Composed of the amino acid sequence Tyr-Gly-Gly-Phe-Met, where the N-terminal tyrosine is essential for receptor binding and activation. The structural similarity to morphine's phenolic group underscores the conserved nature of opioid-receptor interactions (Hruby & Agnes, 1999).
- **[Leu^5]-enkephalin (LE)**: Composed of the amino acid sequence Tyr-Gly-Gly-Phe-Leu, with the N-terminal tyrosine critical for binding to opioid receptors. [Leu^5]-enkephalin plays an important role in modulating pain, mood, and neuroendocrine signaling, similar to [Met^5]-enkephalin (Przewłocki & Przewłocka, 2001).

Endorphins

Beta-endorphin, derived from pro-opiomelanocortin (POMC), is a 31-amino-acid peptide with high affinity for mu-opioid receptors (MORs) (Zagon et al., 2017). It is released in response to stress and pain, contributing to the body's natural analgesic mechanisms. Beta-endorphin also influences immune function and has been implicated in mood regulation (Roth-Deri et al., 2008).

Dynorphins

Dynorphins, originating from the prodynorphin gene, include dynorphin A and dynorphin B peptides. Dynorphin A exhibits high affinity for kappa-opioid receptors (KORs) and is involved in modulating pain perception, stress responses, and emotional states (Chavkin et al., 2014). Activation of KORs by dynorphins often produces dysphoric and aversive effects, contrasting the euphoric effects mediated by MORs (Tejeda et al., 2012).

Nociceptin/Orphanin FQ (OFQ/N)

Discovered in 1995, OFQ/N is a 17-amino-acid peptide that binds to the nociceptin receptor (NOP or ORL-1) (Heinricher, 2003; Meunier et al., 1995). Although structurally similar to dynorphin A, OFQ/N does not bind to classical opioid receptors with high affinity. It modulates pain transmission, learning, and memory, and has complex effects on reward and stress pathways (Mogil & Pasternak, 2001; Toll et al., 2016).

Exogenous Opioids

Exogenous opioids include natural, semi-synthetic, and synthetic compounds that interact with opioid receptors to produce analgesic and psychoactive effects. Their therapeutic use is complicated by their high potential for abuse and dependence.

Natural Opiates

Morphine: The prototypical opioid analgesic, morphine is a principal alkaloid of opium and was first isolated by Friedrich Sertürner in the early 19th century (Schiff, 2002). Its complex pentacyclic structure includes multiple chiral centers and functional groups critical for receptor binding (DeRuiter, 2000). Morphine's pharmacological profile includes potent analgesia, euphoria, sedation, respiratory depression, and gastrointestinal effects.

Codeine: Another natural opiate, codeine is less potent than morphine and is used primarily for mild to moderate pain and as an antitussive. It undergoes metabolic conversion to morphine in the liver, contributing to its analgesic effects (Chen et al., 2013).

Semi-Synthetic Opioids

Heroin (Diacetylmorphine): Synthesized from morphine by acetylation, heroin is more lipid-soluble, allowing it to cross the blood-brain barrier rapidly (Rossi et al., 2012). In the brain, it is metabolized back to morphine, exerting potent effects that contribute to its high abuse potential. It is worth noting that heroin, itself is not an active agonist.

Oxycodone and Hydrocodone: Derived from thebaine, these opioids are widely prescribed for pain management but have been implicated in the prescription opioid abuse epidemic (Compton & Volkow, 2006). They bind primarily to MORs and have pharmacokinetic properties that influence their potency and duration of action.

Synthetic Opioids

Fentanyl: A fully synthetic opioid approximately 100 times more potent than morphine, fentanyl is used clinically for anesthesia and severe pain management (Volpe et al., 2011). Its high potency and rapid onset also contribute to its involvement in overdose deaths, especially when illicitly manufactured variants are introduced into the drug supply.

Methadone: Utilized in opioid replacement therapy, methadone has a long half-life and mitigates withdrawal symptoms in individuals with opioid dependence (Kreek, 2000). It is a racemic mixture, with the R-enantiomer being a potent MOR agonist.

DAMGO: A synthetic peptide agonist specific for MORs, DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) is used extensively in research to study MOR function due to its high selectivity and potency (Handa et al., 1981).

Table 1.1: Summary of Endogenous and Exogenous Opioids, Their Structures, Receptor Affinities, and Physiological Effects

Opioid	Type	General Structure	Primary Receptor Affinity	Physiological Effects
Endogenous Opioids				
Enkephalins	Pentapeptides	Amino acid sequence: Tyr-Gly-Gly-Phe-Met/Leu	High affinity for δ-opioid receptors (DORs); moderate for MORs	Modulate nociception, inhibit neurotransmitter release, regulate pain perception, and influence emotional behaviors

[Met^5]-Enkephalin	(Subtype of Enkephalins)	Tyr-Gly-Gly-Phe-Met	DOR > MOR	Similar to enkephalins; involved in analgesia and modulation of immune responses
Endorphins	Peptides	Derived from pro-opiomelanocortin (POMC); 31 amino acids for β-endorphin	High affinity for μ-opioid receptors (MORs)	Produce potent analgesia, modulate stress and immune responses, influence mood and emotional states
Dynorphins	Peptides	Derived from prodynorphin; includes dynorphin A (17 amino acids)	High affinity for κ-opioid receptors (KORs)	Modulate pain perception, produce dysphoria, influence stress responses, and affect emotional processing
Nociceptin/Orphanin FQ (OFQ/N)	Peptide	Heptadecapeptide (17 amino acids)	High affinity for NOP receptors (ORL-1)	Modulates pain transmission, affects learning and memory, influences reward and stress pathways, often counteracting classical opioid effects
Exogenous Opioids				
Morphine	Natural opiate	Pentacyclic benzylisoquinoline alkaloid with five rings (A-E); key functional groups include phenolic hydroxyl at C3 and alcoholic hydroxyl at C6	High affinity for MORs; moderate for DORs	Provides potent analgesia, induces euphoria, causes sedation, respiratory depression, constipation, and has high potential for dependence and tolerance
Codeine	Natural opiate	Similar to morphine with a methoxy group	Low affinity for MORs (prodrug converted to morphine in vivo)	Acts as a mild to moderate analgesic, antitussive (cough

		at C3 instead of a hydroxyl group		suppressant), less potent than morphine, lower risk but still potential for dependence
Heroin (Diacetylmorphine)	Semi-synthetic opioid	Morphine derivative with acetyl groups at positions C3 and C6, increasing lipid solubility	Rapidly metabolized to 6-monoacetylmorphine and morphine; high MOR affinity	Produces intense euphoria, rapid onset analgesia, high abuse potential, significant risk of overdose due to respiratory depression
Oxycodone	Semi-synthetic opioid	Thebaine derivative with modifications at C14 (hydroxyl group), C6 (carbonyl group), and a methoxy group at C3	High affinity for MORs; some affinity for KORs	Used for moderate to severe pain, potential for abuse and dependence, can cause typical opioid side effects including sedation and constipation
Fentanyl	Synthetic opioid	Phenylpiperidine derivative; highly lipophilic with a flexible structure allowing for strong receptor binding	Very high affinity for MORs	Provides extremely potent analgesia, rapid onset and short duration, high risk of respiratory depression, significant contributor to overdose deaths when misused
Methadone	Synthetic opioid	Diphenylheptane structure; racemic mixture with R-methadone being the active isomer	High affinity for MORs; NMDA receptor antagonism	Used in opioid replacement therapy, long half-life allows for once-daily dosing, mitigates withdrawal symptoms, risk of cumulative toxicity due to long half-life
DAMGO	Synthetic peptide	[D-Ala^2, N-MePhe^4, Gly-ol]-	Highly selective for MORs	Used exclusively in research settings

		enkephalin; modified enkephalin with increased stability and selectivity		to study MOR function, not used clinically, provides insights into receptor pharmacology and signaling mechanisms
Hydrocodone	Semi-synthetic opioid	Similar to codeine with a hydrogenated ketone at C6 and removal of the double bond between C7-C8	Moderate to high affinity for MORs	Prescribed for moderate to severe pain and as an antitussive, potential for abuse and dependence, side effects include sedation, dizziness, nausea, and constipation
Buprenorphine	Semi-synthetic opioid	Derived from thebaine; partial MOR agonist and KOR antagonist; complex structure with high lipophilicity	High affinity but partial agonist at MORs; antagonist at KORs	Used in opioid replacement therapy, lower risk of respiratory depression due to ceiling effect, can precipitate withdrawal in dependent individuals, reduces cravings

Opioid Receptors: Classification and Mechanisms

Opioid receptors are a subset of the G protein-coupled receptor (GPCR) superfamily, characterized by seven transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus (Kieffer & Evans, 2009). They mediate the effects of endogenous and exogenous opioids through metabotropic signaling pathways. The primary opioid receptors include:

1. Mu-Opioid Receptors (MORs)
2. Delta-Opioid Receptors (DORs)
3. Kappa-Opioid Receptors (KORs)
4. Nociceptin/Orphanin FQ Receptors (NOP/ORL-1)

Each receptor type has distinct but sometimes overlapping distributions in the nervous system, unique signaling mechanisms, and specific physiological roles. Understanding these differences is crucial for developing targeted therapies that minimize side effects.

1. Mu-Opioid Receptors (MORs)

Distribution

MORs are widely expressed throughout the central and peripheral nervous systems. Key regions include:

- Brainstem: Nucleus tractus solitarius and periaqueductal gray (PAG), involved in analgesia and respiratory control (Pattinson, 2008).
- Thalamus and Cortex: Mediating sensory perception and pain modulation (Mansour et al., 1995).
- Mesolimbic System: Ventral tegmental area (VTA) and nucleus accumbens (NAc), critical for reward and reinforcement (Fields & Margolis, 2015).

- Peripheral Nervous System: Dorsal root ganglia and peripheral nerve terminals, contributing to peripheral analgesia (Stein & Lang, 2009).

Structure and Subtypes

MORs are encoded by the OPRM1 gene, which undergoes extensive alternative splicing to produce multiple isoforms with varying N- and C-terminal sequences (Pasternak & Pan, 2013). These isoforms may contribute to the functional diversity observed in MOR pharmacology and physiology, however evidence for this has been elusive.

Pharmacological studies have suggested the existence of MOR subtypes:

- **μ1**: Mediates supraspinal analgesia; sensitive to naloxonazine blockade.
- **μ2**: Associated with respiratory depression, spinal analgesia, and gastrointestinal effects; naloxonazine-insensitive.
- **μ3**: Identified based on sensitivity to morphine-6β-glucuronide (M6G); may play roles in immune modulation (Pasternak, 2001; Bohn et al., 2000).

The structural basis for these subtypes remains an area of active research, with implications for developing more selective opioid therapeutics.

Signaling Pathways

Upon activation by an agonist, MORs engage inhibitory G proteins ($G_{i/o}$), which initiates several downstream effects. One major effect is the inhibition of adenylyl cyclase, which reduces the production of cyclic adenosine monophosphate (cAMP) and affects protein kinase A (PKA) activity, ultimately altering downstream phosphorylation events (Williams et al., 2013). Additionally, MOR activation modulates ion channels by activating G protein-coupled inwardly rectifying potassium (GIRK) channels, which increases potassium efflux, hyperpolarizing the neuron and reducing its excitability (North, 1993). It also inhibits voltage-gated calcium channels, decreasing calcium influx and thereby reducing neurotransmitter release (Ikeda et al., 2000). Moreover, MORs can recruit β-arrestins, which contributes

to receptor desensitization and internalization, as well as the activation of mitogen-activated protein kinase (MAPK) pathways (Raehal et al., 2011). These molecular actions culminate in the modulation of neuronal circuits involved in pain perception, reward, and other physiological functions

Physiological and Behavioral Roles

Mu-opioid receptors (MORs) are widely expressed in the central and peripheral nervous systems and mediate a range of physiological processes. One of their key functions is in analgesia, where MOR activation inhibits nociceptive transmission at both spinal and supraspinal levels, providing potent pain relief. This analgesic efficacy has made MOR agonists essential tools in clinical pain management (Fields, 2004). However, MORs are also heavily involved in reward and addiction, particularly through their presence in the mesocorticolimbic pathway, including the ventral tegmental area (VTA) and nucleus accumbens (NAc). Activation of MORs in these regions facilitates dopamine release, reinforcing drug-taking behavior and contributing to the addictive potential of opioids (Fields & Margolis, 2015). Another important role of MORs is in respiratory control, as they are expressed in brainstem respiratory centers, modulating ventilatory responses. This effect underlies opioid-induced respiratory depression, a major risk factor in overdose fatalities (Pattinson, 2008).

MOR activation also affects gastrointestinal function by reducing motility and secretions, which often leads to constipation—a common adverse effect of opioid therapy (Camilleri, 2011). In terms of mood and stress responses, MORs influence affective states, with endogenous opioid release during stress exerting anxiolytic effects, while exogenous opioids tend to produce euphoria (Lutz & Kieffer, 2013). Finally, MOR signaling modulates synaptic plasticity, with acute activation impairing learning and memory, while chronic exposure leads to complex adaptations that can affect cognitive function (Pina & Cunningham, 2014).

2. Delta-Opioid Receptors (DORs)

Distribution

DORs are expressed in both the central and peripheral nervous systems, with notable concentrations in:

- Forebrain Structures: Neocortex, olfactory bulb, and striatum, suggesting roles in cognitive and emotional processing (Mansour et al., 1995).
- Limbic System**:** Nucleus accumbens and amygdala, indicating involvement in mood regulation (Pradhan et al., 2011).
- Spinal Cord: Dorsal horn neurons, contributing to spinal analgesia (Gendron et al., 2016).
- Peripheral Sensory Neurons: Modulating pain at the peripheral level (Cahill et al., 2014).

Structure and subtypes

As with other members of the opioid receptor family, DORs possess the classic seven transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus (Kieffer & Evans, 2009). Structurally, DORs share significant similarity with mu-opioid receptors (MORs) and kappa-opioid receptors (KORs), which is reflected in their ability to bind endogenous opioid peptides such as enkephalins. Though less extensively studied, DORs may exhibit some structural diversity due to alternative splicing, which could lead to subtle differences in receptor function across various tissues. DORs are predominantly found in the forebrain, limbic regions, and spinal cord, contributing to their role in modulating pain, emotion, and neuroendocrine functions (Pradhan et al., 2011). This structural and functional complexity makes DORs a promising target for therapeutic development, particularly in chronic pain and mood disorders, with selective agonists showing antidepressant and anxiolytic potential.

Signaling Pathways

DORs also couple to inhibitory G proteins ($G_{i/o}$), resulting in several downstream effects. Similar to MORs, DOR activation inhibits adenylyl cyclase, thereby reducing cAMP levels (Gendron et al., 2016). This is accompanied by modulation of ion channels, including the activation of GIRK channels, which causes hyperpolarization and decreases neuronal excitability (Waldhoer et al., 2004). In addition, DOR activation inhibits N-type calcium channels, leading to reduced neurotransmitter release (Evans et al., 2010). Beta-arrestin recruitment also plays a role in DOR signaling, contributing to receptor desensitization and internalization, although its involvement is less pronounced compared to MORs (Pradhan et al., 2012).

Physiological Roles

DORs are involved in several important physiological roles, particularly in modulating pain and mood-related behaviors. DORs mediate spinal analgesia and are especially effective in chronic pain models, making them promising targets for treating persistent pain conditions (Gendron et al., 2016). In addition to their role in pain modulation, DOR agonists exhibit antidepressant-like effects in animal models, suggesting potential therapeutic applications for mood disorders (Jutkiewicz, 2006). Activation of DORs also has anxiolytic effects, as it can reduce anxiety-like behaviors (Saitoh et al., 2004). Moreover, DOR activation may provide neuroprotective benefits by protecting neurons from hypoxic or ischemic damage, which could have implications for treating neurodegenerative diseases or stroke (Scherrer et al., 2009). Finally, DORs are expressed on immune cells and may play a role in modulating inflammatory responses, indicating their involvement in immune function (Philipp et al., 2016).

3. Kappa-Opioid Receptors (KORs)

Distribution

KORs are distributed in various brain regions and peripheral tissues:

- Hypothalamus: Regulating neuroendocrine functions (Mansour et al., 1995).

- Periaqueductal Gray: Involved in pain modulation (Bodnar, 2013).
- Amygdala and Nucleus Accumbens: Affecting emotional processing and stress responses (Bruchas et al., 2010).
- Spinal Cord: Modulating nociceptive transmission (Simonin et al., 1995).
- Peripheral Tissues: Including sensory neurons and immune cells (Gross & Ibanez-Tallon, 2008).

Structure

KOR, encoded by the OPRK1 gene, and they share the general structure typical of the opioid receptor family, which includes seven transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus (Kieffer & Evans, 2009). KORs are predominantly expressed in regions of the brain associated with stress, reward, and pain perception, such as the hypothalamus, amygdala, periaqueductal gray (PAG), and spinal cord (Bruchas et al., 2010). KOR subtypes are less clearly defined compared to MORs, but different functional forms of KORs may arise through post-translational modifications, such as phosphorylation, which can alter their signaling properties and desensitization profiles. KORs are distinct in their interaction with the endogenous ligand dynorphin, which contributes to their well-known roles in stress-induced dysphoria, aversion, and modulation of pain (Land et al., 2008). The complexity of KOR signaling and its involvement in aversive states makes it an intriguing target for developing therapeutics aimed at treating addiction, depression, and stress-related disorders.

Signaling Pathways

Kappa-opioid receptors (KORs) similarly couple to $G_{i/o}$ proteins, which leads to a range of downstream effects. Activation of KORs inhibits adenylyl cyclase, resulting in decreased cAMP levels (Bruchas & Chavkin, 2010). KOR activation also modulates ion channels, including the activation of GIRK channels,

which leads to hyperpolarization of neurons (Wagner et al., 2010), and the inhibition of voltage-gated calcium channels, reducing neurotransmitter release (Bodnar, 2013). Moreover, KORs robustly recruit β-arrestins, which not only contribute to receptor desensitization and internalization but also activate MAPK signaling pathways (Bruchas & Chavkin, 2010).

Physiological Roles

KOR activation produces a variety of physiological effects, many of which distinguish it from other opioid receptors. KOR activation provides analgesia, particularly effective in visceral and neuropathic pain models, making it a potential target for treating these types of pain (Chen et al., 2007). However, unlike mu-opioid receptors (MORs), KOR activation often leads to dysphoric and aversive effects, which are mediated through the modulation of dopamine release in the mesolimbic pathway, resulting in negative emotional experiences (Land et al., 2008). KORs also play a critical role in the body's response to stress and are implicated in stress-induced reinstatement of drug-seeking behavior, highlighting their involvement in addiction relapse (Van't Veer & Carlezon, 2013). Additionally, KOR activation has physiological effects beyond the nervous system, such as increasing urine production by inhibiting the release of vasopressin, leading to diuresis (Gutstein & Akil, 2001). Given their unique influence on mood and stress, KOR antagonists are currently being explored as potential treatments for depression and anxiety disorders, with the aim of alleviating negative affective states without the dysphoric side effects of KOR activation (Carlezon et al., 2009).

4. Nociceptin/Orphanin FQ Receptors (NOP/ORL-1)

Distribution

NOP receptors are widely distributed in the central nervous system:

- Cortex and Thalamus: Suggesting roles in sensory processing and cognition (Mollereau & Mouledous, 2000).

- Hippocampus: Implicated in learning and memory (Vaughan & Christie, 1996).
- Amygdala: Involved in emotional regulation (Gavioli & Calo, 2013).
- Spinal Cord: Modulating pain transmission (Xu et al., 1996).
- Peripheral Nervous System: Present in peripheral nerve terminals and autonomic ganglia (Mathis et al., 2011).

Structure

The nociceptin/orphanin FQ receptor (NOP), also known as the opioid receptor-like (ORL-1) receptor, is structurally similar to classical opioid receptors but exhibits unique ligand specificity and functional properties. NOP is encoded by the OPRL1 gene and, like other opioid receptors, features the characteristic seven transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus (Meunier et al., 1995). However, unlike MORs, DORs, and KORs, NOP does not bind traditional opioid peptides like enkephalins or endorphins but is instead activated by the peptide nociceptin/orphanin FQ. NOP receptors are widely distributed throughout the central nervous system, including the cortex, hippocampus, thalamus, and spinal cord, indicating their involvement in diverse physiological processes such as pain modulation, learning, and emotional regulation (Mollereau & Mouledous, 2000). Though NOP subtypes are not well-characterized, variations in receptor expression and signaling properties across different brain regions suggest a level of functional diversity. NOP receptors represent an important therapeutic target, particularly for pain management, anxiety, and substance use disorders, without the risk of dependence commonly associated with classical opioid receptors.

Signaling Pathways

Nociceptin/orphanin FQ receptors (NOP) also couple to $G_{i/o}$ proteins, leading to several downstream effects. NOP receptor activation results in the inhibition of adenylyl cyclase, which reduces cAMP levels (Mollereau & Mouledous, 2000). In terms of ion channel modulation, NOP receptors activate GIRK channels, causing neuronal hyperpolarization and decreased excitability (Vaughan & Christie, 1997). Additionally, NOP receptors inhibit N-type calcium channels, leading to a reduction in neurotransmitter release (Schlicker & Morari, 2000). NOP receptors can also recruit β-arrestins, though the functional consequences of β-arrestin recruitment in NOP signaling are less well understood compared to classical opioid receptors (Mollereau & Mouledous, 2000).

Physiological Roles

NOP receptors are involved in several physiological roles, each contributing to the complex modulation of behavior and bodily functions. In terms of pain modulation, NOP receptor activation produces anti-analgesic effects at supraspinal levels but analgesic effects at the spinal level, indicating a dual role in pain pathways (Lambert, 2008). Regarding stress and anxiety, nociceptin, the endogenous ligand for NOP receptors, can produce anxiolytic effects, thereby modulating stress responses (Gavioli & Calo, 2013). NOP receptors also influence reward pathways, playing a role in modulating the effects of drugs of abuse, including opioids, cocaine, and alcohol, which positions them as important players in addiction biology (Kallupi et al., 2017). In addition to these roles, NOP receptors in the substantia nigra and striatum are implicated in motor control, suggesting their involvement in regulating motor function (Marti et al., 2004). Moreover, nociceptin can stimulate food intake, indicating that NOP receptors are involved in energy homeostasis and feeding behavior (Polidori et al., 2000).

Comparative Summary

Comparatively, all opioid receptors, including MORs, DORs, KORs, and NOP receptors, primarily couple to $G_{i/o}$ proteins, resulting in the inhibition of adenylyl cyclase and the modulation of ion channels. In

terms of analgesic effects, MORs and DORs are the primary mediators of pain relief, with MORs being the most clinically significant. KORs also contribute to analgesia but are limited by their dysphoric effects, while NOP receptors exhibit complex pain-modulating properties, providing both analgesic and anti-analgesic effects depending on the site of action. When it comes to mood and reward, MOR activation promotes reward and euphoria, contributing significantly to addiction potential, while KOR activation typically results in dysphoria and aversion. DOR activation may have antidepressant effects, whereas NOP receptors play a role in modulating both stress responses and reward pathways. Understanding these distinct roles allows for the development of targeted therapeutic interventions. MOR agonists are potent analgesics but come with high abuse potential. DOR agonists show promise for providing pain relief with fewer side effects, and they also have antidepressant effects. KOR antagonists are currently being investigated for treating depression and addiction due to their ability to counteract dysphoria, while NOP receptor modulators have potential in treating conditions such as pain, anxiety, and substance use disorders.

Current Research and Clinical Implications

Recent advances focus on developing biased agonists and allosteric modulators that selectively engage beneficial signaling pathways while avoiding adverse effects:

- G Protein vs. Beta-Arrestin Signaling: Biased agonists that favor G protein signaling over β-arrestin recruitment may provide analgesia with reduced tolerance and respiratory depression (Schmid et al., 2017).
- Allosteric Modulators: Compounds that modulate receptor activity indirectly offer another avenue for selective therapeutic effects (Burford et al., 2015).
- Receptor Heteromers: Opioid receptors can form heteromers (e.g., MOR-DOR heteromers), which have unique pharmacological properties and may be targeted for novel treatments

(Gomes et al., 2013). It is worth noting however, that these hereromers have not been confirmed in any physiological context but instead are seen after over expression in cultured cell lines.

Regulation of Mu-Opioid Receptors

Desensitization and Internalization

Prolonged activation of mu-opioid receptors (MORs) leads to receptor phosphorylation by G protein-coupled receptor kinases (GRKs), which facilitates the recruitment of β-arrestins (Williams et al., 2013). This recruitment process causes the uncoupling of MORs from G proteins, resulting in receptor desensitization, and subsequently targets the receptors for internalization via clathrin-mediated endocytosis. Desensitization can occur through different mechanisms: homologous desensitization is specific to the activated receptor subtype and is a direct result of phosphorylation and β-arrestin binding, whereas heterologous desensitization involves phosphorylation by second messenger-dependent kinases, such as protein kinase C, which affects multiple receptor types (Kelly, 2013). Once internalized, MORs can either be dephosphorylated and recycled back to the plasma membrane, thus resensitizing the receptor, or they can be directed to lysosomes for degradation, which contributes to receptor downregulation and impacts the cell's responsiveness to subsequent opioid exposure (Whistler et al., 1999).

Tolerance Development

Tolerance to opioids develops as a result of repeated drug administration, leading to a diminished response over time. Several mechanisms contribute to opioid tolerance. Receptor downregulation occurs when there is a decrease in receptor expression on the cell surface due to enhanced degradation or reduced synthesis. Additionally, receptor desensitization involves persistent phosphorylation and the uncoupling of receptors from G proteins, which reduces receptor responsiveness. Adaptive cellular

changes also play a role, including the upregulation of adenylyl cyclase and other signaling components that counteract the inhibitory effects of opioids (Ingram et al., 1998). Furthermore, alterations in synaptic plasticity, such as changes in neurotransmitter release and receptor expression at synapses, can affect neuronal circuit function, contributing to tolerance (Christie, 2008).

Withdrawal and Dependence

Withdrawal and physical dependence result from neuroadaptive changes that manifest as withdrawal symptoms when opioid use is discontinued. Withdrawal is characterized by neurochemical imbalances, such as rebound hyperactivity of noradrenergic neurons in the locus coeruleus, which contributes to autonomic symptoms like tachycardia and hypertension (Van Bockstaele et al., 2001). During withdrawal, upregulated adenylyl cyclase activity leads to elevated cAMP levels, enhancing neuronal excitability—a phenomenon known as cAMP overshoot (Nestler & Aghajanian, 1997). Activation of the stress axis is also involved, with increased corticotropin-releasing factor (CRF) activity in the extended amygdala, promoting negative affective states (Koob, 2008). Hyperkatifeia, a state characterized by heightened emotional distress and negative affect, further exacerbates the withdrawal experience and contributes to the negative reinforcement driving continued drug use (Koob, 2013). This increased sensitivity to negative emotional states during withdrawal may play a crucial role in the high relapse rates observed in individuals attempting to quit opioid use. Additionally, epigenetic modifications, such as altered gene expression due to DNA methylation and histone modifications, may contribute to a long-lasting vulnerability to relapse, highlighting the persistent nature of dependence (Robison & Nestler, 2011).

Opioid Effects on Neural Circuits

Understanding how opioids alter neural circuitry is critical for developing effective interventions for addiction and pain management.

Mesocorticolimbic Pathway

Opioids modulate the mesocorticolimbic dopamine system, which includes the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC). Activation of mu-opioid receptors (MORs) in the VTA disinhibits dopaminergic neurons, leading to increased dopamine release in the NAc, which reinforces rewarding behaviors (Fields & Margolis, 2015). In addition, opioids induce synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD) at synapses within this pathway, which alters reward processing and contributes to the development of addiction (Kauer & Malenka, 2007).

Extended Amygdala

The extended amygdala integrates stress and reward signals. Chronic opioid use enhances CRF signaling in this region, contributing to the negative emotional states during withdrawal (Koob & Volkow, 2010).

Thalamocortical Circuits

Thalamocortical circuits are pivotal in regulating sensory processing, arousal and conscious awareness. These networks allow for the seamless integration of sensory inputs with motor and cognitive processes, establishing a foundation for both perception and behavior. Opioids, particularly through their interaction with MORs, modulate thalamocortical networks by affecting synaptic transmission, altering neuronal excitability, and influencing the flow of sensory information across these circuits (Birdsong et al., 2019). The paraventricular nucleus of the thalamus (PVT) is another key node in these circuits, modulating arousal, attention, and emotional behaviors (Hsu et al., 2014).

In conclusion, understanding how opioids influence neural circuits is crucial for unraveling the complex effects of addiction, pain management, and behavioral regulation. Opioids modulate key circuits like the mesocorticolimibic pathway which governs reward and reinforcement behaviors, and the extended

amygdala, which integrates stress and reward signals. Additionally, opioids affect thalamocortical circuits that regulate sensory processing and arousal. The paraventricular nucleus of the thalamus (PVT), with its dense opioid receptor expression, plays a central role in mediating opioid effects on these neural circuits, influencing behaviors related to stress, reward and addiction. Next, we transition into a deeper examination of the PVT where we will explore how this midline thalamic structure integrates visceral and emotional signals and how its modulation has been shown to contribute to normal behavior and opioid usage.

The Paraventricular Nucleus of the Thalamus (PVT)

The PVT, situated beneath the third ventricle and extending along the rostrocaudal axis of the thalamus, has garnered increasing attention due to its multifaceted role in regulating a wide array of behavioral processes. Anatomically, the PVT is uniquely positioned to act as a hub, integrating visceral and emotional information and relaying it to various limbic and cortical regions (Li and Kirouac, 2012; Barson et al., 2020). Functionally, it is critically involved in modulating arousal states (Gao et al., 2020; Bu et al., 2022; Zhao et al., 2022; Eacret et al., 2023; Duan et al., 2024), orchestrating motivated behaviors encompassing both reward and aversion (Penzo et al., 2015; Zhang and van den Pol, 2017; Beas et al., 2020; Petrovich, 2021), and influencing drug-seeking behavior and dependence (Bengoetxea et al., 2020; Chisholm et al., 2020; Keyes et al., 2020; Giannotti et al., 2021; Yu et al., 2021; Babaei et al., 2023; Zhu et al., 2023; McDevitt et al., 2024; Paniccia et al., 2024).

One of the earliest functional studies highlighting the PVT's role in stress responses was conducted by Bhatnagar and Dallman (1998). They demonstrated that chronic stress leads to the facilitation of hypothalamic-pituitary-adrenal (HPA) axis responses to novel stressors and identified the PVT as a key neuroanatomical substrate mediating this effect. Lesions of the PVT attenuated the exaggerated corticosterone responses observed after chronic stress, indicating that the PVT plays a crucial role in the

modulation of HPA axis activity. This foundational work set the stage for subsequent research exploring the PVT's involvement in stress and arousal. The PVT was initially characterized as a stress-responsive region due to increased expression of immediate early genes like c-fos following exposure to various stressors (Bhatnagar and Dallman, 1998; Hsu et al., 2014). These findings suggested that the PVT is activated by aversive stimuli and may contribute to the processing of stress-related information.

However, as highlighted in the comprehensive review by Kirouac (2021), the PVT's role extends beyond stress responses to include the integration of both aversive and rewarding signals. The PVT is activated by rewarding stimuli such as food and drugs of abuse, indicating its involvement in positive valence processing (Barson et al., 2020; Kirouac, 2021). This duality underscores the complexity of the PVT's function and suggests that it does not act as a homogeneous structure.

The heterogeneity in effectively driving valence is what gave rise to the initial compartmentalization of the nucleus into anterior and posterior subregions, known as the anterior PVT (aPVT) and posterior PVT (pPVT), respectively. Further studies indicate that these two regions are distinct in their activations, projection patterns, genetic identities, firing properties, and behavioral outputs (Li and Kirouac, 2012; Kolaj et al., 2012, 2014; McDevitt and Graziane, 2019; Gao et al., 2020).

Many studies have reported the intrinsic properties of neurons in the anterior and posterior PVT, but few have examined both compartments simultaneously. Rat PVT neurons have somata with diameters ranging from 12–20 microns, with 3–7 aspiny main dendrites that extend for several hundreds of microns (Richter et al., 2006; Zhang et al., 2009, 2010). These PVT neurons are primarily glutamatergic, using glutamate for rapid neurotransmission (Christie et al., 1987; Csáki et al., 2000; Hur and Zaborszky, 2005).

Heilbronner and Flügge (2005) conducted a study in which they filled PVT neurons with neurobiotin and assessed morphological parameters such as somatic perimeter and number of primary dendrites. They

also examined neurons in the aPVT, middle PVT (mPVT), and pPVT that either hyperpolarized or depolarized in response to α_2-adrenoceptor activation via α-methyl-norepinephrine. Although both responses were observed in all three subdivisions, aPVT neuron responses were mostly depolarizing, whereas mPVT neuron responses were hyperpolarizing. This suggests that, despite morphological similarities, functional differences exist between neurons in different subregions of the PVT. This study exemplifies the extent of heterogeneity that can be seen across the rostrocaudal aspect of the PVT.

The pPVT has received more attention in the literature, likely due to its sensitivity to bodily distress and its prominent role in stress-related behaviors. Activation of the pPVT occurs in response to threats to homeostasis, such as food restriction, physical restraint, tail suspension, foot shock, and even conditioned cues initially paired with foot shock (Penzo et al., 2015; Beas et al., 2018; Gao et al., 2020). Conversely, stimuli that have a positive valence, such as social interaction or exposure to a thermoneutral environment, decrease pPVT activity (Gao et al., 2020; Kirouac, 2015). This has led to the hypothesis that pPVT neurons are activated to position an animal to take action in response to aversive or threatening stimuli, contributing to adaptive behavioral responses.

The pPVT projects to several brain regions, including the nucleus accumbens (NAc), central nucleus of the amygdala, bed nucleus of the stria terminalis, and prefrontal cortex (Li and Kirouac, 2008; Dong et al., 2017; Do-Monte et al., 2017). Direct stimulation of pPVT neurons projecting to the NAc has been shown to increase aversion in real-time place preference assays (Zhu et al., 2016), while inhibition of these neurons decreases drug-seeking behavior, as measured by the number of lever presses in self-administration paradigms (DeGroote et al., 2021; Vollmer et al., 2022). Moreover, pPVT neurons exhibit diurnal variations in activity; they are mostly silent during the day when rodents are inactive and display tonic or burst firing during the night when rodents are more active (Kolaj et al., 2012). The borders of the pPVT can be defined by the expression of dopamine receptor 2 (Drd2) and orexin/hypocretin 2 receptor (OX2R), which are sparse in anterior sections of the mouse brain but become more prevalent

posteriorly (Marcus et al., 2001; Meffre et al., 2019). This gradient in receptor expression further underscores the molecular heterogeneity along the rostrocaudal axis of the PVT.

Conversely, the aPVT has been somewhat more challenging to study due to several factors. Firstly, the aPVT does not exhibit clear-cut responses to specific stimuli, making it difficult to delineate its functional roles. Additionally, there is a lack of distinct behavioral paradigms that selectively engage the aPVT, and its neuronal populations may display complex and heterogeneous activity patterns. In comparison to the pPVT, the aPVT has a larger percentage of neurons that are active at baseline when an animal is at rest, including "type II neurons," which are characterized by their spontaneous firing rates and specific electrophysiological properties (Gao et al., 2020). Unlike the pPVT, the aPVT does not seem to respond to a particular stimulus valence; both noxious and rewarding stimuli lead to inhibition of aPVT neurons (Gao et al., 2020). Indeed, inhibition of aPVT neurons that project to the NAc is rewarding, suggesting that the aPVT may tonically suppress reward pathways (Lafferty et al., 2020; Barson et al., 2020). Furthermore, activation of the aPVT has been shown to mediate a variety of behaviors, including chronic hyperalgesia and appetitive feeding (Furlong et al., 2009).

It would be an overgeneralization to state that the pPVT exclusively responds to aversive stimuli and positions an animal to take action, whereas the aPVT responds solely to rewarding stimuli. Both regions contribute to a complex interplay of neural circuits that modulate behavior. The aPVT shares many of the same projection targets as the pPVT; however, the terminals of these projections segregate into different compartments within target regions. For example, while both project to the NAc, the pPVT terminates predominantly in the ventral aspect of the NAc shell, associated with aversive processing, whereas the aPVT terminates more in the dorsal aspect of the NAc shell, which is implicated in reward processing (Li and Kirouac, 2008; Dong et al., 2017). This difference in their projection patterns and synaptic connectivity may contribute to their differential behavioral outputs.

Moreover, the expression of neuromodulatory receptors, such as mu-opioid receptors (MORs), varies between the aPVT and pPVT. MORs are known to modulate neuronal excitability and synaptic transmission, influencing behaviors related to reward and addiction (Matzeu et al., 2018; Keyes et al., 2020). The distribution and functional impact of MORs within the PVT are not homogeneous, and understanding these differences is crucial for elucidating how opioids affect PVT-mediated behaviors.

Molecular Understanding of the PVT in the Context of Opioids

At the molecular level, progress has been made in understanding how opioids interact with the PVT to influence behavior. The PVT expresses high levels of opioid receptors, including mu-opioid receptors (MORs), delta-opioid receptors (DORs), and kappa-opioid receptors (KORs), making it a significant site for opioid action (Lodge and Grace, 2011; Lavezzi et al., 2011). Autoradiography and in situ hybridization studies have confirmed the presence of MOR mRNA and protein within the PVT, with a heterogeneous distribution favoring certain subregions (Mansour et al., 1995; Sharif and Hughes, 1989).

Mu-Opioid Receptors (MORs)

MORs are G-protein-coupled receptors that, upon activation by endogenous ligands like endorphins or exogenous opioids like morphine, inhibit adenylate cyclase activity, decrease cAMP levels, and lead to hyperpolarization of neurons by opening potassium channels and closing calcium channels (Williams et al., 2001). In the PVT, MOR activation has been shown to modulate neuronal excitability and synaptic transmission (Matzeu et al., 2018; Keyes et al., 2020).

Molecular Studies on Opioid Effects in the PVT

Recent molecular studies have focused on understanding how opioids affect gene expression and intracellular signaling pathways within the PVT:

- **c-Fos Expression:** Opioid withdrawal induces robust c-Fos expression in the PVT, indicating increased neuronal activity (Zhu et al., 2016; Bechard et al., 2021). This suggests that the PVT is actively involved in mediating the negative affective states associated with withdrawal.
- **Dynorphin/Kappa-Opioid System:** The PVT contains dynorphinergic neurons, and activation of KORs within the PVT has been implicated in stress-induced reinstatement of drug seeking (Matzeu et al., 2018). Molecular studies have shown that stress increases dynorphin expression in the PVT, which can modulate glutamatergic transmission to downstream targets like the NAc (Li et al., 2019). It is also important to mention that KORs have not been proven to be functional in the PVT and it is likely the effect of pre-synaptic KORs or nonselective agonist also activating MORs.
- **Synaptic Plasticity:** Opioid exposure can induce synaptic plasticity within the PVT. Chronic opioid administration alters the expression of AMPA and NMDA receptor subunits, affecting excitatory neurotransmission (Chen et al., 2015). These changes at the molecular level may contribute to the altered neural circuitry associated with addiction.
- **Intracellular Signaling Pathways:** Opioid receptors in the PVT are coupled to intracellular signaling cascades involving MAPK/ERK pathways. Activation of MORs can lead to phosphorylation of ERK1/2, influencing gene transcription and neuronal plasticity (Bertran-Gonzalez et al., 2009). Understanding these pathways is crucial for deciphering how opioids remodel PVT neurons at the molecular level.

Interaction with Other Neurotransmitter Systems

The PVT's interaction with other neurotransmitter systems further complicates its molecular responses to opioids:

- **Glutamatergic Transmission:** The PVT is predominantly glutamatergic, and opioids can modulate glutamate release both presynaptically and postsynaptically (Eppolito et al., 2013). Molecular studies have shown that MOR activation can inhibit glutamate release, affecting excitatory signaling in target regions like the NAc and amygdala.

The intricate interplay between the PVT's glutamatergic signaling and opioid modulation underscores the complexity of its role in mediating neural circuits underlying stress, reward, and addiction, highlighting the necessity for a comprehensive understanding of these mechanisms to inform therapeutic strategies.

Chapter 2: Methods

This chapter details methods, reagents and analysis used in the subsequent chapters.

Animals:

Adult male and female rats with ages between 5 – 8 weeks included both wild-type and μ-opioid receptor knockout (MOR KO) rats on a Sprague-Dawley background. Wildtype Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR KO Sprague-Dawley rats were used from an in-house colony as described in Arttamangkul et al. (2019). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

Microinjections:

Animals (P23-30) were anesthetized with 4% isoflurane and placed in a stereotaxic frame under constant anesthesia (2.5% isoflurane) for viral microinjection of a retrograde adeno-associated virus encoding a green fluorescent protein (AAVrg-CAG-GFP; Catalog # 37825-AAVrg). Injections included the following areas: (1) basolateral amygdala (BLA, anteroposterior: -2.5 mm, mediolateral: ± 3 mm, dorsoventral: -6.95 mm, from bregma; (2) nucleus accumbens (NAc) anteroposterior: +1.7 mm, mediolateral: ± 0.7 mm, dorsoventral: -7 mm, from bregma; and (3) medial prefrontal cortex (mPFC anteroposterior: +1.8 mm, mediolateral: ± 0.5 mm, dorsoventral: -4.25 mm, from bregma). For imaging experiments an adeno-associated virus expressing a SNAP-tagged MOR (pFB-CAG-MOR-snap-SF-P2S-GFP-WPRE-SC40pA, ViroVek) was injected into the aPVT (anteroposterior: -1 mm, mediolateral: ± 0.15 mm, dorsoventral: -5.5 mm, from bregma). A total of 200 nl of each virus was injected at 1 nl/second bilaterally in all regions. All electrophysiology experiments were carried out at least two weeks after viral injection.

Chronic Morphine Treatment:

Rats were treated with morphine sulfate continuously released from osmotic pumps as described previously (Quillinan et al., 2011). Osmotic pumps (2 ML1; Alzet, Cupertino, CA) were filled with the required concentration of morphine in water to deliver 80 mg/kg/day. Each pump has a 2-ml reservoir that releases 10 µl/h for up to 7 days. The dose was chosen to induce the maximum amount of tolerance over the relatively short duration of application. Rats were anesthetized with 4% isoflurane, and an incision was made in the midscapular region for subcutaneous implantation of osmotic pumps under 2.5% isoflurane. The incision was closed with 4-5 stainless steel, ez clip wound closure (Stoelting; #59027). Pumps remained until animals were used for experiments 6 or 7 days later.

Pharmacology:

Drug	Mechanism of Action	Abbreviation	Source
[Met]^5 enkephalin	MOR/DOR agonist	ME	Sigma-Aldrich (St. Louis, MO) [CAS#: 82362-17-2]
Morphine	MOR agonist	-	National Institute of drug abuse (Baltimore, MD)
Nociceptin	OFQR agonist	OFQ	Tocris (Bio-techne Corp., Minneapolis, MN) [CAS#: 170713-75-4]
Naloxone	Antagonist: MOR (full), DOR/KOR (partial)	NLX	Abcam (Cambridge, MA) [CAS#: 357-08-4]

Baclofen	$GABA_B$ agonist	-	Sigma-Aldrich (St. Louis, MO) [CAS: 1134-47-0]
CGP55845	$GABA_B$ antagonist	-	Tocris (Bio-techne Corp., Minneapolis, MN) [CAS#: 149184-22-5]
Picrotoxin	$GABA_A$ antagonist		Tocris (Bio-techne Corp., Minneapolis, MN) [CAS#: 124-87-8]
(+) MK-801	NMDAR blocker	-	Hello Bio (Princeton, NJ) [CAS#: 77086-22-7]
Bestatin	Peptidase inhibitor	-	Sigma-Aldrich (St. Louis, MO) [CAS#: 65391-42-6]
Thiorphan	Enkephalinase inhibitor	-	Sigma-Aldrich (St. Louis, MO) [CAS#: 76721-89-6]
Compound 101	GRK2/3 blocker	CPMD101	Hello Bio (Princeton, NJ) [CAS#: 865608-11-3]
Cyclodextrin			Biotechne (Minneapolis, MN) [CAS#: 7585-39-9]
Barium chloride	Non-selective potassium channel blocker	BaCl2	

TTA-A2	Selective T-type calcium channel antagonist	-	

Electrophysiology:

Tissue Preparation. Rats were deeply anesthetized and euthanized by cardiac percussion. Brains were extracted and blocked, removing the posterior half of the brain. The brain was then fixed onto the vibratome stage (Krazy Glue) and placed in the vibratome chamber with warm (34°C) ACSF containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NAHCO_3$, and D-glucose with +MK-801 (10 µM). Horizontal brain slices (272 µm) containing the locus coeruleus (LC) were then made using a vibratome (VT 1200S; Leica, Nussloch, Germany). The PVT is a midline thalamic area with dense MOR expression, making it a good area to study MORs. The PVT was identified based on its proximity to the 3rd ventricle. Posterior PVT sections could be obtained in both horizontal and coronal preparation. No difference was noted in the quality of recordings or cells based on horizontal versus coronal sectioning though coronal is recommended to maximize the number of sections and clarity of anatomy. Anterior PVT sections could also be prepared as both coronal and horizontal sections however horizontal is recommend to maximize the number of slices and avoid the barrier that are the ependymal cells that line the ventricle. Slices were allowed to recover in warm ACSF containing +MK-801 (10 µM) for at least 30 minutes and then hemisected and stored in glass vials with warm (34°C), oxygenated (95% O_2/5% CO_2) ACSF until used.

Whole cell Recording. Slices were transferred to the recording chamber and continuously superfused with ACSF (1.5–2 ml/min, at 34° C) with morphine (1 µM) where applicable. Recording pipettes (World Precision Instruments, Saratosa, FL) with a resistance of 2-3 MΩ were filled with an internal solution of

(mM) 100 potassium methanesulfonate, 20 NaCl, 1.5 MgCl2, 5 HEPES(K), 2 BAPTA, 2 Mg-ATP, 0.3 NaGTP, pH 7.35, and 275–280 mOsM. Whole-cell recordings from aPVT neurons were obtained using an Axopatch 1D amplifier (Axon Instruments) in either voltage-clamp mode (Vhold = -70 mV) or in a current-clamp mode where the current was manually injected to hold the cell at -70 ± 5 mV. Cells that required more than ± 200 pA of injected current were rejected. Data was collected at 20 kHz and filtered at 10 kHz using AxographX and recordings were continuously monitored using PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO). Basal properties (membrane resistance and capacitance) and series resistance were determined using the average of 20 or more 5 mV pulses following break-in. Series resistance at break-in was <20 MΩ and experiments were terminated if the series resistance increased by >20%.

Drug Perfusions and Incubations. Agonists, including ME, OFQ, and baclofen, and respective antagonists: naloxone, barium chloride (Back), CGP55854 were applied via bath perfusion at a rate of approximately 2 mL/minute. For experiments using the kinase inhibitor compound 101, slices were incubated in a higher concentration (30 μM) in ACSF for at least 1 hour prior to recording. Inhibitors were also included in the bath and drug perfusion solutions (1 μM). For experiments stimulating afferents, picrotoxin (1 μM) and CNQX (10 μM) were used to isolate glutamatergic and GABAergic inputs, respectively.

Measuring Desensitization. The PVT contains multiple $G_{i/o}$ coupled GPCRs that couple to G protein-coupled inwardly rectifying potassium channels (GIRKs). Therefore, GIRK-induced outward current serves as an excellent proxy for GPCR (such as MOR and OFQR) activation. Acute desensitization was measured as the decline in the peak current during the continuous application of a supersaturating concentration of agonist. For example, for MORs, 30 μM ME was superfused for 10 minutes which resulted in a peak response in 2-3 minutes.The peak response was followed by a rapid decline often

eventually reached a steady state in ~7 minutes. This is what I will call acute desensitization. Acute desensitization was measured as a function of 1 minus the ratio of steady state over the peak response.

$$Acute\ desensitization\ (or\ \%\ decline) = 1 - \frac{Steady\ state}{Peak\ response}$$

Chronic morphine treatment. There are two standards for developing tolerance in animal models. 1) Repeated injection of the drug of choice at a fix interval or 2) consistent superfusion via implantation of a pump/pellet diffusing the drug. Both come with benefits and drawback. Repeated injections more accurately recapitulate what an individual would experience and a drug user. That is, multiple "highs" associated with drug taking. This also comes with the benefits of allowing for a cleaner representation of the adaptations that occur as dependence develops. On the contrary, consistent superfusion allows us to assess the effect of chronic MOR activation without the caveat of adaptations seen during the withdrawal phase. It is worth noting that while this is the intention, removing adaptations, even in the face of constant MOR activation, is likely not possible. With these caveats in mind, we elected to use osmotic minipumps superfusing morphine subcutaneously. Rats were treated with morphine sulfate continuously released from osmotic pumps as described previously (Quillinan et al., 2011). Osmotic pumps (2 ML1; Alzet, Cupertino, CA) were filled with the required concentration of morphine in water to deliver 80 mg/kg/day. Each pump has a 2-ml reservoir that releases 10 µl/h for up to 7 days. The dose was chosen to induce the maximum amount of tolerance over the relatively short duration of application. Rats were anesthetized with 4% isoflurane, and an incision was made in the midscapular region for subcutaneous implantation of osmotic pumps under 2.5% isoflurane. The incision was closed with 4-5 stainless steel, ez clip wound closure (Stoelting; #59027). Pumps remained until animals were used for experiments 6 or 7 days later.

Measuring Cellular Tolerance. Chronic morphine treatment decreases MORs efficacy. To assess this, for all experiments using tissue from tolerant animals, brain slices were prepared in morphine (1 µM)

solutions to avoid acute withdrawal during whole-cell recordings. Naloxone (1 µM) was then applied revealing the standing morphine current. A decrease in the standing morphine current reversal by naloxone is indicative of a shift in the concentration-response and thus was taken as an indication of tolerance.

Internalization and Imaging:

To visualize the trafficking of virally expressed MORs, brain slices were prepared and incubated in JF-549 (30 nM, 60 min). JF-549 is a cell-permeable fluorescent dye that labels specific proteins or regions of interest, such as receptors or cellular structures, allowing for visualization under microscopy. It was used here to label MORs in order to track their trafficking within neurons. Images were captured before and after the application of a saturating concentration of ME (30 µM, 10 min) using a 2-photon microscope. A z-series of 10-30 sections was acquired at 1-µm intervals so the whole neuron could be resolved.

Internalization analysis. Images were analyzed using ImageJ software (version 1.53; National Institutes of Health, Bethesda, MD). A custom macro was developed to automate the quantification of mu-opioid receptor (MOR) internalization before and after a 10-minute exposure to a saturating concentration of [Met^5]-enkephalin (ME). The macro streamlined the image processing workflow, ensuring consistency and efficiency across all samples by performing a standardized sequence of operations to measure the distribution of MOR within the cytoplasmic compartment.

The macro executed the following steps:

First, two-photon images acquired from the red channel (MOR label) and the green channel (cell marker) were opened in ImageJ. To enhance image quality and reduce background noise, a Gaussian blur filter with a sigma value of 2 was applied to the entire image stack (Process > Filters > Gaussian Blur; sigma = 2). This step smoothed out minor intensity variations due to imaging artifacts.

The image channels were then split to separate the red (channel 1) and green (channel 2) fluorescence signals (Image > Color > Split Channels). A specific Z-plane that best represented the cell morphology and fluorescence intensity was selected for analysis. This Z-plane was chosen based on consistent cell representation across samples.

In the selected Z-plane, the macro converted the green channel image to an 8-bit grayscale image (Image > Type > 8-bit) and applied an automatic threshold using the default method with a dark background setting (Image > Adjust > Threshold; Method: Default, Dark Background). This thresholding step generated a binary mask that delineated the area of interest, specifically highlighting the cytoplasmic region of MOR-expressing neurons. The binary mask was then converted to a black and white image (Process > Binary > Convert to Mask).

To focus exclusively on the cytoplasmic compartment, the macro excluded the perimeter (membrane) regions from the analysis. This was achieved by eroding the binary mask to eliminate edge pixels that might correspond to membrane-associated MOR (Process > Binary > Erode; repeated as necessary). The resulting mask represented the interior of the cell, ensuring that only cytoplasmic MOR fluorescence was measured.

The interior mask was added to the ROI Manager (Analyze > Tools > ROI Manager) for measurement. The macro then selected the corresponding red channel image and measured the area, mean fluorescence intensity, and integrated density within the interior ROI (Analyze > Measure). These measurements provided quantitative data on the cytoplasmic distribution of MOR.

The red pixel count within the cytoplasm was calculated by multiplying the total number of pixels in the ROI by the normalized mean intensity (mean intensity divided by 255, the maximum intensity value for 8-bit images). To assess changes in MOR internalization, the red pixel count after ME treatment was normalized to the baseline (pre-treatment) red pixel count for each cell. This normalization allowed for the comparison of MOR internalization across different cells and experimental conditions.

By automating these steps with a macro, we ensured consistency and reduced potential user-induced variability in the image analysis process. All image processing steps were performed identically across all samples. Measurements were repeated in at least six technical replicates per group. Data were collected and organized for statistical analysis as described in the Data Analysis section.

Data Analysis:

Sample sizes were not predetermined but for all experiments, animals were used to obtain at least six technical replicates per group. Analysis was performed in AxoGraphX and statistics in GraphPad Prism 9 (GraphPad Software, version 9.0d; San Diego, CA). Values are presented as mean ± S.D. Statistical comparisons were made using unpaired T-test, one-way ANOVA, as well as multiple comparison-adjusted Tukey's post hoc tests and Chi-square as appropriate. For all experiments, $p < 0.05$ was used to define statistical significance.

Chapter 3: Acute Response to [Met]^5 Enkephalin Delineates the Anterior and Posterior PVT

Omar Koita, John T Williams

Vollum Institute, Oregon Health & Science University, Portland, OR 97239

Unpublished

ABSTRACT

The paraventricular nucleus of the thalamus (PVT) is a midline structure spanning the rostro-caudal axis of the thalamus, integrating visceral and emotional signals through projections to multiple brain areas, thereby influencing behavior toward aversive or defensive states. Although mu-opioid receptors (MORs) are expressed throughout the PVT, their distribution is not homogeneous. We hypothesized that specific intrinsic neuronal properties predict the degree of MOR activation in the anterior PVT (aPVT) compared to the posterior PVT (pPVT). To test this, we examined the intrinsic properties and acute opioid responses of neurons in both regions. We found no significant differences in capacitance and membrane resistance between neurons of the two regions. Cell attached recordings of spontaneous firing revealed burst firing is more prevalent in pPVT neurons compared to aPVT neurons. Both populations exhibited a hyperpolarization-activated current (Ih); however, Ih was significantly larger in aPVT neurons compared to pPVT neurons. Activation of MORs with [Met^5]-enkephalin (ME) elicited potassium efflux, with aPVT neurons displaying a larger amplitude current than pPVT neurons. These findings reveal distinct regional differences in MOR-mediated responses within the PVT, providing valuable insights that may inform future research on opioid modulation in the PVT.

INTRODUCTION

The paraventricular nucleus of the thalamus (PVT) is a midline thalamic structure uniquely positioned to integrate visceral, emotional, and arousal-related information and relay it to key limbic and cortical regions involved in regulating behavior (Kirouac, 2015; Li and Kirouac, 2012). Spanning the rostrocaudal axis of the thalamus, the PVT is predominantly composed of glutamatergic neurons and is anatomically subdivided into anterior (aPVT) and posterior (pPVT) regions, each exhibiting distinct connectivity patterns, molecular profiles, and functional roles (Gao et al., 2020; Vertes and Hoover, 2008).

Functionally, the PVT has been implicated in a variety of behaviors, including arousal, stress responses, reward processing, feeding behavior, emotional regulation, and drug-seeking behavior (Barson et al.,

2020; Do-Monte et al., 2017; Kirouac, 2021). Early studies identified the PVT as a stress-responsive region, with increased expression of immediate early genes such as *c-fos* following exposure to stressors (Bhatnagar and Dallman, 1998; Hsu et al., 2014). Subsequent research expanded its role to include the processing of rewarding stimuli, highlighting its involvement in both positive and negative valence (Beas et al., 2018; Kirouac, 2021). This duality underscores the complexity of the PVT's function and suggests that it does not act as a homogeneous structure.

The heterogeneity of the PVT is underscored by differences in neuronal activation patterns, projection targets, genetic profiles, and electrophysiological properties between the aPVT and pPVT (Gao et al., 2020; Li and Kirouac, 2012). The pPVT is particularly sensitive to physiological stressors and threats to homeostasis, such as food restriction, physical restraint, and conditioned fear stimuli (Penzo et al., 2015; Zhu et al., 2018). Activation of pPVT neurons facilitates adaptive responses to aversive conditions, likely through projections to the nucleus accumbens (NAc), central amygdala, and bed nucleus of the stria terminalis, influencing emotional and motivational behaviors associated with stress and aversion (Dong et al., 2017; Do-Monte et al., 2017). In contrast, the aPVT has been associated with reward-related processes and exhibits higher baseline neuronal activity, suggesting a role in modulating reward pathways (Barson et al., 2020; Lafferty et al., 2020). The aPVT projects to regions involved in reward and motivation, such as the dorsal NAc shell and medial prefrontal cortex and is thought to tonically inhibit reward-seeking behavior (Li and Kirouac, 2008; Lafferty et al., 2020).

Importantly, the functional behavioral differences between the aPVT and pPVT may be entirely secondary to their differential inputs and outputs. The distinct afferent and efferent connections of these regions could account for their specific roles in processing stress and reward signals (Li and Kirouac, 2012; Dong et al., 2017). However, the potential contribution of intrinsic neuronal properties to these functional differences has not been fully explored. Understanding whether inherent

electrophysiological characteristics of aPVT and pPVT neurons influence their responsiveness to neuromodulators could provide deeper insight into the PVT's role in regulating behavior.

One neuromodulator of particular interest is the endogenous opioid system. Mu-opioid receptors (MORs) are expressed throughout the PVT but display a non-uniform distribution, raising the possibility of region-specific functions (García-Cara et al., 2020; Matzeu et al., 2018). MORs are G-protein-coupled receptors that, upon activation by endogenous opioids like enkephalins or exogenous opioids such as morphine, modulate neuronal excitability and synaptic transmission (Williams et al., 2001; Lüscher and Slesinger, 2010). Activation of MORs generally results in hyperpolarization of neurons through the opening of G-protein–gated inwardly rectifying potassium (GIRK) channels and inhibition of voltage-gated calcium channels, thereby decreasing neuronal activity and neurotransmitter release (Lüscher and Slesinger, 2010).

Given the PVT's involvement in opioid-related behaviors, examining regional differences in opioid responsiveness can provide insights into how opioids affect the PVT's function at a cellular level (Keyes et al., 2020; Matzeu et al., 2018). This is particularly important in the context of opioid addiction and withdrawal, where the PVT has been shown to play a significant role (Matzeu et al., 2018; Zhu et al., 2016). While differential inputs and outputs of the aPVT and pPVT likely contribute to their distinct functional roles, intrinsic electrophysiological properties may also influence how neurons in these regions respond to neuromodulators like opioids.

Previous studies have indicated that neurons in the aPVT and pPVT exhibit distinct electrophysiological characteristics, such as differences in resting membrane potentials, firing patterns, and the presence of specific ion currents (Gao et al., 2020; Kolaj et al., 2012). In particular, the hyperpolarization-activated cation current (Ih) has been implicated in regulating neuronal excitability and rhythmic firing patterns, which may influence how neurons respond to neuromodulators (Biel et al., 2009; Pape, 1996). However,

whether these intrinsic properties affect MOR-mediated responses in the PVT subregions remains unclear.

We hypothesized that specific intrinsic neuronal properties may inform the degree of MOR activation in the aPVT compared to the pPVT. To test this hypothesis, we conducted a comparative analysis of the intrinsic electrophysiological properties and acute opioid responses of neurons in the aPVT and pPVT. We employed whole-cell patch-clamp recordings in brain slices from mice to assess parameters such as membrane capacitance, input resistance, spontaneous firing activity, and the magnitude of Ih. Additionally, we examined the effects of MOR activation using [Met^5]-enkephalin (ME) to evaluate potassium currents mediated by MORs in both regions.

Our findings reveal that while neurons in the aPVT and pPVT share similar basic electrophysiological properties, significant differences exist in the magnitude of Ih and MOR-mediated potassium currents. Neurons in the aPVT exhibit a larger Ih and greater responsiveness to MOR activation compared to those in the pPVT. These results suggest that, in addition to differential inputs and outputs, intrinsic neuronal properties contribute to region-specific opioid modulation within the PVT. This study lays the groundwork for future investigations into how intrinsic neuronal properties and neuromodulator responsiveness contribute to the functional heterogeneity of thalamic nuclei and other brain regions.

RESULTS

Membrane properties and activity patterns. Whole-cell voltage clamp recordings were obtained from a total of 97 neurons (53 from aPVT and 44 from pPVT). A comparison of the intrinsic membrane properties of neurons in slices from these two subregions is detailed in Table 3.1. Examining the capacitance, used as a measure of cell size, reveals these two subregions have no significant difference in capacitance (aPVT: 22.3 ± 5.8 pF vs pPVT: 21.9 ± 5.7, unpaired t-test, p= 0.36), consistent with Heilbronner and Flügge (2005) Membrane resistance was also examined revealing aPVT neurons had

slightly smaller input resistance (284.0 ± 129.6 MΩ) compared to pPVT neurons (357.2 ± 266.9 MΩ; t-test, unpaired, p = 0.0419).

Cell-attached voltage-clamp recordings were used to assess the intrinsic activity patterns of the anterior and posterior PVT (Figure 3.1). Very few neurons in either subregion exhibited no firing at all, while the majority exhibited sporadic low-frequency spiking (<1hz). Thus, neurons that fired < 1 Hz were grouped as non-tonic firing: 68% of neurons in the aPVT and 47% in the pPVT (N= 16, 14 respectively) exhibited non-tonic firing. Tonic firing, defined as firing > 1 Hz was present in 16% of aPVT neurons and 23% of pPVT neurons (N= 16, 7 respectively). Lastly, burst firing, defined as a doublet or more of action potentials within 10 ms, was present in 16% and 30% of neurons in the aPVT and pPVT respectively (N= 4, 9 respectively). Statistical analysis using a Chi-square test revealed a significant difference in the distribution of firing patterns between the aPVT and pPVT neurons ($\chi^2 = 9.4$, p = 0.0093). Specifically, burst firing was significantly more prevalent in pPVT neurons compared to aPVT neurons. These findings indicate that intrinsic firing patterns differ between the two subregions, with pPVT neurons exhibiting a higher propensity for burst firing.

Intrinsic neuronal excitability. Whole-cell recordings were obtained from 29 neurons (17 from the aPVT and 12 from the pPVT). Using depolarizing current steps (+100 pA), we measured the number of action potentials, latency to firing, and slow afterhyperpolarization (sAHP) (Figure 3.2). On average aPVT neurons fired on average 20.8 ± 12.3 times during a 1-s depolarization at a membrane potential of at -70 ± 5 mV compared to 18.8 ± 12.2 in the pPVT, and these differences were not statistically different (p=0.22). Latency was similar in both populations (aPVT: 34.4 ± 46.9 ms, pPVT: 43.4 ± 34.9 ms; t-test, unpaired, p = 0.2853). Slow afterhyperpolarizations were not present in neurons in which a 100 pA depolarizing current step did not evoke action potentials. However, of the neurons that did spike, no statistical difference was seen in sAHP amplitude (aPVT: -8.4 ± 4.3 mV, pPVT: -8.6 ± 5.3 mV; t-test, unpaired, p = 0.4699). Lastly, we compared the presence of Ih currents in voltage clamp recording with a

hyperpolarizing (-40 mV) voltage step. The difference between the instantaneous current and the steady state during this current step revealed that aPVT neurons had a larger Ih mediated current (aPVT: 220.1 ± 116.9 pA, pPVT: 124.0 ± 49.3 pA; t-test, unpaired, p = 0.04)

Acute opioid response. The PVT is a critical node for the expression of opioid withdrawal symptoms (Zhu et al., 2019), however the effects of opioids are not yet well characterized. Here, we examined the acute response of PVT neurons to [Met^5]-enkephalin (ME). Superfusion of a subsaturating concentration of ME (1 μM) evoked an outward current that peaked after 2-3 minutes. The ME-induced currents were larger in aPVT neurons compared to neurons in the pPVT (aPVT: 66.3 ± 29.0 pA, pPVT: 34.4 ± 21.9 pA; unpaired t-test, p = 0.01). Next, we identify the source of this current by constructing a Current/voltage (IV) plots revealing a reversal potential at ~90 mV indicating activation of a potassium conductance for both populations. The identity of this conductance is further explored in chapter 4.

Presynaptic MOR inhibition. It is well recognized that opioid receptors are densely expressed on presynaptic terminals in the PVT, specifically, MOR and the kappa opioid receptor (KOR). We used electrical stimulation to assess the sensitivity of input to the PVT to MOR activation (Data not shown). In both aPVT and pPVT, GABAergic inputs were insensitive to ME. Glutamatergic inputs however were sensitive in both nuclei though, a complete block of glutamatergic inputs was not seen regardless of how high of a concentration of ME was given indicating not all glutamatergic inputs are sensitive to MOR agonism.

DISCUSSION

In this study, we compared the intrinsic membrane properties, neuronal excitability, and acute opioid responses of neurons in the anterior (aPVT) and posterior (pPVT) paraventricular nucleus of the thalamus. Our findings reveal that while aPVT and pPVT neurons share several electrophysiological characteristics, mild differences exist in their input resistance, presence of hyperpolarization-activated

currents (Ih), and responsiveness to mu-opioid receptor (MOR) activation by [Met^5]-enkephalin (ME). Specifically, aPVT neurons exhibited a larger Ih current and greater ME-induced outward currents compared to pPVT neurons. These results suggest that intrinsic neuronal properties, in addition to differential connectivity, contribute to region-specific opioid modulation within the PVT, which may underlie their distinct roles in regulating stress and reward-related behaviors.

Membrane Properties and Activity Patterns

Our analysis showed no significant difference in capacitance between aPVT and pPVT neurons, indicating similar cell sizes across both regions. This aligns with previous morphological studies reporting comparable somatic dimensions in the PVT (Heilbronner and Flügge, 2005). However, we observed that aPVT neurons had a slightly lower input resistance compared to pPVT neurons. The higher input resistance in pPVT neurons could confer greater sensitivity to synaptic inputs, potentially influencing their excitability and responsiveness to neurotransmitters.

The examination of neuronal firing patterns revealed that the majority of neurons in both subregions exhibited sporadic low-frequency spiking (<1 Hz), categorized as non-tonic firing. Interestingly, burst firing was more prevalent in pPVT neurons (30%) compared to aPVT neurons (16%). Burst firing can enhance synaptic efficacy and is often associated with the encoding of salient or novel stimuli (Lisman, 1997; Sherman, 2001). The higher incidence of burst firing in pPVT neurons suggests a greater capacity for encoding stress-related signals, consistent with the pPVT's role in processing aversive stimuli (Penzo et al., 2015; Zhu et al., 2018).

Intrinsic Neuronal Excitability

When assessing intrinsic excitability through depolarizing current steps, we found no significant differences between aPVT and pPVT neurons in terms of action potential firing frequency, latency to firing, or slow afterhyperpolarization (sAHP) amplitudes. This indicates that basic excitability properties

are largely similar between the two regions under the conditions tested. However, the presence of sAHPs only in neurons that generated action potentials suggests that sAHPs may play a role in regulating firing rates during sustained activity, potentially affecting the output patterns of PVT neurons (Power and Sah, 2007).

Hyperpolarization-Activated Currents (Ih)

A notable finding was the significantly larger Ih current in aPVT neurons compared to pPVT neurons. The Ih current, mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, contributes to setting the resting membrane potential and regulating neuronal excitability and rhythmic firing (Biel et al., 2009; Pape, 1996). A larger Ih in aPVT neurons may promote higher baseline activity and facilitate tonic firing patterns, aligning with observations of higher spontaneous activity in the aPVT (Gao et al., 2020; Lafferty et al., 2020). This intrinsic property could enhance the aPVT's ability to modulate reward-related behaviors by influencing excitability and responsiveness to synaptic inputs.

The differential expression of Ih currents suggests that intrinsic membrane properties may complement the distinct input-output connectivity of the aPVT and pPVT, contributing to their specialized functions. For example, a larger Ih in aPVT neurons could support their role in maintaining tonic inhibition over reward pathways, as proposed in previous studies (Barson et al., 2020; Lafferty et al., 2020).

Acute Opioid Responses

Our examination of the acute effects of MOR activation revealed that ME induced significantly larger outward currents in aPVT neurons compared to pPVT neurons. The ME-induced currents reversed near the equilibrium potential for potassium (~−90 mV), indicating the activation of potassium conductance, likely through G-protein–gated inwardly rectifying potassium (GIRK) channels (Lüscher and Slesinger, 2010; Williams et al., 2001). The greater responsiveness of aPVT neurons to MOR activation suggests higher MOR expression or more efficient receptor coupling in this region.

These findings are consistent with reports of higher MOR density in the aPVT (García-Cara et al., 2020; Matzeu et al., 2018). The enhanced opioid sensitivity of aPVT neurons could lead to greater inhibition of their activity upon MOR activation, thereby modulating reward-related circuits and potentially influencing opioid-seeking behaviors. In contrast, the smaller ME-induced currents in pPVT neurons may reflect lower MOR expression or differences in receptor signaling mechanisms, which could impact how opioids modulate stress-related responses mediated by the pPVT.

Presynaptic MOR Inhibition

Our preliminary observations indicated that glutamatergic inputs to both aPVT and pPVT neurons were sensitive to ME, whereas GABAergic inputs were insensitive. The partial inhibition of glutamatergic inputs suggests that presynaptic MORs modulate excitatory synaptic transmission within the PVT. However, the absence of a complete blockade, even at higher ME concentrations, implies that not all glutamatergic terminals possess functional MORs or that other regulatory mechanisms limit presynaptic inhibition. These results align with studies demonstrating presynaptic opioid modulation of excitatory transmission in other brain regions (Gerachshenko et al., 2009; Hackmack et al., 2003). The insensitivity of GABAergic inputs to ME may be due to the low prevalence of inhibitory synapses in the PVT or a lack of MOR expression on GABAergic terminals in this nucleus (Hur and Zaborszky, 2005; Peng et al., 1996).

Limitations, Future Directions, and Conclusions

These intrinsic differences, combined with the distinct connectivity patterns of the aPVT and pPVT, likely result in region-specific responses to opioids and other neuromodulators. The differential modulation of PVT subregions by opioids could have significant implications for understanding the neural mechanisms underlying addiction, withdrawal, and stress-related disorders.

One limitation of our study is the relatively small sample size for certain measurements, particularly the intrinsic excitability assessments, which may affect the statistical power of our analyses. Additionally, we

did not quantify the expression levels of MORs or HCN channels, which could provide direct evidence linking molecular expression to the observed electrophysiological differences.

Future studies should aim to elucidate the molecular mechanisms underlying the differential MOR responsiveness and Ih currents in the PVT. Investigating the expression patterns of MORs, HCN channel subunits, and associated signaling proteins could enhance our understanding of the intrinsic factors contributing to PVT function. Moreover, exploring the behavioral consequences of manipulating these intrinsic properties in vivo would provide valuable insights into their roles in stress and reward processing.

In conclusion, our study demonstrates that neurons in the aPVT and pPVT exhibit distinct intrinsic membrane properties and differential responsiveness to MOR activation. These differences likely contribute to the specialized functions of each subregion in regulating stress and reward-related behaviors. Understanding the interplay between intrinsic neuronal properties and connectivity in the PVT enhances our comprehension of thalamic function and may inform the development of targeted interventions for neuropsychiatric conditions involving the opioid system.

TABLES AND FIGURES

Table 3.1. Intrinsic properties of PVT neurons while being held at -70 mV

	aPVT	**pPVT**	**p**	**N**
Capacitance (pF)	22.3 ± 5.78	21.9 ± 5.7	.3608	53,44
Membrane resistance (MΩ)	284 ± 129.6	357.2 ± 266.9	* 0.0419	53, 41

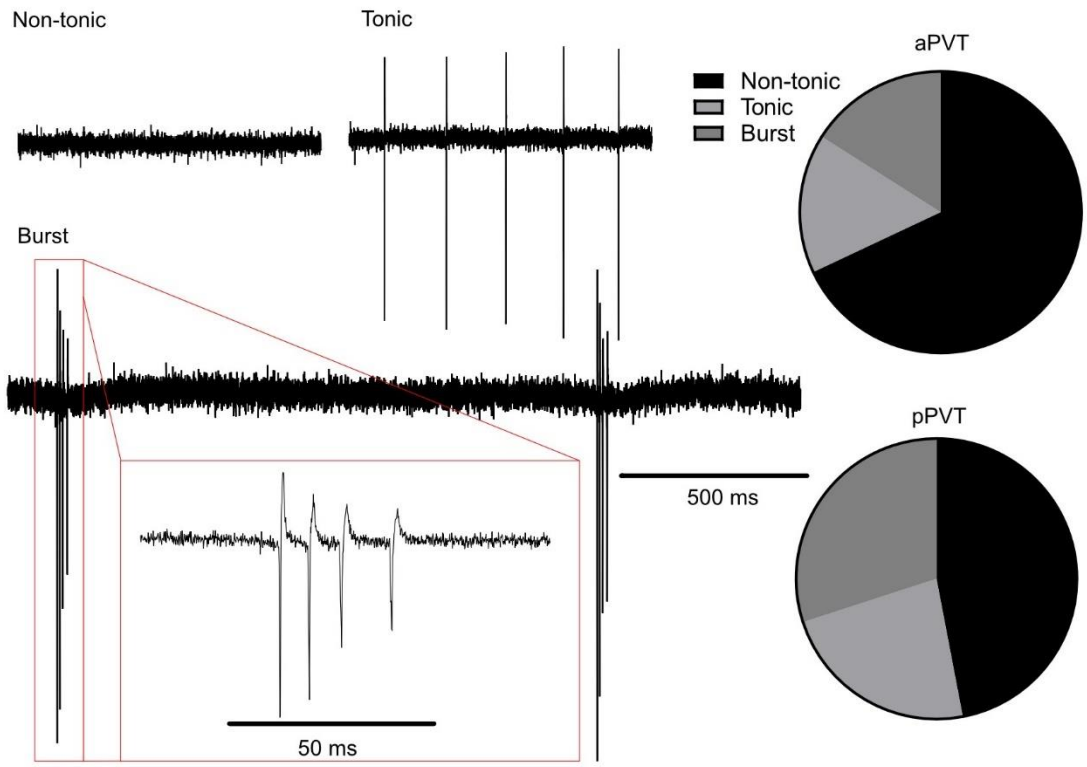

Figure 3.1. The aPVT and pPVT differ in their spontaneous action potentials profiles. Left. representative cell-attached recordings taken from brain slices from PVT neuron. Profiles were defined non-tonic if firing rate was less than 1 Hz (aPVT: 68%; pPVT:47%), Tonic if greater than 1 Hz (aPVT: 16%; pPVT:23%), and bursting if a doublet or more of action potentials were observed within 10 ms (aPVT: 16%; pPVT:30%).

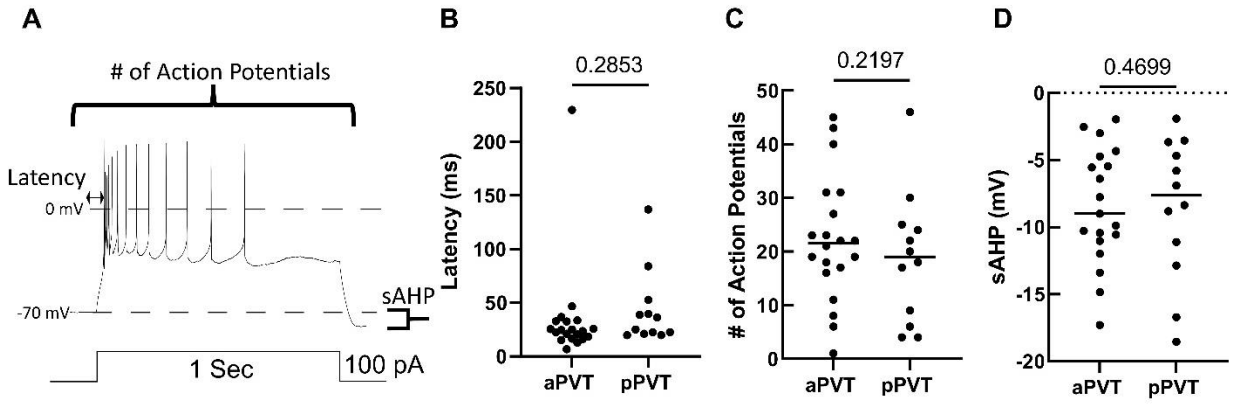

Figure 3.2 Intrinsic excitability in the anterior and posterior paraventricular nucleus of the thalamus. A. Representative trace of an aPVT neurons highlighting the firing properties of a neuron quantified. B. Quantification of latency, measured as the time from depolarization to the first action potential. C. Quantification of the number of action potentials during the 1 second depolarization. D. Quantification of the sAHP, measured as the change from baseline following the cessation of the injecting depolarizing current step.

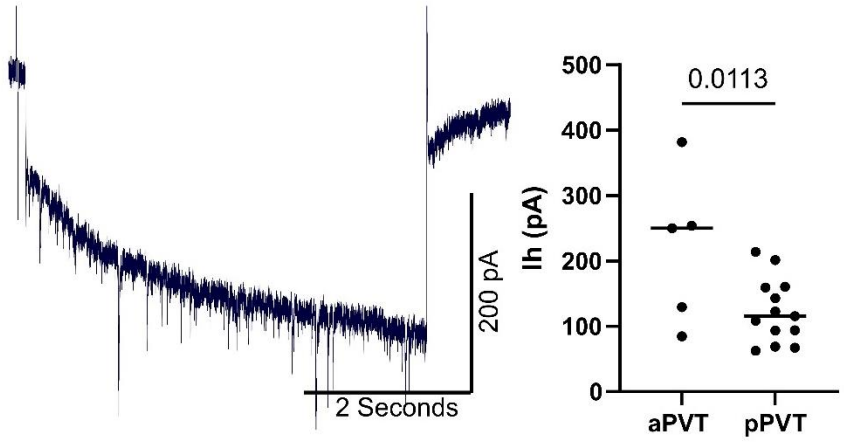

Figure 3.3. Ih currents are larger in the aPVT compared to the pPVT. Left, Example trace of a 40 pA hyperpolarizing voltage step for 4.8 seconds. Right, quantification of the change from the instantaneous current on onset to the steady state.

Table 3.2. Summary of experimental data

Experiment	**aPVT**	**pPVT**	**p**	**N**
Cell attached				24,30
Non-tonic	68%	47%		16,14
Tonic	16%	23%		4,7
Burst	16%	30%		4,9
Whole-cell Voltage clamp				
Ih amplitude to a -40 mV step (pA)	220.1± 116.9	124.0± 49.27	0.0113	13,5
Excitability				
Number of action potentials	22.2 ± 11.7	18.8 ± 12.2	NS	20,12
Latency (ms)	34.4 ± 46.9	43.3 ± 34.9	NS	20,12
Slow afterhyperpolarization (mV)	8.4 ± 4.3	8.6 ± 5.3	NS	19,20
Postsynaptic response				
1 µM ME (pA)	66.3 ± 29.0	34.4 ± 21.9	0.0133	8,8

Author Contributions

Participated in research design: Koita, Williams.

Conducted experiments: Koita.

Performed data analysis: Koita.

Wrote or contributed to the writing of the manuscript: Koita

Chapter 4: Distinct Populations of Neurons in the Anterior PVT Express Tolerance and Withdrawal Following Chronic Treatment with Morphine

Omar Koita, John T Williams

Vollum Institute, Oregon Health & Science University, Portland, OR 97239

ABSTRACT

Neurons in the paraventricular nucleus of the thalamus (PVT) integrate visceral and emotional signals, project to multiple areas, and together bias behavior toward aversive or defensive states. This study examines neurons in the rat anterior PVT that project to the amygdala, nucleus accumbens, and prefrontal cortex. Application of [Met]^5 enkephalin (ME) activated potassium conductance in more than 90% of neurons. The outward potassium current in neurons that projected to the amygdala was larger than those projecting to the nucleus accumbens and medial prefrontal cortex. In all neurons application of a saturating concentration of ME resulted in a peak current that declined during the application (desensitization). Inhibition of the G Protein Receptor Kinase (GRK2/3) with compound101 blocked the desensitization indicating a phosphorylation-dependent process. Thus, the acute actions of opioids was similar in the heterogeneous population of neurons. However, when animals were treated chronically with morphine there were distinct differences in the adaptive processes between neurons. There was a decrease in sensitivity to morphine in neurons projecting to the amygdala and nucleus accumbens (tolerance) and less in the prefrontal cortex. After chronic morphine treatment, a depolarizing current pulse was used to evoke action potentials and the number of action potentials was increased in neurons that projected to the nucleus accumbens (withdrawal). There was however little change in the number of action potentials in neurons projecting to the prefrontal cortex and amygdala. Thus, while separable projection neurons in the PVT exhibit differential tolerance and withdrawal, the result is the same, a disinhibited PVT and therefore an increase in glutamatergic drive onto key regions associated opioid use disorder.

SIGNIFICANCE STATEMENT

The PVT connects introspective cues to multiple limbic areas with a particularly strong role in pain processing. A high percentage of neurons in this area express opioid receptors, suggesting that this brain region is well-suited to maintain many facets of opioid use and dependence. Understanding the cellular actions of opioids in the PVT may therefore shed light on both the rewarding actions of opioids and the withdrawal symptoms in opioid use disorder. Measuring the acute and chronic actions of opioids on single neurons in the PVT is an important step in understanding how chronic opioid use regulates activity in this critical node.

INTRODUCTION

Opioid tolerance that results from repeated or sustained opioid receptor activation is mediated by both receptor-dependent tolerance and adaptive processes that counter the continued activation of the receptor. Receptor-dependent tolerance is marked by a decrease in the coupling of receptors to effectors, such that receptor activation becomes less effective. This involves molecular mechanisms such as phosphorylation of μ-opioid receptors (MORs) by G protein-coupled receptor kinases (GRKs), leading to the recruitment of β-arrestins, receptor desensitization, and internalization (Williams et al., 2013). Receptor-dependent tolerance is cell type-specific, with different neurons displaying vastly different degrees of tolerance (Levitt et al., 2018; Birdsong and Williams, 2020). Adaptive processes include changes in neuronal excitability, alterations in signaling pathways, and synaptic plasticity that oppose the effects of continued opioid exposure. Upon termination of opioid treatment, these adaptive processes are unmasked, resulting in withdrawal symptoms. The untoward effects of withdrawal are a major contributor to opioid use disorder (OUD) (Koob, 2013; Koob & Volkow, 2016). Adaptations occur in multiple brain areas, including those involved in the perception of pain and stress, leading to hyperalgesia and hyperkatifeia (an increased intensity of negative emotion) during withdrawal.

The paraventricular nucleus of the thalamus (PVT), a midline thalamic structure that integrates information from subcortical and cortical nuclei (Li and Kirouac, 2012; Kirouac, 2015; Otis et al., 2019; Penzo and Gao, 2021), plays a crucial role in emotional and motivational aspects of behavior, including arousal, stress, and reward processing. The PVT has been implicated in the actions of opioids and opioid dependence (Bengoetxea et al., 2020; Chisholm et al., 2020; Keyes et al., 2020; Giannotti et al., 2021; Yu et al., 2021; Babaei et al., 2023; Zhu et al., 2023; McDevitt et al., 2024; Paniccia et al., 2024). MOR activation in the PVT affects neuronal activity by modulating excitability and synaptic transmission, which can influence the expression of withdrawal symptoms. Indeed, the PVT modulates relevant aspects of opioid withdrawal such as arousal (Gao et al., 2020; Bu et al., 2022; Zhao et al., 2022; Eacret et al., 2023; Duan et al., 2024) and pain awareness (Chang et al., 2019; Zhang et al., 2022, 2023). Previous studies have shown that both spontaneous and antagonist-induced withdrawal increase c-fos expression in PVT neurons (Chahl et al., 1996; Zhu et al., 2016), suggesting heightened neuronal activity during withdrawal. Thus, adaptations in the PVT potentially contribute to the symptomatology of opioid withdrawal. However, despite these insights, the acute activation of MORs and the adaptive processes resulting from chronic morphine treatment in the PVT remain to be fully characterized.

The PVT is a heterogeneous population of neurons commonly divided into the anterior (aPVT) and posterior PVT (pPVT) (Li and Kirouac, 2012; Gao et al., 2020; Rivera-Irizarry et al., 2023; Shima et al., 2023). Firing properties (Kolaj et al., 2012, 2014; McDevitt and Graziane, 2019) and gene expression profiles (Gao et al., 2020) further distinguish PVT neurons. Despite this heterogeneity, there is a dense expression of opioid receptors (Eacret et al., 2023; Hou et al., 2023). While multiple projections from the PVT play key roles in the acute and chronic actions of opioids, the field has taken a particular interest in the PVT's projections to the nucleus accumbens (NAc), as projections from the PVT to the NAc mediate active avoidance and gate the expression of opioid withdrawal behaviors (Zhu et al., 2016; Dong et al., 2020; Ma et al., 2021; Kanai et al., 2022; McDevitt et al., 2024). However, efferent projections from the

PVT also include the amygdala (AM) and medial prefrontal cortex (mPFC) (Li and Kirouac, 2008; Gao et al., 2020), all of which have been shown to contribute to the incubation of drug craving (Pickens et al., 2011). The amygdala encodes emotional valence (Barson et al., 2020; Kirouac, 2015; O'Neill et al., 2023; Penzo et al., 2015; Piantadosi et al., 2024), and PVT projections to the basolateral amygdala (BLA) modulate neuropathic pain and emotional anxiety (Tang et al., 2023). The mPFC is known to play a role in the preoccupation phase of opioid addiction (Koob and Volkow, 2016), and increased activity from PVT projections to the mPFC has been shown to increase arousal and fear memory retrieval (Huong et al., 2006; Padilla-Coreano et al., 2012). Despite the recognized importance of these projections, the acute activation of MORs and the adaptive processes resulting from chronic morphine treatment in projection-defined neurons of the PVT have not been fully characterized. Understanding these projection-specific responses is crucial for elucidating the neural mechanisms underlying opioid tolerance and withdrawal.

The present investigation examines the acute and chronic activation of μ-opioid receptors on aPVT neurons with defined projections to the NAc, amygdala, and mPFC. Given the distinct roles of these projection targets in opioid addiction and withdrawal, examining projection-specific responses is critical for understanding how MOR activation contributes to these processes. We hypothesized that neurons projecting to different areas would exhibit distinct responses to acute and chronic MOR activation, reflecting their unique roles in behavior. Indeed, both the acute and chronic activation of MORs showed distinct responses based on the projection area. Neurons that project to the amygdala and NAc developed an increased level of receptor-dependent tolerance to morphine, as measured by the activation of potassium conductance, compared to cells projecting to the mPFC. Additionally, the increase in action potential generation in the presence of morphine following chronic morphine treatment was taken as another form of tolerance. In neurons that projected to the amygdala, the current induced by morphine was smaller in slices taken from morphine-treated animals, indicating the

development of tolerance, but there was no change in excitability as determined by the number of action potentials induced by a depolarizing current. Neurons that projected to the mPFC exhibited some tolerance to morphine, and the excitability was increased as measured by the increase in action potentials induced by a depolarizing current. These results indicate that chronic morphine treatment resulted in varying degrees of tolerance and withdrawal that were distinguished by the projections to three brain areas.

By investigating both acute and chronic MOR activation in projection-defined neurons of the aPVT, this study contributes to a deeper understanding of the neural mechanisms underlying opioid tolerance and withdrawal. The identification of projection-specific adaptations highlights the complexity of opioid effects on neural circuits and emphasizes the importance of neuronal identity and connectivity.

RESULTS

MOR expression in the aPVT

There is a high density of neurons that express OPRM1 and the μ-opioid receptor (MOR) in the PVT (Mansour et al., 1994, Arvidsson et al., 1995, Le Merrer et el., 2009), however, the presence of MORs on neurons with distinct projections has not been explored. The present study examined neurons in the aPVT with projections to the amygdala (AM), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC, Figure 4.1a). Retrograde GFP (AAVrg-CAG-GFP) was microinjected into each ares, horizontal sections were prepared after 2 weeks and images were taken of the aPVT. Labeled neurons in the aPVT were identified using a epifluorescent dissection macroscope to grossly verify the presence of projection-defined neurons (Figure 4.1b). Single neurons that projected to each area were then imaged using a two-photon microscope. Opioid receptors were identified by incubating the preparation with naltrexamine-acylimidazole-A594 (NAI594, 100 nM, 45 min) which covalently binds a fluorescent dye (AlexaFluor-594) to opioid receptors (Adoff et al., 2023). Opioid receptor-expressing neurons were observed in all projection-defined subpopulations. The images were marked by the presence of NAI-594

fluorescence in both pre- and postsynaptic structures. Postsynaptic receptors were functionally identified using whole-cell recordings from aPVT neurons and applying [Met]^{5}enkephalin (ME). ME induced an outward current in neurons that projected to each area that ranged from 20-150 pA in amplitude. The amplitude of the outward current induced by ME (1 μM) varied depending on the projection target (Figure 4.1). The outward currents induced by ME in recordings from neurons that projected to the amygdala (aPVT-AM) were larger compared to those projecting to the NAc and mPFC. The capacitance and resting membrane resistance were similar in each projection indicating that the size and conductance of the neurons were not the determinants of the variable amplitude of the opioid current (Figure 1-1).

Activation of potassium conductance

The underlying conductance induced by MOR activation was examined following the construction of current voltage plots. Two methods were used, (1) voltage steps (10 mV) were made from -45 to -125 mV and the current at each step was measured in the absence and presence of ME (10 μM, Figure 4.2-2) and (2) the currents induced by ramp potentials from -55 to -125 mV were measured before and following photoactivation of caged [Leu]^{5}enkephalin (CYLE, Figure 2, Banghart, Sabatini, 2012). A solution containing CYLE (50 μM) was recycled for a minimum of 5 min. Two 1 s ramp potentials from -55 to -125 mV were made 15 s apart. Before the second ramp a 50 ms pulse of light (356 nm, 10 mW) was applied to photoactivate CYLE (Figure 4.2C). Photoactivation resulted in an outward current measured at -55 mV. The current activated by CYLE during the potential ramp was subtracted from the control ramp to obtain the CYLE-induced current (Figure 4.2D, E). As was observed with the voltage step protocol the opioid current reversed at the potassium equilibrium potential. Unexpectedly the opioid induced conductance at negative potentials did not rectify inwardly. To further characterize the current, the extracellular concentration of potassium was changed from 2.5 to 6.5 and to 10.5 mM KCl. The

reversal potential of the opioid current was determined at each extracellular concentration of potassium (2.5 mM: -95.5 ±5.1 mV; 6.5 mM -74.8 ±3.0; 10.5 mM -62.5 ±4.1 mV; slope = 52.5). Thus, the reversal potential was predicted by the Nernst equation for potassium indicating an increase in potassium conductance. The application of $BaCl_2$ (1 mM) blocked the opioid current (Figure 4.2-3) further indicating an increase in potassium conductance. To further characterize the underlying conductance the action of the GIRK1 activator, ML297, was examined (Wydeven. et al., 2014). Application of ML297 (10 µM) did not affect the resting conductance but increased the outward opioid current suggesting that that GIRK1 was involved (Figure 4.2-4). In the presence of ML297 the overall shape of the current voltage plot did not change even at potentials more negative than the potassium equilibrium potential. Thus, this opioid-sensitive potassium conductance differed from the activation of GIRK that has been observed in other neurons.

Acute desensitization

Acute desensitization induced by a saturating concentration of potent agonist varies among different neurons. For example, desensitization in the LC is considerable larger than in neurons of the Köllikier Fuse (Levitt and Williams, 2018). In the present study application of a saturating concentration of ME (30 µM) results in a peak outward current that declined to 65.4 ± 19.9% of the peak in 10 min (n=14 from 11 animals, Figure 3C). Although the peak amplitude of the ME-induced current in neurons with different projection targets varied, the extent of desensitization was similar indicating that the size of the current was not a determining factor in acute desensitization. Acute desensitization is known to be dependent on phosphorylation of the C-terminus of MOR by G protein kinase (GRK, Lowe et al., 2015). Acute desensitization was examined in slices incubated in the GIRK2/3 blocker, compound 101 (30 µM, 1 hour) that completely blocked the desensitization induced by ME (30 µM, 10 min, Figure 4.3C). Thus, the desensitization of MORs in the aPVT is dependent on phosphorylation induced by GRK2/3.

MOR trafficking

Multiple mechanisms contribute to desensitization and tolerance, such as a reduction in surface expression of MORs (Koch, T., & Höllt, V. 1998). Thus, we examined the trafficking of mu-opioid receptors (MORs) using virally expressed SNAP-tagged MORs. Slices containing SNAP-tagged MORs were prepared and incubated with SNAP Surface 594 (JF-549, 30 nM, 60 min), followed by live imaging using a 2-photon microscope (Figure 4.4A). Images were captured before and after the application of ME (30 μM, 10 min). Prior to ME application, SNAP-MORs were localized primarily at the plasma membrane (Figure 4A). Following ME treatment, the receptors were redistributed into puncta, observed both at or near the plasma membrane and within the cytoplasm (Figure 4.4B). The extent of receptor internalization used a custom FIJI macro to measure fluorescence within the plasma membrane. Six cells from two animals were analyzed. The increase in mean fluorescent intensity within the cytosolic region of interest was normalized to baseline expression by calculating the ratio of post-ME treatment fluorescence to baseline fluorescence. For comparison, baseline expression was represented by a ratio of baseline fluorescence to itself, effectively setting it to zero. Thus, receptor internalization relative to the baseline expression levels was determined (Figure 4C). These findings confirm that MOR trafficking was induced by the application of a saturating concentration of ME, leading to receptor internalization.

Chronic morphine treatment

The development of tolerance and the expression of acute withdrawal were investigated following the treatment of animals with morphine for 6-7 days using osmotic mini pumps (80 mg/kg/day). Receptor-dependent tolerance was investigated using a protocol where slices were prepared and maintained in a concentration of morphine (1 μM) that approximated the circulating concentration (Quillinan et al., 2011). Slices from untreated animals were also prepared and maintained in the presence of morphine (1

μM). Recordings were made from neurons with identified projections. In each experiment recording of a steady baseline of 2-5 min was obtained prior to the application of naloxone (1 μM). The inward current induced by naloxone was taken as a measure to the current induced by morphine. The current induced by a saturating concentration of baclofen (10 μM, Figure 4.5A) was also measured and was used to normalize the morphine induced current. The morphine dependent current in slices from morphine treated animals was decreased relative to that found in slices from untreated controls and was taken as a measure to receptor dependent tolerance (Figure 4.5B). Tolerance as measured with this protocol was greater in neurons that projected to the amygdala and NAc than that observed in neurons that projected to the mPFC (Figure 4.5B). The current induced by baclofen was not different in experiments in slices taken from untreated and morphine treated animals indicating that the tolerance to morphine was homologous (Figure 4.5).

Withdrawal

To investigate potential signs of withdrawal the excitability of neurons during naloxone-precipitated withdrawal was examined in brain slices from morphine treated and untreated animals. Slices were prepared and maintained in morphine (1 μM). A depolarizing current (100 pA, 1 s) was applied to induce action potentials (Figure 4.6A) in the presence of morphine (1 μM) and following the application of naloxone (1 μM). This approach was used to examine the tolerance to morphine and acute withdrawal induced by naloxone. The number, latency from onset of the depolarization and instantaneous frequency of action potentials were measured (Figure 4.6B, 4.6-1) of neurons that fired during said depolarizing step. In recordings from neurons that projected to the NAc and mPFC the number of action potentials induced by the depolarizing current in the presence of morphine was larger in slices taken from morphine treated animals relative to untreated controls (Figure 4.6). However, the number of action potentials measured in neurons that projected to the amygdala was not changed. The results

indicate that chronic morphine treatment resulted in an increase in excitability even in the presence of morphine (1 μM). This was taken as a sign of tolerance in neurons projecting to the NAc and mPFC. The instantaneous firing rate and latency to the onset of firing during the depolarizing current were not different in neurons projecting to all three areas. Finally, naloxone precipitated withdrawal was examined. NAc-PVT neurons showed further excitation following chronic morphine treatment indicating further adaptations unmasked by withdrawal. No further increase was observed in AM or mPFC projecting aPVT neurons.

DISCUSSION

This study examined the acute and chronic action of opioids in the aPVT. Opioids induced an outward current in neurons that projected to three key areas involved in different cognitive and behavioral aspects of opioid use disorder and withdrawal. The outward current was mediated by a potassium conductance that was distinctive in that the current/voltage relationship lacked the typical inward rectification of GIRKs but was augmented by a positive modulator of GIRK1 channels, ML297. Acute desensitization of the current induced by opioids was dependent on phosphorylation by GRK2/3, as determined using the GRK2/3 blocker, compound 101. Phosphorylation of MORs by GRK2/3 leads to the recruitment of β-arrestins, which uncouple the receptors from G proteins, leading to receptor internalization (Williams et al., 2013). Neurons in the aPVT exhibit an intermediate level of desensitization relative to that found in the locus coeruleus where acute desensitization is prominent (Bailey et al., 2009), and the Kölliker-Fuse nucleus where desensitization and tolerance are limited (Levitt and Williams, 2018). Following chronic morphine treatment, tolerance to morphine was observed in neurons that projected to all three regions. Taken together, the results show that the aPVT exhibits heterogeneous acute and adaptive responses following chronic morphine treatment that are distinguished by the projection target of individual neurons.

A common problem with repeated treatments with opioids is the development of tolerance to some but not all actions. For example, tolerance to the analgesic actions of opioids limits therapeutic treatment; however, not all opioid-sensitive neurons develop the same degree of tolerance. For example, neurons in the Kölliker-Fuse that regulate respiration express MORs but show limited acute desensitization and tolerance following chronic morphine treatment (Levitt and Williams, 2018). Thus, the degree of tolerance and downstream adaptive responses resulting from chronic opioid treatment is diverse across brain regions.

Neurons in the PVT contribute to multiple behaviors. For example, the PVT–CeA pathway drives conditioned freezing responses, whereas the PVT–NAc pathway signals active avoidance events (Choi et al., 2010; Olson et al., 2012; Li et al., 2013; Bravo-Rivera et al., 2014; Penzo et al., 2015; Ramirez et al., 2015; Fadok et al., 2017). In addition, the aPVT and pPVT send non-overlapping projections to areas including the NAc, amygdala, and mPFC, further highlighting the heterogeneity of the PVT (Gao et al., 2020; Shima et al., 2023). The results from the present study indicate that amygdala-projecting neurons have a larger potassium-mediated conductance when compared to those projecting to the NAc and mPFC. Given that the cell capacitance and input resistance were similar between neurons in each of the projection areas, it is unlikely that cell size and resting conductance played a role in the different amplitudes of the current induced by ME. Thus, the coupling of MORs to the potassium conductance is likely the determinant of the current amplitude. Functionally, the larger opioid-induced currents in amygdala-projecting neurons may influence emotional processing during withdrawal, potentially enhancing aversive states. In contrast, the increased excitability and tolerance observed in neurons projecting to the NAc and mPFC may facilitate motivated behavior towards drug seeking and altered cognitive processes, respectively. These projection-specific adaptations suggest that chronic opioid exposure differentially affects neural circuits involved in addiction, contributing to the complex behavioral manifestations of opioid use disorder.

The outward current was mediated by a potassium conductance that was distinctive in that the current/voltage (I/V) relationship lacked a typical inward rectification. A previous study indicated that a MOR mediated hyperpolarization was not solely determined by GIRKs as indicated by inability of tertiapin-Q, a GIRK blocker, to completely bock the opioid current or a MOR mediated hyperpolarization (Hou et al., 2023). The results from that study along with the current/voltage plots of the opioid current made in the present investigation, suggest that the underlying conductance is not dependent solely on GIRK channels. Single-cell RNA sequencing revealed KCNJ3, KCNJ6, KCNJ9, and KCNJ5 expression in the aPVT (Gao et al., 2020; Allen Brain Atlas). Thus, neurons in the PVT are capable of expressing GIRKs. The fact that ML297 increased the amplitude of the opioid current suggests that GIRK1 could be involved; however, ML297 did not increase the current at potentials more negative than the potassium equilibrium potential. This suggests a complex interaction between GIRK channels and other potassium channels, affecting neuronal excitability. The identity of the potassium conductance activated by opioids remains a question. One possibility is the two-pore domain potassium (K2P) channel family. Knockout mice lacking TREK-1 channels were significantly less sensitive to morphine, indicating that opioids could act through this channel (Devilliers et al., 2013). In addition, morphine caused an outward current in COS cells expressing TREK-1 channels, and the current voltage plot was similar to that found in the present study (Devilliers et al., 2013). Thus, the conductance underlying the outward current in the PVT may involve K2P channels, potentially in conjunction with GIRK channels, reflecting a complex interplay that affects neuronal excitability.

Withdrawal

The untoward symptoms of acute withdrawal are thought to contribute to opioid addiction in that continued drug seeking is maintained in order to avoid aversive symptoms (Koob and Volkow, 2016; Koob, 2020). The aPVT is a central link between afferent and efferent connections that contribute to the emotional symptomatology of opioid withdrawal. Its connections to limbic structures, such as the

amygdala and NAc, play critical roles in processing negative emotional states. Projections to the NAc, mPFC, and amygdala are all key areas in both the positive and negative aspects of acute and chronic actions of opioids. Naloxone-induced withdrawal resulted in a substantial increase in c-fos expression in the PVT, although it was unclear if the increase resulted from an increased intrinsic excitability or an increase in synaptic drive (Zhu et al., 2016). Further, silencing the projection to the NAc blocked relapse, highlighting the importance of this pathway in withdrawal-induced drug seeking. The concept of hyperkatifeia, or increased sensitivity to emotional distress, is central to understanding the motivational aspects of withdrawal (Koob, 2020). The aPVT may mediate hyperkatifeia through its influence on limbic circuits, reinforcing drug-seeking behavior as a means to alleviate negative emotional states.

An increase in intrinsic excitability has been proposed with the use of depolarizing current steps using whole-cell recordings of action potentials in PVT neurons (McDevitt and Graziane, 2019). The number of spontaneous action potentials in unidentified neurons measured with cell attached recordings was greater in slices from morphine treated animals than untreated controls. Neurons that fired at rates greater than 1 Hz made up 54% of recordings in morphine treated slices and only 17% of recordings from untreated controls. (data not shown). Further, using whole cells recordings that compared the number of action potentials induced by a depolarizing current step revealed an increase in action potentials in neurons that project to the NAc and mPFC. Thus, suggesting an increase in intrinsic excitability following chronic morphine treatment. There was no change in the number of action potentials in recordings made from neurons that projected to the AM. The increased number of action potentials in the presence of morphine (1 μM) in neurons projecting to the NAc also suggests a degree of tolerance or adaptations that preclude withdrawal. The results support the notion that the aPVT plays a significant role in the emotional and motivational aspects of opioid withdrawal.

Taken together, the results indicate that chronic morphine treatment results in adaptive processes in the aPVT that contribute to dysregulation of multiple circuits in different ways depending on the

differential projections. The increased activity of neurons projecting to the NAc could promote motivated behavior towards drug seeking. An increase in the excitability of neurons projecting to the BLA likely drives aversion and may contribute to the hyperkatifeia associated with opioid withdrawal. Increases in glutamate drive into the mPFC following chronic morphine treatment can contribute to increased arousal or fear memory retrieval (Huong et al., 2006; Padilla-Coreano et al., 2012). In addition to the postsynaptic adaptations, the afferent regulation is particularly important given the number of afferents that are directly or indirectly regulated by opioids (Barson et al., 2020; Kooiker et al., 2021). Changes in afferent inputs to the aPVT following chronic opioid treatment could contribute to altered excitability. The adaptive mechanisms acting at the circuit level are likely an important component in the overall activity of this area of convergent input, which can drive withdrawal and subsequent drug seeking.

The results align with previous studies demonstrating that chronic morphine treatment leads to adaptive changes in MOR function and neuronal excitability. The identification of projection-specific adaptations in the aPVT adds to the understanding of opioid effects on neural circuits, emphasizing the importance of neuronal identity and connectivity in the development of opioid tolerance and withdrawal symptoms.

There are limitations to consider with the use of brain slices, as the complex network interactions and neuromodulatory influences present *in vivo* are absent. Additionally, the specificity of the pharmacological agents used, such as compound 101 and ML297, should be considered. Although compound 101 is a GRK2/3 inhibitor, off-target effects cannot be entirely ruled out, which might influence the interpretation of our results regarding acute desensitization. Similarly, ML297 is a GIRK1-selective activator, but its effects on other ion channels may complicate the conclusions about the identity of the potassium conductance. Future studies employing more selective genetic or pharmacological tools could help clarify these issues.

In conclusion, this study highlights the unique contributions of the aPVT in opioid tolerance and withdrawal, particularly emphasizing the projection-specific adaptations of MOR function and neuronal excitability. The identification of a potassium conductance underlying the opioid-induced outward current that is distinct from typical GIRK-mediated currents suggests the involvement of non-GIRK potassium channels, such as K2P channels. The observations contribute to a deeper understanding of the neurobiological mechanisms underlying opioid tolerance and withdrawal. By elucidating the specific adaptations in projection-defined neurons of the aPVT, the results may contribute to the development of targeted therapies aimed at mitigating withdrawal symptoms and reducing the propensity for relapse in opioid use disorder.

FIGURES

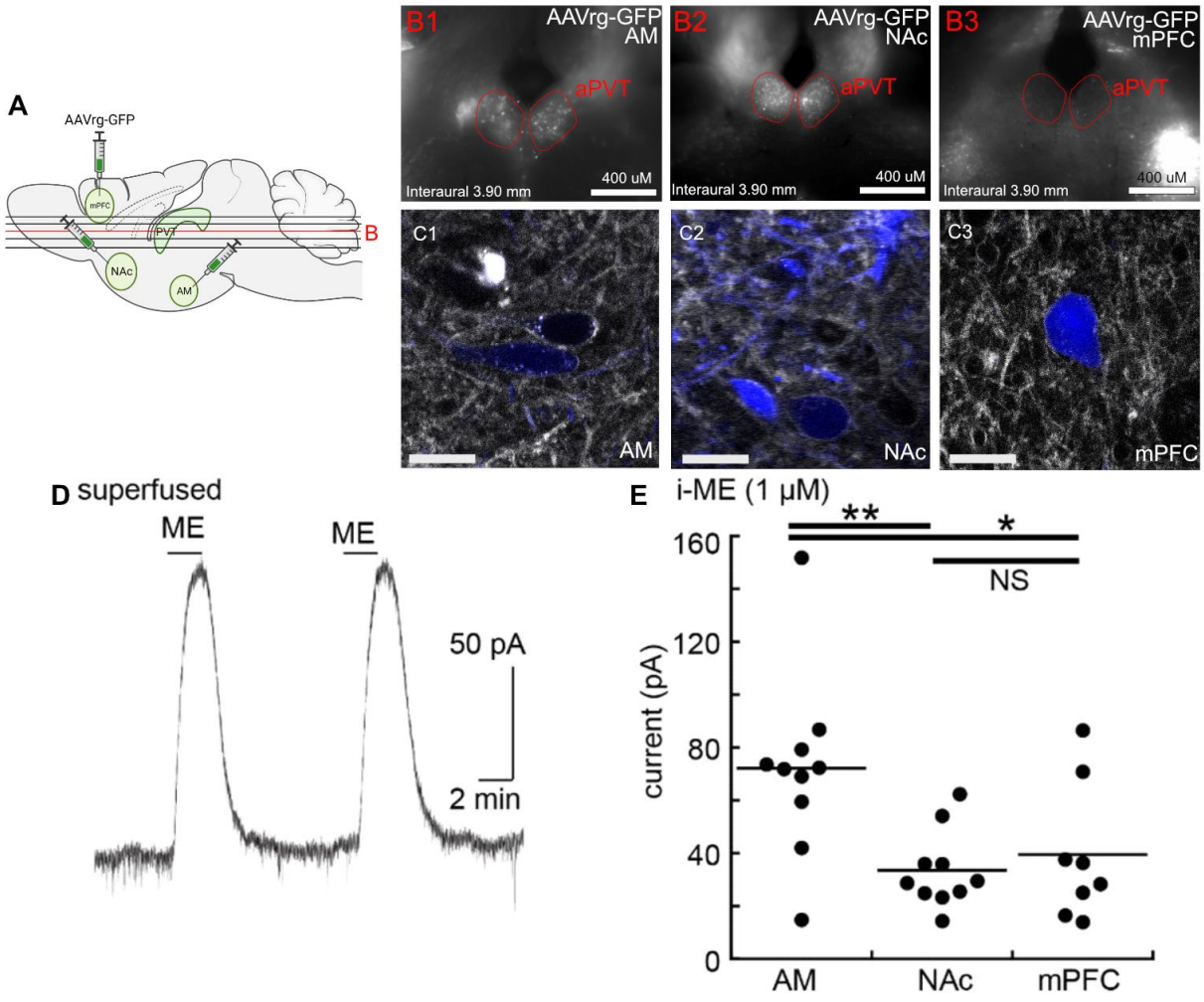

Figure 4.1. Mu-opioid receptors are expressed on neurons of anterior paraventricular nucleus of the thalamus (aPVT) that project to three areas. (A) Schematic of stereotaxic injection for targeting 3 targets of the aPVT. Injections of AAV2-retro-GFP into the amygdala, (AM), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) were made. Horizontal black lines represent how the brain slices were prepared. The red line indicates the section illustrated in panel B. (B) Macroscopic representation of retrogradely labeled projections. Left: Representative image from injection in the Amygdala. Middle: injection in the nucleus accumbens. Right: injection in the medial prefrontal cortex. (C) 2-photon representative image of retrogradely labeled neuron shown in blue. Sections were stained with NAI-

A594, shown in white. Left: Amygdala; Middle: nucleus accumbens; Right: medial prefrontal context. ME-induced potassium conductances in the anterior paraventricular nuclear of the thalamus. Scale bar: 20 µm. (D) Whole-cell voltage-clamp recording of an aPVT neuron. Lines indicate a 2-minute superfusion of [Met]^{5}enkephalin (ME 1 µM). (E). Summary of the amplitude of the outward current induced by ME (1 µM) in neurons projecting to the three different areas (AM; n=10 from 5 animals. NAc; n=10 from 3 animals. mPFC; n=8 from 3 animals). Superfusion of ME (1 µM) causes an outward current of 72.2±11.0 (SEM) pA in amygdala-projecting aPVT neurons significantly larger than NAc-projecting (33.5±4.6 pA) and mPFC-projecting aPVT neurons (39.4±9.2 pA). AM-PVT neurons had a larger amplitude response compared to mPFC-PVT and NAc-PVT ($P < .05$, one-way ANOVA) (*, $p \leq 0.05$. ** $p \leq 0.01$).

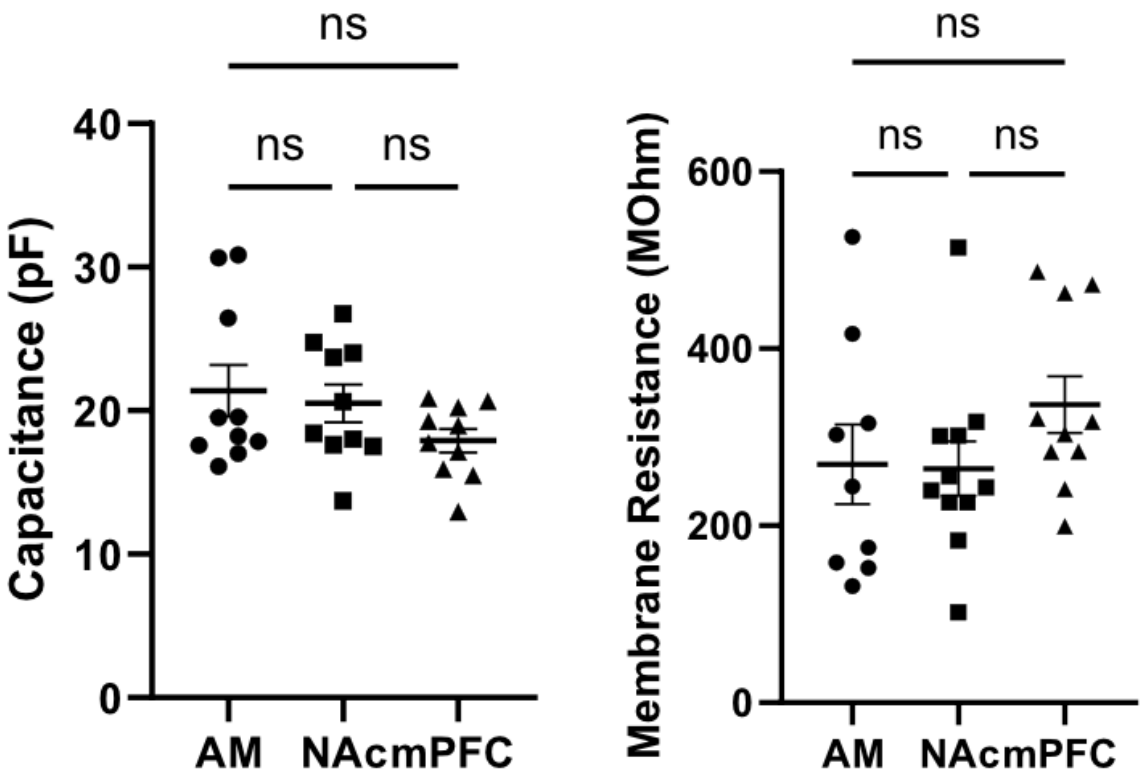

Figure 4.1-1. Projection-specific capacitance and membrane resistance. A. Cumulative data of cell capacitance in Figure 2B ($P > .05$, one-way ANOVA). B. Cumulative data of membrane resistance in Figure 2B ($P > .05$, one-way ANOVA).

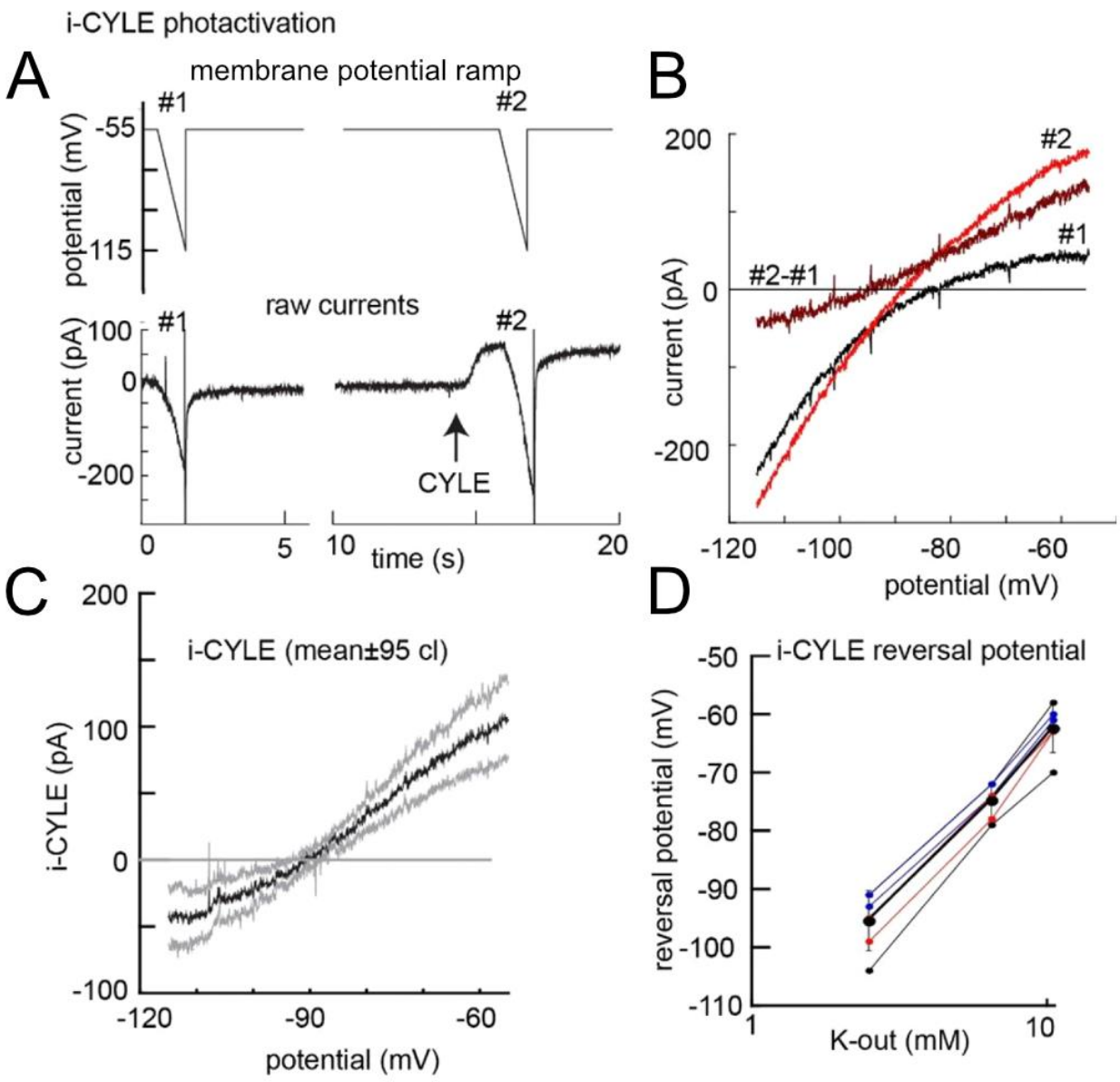

Figure 4.2. (A). top trace: voltage ramp protocol. Bottom: Example trace the current induced during the voltage ramp before and just after photoactivation of [(α-carboxy-2-nitrobenzyl)-Tyr1]-[Leu^5]-enkephalin (CYLE). Arrow indicated where UV light flash was applied (50 ms, 10 mW). Trace labeled #1 is in control, and #2 is during the outward current induced by CYLE. Right side - current-voltage plots obtained from the ramp experiment. #1 represents the control #2 is during the activation of current induced by CYLE. The current induced by CYLE is obtained by subtraction of the control (#1) from the current during the

activation induced by CYLE (#2). B. The average IV plot of the current induced by photoactivation of CYLE (n= 6 from 5 animals). This shows the reversal potential (-91.9±2.9 mV) and the outward reactivation of the opioid-induced current. F. Summarized results showing the change in reversal potential in experiments using different concentrations of extracellular potassium (2.5 mM: -95.5 ± 5.1 mV; 6.5 mM -74.8 ± 3.0; 10.5 mM -62.5 ± 4.1 mV; N=6, 5 animals, slope = 52.5). The dark line indicates the average (Mean±SE) of the experiments, thin lines illustrate individual neurons.

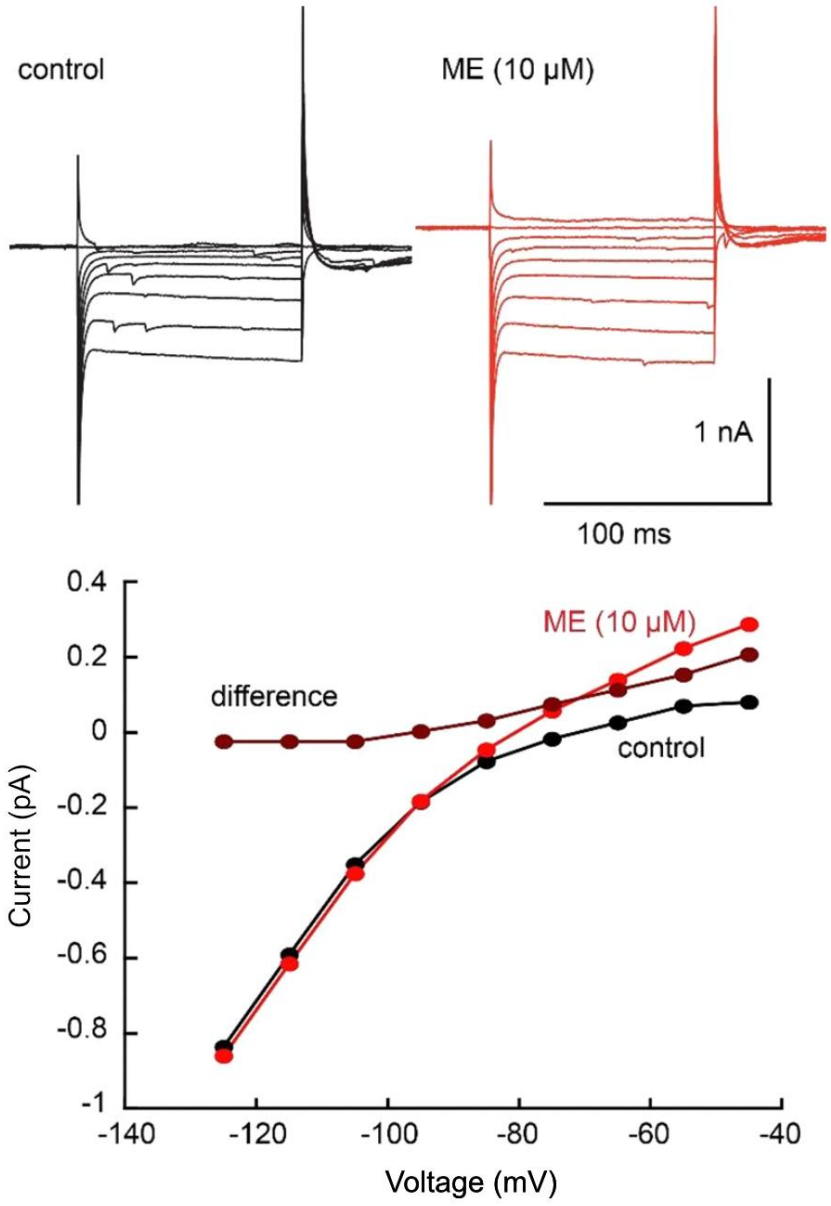

Figure 4.2-1 Current-voltage plot obtained using a voltage step protocol. Neurons were held at -55 mV and current steps from -45 to -125 mV were applied. Bottom graph shows the amplitude of the current steps in control (black) in the presence of ME (10 µM, red) and the ME induced current obtained by subtracting the step currents in control from those in the presence of ME (difference). The experiment illustrates the reversal potential and the outward rectification of the current induced by ME.

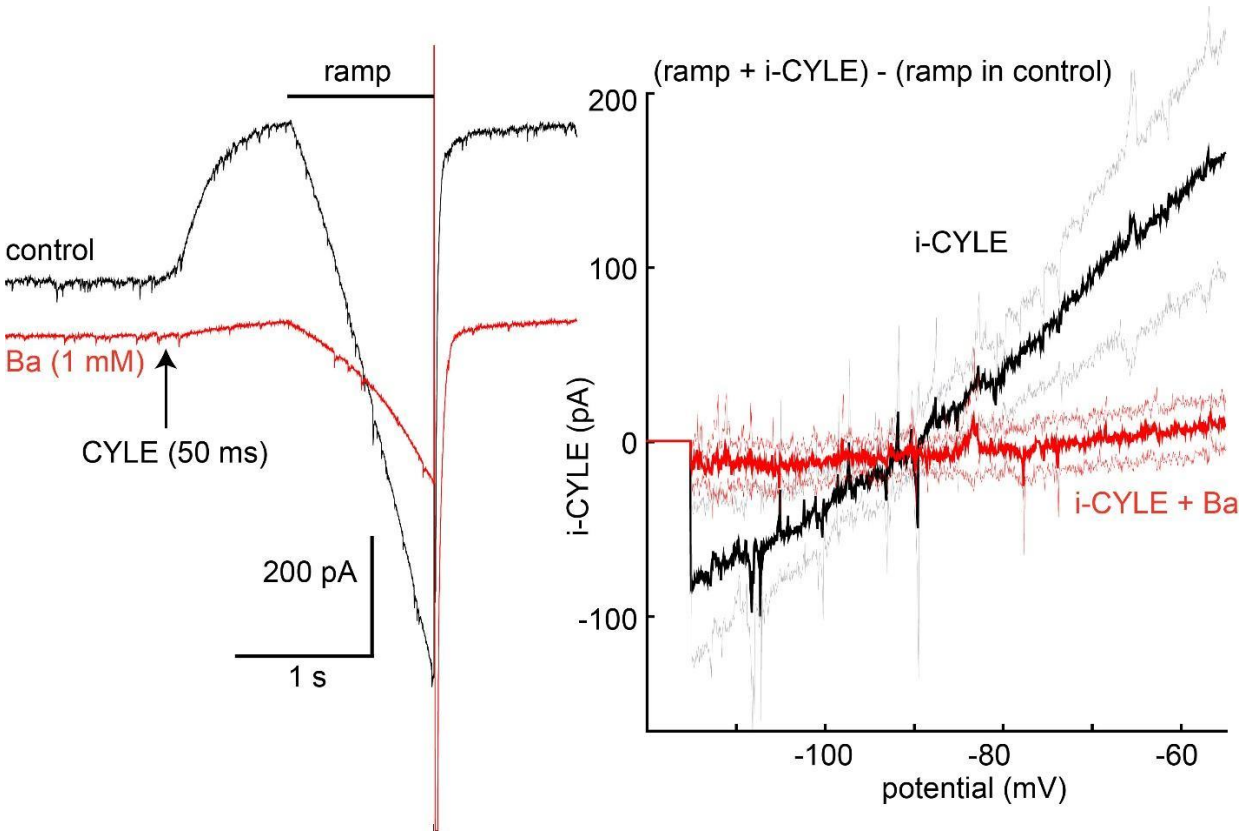

Figure 4.2-2 $BaCl_2$ (1 mM) blocks the CYLE induced current. Left shows current traces in control (black) and in the presence of $BaCl_2$ (red). At the arrow a UV flash was used to photoactivate CYLE. The outward current was almost completely eliminated in the presence of $BaCl_2$. At the peak of the outward current induced by CYLE a ramp potential was applied. Right, Current-voltage plots of summarized experiments dark lines indicate the averaged results faint lines are the 95% confidence limits (black – control, Red – in $BaCl_2$.

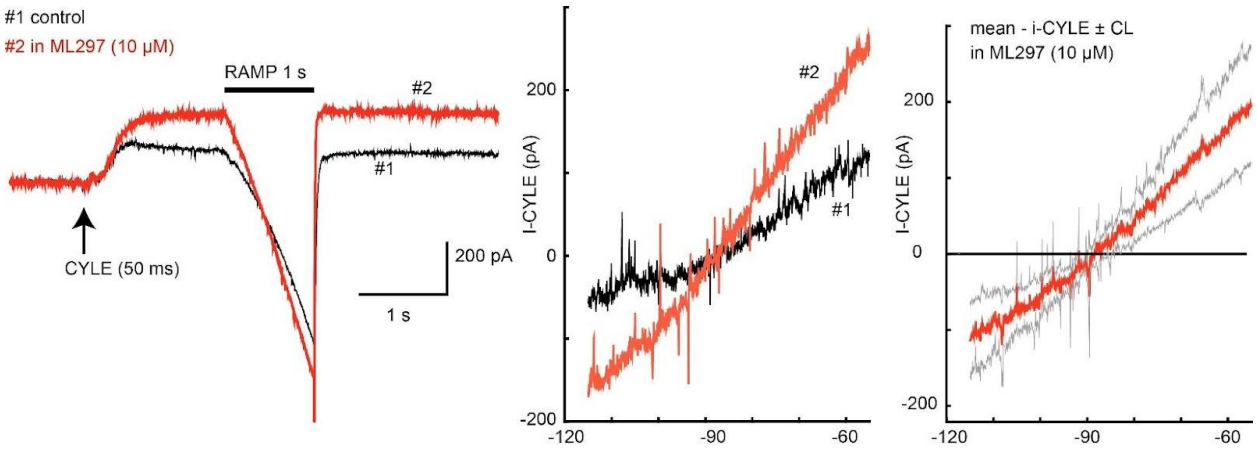

Figure 4.2-3 ML297 increases CYLE induced outward current. Left, shows current traces in control (black) and in the presence of ML297 (10 µM, red). At the arrow, a UV flash was used to photoactivate CYLE. The outward current was almost increased by about 100 % in the presence of ML297. At the peak of the outward current induced by CYLE a ramp potential was applied. Middle, current voltage plot of the CYLE induced current in control (black) and in the presence of ML297 (red). Right, summarized experiments showing the averaged CYLE induced current in the presence of ML297 (dark red line is the mean and the faint lines are the 95% confidence limits. The experiment shows that although ML297 increased the overall CYLE induced current, the outward rectification was not changed.

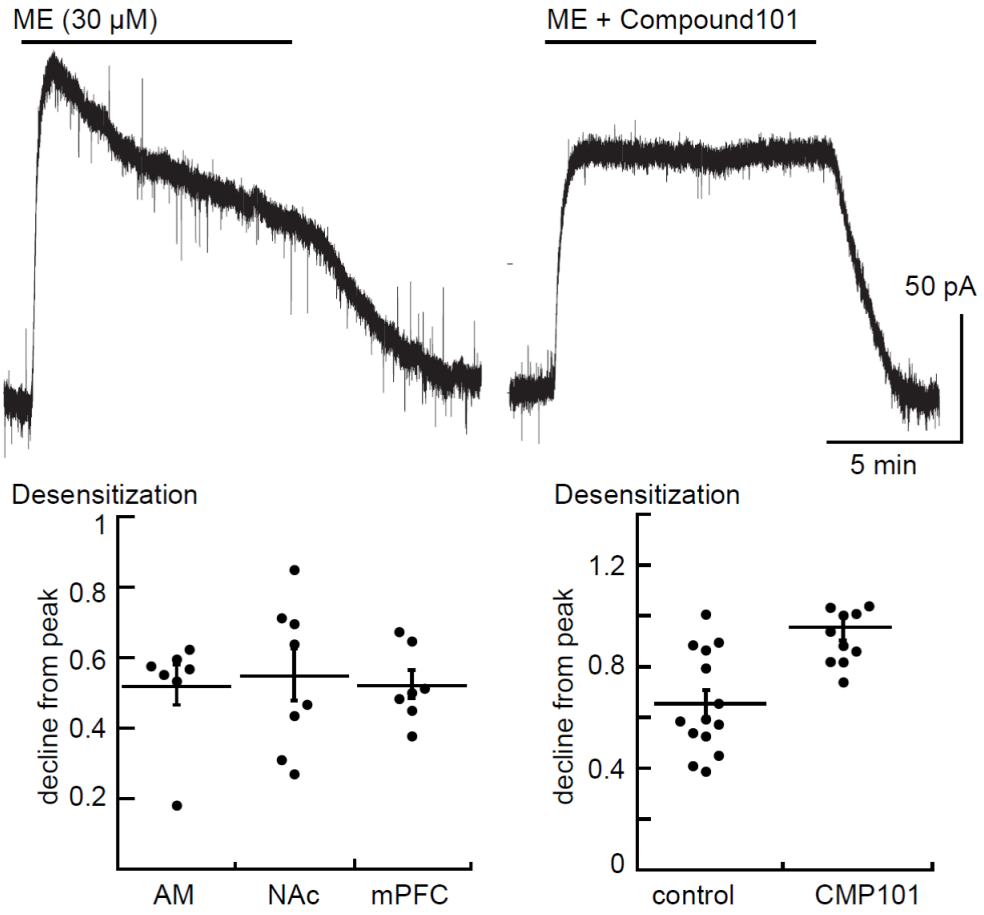

Figure 4.3. Acute desensitization of neurons in the aPVT. A. Left, an example of the acute desensitization induced by a saturating concentration of ME. Right, an example of the acute desensitization induced by a saturating concentration of ME after 45 min incubation in compound 101. B. Summary of the percent decline from peak induced by a 10-minute incubation of ME (30 µM) in neurons projecting to the three different areas (AM; n=7 from 3 animals. NAc; n=8 from 5 animals. mPFC; n=7 from 3 animals) no difference was seen between the three populations ($P > .05$, one-way ANOVA). C. Representative trace demonstrating acute desensitization under a saturating concentration of Met^5-Enkephalin (30 µM) post CMPD101 incubation. D. Quantification of desensitization; naïve (0.65 ± 0.19) (N=15, from 10 animals), CMPD101 (.91 ±.10) (N=10, from 7 animals) mean ± SE (p-value = 0.0011, one-way ANOVA).

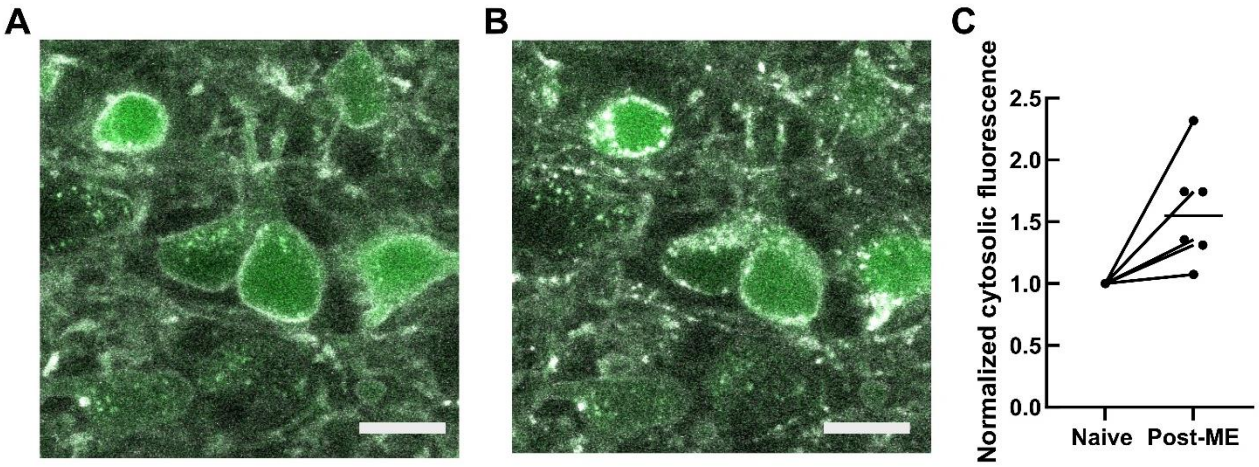

Figure 4.4. MOR is trafficked following application of a saturating concentration of ME (30 µM, 10 min). SNAP-MOR was virally expressed in MOR Knockout rats and imaged using a 2-photon microscope. A. An image taken before application of ME. Scale bar: 20 µm. B. Image of the same area following application of ME (30 µM, 10 min). The labeled SNAP-MORs move from a smooth distribution along the plasma membrane to a distinctly punctate distribution. Scale bar: 20 µm. C. Quantification of SNAP expression before and after a saturating concentration of ME normalized to expression in naïve state.

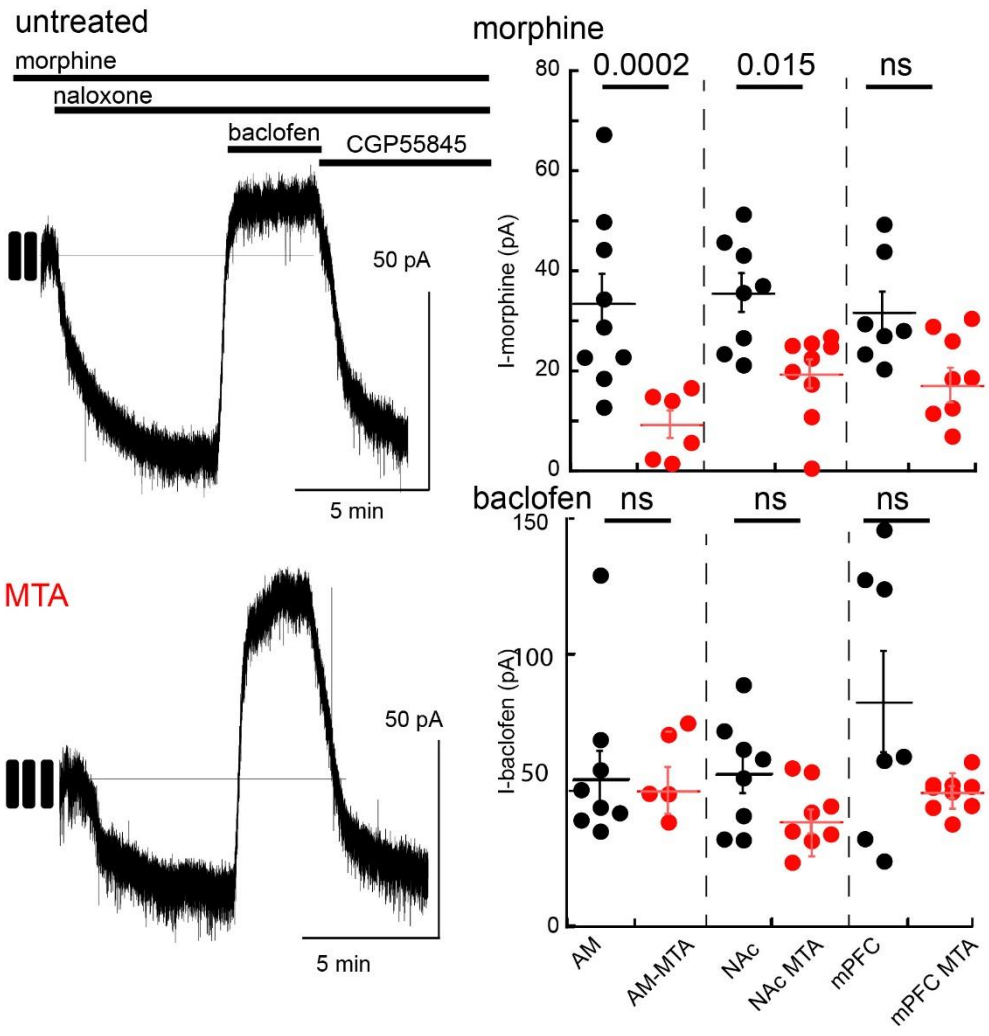

Figure 4.5. Tolerance to morphine. (A). protocol to determine the morphine-induced current in slices from untreated and morphine treated animals. Brain slices were prepared and maintained in morphine (1 µM) in untreated and morphine treated animals. Stable recordings were established followed by superfusion with naloxone (NLX 1 µM). The amplitude of the inward current induced by naloxone is the reversal of the morphine induced current and is smaller in slices from morphine treated animals. Baclofen (10 µM) was used as a control for potential heterologous actions and a measure of cell viability following the chronic treatment. (B) The inward current induced by naloxone was smaller in neurons projecting to the AM

(control 42.5 ± 5.4; MTA 8.7 ± 2.7), NAc (control 36.4 ± 6.4; MTA 16.0 ± 3.5) and minimal in the mPFC: 29.6 ± 14.1; MTA: 17.6 ± 3.9). (C). Quantification of baclofen mediated current. There was no significant difference in the current induced by baclofen in all neurons in slices from untreated and morphine treated animals. ±SD, All data are presented as mean with individual data points displayed. Statistical significances are shown as p-values.

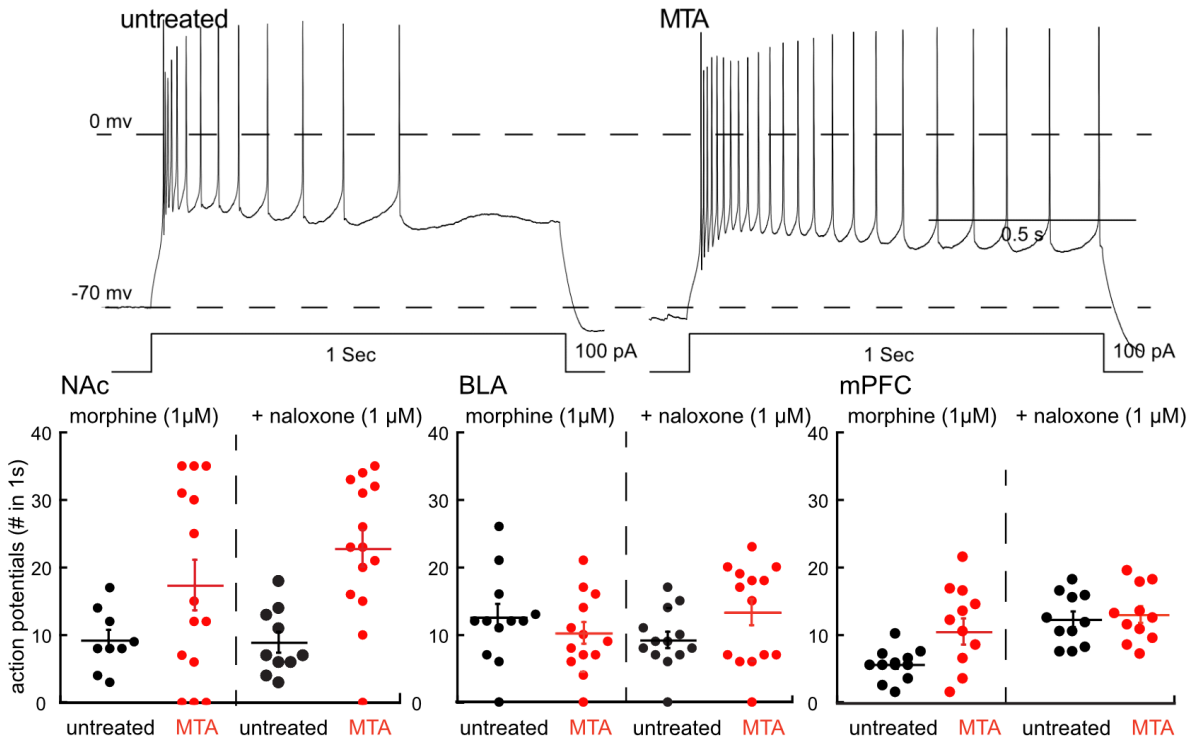

Figure 4.6. aPVT neurons fire more after chronic morphine treatment. Sections were prepared and maintained in morphine following chronic morphine treatment or in naïve animals. Whole-cell current-clamp recordings were used to measure action potentials induced by a depolarizing current step (100 pA). The number of action potentials were measured. A. representative trace illustrating action potentials during a depolarizing current step in morphine of a naïve/untreated animal (left) and morphine-treated animal (MTA) (right). B. Quantification of the number of action potentials in treated and untreated cell. Cells are unpaired and represented by four states, in morphine (untreated; inhibited, MTA; tolerant) and in naloxone (untreated; disinhibited, MTA; withdrawn). NAc: morphine/untreated: 9.2 ± 4.5, morphine/MTA: 17.4 ± 10.2, Naloxone/untreated: 17.4 ± 14.0, Naloxone/MTA 22.8 ± 10.2. AM: morphine/untreated: 13.5 ± 5.8, morphine/MTA: 10.8 ± 5.1, Naloxone/untreated: 10.1 ± 3.5, Naloxone/MTA 14.1 ± 6.4. mPFC: morphine/untreated: 6.1 ± 2.3, morphine/MTA: 12.7 ± 3.8,

Naloxone/untreated: 10.9 ± 6.7, Naloxone/MTA 13.4 ± 4.1. ± SE. All data are presented as mean with individual data points displayed. Statistical significances are shown as p-values.

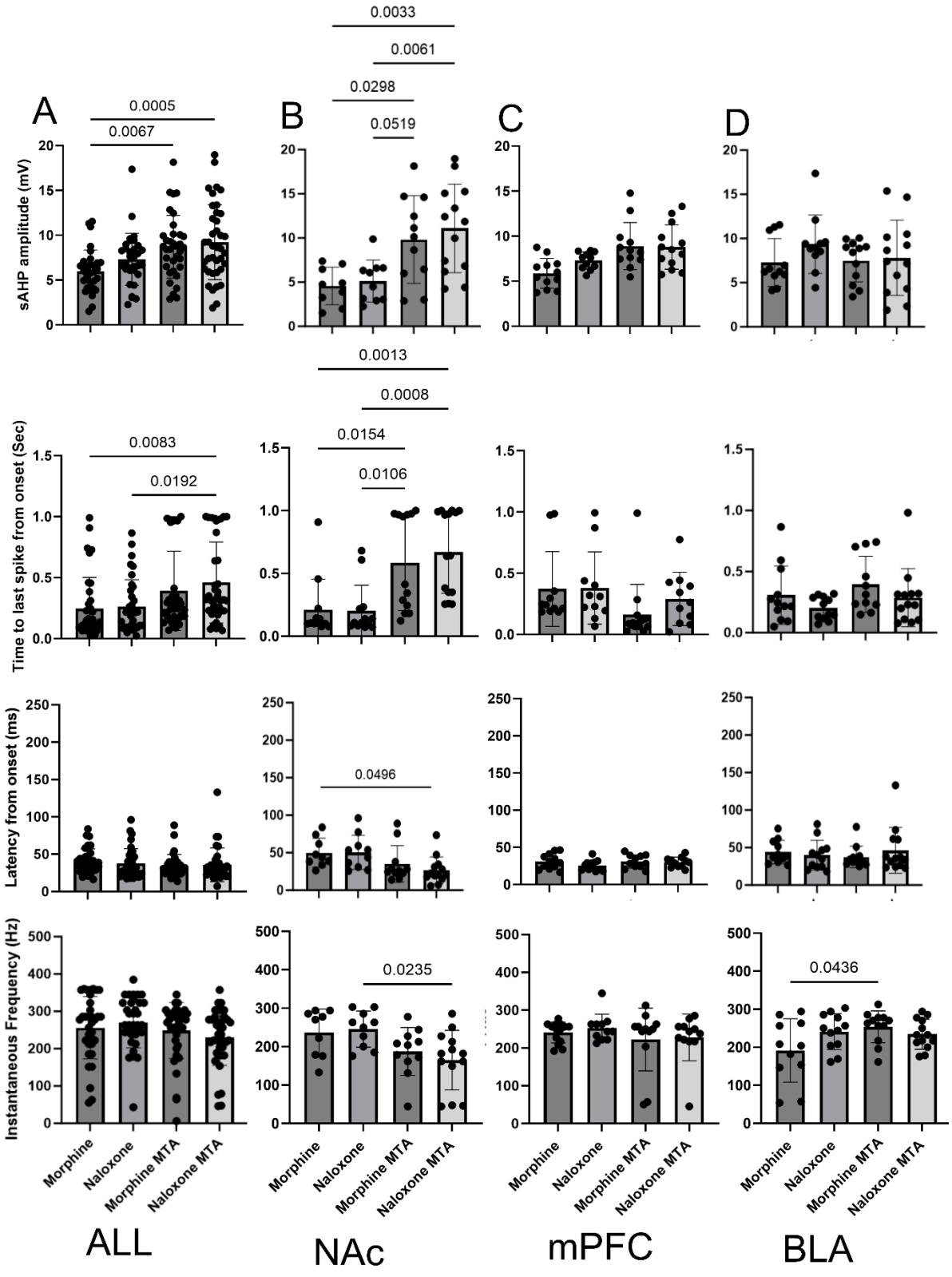

Figure 4.6-1. Depolarized aPVT neurons firing properties following chronic morphine treatment.

Sections were prepared and maintained in morphine following chronic morphine treatment or in naïve animals. Whole-cell current-clamp recordings were used to measure action potentials induced by a depolarizing current step (100 pA). The amplitude of the slow afterhyperpolarization, latency to spike cessation, latency from onset, and instantaneous frequency were recorded. (A). consolidation of all three projections. Slow afterhyperpolarization (sAHP) amplitude (mV): morphine/untreated: 5.99 ± 2.38, Naloxone/untreated: 7.32 ± 2.9, morphine/MTA: 8.70 ± 3.5, Naloxone/MTA 9.23 ± 4.18. Time to last spike (ms): morphine/untreated: 247 ± 255, Naloxone/untreated: 264 ± 219, morphine/MTA: 392 ± 325, Naloxone/MTA 460 ± 332. Latency from onset (ms): morphine/untreated: 40.8 ± 16.6, Naloxone/untreated: 37.9 ± 19.8, morphine/MTA: 34.4 ± 15.2, Naloxone/MTA 35.8 ± 22.5. Instantaneous frequency (Hz): morphine/untreated: 224± 62.9, Naloxone/untreated: 246.4 ± 42.6, morphine/MTA: 222.3 ± 68.4, Naloxone/MTA 208.5 ± 67.9. (B) **Nucleus accumbens**. Slow afterhyperpolarization (sAHP) amplitude (mV): morphine/untreated: 4.57 ± 2.12, Naloxone/untreated: 5.13 ± 2.38, morphine/MTA: 9.83 ± 4.97, Naloxone/MTA: 11.09 ± 5.01. Time to last spike (ms): morphine/untreated: 0.21 ± 0.24, Naloxone/untreated: 0.20 ± 0.20, morphine/MTA: 0.59 ± 0.38, Naloxone/MTA: 0.67 ± 0.33. Latency from onset (ms): morphine/untreated: 49.84 ± 19.36, Naloxone/untreated: 50.36 ± 22.85, morphine/MTA: 37.06 ± 24.78, Naloxone/MTA: 29.33 ± 17.90. Instantaneous frequency (Hz): morphine/untreated: 236.80 ± 61.07, Naloxone/untreated: 245.60 ± 46.94, morphine/MTA: 187.50 ± 62.37, Naloxone/MTA: 164.70 ± 77.54. (C) **medial prefrontal cortex**. Slow afterhyperpolarization (sAHP) amplitude (mV): morphine/untreated: 5.87 ± 1.65, Naloxone/untreated: 7.29 ± 0.95, morphine/MTA: 8.88 ± 2.62, Naloxone/MTA: 8.79 ± 2.46.

Time to last spike (ms): morphine/untreated: 161.8 ± 247.2, Naloxone/untreated: 290.8 ± 217.1, morphine/MTA: 371.7 ± 305, Naloxone/MTA: 379.6 ± 295.2 Latency from onset (ms): 30.98 ± 10.22, Naloxone/untreated: 25.53 ± 6.98, morphine/MTA: 30.90 ± 8.87, Naloxone/MTA: 29.89 ± 6.84 Instantaneous frequency (Hz): morphine/untreated: 241.30 ± 27.60, Naloxone/untreated: 252.90 ± 36.24, morphine/MTA: 222.80 ± 82.93, Naloxone/MTA: 228.20 ± 61.62 (D) **Amygdala**. Slow afterhyperpolarization (sAHP) amplitude (mV): morphine/untreated: -7.27 ± 2.71, Naloxone/untreated: -9.35 ± 3.30, morphine/MTA: -7.49 ± 2.40, Naloxone/MTA: -7.80 ± 4.25 Time to last spike (ms): morphine/untreated: 0.40 ± 0.23, Naloxone/untreated: 0.31 ± 0.24, morphine/MTA: 0.20 ± 0.09, Naloxone/MTA: 0.29 ± 0.24 Latency from onset (ms): morphine/untreated: 44.11 ± 15.62, Naloxone/untreated: 39.83 ± 19.76, morphine/MTA: 35.38 ± 8.99, Naloxone/MTA: 46.32 ± 30.67 Instantaneous frequency (Hz): 191.50 ± 83.39, Naloxone/untreated: 241.00 ± 46.96, morphine/MTA: 253.80 ± 42.07, Naloxone/MTA: 234.20 ± 39.20. ±SD. All data are presented as mean with individual data points displayed. Statistical significances are shown as p-values.

Chapter 5: Additional Data

Heterologous Regulation of the Nociceptin Receptor after Mu-Opioid activation

PREFACE

This chapter describes work directed towards understanding the adaptations of class A G protein-coupled receptors (GPCRs) following activation of another GPCR, specifically, the mu-opioid receptor (MOR) and orphanin FQ opioid peptide receptor (OFQR) in the paraventricular nucleus of the thalamus (PVT). This study acts as a prelude of experiments to investigate if acute GPCR activation can alter kinase activity enough to alter the desensitization of another. Then experiments were done to assess if chronic morphine treatment changes either MOR or OFQR desensitization.

I conducted this study under the mentorship and assistance of Dr. John T. Williams and was inspired by the findings of Dr. Sweta Adhikary. This chapter details attempts to measure acute crosstalk by 1) using a saturating concentration of OFQ to see if OFQ indirectly desensitize MOR. 2) measuring the rate of MOR and OFQR desensitization following chronic morphine treatment. This study aims to examine how rapidly kinase recruitment by class A GPCR activation can affect another class A GPCR. Interestingly, chronic activation of MORs with morphine did not augment the rate of desensitization for either MOR or OFQR. However, these experiments do highlight potential experiments to assess acute adaptation in more sensitive nuclei.

RESULTS

Acute activation of OFQr is not sufficient to induce heterologous desensitization of MORs in the aPVT.

Considering sustained signaling at the plasma membrane is the underlying process that initiates heterologous desensitization, I aimed to assess how quickly this occurs. Acute desensitization following sustained activation (longer than 2 minutes) by a saturating concentration of agonist leads to decreased sequential amplitude responses to the same concentration. A brief (<2 min) pulse of ME was given to

establish the baseline amplitude. This was followed by a saturating concentration of nociception, once the cell had completely desensitized (7 min), another brief pulse of ME was given (Figure 5.1), which did not change the peak amplitude. Lastly, we assessed if chronic activation of MORs with morphine was sufficient to increase the rate of desensitization of MORs (as seen in the LC – Arttamangkul et al., 2019; Leff et al., 2020) and OFQr. Desensitization of both receptors remained unchanged following chronic morphine treatment (Figure 5.2).

OFQR desensitization is dependent on GRK2/3 phosphorylation. Acute desensitization depends on phosphorylation of the C-terminus of MOR by G protein kinase (GRK, Lowe et al., 2015). Figure 4.3 illustrates the importance of GRK2/3 for the acute desensitization of MORs. Thus, acute desensitization of OFQR was examined in slices incubated in the GIRK2/3 blocker, compound 101 (30 µM, 1 hour) that completely blocked the desensitization induced by OFQ (30 µM, 10 min, Figure 5.2). Thus, the desensitization of MORs and OFQR in the aPVT is dependent on phosphorylation induced by GRK2/3.

DISCUSSION

In this section, we begin to explore the heterologous regulation of OFQr following activation of MORs. The findings suggest that MOR activation has no effect on the OFQ system of expressing neurons. NOP is generally recognized for its role in modulating pain and stress responses without the rewarding effects typically associated with MOR activations. Studies have even postulated and anti-MOR function for this receptor as pretreatment of OFQr agonist have been shown to reduce the potency of MOR agonist. This study aimed to assess if there are regulatory mechanisms that could contribute to this phenomenon. While we no adaptations with our assays, we cannot rule out the possibility that these two systems interact. More studies, perhaps utilizing a faster delivery system may be better suited to discern any differences.

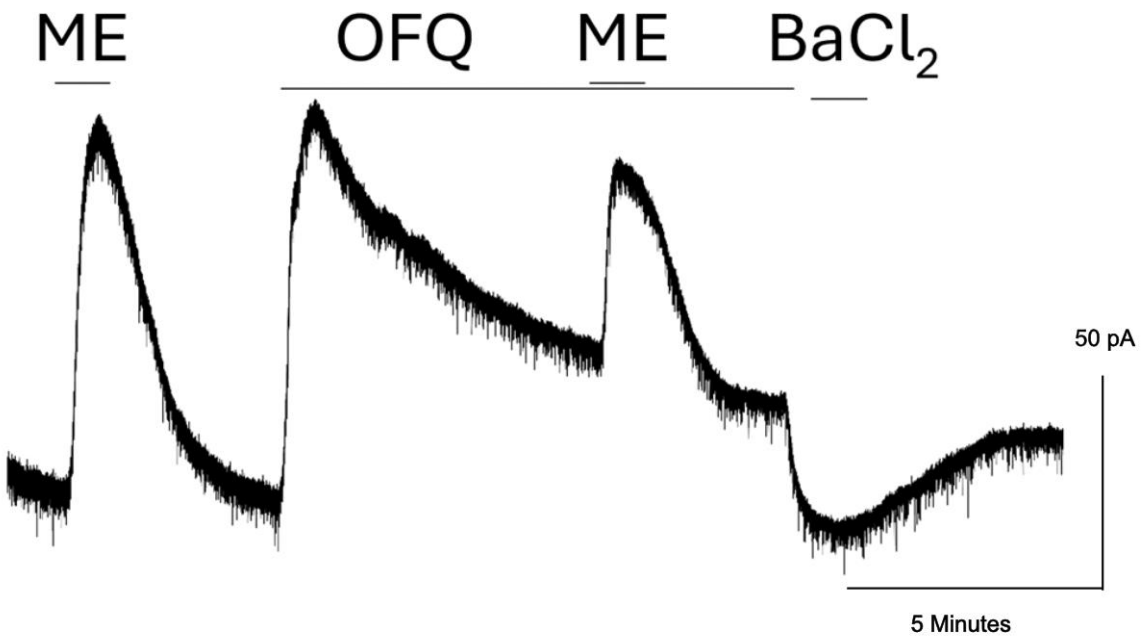

Figure 5.1. **Example trace of paradigm to assess heterologous desensitization**. A brief (<2 min) pulse of ME was given to establish the baseline amplitude. This was followed by a saturating concentration of nociception, once the cell had completely desensitized (7 min), another brief pulse of me was given. Upon wash with OFQ, $BaCl_2$ was given to block all potassium conductances. OFQ shows a slow wash from the section so upon the cessation of BaCl, we see a gradual increase in baseline.

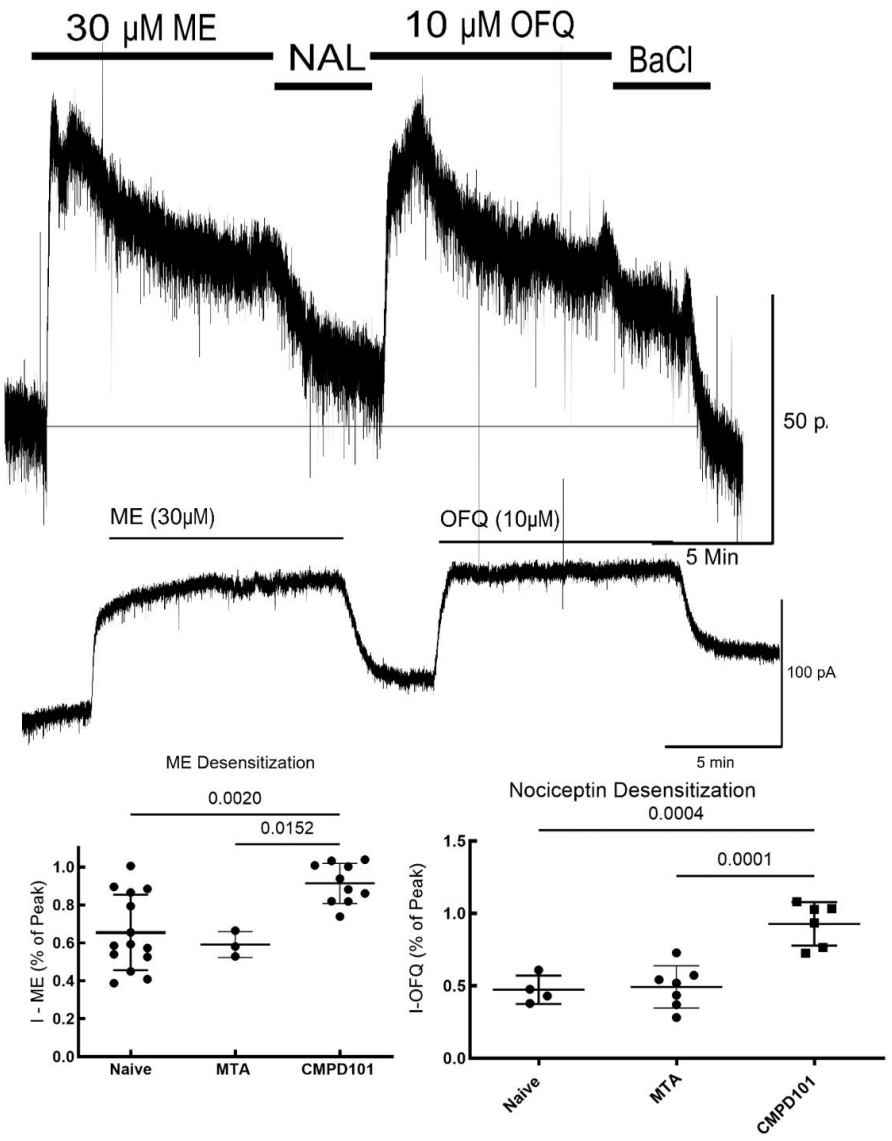

Figure 5.2. **OFQR desensitization is dependent on GRK2/3 phosphorylation.** A. Top, an example of the acute desensitization induced by a saturating concentration of ME and then OFQ. Bottom, an example of the acute desensitization induced by a saturating concentration of ME and then OFQ after 45 min incubation in compound 101. B. Quantification of desensitization; ME: naïve (0.65 ± 0.19)(N=15, from 10 animals), MTA (.59 ±.07)(N=3, from 2 animals) CMPD101 (.91 ±.10) (N=10, from 7 animals) ±SD (p-value = 0.0020, one-way ANOVA) / OFQ: naïve (0.47 ± 0.10)(N=4, from 3 animals), MTA (.49 ±.15)(N=7, from 3 animals), CMPD101 (.91 ±.10) (N=6, from 4 animals) ±SD (p-value = 0.0004, one-way ANOVA).

Adaptation of Glutamatergic inputs into the Paraventricular nucleus of the thalamus following chronic morphine treatment.

PREFACE

The PVT has been squarely placed as a circuit critical for the expression of opioid withdrawal symptoms, however, the adaptation that drives this response remains unknown. To further complicate its role in opioid withdrawal, the PVT is dense with MORs pre- and postsynaptically. All considered, one fact remains, when projections from the PVT to the NAc are inhibited, the expression of opioid withdrawal symptoms is blocked. Thus, gating of these symptoms relies on activity from the PVT. Considering MORs are expressed both pre- and postsynaptically, adaptations on both sides of the synapse could contribute to opioid withdrawal symptoms. Chapter 4 described the adaptations that follow chronic opioid exposure in PVT neurons thus I aimed to assess the presynaptic adaptation that is unmasked by withdrawal.

RESULTS

Glutamatergic inputs to the aPVT are sensitive to opioid inhibition. To assess if there was an increase in the glutamatergic drive onto aPVT neurons following chronic morphine treatment, we first assessed the sensitivity of afferents to the PVT to opioid inhibition. We used local electrical stimulation to stimulate afferents into the PVT and 1 µM picrotoxin (PTX) and 10 µM CNQX, to isolate GABAergic and glutamatergic inputs, respectively. GABAergic IPSCs (GABAergic) exhibited no sensitivity to [MET]^{5}Enkephalin (ME). Conversely, PTX-insensitive responses (glutamatergic) exhibited a significant decrease in amplitude following the superfusion of both ME and morphine (FIGURE 5.3).

Adaptations of glutamatergic inputs to the aPVT following chronic morphine treatment. Next, to assess the adaptations that follow chronic morphine treatment, animals were chronically treated via subcutaneous implantation of osmotic minipump as described in Chapter 4. Sections were given 1 hour to recover, thus allowing for morphine to be washed from the tissue. Bath application of 1 µM morphine

decreased in EPSC amplitude in naïve slices (78% of baseline) but not in slices prepared from morphine-treated animals (MTA) (98% of baseline). This morphine-induced inhibition was reversed with 1 μM naloxone (NLX), returning naïve cells to baseline (91% of baseline) and leading to an overshoot in MTA (141% of baseline). A saturating concentration of baclofen (30 μM) was used as a control of cell health. Both naïve and MTA exhibited profound inhibition of stimulated EPSCs (Naïve: 25% of baseline; MTA: 33% of baseline). There was not a statistically significant difference between the measured EPSCs in naïve and MTA to morphine however, the lack of inhibition in MTA is striking. Should it be taken at face value, this would suggest that MOR-sensitive presynaptic terminals develop a robust tolerance following chronic opioid treatment. Another explanation is that 1 hour is not a sufficient to remove residual morphine; however, this effect was seen in recordings from the end of the recording day (5+ hours after preparation of the slices).

DISCUSSION

Pre- and postsynaptic adaptations to chronic opioids likely underlie the complex signals that contribute to opioid withdrawal. Here we show preliminary evidence that glutamatergic inputs, not GABAergic, are not only sensitive MOR agonism but also show adaptations that increase glutamatergic drive onto PVT neurons. McDevitt and Graziane (2019) showed that tolerance induced by an escalating dose of morphine for 5 days via i.p. injection led to an increase in excitability as measured as an increase in the AMPA/NMDA ratio. This finding inspired an assessment of postsynaptic adaptations in the face of chronic opioid exposure (figure 4.5). One important caveat of this approach comes from the mode of inducing tolerance. Our approach reduces the influence of adaptations secondary to withdrawal (or an unmasking of the adaptive process once MOR signaling is ceased) thus, our approach tests the hypothesis that adaptations of MOR sensitive presynaptic inputs to the PVT undergo adaptations that increase their glutmatergic drive in the face of naloxone precipitated withdrawal. Our results

demonstrate that MOR sensitive glutamatergic inputs exhibit both a tolerance, decreased responsiveness, to morphine and a large rebound in glutamatergic drive during withdrawal.

These findings, suggest that MOR sensitive glutamatergic inputs to the PVT are primed to further excite the PVT and potentially exacerbate the symptomology of opioid withdrawal via increased glutamatergic drive alone.

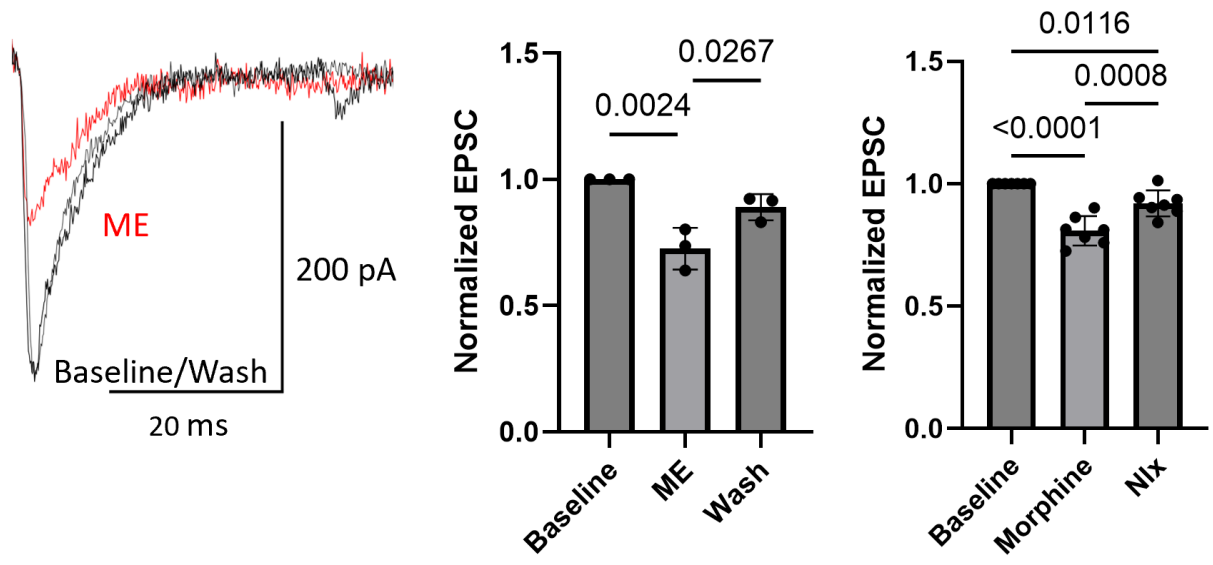

Figure 5.3. **Glutamatergic input into the aPVT are sensitive to opioid inhibition**. A. representative trace of electrically stimulated EPSC at baseline (black), ME (red), and wash (grey). B. Quantification of the normalized EPSC amplitude in response to ME. C. Quantification of the normalized EPSC amplitude in response to morphine.

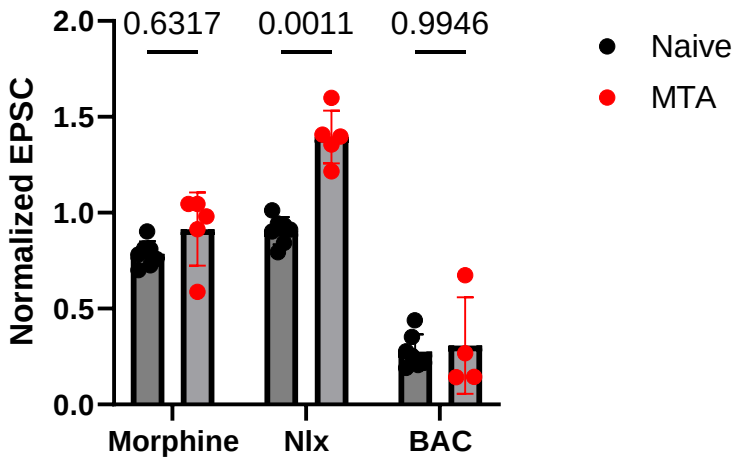

Figure 5.4. **Chronic morphine treatment increases EPSC rebound during naloxone-precipitated withdrawal.** Quantification of normalized EPSC amplitude in response to inhibition.

Chapter 6: Discussion and future directions

Discussion

The results of this thesis provide critical insights into the functional adaptations of the paraventricular nucleus of the thalamus (PVT) following chronic morphine treatment, highlighting how opioid-induced changes within this region contribute to withdrawal-related behaviors. The PVT is an integrative hub, linking limbic regions involved in stress, motivation, and emotion, and our findings demonstrate that opioid receptor regulation in this region is projection specific. A key strength of this work is the detailed exploration of both the anterior (aPVT) and posterior (pPVT) subregions, two subregions with accumulating evidence suggesting their distinct roles in modulating behavior.

Anterior versus the posterior PVT

Our research demonstrates that neurons in the aPVT and pPVT exhibit distinct electrophysiological and functional properties, particularly in their responsiveness to opioid exposure and their contributions to withdrawal behaviors. This distinction between aPVT and pPVT becomes particularly important in the context of opioid withdrawal, where negative affect, stress and reward seeking likely drive continued drug-seeking behavior. Our data highlights that both are sensitive to opioids however given the significantly smaller potassium mediated MOR response in the pPVT, study of MOR desensitization and tolerance is difficult.

The functional differences between the two regions are reflected in their intrinsic electrophysiological properties. For example, the hyperpolarization-activated cation current (Ih) was significantly larger in aPVT neurons compared to pPVT neurons, which may account for differences in baseline excitability and firing patterns between these subregions. This is consistent with previous studies suggesting that the aPVT plays a more active role in regulating tonic activity in circuits related to reward, while the pPVT is more reactive to acute stressors.

The markedly smaller potassium-mediated MOR response observed in the pPVT presents a significant challenge in studying MOR desensitization and tolerance within this subregion. This attenuated response may stem from lower expression levels of MORs or differences in G-protein coupling efficiency specific to pPVT neurons. Additionally, variations in the subunit composition of GIRK channels between the aPVT and pPVT could contribute to the differential responsiveness to opioids. Understanding these mechanistic underpinnings is crucial, as they suggest that pPVT neurons may rely less on MOR-mediated inhibitory signaling, rendering them more susceptible to excitatory inputs during withdrawal states. This heightened excitability could exacerbate stress and aversive behaviors, which are hallmark symptoms of opioid withdrawal.

The inability to effectively study MOR desensitization in the pPVT due to the minimal baseline response limits our comprehension of how chronic opioid exposure affects this region. Alternative approaches, such as examining intracellular signaling pathways or enhancing GIRK signaling with ML297, may be necessary to elucidate the pPVT's role in opioid tolerance and dependence. If ML297 is used, careful consideration must be given to the potential of additional adaptations caused by increased potassium conductance.

Our findings align with previous studies that have highlighted functional distinctions between the aPVT and pPVT. For instance, Zhu et al. 2016 demonstrated that the aPVT is more actively involved in reward-related behaviors, while the pPVT is more responsive to stress-related stimuli. The larger Ih current and greater MOR-GIRK coupling in the aPVT support the notion of its role in modulating tonic activity within reward circuits. In contrast, the pPVT's reduced MOR responsiveness may predispose it to facilitate stress and aversive responses during opioid withdrawal. Moreover, our observation that chronic morphine treatment leads to decreased GIRK channel responsiveness in the aPVT extends the work of Matzeu et al. 2018, who reported that alterations in PVT activity contribute to the negative emotional states associated with withdrawal. By highlighting the circuit-specific adaptations within the PVT, our

study underscores the importance of considering regional heterogeneity when developing therapeutic strategies for opioid addiction.

These findings challenge the traditional view of the PVT as a homogeneous structure and emphasize the need for more nuanced research that considers the distinct roles of its subregions. Future studies should explore the molecular and electrophysiological differences between aPVT and pPVT neurons, particularly in the context of chronic opioid exposure, to develop targeted interventions that address the specific mechanisms underlying withdrawal and relapse.

G-protein–gated inwardly rectifying potassium channel

A major focus of our work was examining the role of G-protein–gated inwardly rectifying potassium (GIRK) channels in modulating opioid receptor (MOR) activity within the PVT. GIRK channels are a critical component of MOR signaling, as their activation leads to neuronal hyperpolarization and decreased excitability, effectively dampening the cell's responsiveness to further stimuli. In this context, our findings provide new insights into how MORs in the PVT are regulated through GIRK channel activity.

Specifically, we observed that the activation of GIRK channels in the aPVT following the application of [Met^5]-enkephalin (ME) resulted in a larger potassium current compared to the pPVT. This suggests that neurons in the aPVT are more responsive to MOR activation than those in the pPVT, which may underlie the greater influence of aPVT circuits on opioid-related behaviors. The larger GIRK-mediated currents in aPVT neurons could lead to greater inhibition of these neurons in response to opioid exposure, contributing to the regulation of reward pathways and potentially reducing stress responses in the short term.

However, the chronic exposure to opioids seems to disrupt this regulatory mechanism. Following prolonged morphine treatment, the responsiveness of GIRK channels to MOR activation was diminished, indicating a form of desensitization or tolerance. This decreased GIRK activity in the face of chronic

opioid exposure likely contributes to the increased excitability observed in PVT neurons, particularly those projecting to reward-related regions like the NAc. This finding aligns with the broader literature on opioid tolerance, where prolonged MOR activation leads to diminished receptor efficacy, contributing to both tolerance and dependence.

The differential activation of GIRK channels in the aPVT versus pPVT also has important implications for understanding the circuit-specific effects of opioids. In the pPVT, where GIRK-mediated currents are smaller, the effects of opioid withdrawal may be more strongly driven by enhanced excitatory inputs rather than changes in intrinsic inhibitory mechanisms. This could explain why the pPVT is more involved in stress and aversive behaviors, as its neurons are less effectively inhibited by MOR activation and more susceptible to excitatory drive during withdrawal.

Our observation that GIRK-mediated currents are significantly larger in aPVT neurons suggests a higher efficiency of MOR-GIRK coupling or greater expression of GIRK channels in this subregion. This enhanced inhibitory capacity may play a crucial role in regulating the activity of neurons involved in reward processing. In contrast, the smaller GIRK currents in pPVT neurons indicate a reduced inhibitory influence, which may make these neurons more responsive to excitatory inputs, aligning with their proposed role in stress and aversive processing.

Chronic morphine treatment appears to disrupt GIRK channel function in the PVT, as evidenced by the diminished responsiveness to MOR activation following prolonged exposure. This desensitization could be due to several mechanisms, including phosphorylation of GIRK channels by protein kinases activated during chronic opioid exposure (Levitt & Williams, 2012), or downregulation of GIRK channel expression. The resultant decrease in inhibitory control may lead to heightened neuronal excitability, particularly in PVT neurons projecting to the NAc and medial prefrontal cortex (mPFC), thereby contributing to withdrawal symptoms and increased relapse risk.

These mechanistic insights highlight the critical role of GIRK channels in modulating PVT neuron activity and suggest that targeting GIRK channel function could be a viable strategy for mitigating opioid withdrawal symptoms. Restoring normal GIRK-mediated inhibitory signaling may help rebalance the excitatory-inhibitory dynamics disrupted by chronic opioid use.

Our findings contribute to a growing body of literature emphasizing the importance of GIRK channels in opioid receptor signaling and addiction-related neuroadaptations. Previous research has extensively characterized MOR-GIRK interactions in regions such as the locus coeruleus and ventral tegmental area, where alterations in GIRK function have been linked to opioid tolerance and dependence (Williams et al., 2001). However, the role of GIRK channels within the PVT has been less well-defined.

By demonstrating that the aPVT exhibits larger GIRK-mediated currents in response to MOR activation, our study aligns with the work of Kolaj et al. (2014), who reported region-specific electrophysiological properties within the PVT. Additionally, our observation that chronic morphine exposure leads to GIRK channel desensitization extends the findings of Arora et al. (2011), who showed similar adaptations in other brain regions associated with addiction.

These results support the theoretical framework that posits opioid tolerance and dependence arise from cellular adaptations that diminish inhibitory G-protein signaling pathways (Christie, 2008). Specifically, the decreased GIRK channel responsiveness in the PVT may represent a compensatory mechanism that contributes to the hyperexcitability observed during withdrawal. This hyperexcitability could enhance glutamatergic transmission to downstream targets like the NAc and mPFC, exacerbating withdrawal symptoms and promoting relapse.

Furthermore, our study underscores the functional heterogeneity within the PVT, reinforcing the idea that the aPVT and pPVT have distinct roles in modulating behavior (Hsu et al., 2014). The differential

GIRK channel function between these subregions may help explain their unique contributions to the neural circuits underlying addiction and stress responses.

In light of these findings, future research should focus on elucidating the molecular mechanisms governing GIRK channel regulation in the PVT during chronic opioid exposure. Understanding how these channels are modulated could reveal novel therapeutic targets for treating opioid addiction. For example, pharmacological agents that enhance GIRK channel activity might restore inhibitory control over hyperexcitable PVT neurons, alleviating withdrawal symptoms and reducing the likelihood of relapse.

Circuit specific adaptations to chronic opioid exposure

One of the most significant findings from this research is the differential increase in excitability of neurons that project to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). These areas are key nodes in reward and motivational circuits, suggesting that chronic morphine treatment amplifies the influence of these PVT projections on circuits that drive drug-seeking behavior. The increased firing to a depolarizing current step observed in these neurons point to a heightened baseline excitability, which could prime the system for relapse in withdrawal conditions. Importantly, the fact that these changes are not observed in neurons projecting to the amygdala (AM) underscores the circuit-specific nature of opioid-induced adaptations within the PVT. This finding suggests that withdrawal behaviors may be governed by selective changes in neuronal excitability, where certain projections (e.g., NAc, mPFC) facilitate the compulsive drive to avoid withdrawal symptoms, while others (e.g., AM) may not be as directly involved.

Another important aspect of our study is the increase in glutamatergic drive into the mPFC following chronic opioid exposure. The mPFC plays a central role in executive function, stress regulation, and decision-making, and increased excitatory input from the PVT may disrupt these functions during

withdrawal. Enhanced glutamatergic signaling could contribute to heightened arousal and anxiety, which are common in withdrawal states. These findings are consistent with the broader hypothesis that chronic opioid use induces a hyperactive state within circuits that govern both emotional and cognitive responses to stress, thus reinforcing the cycle of addiction by increasing the salience of negative emotional states.

The implications of these findings extend beyond understanding the acute symptoms of withdrawal. The PVT's role in mediating stress-related behaviors makes it a key target for understanding the long-term effects of chronic opioid use, especially in the context of relapse vulnerability. Our findings suggest that PVT circuits, particularly those projecting to the NAc and mPFC, may serve as critical nodes for intervention. Targeting these circuits could potentially disrupt the maladaptive behaviors associated with opioid addiction, such as heightened stress sensitivity and compulsive drug-seeking.

The complexity of opioid receptor regulation in the PVT also raises interesting questions about the interplay between intrinsic and extrinsic factors in withdrawal and tolerance. For example, the differential changes in excitability across PVT projections suggest that opioid-induced adaptations are not uniform but are influenced by the specific output targets of PVT neurons. This supports the idea that the PVT does not function as a homogeneous entity but is instead composed of functionally distinct populations of neurons that differentially regulate reward, stress, and emotional circuits.

Lastly, this study underscores the importance of considering both pre- and postsynaptic mechanisms in understanding opioid receptor regulation. While much of the focus in the opioid field has been on postsynaptic changes, such as receptor desensitization and downregulation, our findings highlight the role of presynaptic adaptations, particularly in glutamatergic input to the PVT. These adaptations may play a critical role in shaping the overall excitatory tone of PVT circuits, particularly during opioid

withdrawal. Future work should aim to disentangle the specific contributions of pre- and postsynaptic changes to the overall dysregulation of PVT circuits in opioid addiction.

The circuit-specific adaptations to chronic opioid exposure within the paraventricular nucleus of the thalamus (PVT) are illuminated by our findings on neuronal excitability changes. Specifically, chronic morphine treatment resulted in a pronounced increase in the excitability of PVT neurons projecting to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), as evidenced by their heightened firing in response to depolarizing current steps. This increased excitability suggests several underlying mechanistic changes:

1. **Altered Ion Channel Functionality**: Chronic opioid exposure may modulate the expression or activity of voltage-gated sodium and potassium channels in these neurons. Upregulation of sodium channels or downregulation of potassium channels could lower the threshold for action potential initiation, leading to increased neuronal firing rates.

2. **Synaptic Plasticity Enhancements**: There may be an increase in excitatory synaptic inputs onto PVT neurons projecting to the NAc and mPFC. This could involve enhanced glutamate release from presynaptic terminals or increased postsynaptic sensitivity due to upregulation of AMPA and NMDA glutamate receptors.

3. **Reduced GABAergic Inhibition**: Chronic morphine treatment might decrease inhibitory GABAergic inputs or impair GABA receptor function in these projection-specific neurons. A reduction in inhibitory tone would result in disinhibition, contributing to increased excitability.

4. **Intracellular Signaling Pathway Modifications**: Opioids can influence intracellular signaling cascades, such as the cAMP-PKA pathway, leading to phosphorylation of ion channels and receptors that alter neuronal excitability.

5. **Gene Expression Changes**: Prolonged opioid exposure may induce changes in gene expression that promote a hyperexcitable state. For example, upregulation of immediate early genes or transcription factors like ΔFosB could result in long-term alterations in neuronal function.

These mechanistic adaptations collectively enhance the output of PVT neurons to the NAc and mPFC, regions integral to reward processing, motivation, and executive function. The increased excitatory drive to the NAc could potentiate reward-seeking behaviors, while heightened input to the mPFC may disrupt cognitive control and decision-making processes. Such changes are likely to contribute to the compulsive drug-seeking and relapse observed during opioid withdrawal.

Our observations are in concordance with previous studies emphasizing the role of the PVT in addiction and stress-related behaviors. The PVT-NAc pathway has been implicated in mediating the reinforcing effects of drugs and cue-induced relapse. Zhu et al. (2016) demonstrated that activation of PVT projections to the NAc promotes reinstatement of cocaine-seeking behavior, highlighting the pathway's significance in relapse vulnerability. Our finding of increased excitability in PVT neurons projecting to the NAc suggests that chronic opioid exposure sensitizes this circuit, potentially exacerbating the risk of relapse during withdrawal.

Similarly, the PVT-mPFC projection plays a crucial role in stress responses and executive functions. Hsu et al. (2014) reported that the PVT sends dense glutamatergic projections to the mPFC, influencing stress-induced reinstatement of drug-seeking behavior. The enhanced glutamatergic drive from the PVT to the mPFC observed in our study aligns with this, indicating that chronic opioids may disrupt prefrontal cortical regulation, contributing to the impaired decision-making and increased stress sensitivity characteristic of withdrawal states.

The lack of significant excitability changes in PVT neurons projecting to the amygdala (AM) underscores the circuit-specific nature of opioid-induced adaptations. This specificity supports the notion of

functional heterogeneity within the PVT, as proposed by Li and Kirouac (2012), who highlighted the distinct roles of PVT subregions and their projections in modulating emotional and motivational behaviors.

Our findings also contribute to the broader understanding of synaptic plasticity in addiction. Chronic exposure to drugs of abuse is known to induce long-term potentiation-like changes in neural circuits, altering synaptic strength and neuronal excitability (Kauer & Malenka, 2007). By identifying specific PVT circuits that undergo such adaptations following chronic opioid exposure, our study provides valuable insights into the neural mechanisms underlying addiction-related behaviors.

These results emphasize the importance of targeting specific neural circuits in developing therapeutic strategies for opioid addiction. Interventions aimed at normalizing the excitability of PVT neurons projecting to the NAc and mPFC could potentially mitigate withdrawal symptoms and reduce relapse risk. For example, pharmacological agents that modulate ion channel function or synaptic transmission within these circuits may restore balance and improve outcomes for individuals struggling with opioid dependence.

In light of these findings, future research should focus on elucidating the precise molecular mechanisms driving the increased excitability in these projection-specific neurons. Understanding the interplay between chronic opioid exposure, ion channel regulation, and synaptic plasticity will be crucial for developing targeted therapies that address the root causes of addiction and withdrawal.

Future Directions

There are several exciting avenues for future research that could build on the findings of this thesis and deepen our understanding of opioid receptor regulation in the PVT. Below, we outline some potential directions:

Comprehensive Morphological and Functional Characterization of PVT Neurons: Our study suggests that neurons in the anterior and posterior PVT (aPVT and pPVT) exhibit distinct functional properties, particularly in relation to their projection targets. However, a more comprehensive characterization of these neurons is needed to fully understand the morphological and functional differences between aPVT and pPVT populations. Future studies should focus on detailed morphological analyses using techniques such as single-cell RNA sequencing, in combination with electrophysiological recordings, to determine whether specific projection neurons share distinct morphological or molecular signatures. Additionally, it would be informative to investigate whether neurons that project to the same region (e.g., NAc) from the aPVT and pPVT exhibit similar or distinct properties. Such studies could reveal new insights into the functional heterogeneity of the PVT and its role in opioid addiction.

Glutamatergic Input Modulation and Circuit-Specific Effects: The increased glutamatergic drive to the PVT following naloxone precipitated withdrawal in chronic morphine animals points to a critical area of future investigation. It would be valuable to explore whether this increased excitatory input is driven by specific upstream nuclei or by intrinsic adaptations within the PVT itself. McDevitt and Graziane 2019 found an increase in the AMPA/NMDA ratio of PVT neurons following chronic treatment with morphine via escalating daily i.p. injections. Repeating this experiment with our approach of using an osmotic mini pump would tell us if this increase in AMPA/NMDA ratio is secondary to receptor level adaptations (i.e. MOR signaling leads to changes in the AMPA/NMDA ratio) or system level adaptations (i.e. increased glutamatergic inputs during withdrawal potentiate this synapse leading to an increase in the AMPA/NMDA ratio). Techniques such as optogenetics or chemogenetics could be used to selectively activate or inhibit specific inputs to the PVT, providing a more precise understanding of how these inputs contribute to opioid withdrawal and relapse. Additionally, future studies could employ transgenic animal models (e.g., OPRM1-Cre rats) to selectively manipulate MOR-expressing neurons and assess their role in modulating glutamatergic transmission. This line of research could identify potential

therapeutic targets for reducing the hyperarousal and stress responses associated with opioid withdrawal.

Investigating the Mechanisms of Cross-Regulation Between GPCRs: Our findings suggest that opioid receptor regulation may extend beyond classical receptor downregulation and desensitization, particularly in the context of cross-regulation between GPCRs. Future research should explore how chronic morphine exposure affects the interaction between MORs and other GPCRs, such as nociceptin receptors (OFQ). Given that nociceptin signaling has been implicated in opioid tolerance, it would be valuable to investigate whether chronic opioid treatment enhances or diminishes cross-desensitization between MORs and OFQ receptors. This could provide new insights into the molecular mechanisms underlying opioid tolerance and identify novel strategies for preventing tolerance in opioid therapies.

Behavioral Correlates of Electrophysiological Changes in PVT Neurons: One of the most important future directions is to link the electrophysiological changes observed in PVT neurons with specific behavioral outcomes in animal models of opioid addiction. For example, does increased excitability in PVT neurons that project to the NAc directly correlate with heightened drug-seeking behavior? Can reducing glutamatergic input to the mPFC mitigate withdrawal-related anxiety and hyperarousal? Combining electrophysiological recordings with behavioral assays, such as self-administration or stress-induced relapse paradigms, would provide critical insights into the functional relevance of the observed neuronal changes. These experiments could also help determine whether targeting specific PVT circuits could reduce withdrawal symptoms and prevent relapse in opioid addiction.

In conclusion, this thesis has begun to lay a foundation for a deeper understanding of opioid receptor regulation in the PVT and its role in mediating the negative emotional states associated with opioid

withdrawal. Future research should aim to further elucidate the molecular, cellular, and circuit-level changes that occur in the PVT during opioid addiction.

Appendix: Recipes

Modified Krebs Buffer Solution

1. Add the following to 1 L nanopore H2O to obtain 2 L modified Krebs buffer
a. 4.0 g D-glucose (11 mM)
b. 3.6 g NaHCO3 (25 mM)
c. 200 mL 10x stock solution (1 L 10x stock recipe below)
i. 7.363 g NaCl (126 mM)
ii. 0.186 g KCl (2.5 mM)
iii. 0.244 g MgCl2 (1.2 mM)
iv. 0.353 g CaCl2 (2.4 mM)
v. 0.166 g NaH2PO4 (1.2 mM)
vi. 1 L ddH2O
d. Constitute to a final volume of 2 L with nanopore H2O
e. Incubate in a 35° C water bath, while oxygenating with 95%/5% O2/CO2 gas

Compound 101

KMS-based BAPTA internal solutions

1. Add the following to 10 mL nanopure H2O to obtain 50 mL of KMS 10 BAPTA intracellular solution
a. 770 mg potassium methanesulfonate (115 mM)
b. 0.5 mL 2M NaCl (20 mM)
c. 75 µL 1M MgCl2 (1.5 mM)
d. 69 mg HEPES (K) (10 mM)
e. 328 mg BAPTA (K4) (10 mM)
2. Constitute to a final volume of 50 mL
3. Filter with a 0.45 µm filter into 10 mL aliquots
4. Store aliquots at -20 C until needed

5. Before use, thaw a 10 mL aliquot and add the following:
a. 1.6 mg Na-GTP (0.3 mM)
b. 10 mg Mg-ATP (2 mM)
c. 25.5 mg phosphocreatine (10 mM)
6. Add pH until 7.35
7. Adjust osmolarity until between 275-280 mOsm.
8. Aliquot into 1 mL fractions
9. Store unused aliquots at -20 C

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