# Kappa opioid receptor modulation of nucleus accumbens feedforward inhibitory microcircuits

By

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# CHAPTER 1

## Introduction

### 1.1. The opioid epidemic: a historical prospective

The use of opium dates back to 3,400 BC, when the Sumerians cultivated poppies and isolated opium in the region now known as Iraq (Brownstein, 1993). They called opium "gil," or joy, and likely used it during religious rituals for the euphoric state it induced. From the eighth to thirteenth centuries, opium was traded along the silk road, spreading its use to all of Asia and Europe. Morphine was first purified from opium by the Prussian (present-day Austria) pharmacist Friedrich Wilhelm Adam Sertürner. He reported his discovery of a sleep inducing molecule to the Trommsdorffs Journal der Pharmacie in 1805 and 1806, naming it morphium after the Greek god of sleep, Morpheus (Krishnamurti & Rao, 2016). It was hoped that morphine would not have the same addictive properties as opium, but this was not the case. In another attempt to produce a less addictive opioid, heroin was synthesized in 1874 by C.R.A. Wright and mass produced by the Bayer Company Germany as a more potent and less addictive alternative to morphine. Obviously, this misinformation has had drastic consequences as heroin remains a dangerous illicit drug today, contributing to over 14,000 overdose deaths in 2019 in the United States alone (Abuse, 2021). This would not be the last time the pharmaceutical industry misrepresented opioids for financial gain.

The first records of opium addiction and tolerance date back to the sixteenth century, and no region felt these harmful side effects more than China. Tobacco had been banned in China but was quickly

replaced with the smoking of opium, leading to widespread opium addiction across the region. Despite the Chinese government subsequently banning opium, Britain, through the East India Trading Company, illegally traded opium to counter the imbalance in trade between Britain and China. This ultimately led to the opium wars in the early to mid-nineteenth century, both of which China lost (*Opium Trade | History & Facts*, 2021). It was not until 1910 that the Chinese were successful in dismantling the opium trade. By this time a staggering 13.5 million Chinese citizens, 27% of adult males, suffered from opiate addiction.

In the United States, opiate addiction increased in the late nineteenth century with an estimated 200,000 people suffering from addiction by 1900 (Courtwright, 1978). This has largely been attributed to the use of morphine during the civil war, as well as the large influx of addicted Chinese immigrants who worked on the transcontinental railroad. These events led to the banning of opium in the United States in 1905. Despite the gradual increase in persons suffering from opiate addiction in the United States, opioid use remained comparatively under control throughout most of the 1900s. The FDA approved extended-release forms of morphine (MS Contin) in 1985 and oxycodone (OxyContin) in 1995 (National Academies of Sciences et al., 2017). These new opioid formulations paired with the unethical sales approach of Purdue Pharma led to the modern opioid epidemic, a mirror image of what the Bayer Company had done with heroin one hundred years earlier.

The United States has seen an increase in opioid related overdose deaths from less than 10,000 in the year 2000 to 49,860 in 2019 (Abuse, 2021). Opioid related overdoses made up 70% of all drug related overdose deaths in 2019. Recently, the opioid epidemic has been exacerbated by the

COVID-19 pandemic. While the data is still coming in, estimates place opioid related overdoses in 2020 close to 70,000, an increase of 20,000, with estimates for 2021 even higher (*Products - Vital Statistics Rapid Release - Provisional Drug Overdose Data*, 2021). This increase is likely due to the introduction of numerous new stressors such as social isolation, job loss, increased anxieties, or loss of loved ones, leading many to relapse in their drug abstinence. As we will discuss, it is our hope that the work presented here may inch the field forwards towards a therapeutic target for the treatment of stress induced relapse.

Clearly the opioid epidemic we find ourselves in is the culmination of a millennia of complex social, economic, and clinical issues. To devise solutions for this epidemic it is important to know how we got here. No one solution will solve the epidemic but putting our skills and efforts to their best use will allow us to chip away. As scientists, I believe understanding how the brain's endogenous opioid system functions is paramount to successfully combating this epidemic.

## 1.2. Endogenous opioid signaling, pharmacology, and behavior

Opioids, such as morphine and heroin, exert their effects on the body through signaling events controled by opioid receptors. There are four primary opioid receptors, μ (MOR), κ (KOR), δ (DOR), and nociceptin opioid peptide receptor (NOP). Despite high sequence homology with the other three receptors, NOP has little to no affinity for opioid peptides or morphine and its structural derivatives (Meunier et al., 1995). Because of this, NOP will not be reviewed in this dissertation. The opioid receptors are Gαi coupled GPCRs and negatively regulate adenylyl cyclase (Figure 1.1). Their seven transmembrane domains share 70% sequence homology, while their extracellular

domains and c/n-terminal tails share little homology allowing for differential ligand binding and intracellular signaling partners. Canonically, these receptors inhibit neurotransmission by activating G-protein gated inward rectifying potassium channels or inhibiting voltage gated calcium channels (Figure 1.1). These signaling events lead to hyperpolarization of the neuron and the disruption of presynaptic vesicular release, inhibiting signal transduction. In the spinal cord, opioids inhibit neurotransmitter release of primary afferent terminals, inhibiting the communication of sensory information from the peripheral nervous system to the central nervous system (CNS), cutting off pain signals before they can reach the brain. All three opioid receptors exhibit analgesic properties, but also modulate distinct behaviors through their signaling in the brain; MOR activation leads to euphoria, DOR to antidepressant and anxiolytic effects, and KOR to negative affective states.

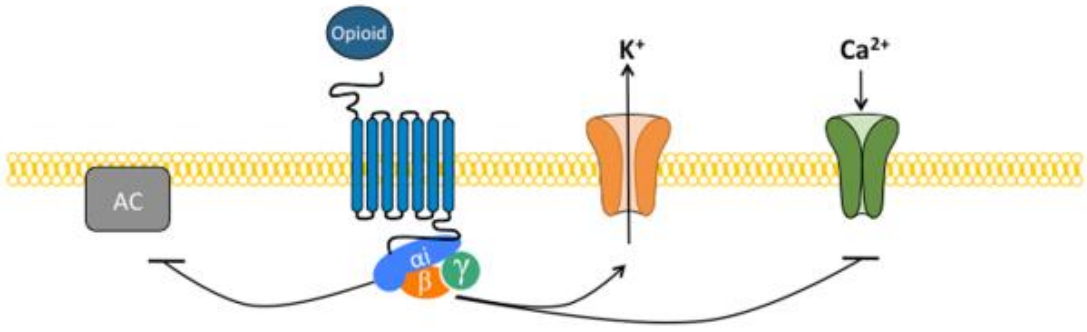


**Figure 1.1. Canonical opioid receptor signaling pathways.** The μ, κ, and δ opioid receptors are 7 transmembrane domain G-protein coupled receptors. The $G_{i\alpha}$ subunit inhibits adenylyl cyclase activity and decreases cAMP – PKA signaling. The Gβγ subunit directly interacts with G-protein-gated inward rectifying potassium channels and N-type Ca2+ channels to activate and inhibit conduction, respectively. Together, these mechanisms inhibit kinase activity, hyperpolarize the neuron, and interrupt vesicular neurotransmitter release to inhibit neuronal activity.

Endogenous opioids are produced within the brain and circulate throughout the major organ systems. There are three families of endogenous opioids which consist of neuropeptides proteolytically cleaved from large precursor proteins. These proteins are proopiomelanocortin (POMC), preproenkephalin (PENK), and preprodynorphin (PDYN), which are the precursors for endorphins, enkephalins, and dynorphins, respectively. Endorphin acts primarily through MORs and DORs, enkephalins through DORs, and dynorphins through KORs. These peptides also differ in where they are synthesized. POMC synthesizing neurons are restricted to the arcuate nucleus of the hypothalamus and the nucleus tractus solitarius in the dorsal medulla where they send projections to much of the CNS. Conversely, PENK and PDYN synthesizing neurons are located in many brain regions (Benarroch, 2012).

Opioid receptors are expressed widely throughout the peripheral and CNS which leads to many unintended, acute negative side effects when using opioids for analgesia. In the small intestine, activation of MORs leads to a decrease in gut motility and constipation, a common side effect seen with prolonged opioid use. Other common side effects are itching, decreased blood pressure, and nausea. The most common cause of opioid related overdose death is opioid-induced respiratory depression (OIRD). Though a peripheral response, OIRD is triggered by opioids affecting the cells within the brainstem that respond to changes in carbon dioxide and oxygen in the blood (Bubier et al., 2020).

Opioid receptors undergo extensive tolerance when repeatedly activated, presenting a challenge for clinicians when trying to maintain analgesia. Tolerance is defined as the need to increase the dose to maintain efficacy. Opioid receptors are prone to tolerance and the dose must be increased to maintain analgesia over time. However, doing so also increases the likelihood and severity of the negative side effects listed above. This makes maintaining analgesia while mitigating negative side effects difficult, causing opioids to become less efficacious over time. To combat tolerance as well as other side effects of opioid use, we need to better understand the signaling mechanisms that cause them to develop opioids that are less likely to recruit them. Current strategies under development to prevent the negative side effects of opioids such as peripherally restricted opioids and biased agonists are being pursued.

Peripherally restricted opioids have been developed by screening for hydrophilic compounds that have difficulty crossing the blood brain barrier. This would allow for opioids to exert their analgesic effects while limiting their side effects in the brain, such as euphoria, decreasing their abuse potential. Many peripherally restricted opioids show preclinical efficacy for the treatment of pain; however, most perform no better than placebo in clinical trials. There is reason for hope, as CR845, a peripherally restricted KOR agonist, has performed well in phase II clinical trials and is under development by Cara Therapeutics for the treatment of postoperative and osteoarthritic pain (Albert-Vartanian et al., 2016). The early success of CR845 in clinical trials, and others preclinically, provides initial proof of concept for the efficacy of peripherally restricted opioids and warrants further investigation.

Opioid receptors signal through multiple effector arms, even concurrently. This allows them to recruit various effector systems and differentially impact the cell and circuit depending on the system recruited. For example, upon ligand binding and activation of G-protein signaling via GTP hydrolysis, the Gα subunit can interact directly with the GIRK channel, $K_{ir3}$, activating it and hyperpolarizing the cell. Conversely, the Gβγ subunit can interact directly with $Ca^{2+}$ channels and inhibit $Ca^{2+}$ conductance as well as activate the MAPK pathway (Al-Hasani & Bruchas, 2011). Following activation, G-protein receptor kinase 2 or 3 phosphorylates the receptor and recruits arrestin 2/3. Arrestin recruitment may lead to the sequestration and desensitization of the receptor, contributing to tolerance as evidenced by arrestin-3 KO mice showing decreased tolerance to morphine (Bohn et al., 2000). The arrestin bound opioid receptor also has the ability to recruit signaling transduction pathways such as MAPKs, further diversifying the many pathways opioid receptors can recruit (Lefkowitz & Shenoy, 2005). A current hypothesis within the field is that opioid receptors recruit specific signaling arms to mediate distinct physiological effects, and that biased agonists may be able to circumvent some of the negative side effects seen with current opioids by only activating specific signaling arms. The development of biased opioid receptor agonists is an ongoing area of research for the field, with many compounds showing preclinical utility (Mores et al., 2019; Schmid et al., 2017).

*Dynorphin/KOR system*

The endogenous ligand for the KOR is dynorphin, and in the striatum dynorphin is released by D1+ MSNs. Prodynorphin is cleaved by a series of non-selective proteases cathepsin L, PC1, 2 and 3 and CPE to create dynorphin A, dynorphin B, big dynorphin (dynorphin A + dynorphin B), α-neoendorphin, and β-neoendorphin (Berman et al., 2000; Boudarine et al., 2002; Kangawa et al.,

1979; Minamino et al., 1981; Minokadeh et al., 2010). Interestingly, prodynorphin cleavage products are differentially expressed in the mesocortical dopamine system, though basal expression of prodynorphin is highest in the ventral striatum (Willuhn et al., 2003; Zamir et al., 1984). The *in-vivo* release of these peptides remains unclear and it is an intriguing idea that various brain regions may produce specific prodynorphin cleavage products to elicit different KOR signaling cascades to support varying behavioral states. In contrast to MOR and DOR agonists, KOR agonists do not induce euphoria and exhibit low abuse potential. Initially, this steered researchers towards MORs and DORs for their roles in the positive reinforcement of opiates (Le Merrer et al., 2009). However, interest in KOR regulation of the negative reinforcing properties of drugs, paired with its low abuse potential has triggered a rush in KOR research.

As mentioned, activation of KORs is inhibitory, but depending on the cell type on which they are expressed could lead to circuit activation. For instance, activation of a $G_{i\alpha}$ GPCR on an inhibitory cell could lead to downstream disinhibition and signal facilitation. In rodent models, KOR signaling generally promotes negative affective states. For example, salvinorin A, a KOR agonist, dose dependently increased intracranial self-stimulation thresholds, interpreted as an increase in anhedonia (Carlezon et al., 2006). Early studies by Mucha and Herz described the KOR agonist, U-50488, to induce robust conditioned place aversion (Mucha & Herz, 1985). While KOR agonists promote negative affective states, KOR antagonists block them. U69593 increased immobility in the forced swim test while the KOR antagonist, nor- binaltorphimine, decreased immobility representative of prodepressive and antidepressive effects, respectively (Mague et al., 2003). Moreover, prodynorphin knockout mice exhibit an anxiolytic phenotype that is reversed by U-50488 (Wittmann et al., 2009). The effects listed in this section have been repeated by a number

of groups across multiple genetic backgrounds with various pharmacology. Taken together, there is strong evidence that KOR signaling promotes negative affective states.

KOR signaling has important implications for drug addiction. KOR activation mirrors the effect of stress on potentiating drug reward. Forced swim test is often used as a measure for depression, but it also an intense stressor. Forced swim test administered prior to cocaine conditioned place preference (CPP) enhances place preference. KOR antagonists block this enhancement, while KOR agonists alone can recapitulate the enhancement (McLaughlin et al., 2006). Negative affective states during withdrawal and stress are critical behavioral states that drive drug relapse (Koob & Le Moal, 2001; Mantsch et al., 2016). In this context, KOR signaling may play an important role in withdrawal induced negative affect and stress induced relapse. In animal models, relapse is modeled by the reinstatement of drug seeking behavior. Mice can be conditioned to associate a behavior or context with a rewarding substance (drug) to reinforce that behavior. Over time this association can be extinguished and the animal ceases to perform the behavior. This mirrors abstinence in humans. Re-exposure to the drug rapidly reinstates drug seeking behavior. Interestingly, stress is also capable of reinstating drug seeking (Mantsch et al., 2016). This is called stress-induced reinstatement and models human relapse. Several lines of evidence show that KORs mediate stress-induced reinstatement to a variety of drugs of abuse including nicotine and cocaine (Graziane et al., 2013; Jackson et al., 2013; Polter et al., 2014). Prior administration of nor-BNI blocks stress-induced reinstatement, and one study found that direct infusion of nor-BNI into the ventral tegmental area (VTA) was sufficient to block stress-induced reinstatement of cocaine self-administration (Graziane et al., 2013). Multiple studies have also implicated KOR signaling in drug withdrawal-induced negative affect. KOR antagonism in the bed nucleus of the stria

terminalis (BNST) attenuates alcohol withdrawal-induced negative affective behavior and systemic administration of a short-acting KOR antagonist reduces anxiety and depressive-like behaviors following cocaine self-administration (Erikson et al., 2018; Valenza et al., 2017). Likewise, KOR blockade also reduces physical signs of nicotine withdrawal (Tejeda et al., 2012). Given the critical role negative affective states and stress play in relapse, KOR signaling represents an appealing therapeutic target to prevent relapse.

KOR signaling strongly regulates dopamine dynamics in the midbrain. Ultrastructural studies show that KORs tend to localize along dopaminergic varicosities (Svingos et al., 1999, 2001). In agreement, KOR agonists inhibit dopamine release in the VTA, nucleus accumbens (NAc), dorsal striatum, and medial prefrontal cortex (mPFC) (Ehrich et al., 2015; Ford et al., 2007; Gehrke et al., 2008; Tejeda et al., 2013). Importantly, KOR signaling in the VTA, NAc, mPFC, and lateral hypothalamus all induce conditioned place aversion (CPA) (Bals-Kubik et al., 1993). This led to the hypothesis that KOR mediated decrease in dopamine release in these brain regions leads to aversion. Indeed, conditional KOR knockout (KO) from dopaminergic neurons abolished U69593 conditioned place aversion and could be rescued by viral re-expression in the VTA (Chefer et al., 2013). However, recent studies from the Chavkin group have challenged the notion that KOR inhibition of dopamine is required for aversion. They first found the KOR agonist dependent CPA was intact in dopamine deficient animals (Land et al., 2009). It is possible that this could be explained by compensatory mechanisms that develop in the dopamine deficient mice. They followed this work with an intricate study using various genetic KO models of the KOR and p38α MAPK to show that it is KOR signaling through p38α in dopaminergic neurons that is required for KOR mediated aversion, not KOR inhibition of dopamine release (Ehrich et al., 2015).

Recently, evidence for KOR mediated disinhibition of corticotopin releasing factor neurons in the central nucleus of the amygdala underlying aversive behaviors has emerged (Hein et al., 2021). More targeted studies are required to determine the exact mechanisms by which KORs mediate aversion.

KOR agonists were initially pursued as a target for analgesia as they do not exhibit the same addictive properties as MOR agonists. KOR agonists have shown efficacy in models of neuropathic, thermal, and inflammatory pain (Paton et al., 2020). However, they have been severely limited in their use by their off-target effects. In the clinic, KOR agonists induce psychomimetic effects, sedation, and dysphoria (Pfeiffer et al., 1986; Wadenberg, 2003). Medicinal chemistry strategies underway to combat these negative side effects are the development of biased, mixed, and peripherally restricted agonists. In terms of biased agonists, multiple studies have found that KOR ligands that preferentially signal through G-proteins exhibit analgesic properties while not eliciting sedation or dysphoria (Brust et al., 2016; White et al., 2015). This suggests that KOR mediated negative affect is likely produced by G-protein independent mechanisms, such as beta-arrestin signaling, opening the door for the use of biased KOR agonists for the treatment of pain (Table 1.1).

| Drug | Target | Biased |
|---|---|---|
| U69593 | KOR | No |
| U50488 | KOR | No |
| Salvinorin A | KOR, partial D2 receptor agonist | No |
| Dynorphin A | Endogenous KOR agonist | Moderate G-Protein Biased |
| BPHA | KOR, some affinity for MOR & DOR | G-Protein Biased |
| RB-64 | KOR | G-Protein Biased |
| Triazole 1.1 | KOR | G-Protein Biased |
| 6'GNTI | KOR Partial Agonist | G-Protein Biased |

**Table 1.1. Common KOR agonists and their signaling biases.** Commonly used KOR agonists that have been well characterized in the context of biased G-protein or beta-arrestin signaling. Note that very few agonists have been characterized to exhibit strong beta-arrestin bias. (Brust et al., 2016; Dunn et al., 2018; Rives et al., 2012; White et al., 2014, 2015)

Recently, attention has moved towards KOR antagonists for the treatment of negative affective disorders such as anxiety, depression, and PTSD (Carlezon Jr. & Krystal, 2016). Many early clinical trials with KOR antagonists such as nor-BNI and JDTic were halted due to toxicity. Pharmacodynamic properties of these drugs also made them unamenable to clinical trials. Nor-BNI antagonism of KOR signaling can last for weeks, and though JDTic is relatively shorter, antagonism of the KOR still persists up to 11 days post JDTic administration. In clinical trials that may need to be halted quickly for various reasons, long lasting effects as seen with these antagonists are not ideal, at least in early studies. Exciting developments are on the way. The first short acting KOR antagonist was developed by Eli Lilly and showed promising preclinical effects in mood and addictive disorders (Mitch et al., 2011; Rorick-Kehn et al., 2014). This compound has since been taken over by Janssen and named JNJ-67953964. JNJ-67953964 recently successfully completed the first proof of mechanism study in the Fast-Fail Trials Program and has been given the go ahead for continued clinical evaluation (Krystal et al., 2020). Continued

incentives like the FAST-FAIL program that provide support for the rapid testing of compounds in psychiatric disorders will be imperative for the successful development of CNS drugs.

## 1.3. The nucleus accumbens as a reward hub

### *Drugs of abuse modify NAc physiology*

As many of the behavioral effects of KOR ligands occur within the brain's reward circuitry, we will now review the NAc as a reward hub. The NAc is a key node within the mesolimbic reward pathway, integrating salient information from glutamatergic afferents and neuromodulatory dopaminergic projections from the VTA to drive motivated behavior. Synaptic changes within the NAc underlies many maladaptive motivational states including addiction, anxiety, depression, and autism. In the context of addiction, many early studies focused on changes in dopamine signaling in the NAc, as a commonality between many drugs of abuse is their ability to increase dopamine in the NAc. This led to the hypothesis that dopamine is a "pleasure" molecule that is increased to reinforce pleasurable experiences, or more recently to encode reward prediction error. This notion has been challenged by studies characterizing the role of dopamine signaling in the NAc as assigning saliency to a signal (Kutlu et al., 2021). As reviewed above, KOR signaling decreases dopamine release in the accumbens. However, the behaviors that are supported by this mechanism remain unclear, as dopamine inhibition is not required for KOR mediated aversion in the NAc. Regardless, dopamine plays an important neuromodulatory role in NAc circuit integration and reward processing.

Over the last two decades, changes in the synaptic strength of glutamatergic afferents onto NAc medium spiny neurons (MSNs) have been shown to be necessary and sufficient for many drug withdrawal related behaviors, such as craving, negative affect, and relapse (Conrad et al., 2008; Ebner et al., 2018). A seminal study by Mark Thomas from the Malenka lab was the first to show drug dependent changes in synaptic strength in the NAc when they found that glutamatergic transmission onto NAc shell MSNs was decreased following a challenge dose of cocaine in mice previously exposed to cocaine and suggested that this molecular mechanism may underlie behavioral sensitization (Thomas et al., 2001). This triggered a rush of research into drug induced synaptic alterations in the NAc, and specifically changes in glutamatergic strength. Much of the focus has been on changes in MSN AMPAR expression, with changes in glutamatergic strength in the NAc having been shown to underlie drug craving and reinstatement (Conrad et al., 2008; Ebner et al., 2018). While the underlying mechanisms and timing may differ between cocaine, opioid, and amphetamine induced synaptic changes in the NAc, evidence suggests that the different drug classes ultimately overlap in their ability to potentiate excitatory synapses (specifically onto D1+ MSNs) during withdrawal (Hearing et al., 2018; Jedynak et al., 2016; Madayag et al., 2019). As genetic tools have improved, we have also begun to distinguish between drug induced plasticity mechanisms at direct and indirect pathway MSNs (Grueter et al., 2010; Madayag et al., 2019; Turner, Kashima, et al., 2018; Zhu et al., 2016). Interestingly, many of these forms of plasticity overlap with stress induced alterations in NAc glutamatergic transmission (Bagot et al., 2015; Christoffel et al., 2011; Lim et al., 2012). Investigating how these forms of plasticity interact and influence behavior will be a key future direction in understanding how stress modifies drug use.

*Anatomy*

The NAc is a heterogenous brain structure consisting of multiple distinct neuronal cell types. MSNs make up 90-95% of the neurons in the NAc, with the remaining being made up by γ-aminobutyric acid (GABA)-ergic and cholinergic interneurons. MSNs get their name from their medium sized soma (~15μM diameter) and high number of dendritic spines. MSN populations can be subdivided based on the expression of either the dopamine D1 or D2 receptors. D1 expressing MSNs (D1 MSNs) colocalize with dynorphin and substance P while D2 expressing MSNs (D2 MSNs) colocalize with enkephalin and the adenosine 2a (A2a) receptor. MSNs are GABAergic and are the primary projection neurons from the NAc with D1 MSNs primarily projecting to midbrain DA nuclei (VTA and substantia nigra [SN]) as well as the ventral pallidum (VP) while D2 MSNs project exclusively to the VP (Figure 1.2). These diverging projection pathways have been labeled the direct (D1) and indirect (D2) pathways and have historically been characterized to direct opposing behaviors where the direct pathway promotes reward related behaviors while the indirect pathway promotes aversive behaviors. In support of this model, cocaine self-administration leads to circuit specific synaptic changes onto D1 MSNs but not D2 MSNs that underlie reward seeking (Pascoli et al., 2014). Conversely, the strengthening of thalamic to D2 MSN projections underlie negative affective states during morphine withdrawal (Zhu et al., 2016). D1 and D2 MSNs regulating opposing behaviors in the NAc mirrors the role of MSNs in the dorsal striatum where D1 MSNs promote locomotor activity while D2 MSNs inhibit it. However, recent studies challenge this simplicity, showing that depending upon the stimulation pattern, both MSN subtypes can regulate reward and aversion (Soares-Cunha et al., 2020). It is also difficult to reconcile the overlapping projection pattern to the VP by both cell types, such that MSN mediated

behavioral outcomes cannot be explained by projection pattern alone. It is likely that MSNs work in concert to execute motivated behavior.

While MSN projections primarily synapse outside of the striatum, D1 and D2 MSNs are locally connected through lateral inhibition (Dobbs et al., 2016) (Figure 1.2). Fast ionotropic $GABA_AR$-mediated inhibitory current between MSNs exhibits low amplitude and slow activation kinetics. Along with ultrastructural imaging studies, this implies MSN-to-MSN lateral inhibition occurs at distal dendrites. MSN-to-MSN lateral inhibition also appears to be asymmetric, with reports that D2 MSNs form more frequent and stronger synapses onto D1 MSNs (Planert et al., 2010). Though weaker than other forms of local inhibition in the NAc, lateral inhibition may function to inhibit competing pathways and synchronize output from distinctly functional MSN ensembles (Cruz et al., 2013, 2014; Moyer et al., 2014).

The NAc itself can be anatomically divided into the core and shell subregions, with the shell often further divided into lateral and medial shell. The core encircles the anterior commissure and extends ventrolaterally from the lateral ventricle and the shell envelopes the medial, lateral, and ventral borders of the core (Heimer et al., 1991). The NAc core (NAcc) and shell (NAcsh) differ in their innervation patterns, with the core being targeted by glutamatergic projections from the prelimbic PFC, midline nuclei of the thalamus (mThal), dorsal hippocampus, and basolateral amygdala (BLA) while the shell receives projections from the infralimbic PFC, mThal, ventral hippocampus, and BLA. The core also receives DA input from both the VTA and SN, while the shell is exclusively innervated by the VTA (Figure 1.2). As for projections, core efferents primarily project to motor regions of the cortex while shell projections lead to the PFC and various

subcortical motor areas, such as the lateral hypothalamus and extended amygdala (Alexander et al., 1986, 1990). Morphologically, MSNs in the shell are smaller with fewer dendritic spines than the MSNs in the core (Meredith et al., 1992). The core and shell can also be distinguished based on their behavioral outcomes. The shell has been implicated in the reinforcement of associative learning, feeding behavior, novelty, and relapse (Bossert et al., 2007; Parkinson et al., 1999; Plasse et al., 2012). The core has been implicated in spatial learning, conditioned responding, and impulsivity (Caprioli et al., 2014; Hernandez et al., 2002; Ito et al., 2004; Maldonado-Irizarry & Kelley, 1995; Pisansky et al., 2019). On a circuit level, the core and shell regions appear to work in concert with the dorsal striatum through a series of ascending loops that end in the motor cortex to transform motivational state into action (Haber et al., 2000).

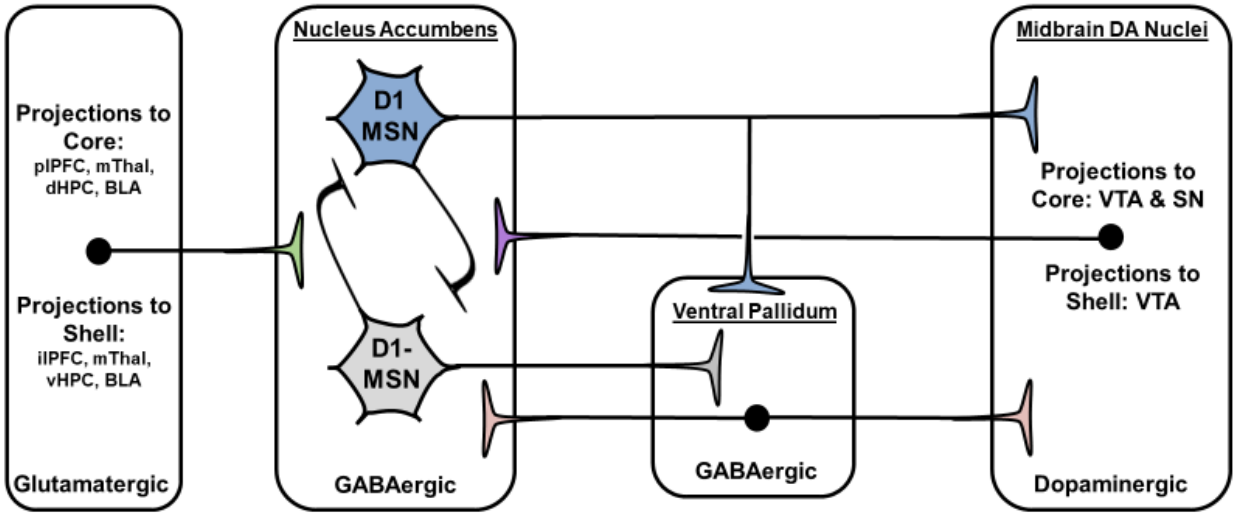


**Figure 1.2. Final common pathway for reward.** Glutamatergic projections into the nucleus accumbens ramify equally onto D1+ and D1- MSNs, with slight differences between core and shell afferents. D1 MSNs project directly to midbrain dopamine nuclei while D1- MSNs project indirectly to midbrain dopamine nuclei through the ventral pallidum. The nucleus accumbens core is reciprocally innervated by both the VTA and SN while the shell is exclusively innervated by the VTA. Abbreviations: plPFC=prelimbic prefrontal cortex, ilPFC=infralimbic prefrontal cortex, mThal=mediodorsal nuclei of the thalamus, dHPC=dorsal hippocampus, vHPC=ventral hippocampus, BLA=basolateral amygdala, VTA=ventral tegmental area, SN=substantia nigra.

MSNs have a hyperpolarized resting membrane potential of -85mV driven by constitutively active inwardly rectifying potassium channels (Kir2) and the absence of HCN channels, which also renders MSNs quiescent at baseline (Mermelstein et al., 1998; Wilson & Kawaguchi, 1996). Glutamatergic drive onto MSNs pushes them from their downstate into an upstate of ~-55mV, after which coincident glutamatergic drive may elicit action potential firing. Therefore, MSN activity is dependent upon coincident glutamatergic afferents in for signal transduction to occur. This renders MSN activity highly susceptible to circuit elements that modulate glutamatergic drive in the NAc. One such circuit element is feedforward inhibition (FFI).

## 1.4. Feedforward inhibition as a vital microcircuit element

FFI will herein be defined as the process by which local interneurons, namely parvalbumin positive fast-spiking interneurons (PV-FSIs), receive glutamatergic input and in turn send inhibitory projections onto MSNs to spatiotemporally restrict their output (Figure 1.3). Feedforward inhibitory microcircuits are becoming increasingly appreciated for their role as a computational circuit element within the NAc that allows for the appropriate propagation of a multitude of neurochemical signals. PV-FSI – to – MSN FFI is more robust than MSN – to – MSN lateral inhibition and PV-FSIs synapse more reliably onto both MSN subtypes, positioning FFI as the primary inhibitory regulator of MSN activity (Planert et al., 2010). There are three primary interneuron subtypes in the NAc: the PV-FSI and the low threshold spiking somatostatin (SST) interneuron, both of which are GABAergic, and the tonically active acetylcholine releasing cholinergic interneuron. Of the three populations, the unique structural, physiological, and

molecular profile of PV-FSIs imparts the ability to efficiently perform FFI and will be reviewed below.

*PV-FSI electrophysiology*

Similarly to the dorsal striatum, PV-FSIs in the ventral striatum can sustain high frequency action potential firing between 150-200 Hz compared to the peak 30 Hz firing rate of MSNs (Taverna et al., 2007) (Figure 1.3). This is due in large part to a rapid afterhyperpolarization that is ten-fold faster in PV-FSIs than MSNs. Similar to MSNs, PV-FSIs exhibit no autonomous action potential firing, but do have a slightly more depolarized resting membrane potential around 75-80mV (Yu et al., 2017). Other unique electrophysiological characteristics of PV-FSIs are their ability to undergo burst firing and subthreshold membrane oscillations referred to as "chattering", distinct modes of activity that allow for easy identification (Scudder et al., 2018; Taverna et al., 2007). PV-FSI excitatory postsynaptic currents EPSCs also display markedly fast decay kinetics, likely due to their aspiny dendrites and high expression of GluA2-lacking Ca2+-permeable AMPARs (CP-AMPARs) (Manz et al., 2020; Yu et al., 2017). One of the more intriguing electrophysiological characteristics of PV-FSIs is that they are electrically connected by gap junctions. These gap junctions allow PV-FSIs to rapidly recruit and synchronous nearby PV-FSIs to robustly inhibit large populations of MSNs (Lau et al., 2010). These unique electrophysiological characteristics allow PV-FSIs to rapidly adapt to changes in circuit activity, fine tuning their inhibition of MSNs.

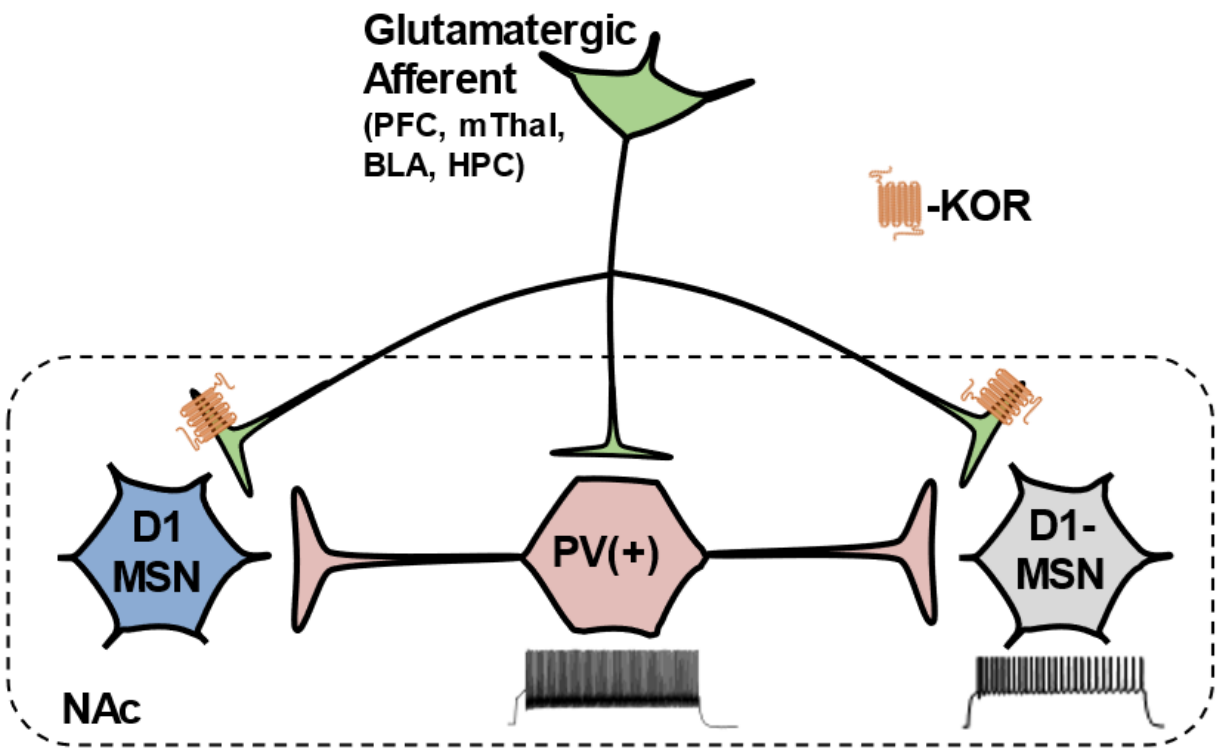


**Figure 1.3. Nucleus accumbens feedforward inhibitory microcircuit.** Parvalbumin fast-spiking interneurons (PV-FSIs) receive collateralizing glutamatergic input and in turn send robust inhibitory GABAergic projections to nearby medium spiny neurons (MSNs). This provides synchrony, action potential timing, and ensemble recruitment in the nucleus accumbens. KORs have been shown to decrease glutamatergic drive onto MSNs, however their contribution to feedforward inhibitory drive is unknown. Also depicted are representative current step traces from PV-FSIs and MSNs exemplifying the fast action potential kinetics of PV-FSIs. Abbreviations: PFC=prefrontal cortex, mThal= mediodorsal nucleus of the thalamus, BLA=basolateral amygdala, HPC=hippocampus.

*PV-FSI connectivity*

PV-FSIs make up roughly 1% of the neurons within the NAc, but have extremely dense axonal arbors that project to a diameter of 400-600 µm and innervate a larger number of MSNs (Kawaguchi, 1993). Dual recordings have found that PV-FSIs synapse onto ~55% of MSNs within their dendritic area, however the proportion of D1- vs D2-dopamine receptor expressing MSNs that are innervated is unknown, but appears to be homogonous (Wright et al., 2017; Yu et al., 2017). Given the density of MSNs within the NAc, a single PV-FSI can innervate roughly 15,000 MSNs (Schall et al., 2021). PV-FSIs also deliver inhibitory postsynaptic currents (IPSCs) to MSNs

of over 1000pA in amplitude (Yu et al., 2017). This inhibitory current is stronger than IPSCs originating from SSTs to MSNs and lateral inhibition between MSNs (Scudder et al., 2018; Wright et al., 2017). PV-FSIs also synapse on the soma or proximal dendrites of MSNs (Qi et al., 2016). This positioning may allow PV-FSI synapses to escape synaptic filtering by MSN synaptic mechanisms, allowing for rapid and potent inhibition compared to the distal synapses formed by MSN lateral inhibition. In fact, PV-FSIs have been shown to inhibit MSN action potential firing in response to the same glutamatergic input before the MSN fires the first action potential (Yu et al., 2017). Their sparse expression paired with their unique ability to rapidly inhibit many MSNs within their vicinity has lead to the hypothesis that PV-FSIs contribute to the activation of distinct MSN ensembles. Whether PV-FSIs overlap in their inhibition of MSNs is unknown but would be highly informative to this hypothesis.

*PV-FSI molecular profile*

Due to the specificity of the promoter for the PV gene, PV-FSIs are easily targeted by genetic strategies, such as fluorescent tagging and optogenetics, allowing for easy identification and specificity (Coleman et al., 2021; Manz et al., 2020; Manz, Coleman, Grueter, et al., 2021). PV is a low molecular weight molecule (9-11kDa) with three Ca2+-chelating EF hand motifs. PV is expressed highest in fast contracting muscle tissue, as well as in the brain and endocrine tissues. While its exact role in PV-FSIs is unclear, it is thought to regulate Ca2+ homeostasis to aid in their fast-spiking ability. In contrast to MSNs, PV-FSIs express high levels of GluA2-lacking CP-AMPARs, contributing to upwards of 60% of the EPSC (Manz et al., 2020). CP-AMPARs exhibit faster EPSC kinetics, greater conductance, and inward rectification compared to calcium impermeable AMPARs (CI-AMPARs), contributing to the heightened sensitivity of PV-FSIs to

changes in glutamatergic input (Liu & Cull-Candy, 2000; Manz et al., 2020; Nissen et al., 2010). Recently, the basal expression of CP-AMPARs has been shown to contribute to forms of endocannabinoid synaptic plasticity through their role in Ca2+ signaling in PV-FSIs (Manz et al., 2020). While PV-FSIs are often largely referred to as a uniform population, not all PV-FSIs are homogonous. For example, a subset of PV-FSIs have been found to exclusively express cannabinoid receptor 1 (CB1) (Winters et al., 2012). It is possible that CB1 expressing FSIs represent functionally distinct PV-FSIs within the NAc. Interestingly, there is a population of FSIs that express calretinin in more caudal regions of the NAc as opposed to the more rostral caudal gradient seen with PV-FSIs (Tepper et al., 2010). It is possible that calretinin interneurons function similarly to PV-FSIs, however little characterization of calretinin expressing interneurons in the NAc has been performed and represents an important gap in our understanding of NAc microcircuits.

*PV-FSIs in behavior*

The behavioral role of FFI within the NAc is still up for debate. The prevailing functional hypotheses is that PV-FSI activity orchestrates the recruitment of functionally distinct MSN ensembles to allow for the appropriate behavioral outcome by silencing competing ensembles (Schall et al., 2021). Activation of glutamatergic afferents from the VTA to NAc PV-FSIs with *in-vivo* optogenetics promotes conditioned place aversion (Qi et al., 2016). While informative, it is more likely that PV-FSIs are dynamically engaged by multiple circuit elements during complex behaviors, as evidenced by the asynchronous activity of dorsal striatal PV-FSIs during a reward seeking task (Berke, 2008). In contrast to Qi et al., 2016, selective potentiation of BLA-to-PV-FSI transmission expedited the acquisition of cocaine self-administration (Yu et al., 2017), indicating

that PV-FSIs may support associative reward learning. In agreement with this hypothesis, targeted silencing of NAc PV-FSIs with a cre-inducible tetanus toxin light chain strategy inhibited the expression of locomotor sensitization following repeated injections of amphetamine and blocked amphetamine-induced CPP (Wang et al., 2018). Similarly to PV-FSIs in the PFC, PV-FSIs in the NAc have been implicated in impulsivity. Optogenetic and chemogenetic inhibition of NAc core PV-FSIs increased the rate of premature responding during a 5-choice serial reaction time task (Pisansky et al., 2019). Given the variety of behaviors NAc PV-FSIs have been implicated in, it is likely that they function to support a wide array of NAc dependent motivated behaviors. It is possible that separate populations of PV-FSIs govern functionally distinct behaviors.

*Neuromodulation of feedforward inhibition*

For many years it was assumed that changes in excitatory inputs onto PV-FSIs would not occur as they do onto MSNs. It is easy to picture how the lack of compartmentalization in the aspiny dendrites of PV-FSIs could be prohibitive to complex neuromodulation compared to the spiny synapses of MSNs. Indeed, in the cortex PV-FSIs appear to simply integrate inputs from the local network without specificity, leaving complex computational elements to pyramidal neurons (Atallah et al., 2012; Cossell et al., 2015; Scholl et al., 2015; Trachtenberg, 2015). However, several recent studies have highlighted major differences between cortical and ventral striatal PV-FSIs. One key finding was the difference in PV-FSI-to-primary output neuron connectivity between the cortex and NAc. In the cortex, PV-FSIs connect with almost every primary neuron within 200 µm of their somas (Packer & Yuste, 2011). This connectivity is better suited for blanket inhibition of the local neuronal population. In the NAc, multiple studies have shown that connectivity between PV-FSIs and MSNs is between 50-60% (Wright et al., 2017; Yu et al., 2017).

This positions NAc PV-FSIs to selectively inhibit specific neuronal populations rather than blanket inhibition of all nearby cells as in the cortex. Based on connectivity alone, we can see that NAc PV-FSIs more dynamically influence circuit activity compared to cortical PV-FSIs.

An exciting study out of the lab of Yan Dong was the first to show experience dependent plasticity of excitatory inputs onto PV-FSIs (Yu et al., 2017). They found that cocaine self-administration enhanced presynaptic release probability from the BLA to NAcsh PV-FSIs and that selective potentiation of this feedforward circuit promoted cocaine self-administration. They also found that the BLA more strongly innervates NAcsh PV-FSIs than MSNs, suggesting that some afferents may preferentially target NAc FFI to influence signal transduction. These findings highlight the importance of the FFI circuit in mediating NAc dependent behaviors, as well as the maladaptive neuromodulatory adaptations that can selectively occur within the circuit to alter behavioral outcomes. The Grueter lab has pioneered the study of synaptic plasticity mechanisms in NAc FFI circuits by providing evidence of modulation by endocannabinoid, adrenergic, and GABA(B) signaling (Manz, Coleman, Grueter, et al., 2021; Manz, Coleman, Jameson, et al., 2021; Manz et al., 2019, 2020). Clearly, excitatory drive onto NAc PV-FSIs undergoes extensive neuromodulation through a myriad of molecular mechanisms as well as experience dependent modulation. While we have learned much regarding FFI neuromodulatory mechanisms, numerous receptor systems have yet to be explored. Most importantly, the behaviors that recruit these signaling mechanisms remain completely unknown. This dissertation investigates the signaling mechanisms by which KORs regulate NAcc FFI microcircuits and the behavioral states that recruit these signaling mechanisms.

## 1.5. Role of KORs in the nucleus accumbens

Early studies investigating the function of KORs in the NAc examined their contribution to negative affective states and aversion by locally infusing KOR agonists and antagonists. The Shippenberg group mapped multiple nuclei as being important for KOR mediated aversion, including the NAc by injecting U-50488 into the NAc during a conditioned place preference assay (Bals-Kubik et al., 1993). Directly infusing nor-BNI into the NAc also blocks the aversive effects of systemic KOR agonists, highlighting the NAc as an integral locus for KOR mediated aversion (Land et al., 2009). This study also implicated KOR signaling in serotonergic projections from the dorsal raphe to the NAc as being required for KOR mediated aversion. Systemic KOR agonists decrease dopamine overflow in the NAc, an effect shown to be due to KOR expression on dopamine terminals from the VTA (Chefer et al., 2013). KOR expression in NAc dopamine terminals was also shown to be required for U-50488 conditioned place aversion. However, it is still unclear if KOR dependent decrease in NAc dopamine is required for aversion. Ehrich et al. found that somatic KOR signaling effects on the excitability of VTA dopamine neurons was responsible for aversion independent of dopamine release in the NAc. Regardless of the mechanism, it is likely the KOR mediated aversion is due to the convergence of its signaling effects in multiple neuromodulatory systems converging on NAc MSN regulation. A major limitation of the current studies is the use of the CPA assay to measure KOR triggered aversion. Aversion can be mediated by disparate mood states, such as anxiety, depression, or dysphoria. Determining whether distinct neuromodulatory systems in the NAc mediate disparate negative affective states is an important future direction.

To further complicate the mechanisms by which KOR signaling in the NAc leads to negative affect, multiple recent studies have characterized a rostral caudal gradient along which KOR signaling elicits opposing behavioral effects. This was first reported by Castro et al. when they found that injecting U-50488 across the NAc led to mixed effects on CPA and sucrose preference (Castro & Berridge, 2014). In the majority of the NAc, U-50488 induced CPA as well as a decrease in sucrose preference. However, in the rostrodorsal NAcsh, U-50488 induced a place preference as well as doubled the hedonic reaction score in the sucrose preference test. This region has been termed the opioid hedonic hotspot, as MOR and DOR agonists also elicited much stronger effects on preference and "liking" when injected into this region compared to elsewhere in the NAc. The following year, Al-Hasani et al. provided the first evidence for endogenous dynorphin release in these distinct subregions similarly regulating aversion and reward (Al-Hasani et al., 2015). By optogenetically stimulating dynorphin release from dynorphin-expressing cells, they found dorsal dynorphin release to be rewarding in multiple assays including operant self-stimulation while ventral dynorphin release was aversive. This was dependent upon KOR signaling as locally infused nor-BNI blocked these effects. While the mechanisms that allow for KOR signaling to elicit opposing effects on reward behavior are unknown, both Al-Hasani and Castro surmise that it could be due to anatomical difference between these subregions, such as afferent projections. These differences may partially be explained by the regional dynamics of KOR regulation of dopamine release. KOR inhibition of dopamine release has been shown to be greater in the caudal versus rostral NAcsh (Pirino et al., 2020). The same study found that KOR activation along this gradient differentially regulated anxiety-like or approach-avoidance behaviors similarly to Al-Hasani and Castro. However, these findings are correlative and show no direct link between dopamine dynamics and behavioral outcomes.

There is evidence that KORs differentially regulate circuit elements within the NAc, providing support for the hypothesis that KOR regulation of distinct anatomical circuits underlies KORs opposing roles in behavior. Hjelmstad and Fields were the first to examine the effect of KOR signaling on noncatecholaminergic transmission, finding that KOR activation decreases both GABA and glutamate release in the NAcsh (Hjelmstad & Fields, 2001, 2003). Interestingly, Hjelmstad and Fields found that KORs use different signaling mechanisms to regulate GABA and glutamate release, with GABA inhibition requiring reductions in N-type $Ca^{2+}$ channel conduction and inhibition of glutamate occurring downstream of $Ca^{2+}$ entry. Another mechanism by which KORs influence NAc circuit dynamics is through heterosynaptic suppression of excitatory inputs. Burst firing from PFC inputs strongly attenuates hippocampal evoked excitatory post-synaptic potentials, partially through the recruitment of KORs (Brooks & O'Donnell, 2017). This would effectively shift NAc circuit elements away from hippocampal driven signal transduction. KOR signaling also differentially modulates excitatory inputs directly. In mice, U69593 selectively inhibits BLA afferents onto D1+ MSNs and not hippocampal afferents, while also more strongly inhibiting lateral inhibition onto D2+ MSNs, biasing circuit activity towards D2+ MSN activity (Tejeda et al., 2017). This example of circuit biasing following KOR activation provides an excellent framework for how KOR signaling could differentially influence negative affective behaviors via anatomical circuit elements. In this model, release of dynorphin would decrease BLA – D1+ MSN transduction while leaving BLA – D2+ MSN signaling intact. Dynorphin mediated lateral disinhibition of D2+ MSNs would further bias signaling through BLA – D2+ MSNs circuits. As D2+ MSN activation in the NAc is broadly considered to be aversive, we could extrapolate that dynorphin release may bias BLA signal transduction in the NAc toward aversive

promoting pathways. While KOR regulation of glutamate and GABA signaling in the NAc represents an important mechanism by which KOR signaling may influence behavior independent of catecholamine regulation, studies examining the direct impact on behavior are lacking.

Having reviewed the behavioral and electrophysiological impact of pharmacological recruitment of the dynorphin/KOR system in the NAc, I will now cover the behavioral states that have been shown to recruit this system. Many forms of stress have been shown to recruit dynorphin and KOR signaling in the NAc. Patients suffering from chronic pain often experience altered emotional states such as increased anxiety and stress leading to an increased rate of opioid overdoses as a result (Volkow & McLellan, 2016). A recent study found that KORs exhibit increased functionality in the NAc following an inflammatory pain model (CFA) and that dynorphin expressing neurons are recruited through a disinhibition mechanism to mediate pain induced negative affective states (Massaly et al., 2019). Early life stress also increases KOR functionality in the NAc, a mechanism that has been hypothesized to predispose individuals to higher ethanol intake, possibly due to a KOR mediated hypodopaminergic state in the NAc (Karkhanis et al., 2016). Physical stressors such as restraint stress and forced swim test have been shown to recruit the dynorphin/KOR system (Pliakas et al., 2001; Shirayama et al., 2004a). In the case of the forced swim test, Pliakas et al. found that CREB function is increased following forced swim test and provide evidence for CREB mediated induction of dynorphin. CREB activity is increased in the NAc following acute cocaine, morphine, or amphetamine (Konradi et al., 1994; Terwilliger et al., 1991). Viral overexpression of CREB decreases the rewarding properties of cocaine, perhaps by enhancing the anxiogenic effects of cocaine through the upregulation of dynorphin (Carlezon et al., 1998). CREB mediated induction of dynorphin represents a possible molecular mechanism by

which drugs of abuse recruit and alter the dynorphin/KOR system, leading to long lasting negative affective states that promote relapse.

Multiple drugs of abuse recruit and alter the dynorphin/KOR system. Studies reporting the effects of cocaine on KOR function and expression are mixed, ranging from no effect (Schroeder et al., 2003), upregulation (Collins et al., 2002; Unterwald, 2001), or down regulation (Rosin et al., 1999). It is important to note that KOR expression is increased in patients who suffered from cocaine use disorder, though the direct relation to cocaine use is unclear (Hurd & Herkenham, 1993). Increases in NAc dynorphin following cocaine exposure, however, have consistently been reported (Hurd et al., 1992; Spangler et al., 1993). It is possible that prolonged enhancement of dynorphin release could lead to desensitization of KORs, and that the mixed effects of cocaine on KOR expression are due to different cocaine administration paradigms. Prior exposure to cocaine completely blocks dynorphin A induced depression of EPSCs onto NAc MSNs but not dynorphin B (Mu et al., 2011). Dynorphin A and B have both been shown to decrease excitatory transmission onto NAc MSNs, though through seemingly different mechanisms. Dynorphin A induced depression is reversible by nor-BNI, but dynorphin B induced depression is only partially reversed. This study did not block the effect of dynorphin B with prior application of the KOR antagonist, so it is unclear whether the effects of dynorphin B are due to different KOR signaling arms, or if the effect is from activity at non-KORs. Regardless, the disruption of Dynorphin A dependent depression following cocaine provides a possible molecular substrate for KOR dependent negative affective states following drug exposure.

The role of the dynorphin/KOR system in mediating morphine related negative affective states is less understood. 48 hours after non-contingent morphine administration, dynorphin is decreased in the NAc (Yukhananov et al., 1993). However, Prodynorphin mRNA is increased in the NAc following 8 days of morphine withdrawal, suggesting dynorphin signaling may underlie negative affective states that develop following prolonged withdrawal from morphine (Nylander et al., 1995). Indeed, following protracted morphine withdrawal (4 weeks) dynorphin levels are elevated in the NAc and depressive-like behaviors can be blocked by infusing nor-BNI into the NAc (Zan et al., 2015). Though more studies are necessary, initial evidence points towards the NAc dynorphin/KOR system as a key therapeutic target for morphine withdrawal induced negative affective states.

KOR dynamics are also altered following ethanol consumption. KOR dependent inhibition of dopamine release in the NAcc following ethanol self-administration is increased and correlated to the total amount of ethanol consumed in non-human primates (Siciliano et al., 2015). This shows that increased alcohol intake is correlated to increased KOR activity and suggests KOR antagonists may promote alcohol abstinence. Results from a microdialysis study found that dynorphin A is dose dependently increased in the NAc 30 minutes following ethanol exposure and prodynorphin mRNA levels are increased during 24 and 48 hour ethanol withdrawal (Marinelli et al., 2006; Przewłocka et al., 1997). This highlights a potential difference in the timing of dynorphin/KOR system recruitment between and within drug classes. While studies examining dynorphin release throughout the addiction cycle and are limited, an intriguing possibility is that various prodynorphin cleavage products are released during different stages of drug consumption and mediate negative affective states throughout the addiction cycle. This idea is congruent with the

opponent-process theory of motivation that states that in order to maintain homeostasis, an increase in hedonic state will be followed by a compensatory decrease in hedonic state (Solomon & Corbit, 1974). Under this model, the dynorphin/KOR system is recruited as an "anti-reward" mechanism to balance the euphoric properties of drugs. However, when taken repeatedly, these drugs can alter the KOR/system leading to maladaptive changes in negative affective states that may ultimately reinforce drug consumption. In support of this, intra-NAcsh infusion of nor-BNI attenuates ethanol self-administration in ethanol dependent animals, but not in nondependent animals (Nealey et al., 2011).

It is clear that the dynorphin/KOR system plays an important role in regulating NAc circuit activity, and that drugs of abuse recruit and adapt this system to support negative emotional states. A notable circuit element that has not been explored for KOR regulation of NAc signal transduction is FFI. NAc PV-FSIs have been implicated in modulating anxiety (Xiao et al., 2020). In the shell, PV-FSIs exhibit increased excitability following chronic stress, and chemogenetic inhibition of PV-FSIs can decrease anxiety-like behaviors while optogenetically driving their activity is anxiogenic in mice. As covered in the *PV-FSIs in behavior* section, NAcc PV-FSIs also gate impulsive decision making. While these data implicate PV-FSIs as a possible player in withdrawal behavior, this has not yet been explored. Bringing these lines of evidence together, KOR regulation of PV-FSI FFI represents an excellent candidate for drug adaptations underlying stress induced relapse. Understanding how KORs regulate FFI circuits in the NAc is a key first step in exploring this possibility.

## 1.6. Aims

The overall goal of this dissertation is to understand how KORs regulate FFI in the NAcc and the behaviors that this regulation is important for. Signal transduction through the NAc is much more complex than signal in – signal out. Dynamic synaptic and circuit elements are at play that allow for the correct integration of multiple excitatory, inhibitory, and modulatory signals. We know that changes in these elements underlie many facets of addiction and other motivated behaviors. However, we still don't understand how various inputs are integrated into coordinated efferents. We can think of the process of signal transduction through the NAc like our road systems. You are in your car sitting at a major highway intersection. The road you just travelled on is the afferent projection into the NAc, the intersection is the NAc, and the roads leading away from the intersection are the efferents leaving the NAc. You have the choice to go straight, left, or right and there are signs and signals guiding you. FFI might look like the turning of a stoplight red. FFI may turn one, multiple, or even all directions of travel red, guiding you towards your direction of travel or stopping it. We know FFI circuits undergo state dependent neuromodulation that can bias FFI. Therefore, certain factors will dictate whether the stoplight you find yourself will be red, green, or a turn signal. For us those factors are receptor signaling. When the dynorphin/KOR system is recruited the stoplights may change and guide you in a specific direction. Depending on which road you find yourself leaving the intersection, you will find yourself in a distinct location specific to that road. These distinct locations represent different behavioral outcomes. The question I am asking is, "How does recruitment of KORs change the stoplights (FFI), and what direction of travel does this guide me (behavioral responses)?"

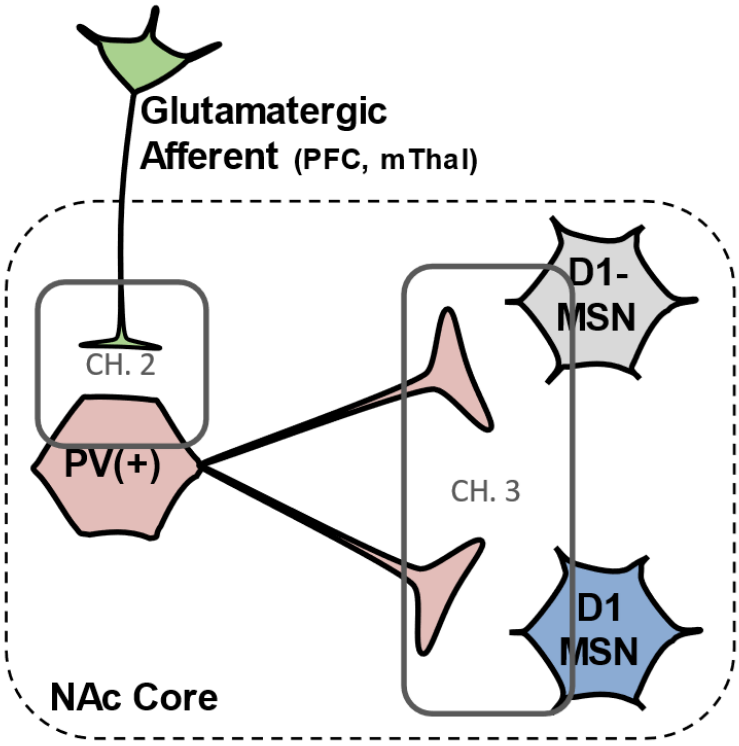


**Figure 1.4. Synaptic focus within the feedforward inhibitory circuit of Chapters 2 and 3.** Ch. 2 of this dissertation will focus on excitatory drive onto NAc core PV-FSIs. It will examine PFC and mThal afferent regulation by KORs, provide a detailed mechanism by which KORs decrease excitatory drive at this synapse, and present data for the involvement of this mechanism in the response to stress. Ch. 3 will examine the effect KOR signaling has on FFI onto D1+ and D1- MSNs. Again, PFC and mThal driven feedforward inhibitory microcircuits will be tested.

In this dissertation I dissect how KORs regulate and bias NAcc FFI. This body of research can be broken into two aims: First, to determine how the recruitment of KORs modulates NAcc FFI, and second, what are the behaviors in which this mechanism is recruited. Chapter 2 describes a mechanism by which KORs decrease excitatory drive onto FFI circuits (Figure 1.4). It also provides evidence for the recruitment of this mechanism following restraint stress. Chapter 3 describes a biasing of FFI following KOR activation and provides preliminary evidence for the role of KORs on PV-FSIs in stress (Figure 1.4). A key future direction of this work that is addressed in Chapter 4 is to determine if and how KOR modulation of FFI is altered in pathological states such as addiction.

# CHAPTER 2

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## Kappa opioid receptor modulation of excitatory drive onto nucleus accumbens fast-spiking interneurons

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## 2.1. Abstract

The dynorphin/kappa opioid receptor (KOR) system within the nucleus accumbens (NAc) contributes to affective states. Parvalbumin fast-spiking interneurons (PV-FSIs), a key component of FFI, participate in integration of excitatory inputs to the NAc by robustly inhibiting select populations of medium spiny output neurons, therefore greatly influencing and NAc dependent behavior. How the dynorphin/KOR system regulates FFI in the NAc remains unknown. Here, we elucidate the molecular mechanisms of KOR inhibition of NAc PV-FSI excitatory transmission using a combination of whole-cell patch-clamp electrophysiology, optogenetics, pharmacology, and a parvalbumin reporter mouse. We find that postsynaptic KOR stimulation induces long-term depression (LTD) of PV-FSI excitatory synapses by stimulating the endocytosis of AMPARs via a PKA and calcineurin-dependent mechanism. Furthermore, KOR regulation of PV-FSI synapses are input specific, inhibiting thalamic but not cortical inputs. Finally, following acute stress, a protocol known to elevate dynorphin/KOR signaling in the NAc, KOR agonists no longer inhibit PV-FSI excitatory transmission. In conclusion, we delineate pathway-specific mechanisms mediating KOR control of feedforward inhibitory circuits in the NAc and provide evidence of the recruitment of this system in response to stress.

## 2.2. Introduction

The nucleus accumbens (NAc) is a key node within the mesolimbic dopamine system, integrating glutamatergic input from limbic, cortical, and thalamic brain regions to drive motivated behavior (Grueter et al., 2012; Joffe et al., 2014). Experience dependent plasticity of these inputs has been shown to underlie maladaptive motivational states (Grueter et al., 2012; Pascoli et al., 2012, 2014; Zhu et al., 2016; Creed & Lüscher, 2013; Turner, Kashima, et al., 2018). NAc-dependent motivational output is organized by networks of parvalbumin-expressing fast-spiking interneurons (PV-FSIs) that gate medium spiny neuron (MSN) output via FFI (Manz, Coleman, Grueter, et al., 2021; O'Donnell & Grace, 1995; Scudder et al., 2018; Trouche et al., 2019; Wright et al., 2017; Yu et al., 2017). For example, inhibition of NAc PV-FSIs increases impulsivity and decreases psychostimulant induced associative learning (Pisansky et al., 2019; Wang et al., 2018). Studies from our lab and others indicate that feedforward glutamatergic synapses onto PV-FSIs exhibit multiple molecularly distinct modes of plasticity, each proceeding via G-protein-coupled receptor (GPCR)-dependent mechanisms (Manz, Coleman, Grueter, et al., 2021; Manz et al., 2019, 2020; Scudder et al., 2018). However, it is unknown whether endogenous opioid signaling, a neuromodulatory system critically involved in adaptive and pathological reward behavior, modulates PV-FSI-embedded feedforward microcircuits.

The dynorphin/kappa opioid receptor (KOR) system is heavily implicated in stress, stress induced reinstatement of drug seeking, as well as negative affective states during withdrawal (Knoll & Carlezon, 2010; Tejeda & Bonci, 2019; Valenza et al., 2017; Van't Veer & Carlezon, 2013). Importantly, recruitment of the dynorphin/KOR system in the NAc is aversive (Al-Hasani et al.,

2015; Bals-Kubik et al., 1993; Massaly et al., 2019), and dynorphin is increased in the NAc of individuals with cocaine use disorder and suicidal ideologies, as well as in rodent models of addiction and depression. (Carlezon et al., 1998; Hurd et al., 1997; Hurd & Herkenham, 1993; Pliakas et al., 2001). KORs are Gαi-coupled GPCRs that dynamically and heterosynaptically regulate glutamate release in the NAc, highlighting the importance of KORs as key mediators of NAc signal transduction (Brooks & O'Donnell, 2017; Hjelmstad & Fields, 2003; Svingos et al., 1999, 2001; Tejeda et al., 2017). How KORs modulate PV-FSI-embedded feedforward microcircuits, and PV-FSI relevant behaviors, represent an important gap in understanding how this receptor influences NAc circuit activity. We examined PFC and mThal inputs into the NAc as they regulate opposing behaviors. Stimulation of PFC – NAc afferents promotes real-time place preference, and stimulation of mThal – NAc afferents promotes real-time place aversion (Britt et al., 2012; Zhu et al., 2016).

We used whole-cell patch-clamp electrophysiology, transgenic mice, and behavioral manipulations paired with pharmacology to probe the dynorphin/KOR system at excitatory inputs onto NAc core (NAcc) PV-FSIs. We report that postsynaptic KORs diminish feedforward drive onto NAcc PV-FSIs via AMPAR endocytosis through a PKA/calcineurin-dependent mechanism. Interestingly, we also find that mThal afferents onto PV-FSIs are preferentially regulated by KORs over PFC afferents. To test for *in vivo* recruitment of the KOR system we used immobilization stress. Acute immobilization stress prevented KOR-induced depression of excitatory transmission onto NAcc PV-FSIs. Importantly, KOR-dependent long-term depression (LTD) was recovered by pre-exposure to the KOR antagonist nor-BNI, suggesting the involvement of this mechanism in response to acute stress. Together, these findings provide novel insight into mechanisms by which

the endogenous dynorphin/KOR system regulates feedforward drive in the NAcc and implicates KORs on NAcc PV-FSIs in acute stress.

## 2.3. Materials and Methods

### *Animals*

Mice were housed in the Vanderbilt University Medical Center animal care facility in accordance with Institutional Animal Care and Use Committee guidelines. Mice were group housed 2-5/cage with *ad lib* access to food and water and kept on a 12-hr light-dark cycle. Male and female mice ages 7-12 weeks were used for all experiments. No differences were found between sexes, so data was combined. To allow for fluorescent visualization of PV-FSIs in electrophysiology experiments, Cre-induced STOP$^{fl/fl}$-tdTomato mice (Ai9, *Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$) (Jackson Laboratory Stock No.: 007909) were crossed with parvalbumin (PV) PV-IRES-Cre mice (PV$^{Cre}$, *Pvalb*$^{tm1(cre)Arbr/J}$, Stock No.: 008069), generating PV$^{Cre}$-tdTomato$^{fl/fl}$ (PV$^{tdT}$) mice.

### *Electrophysiology*

For detailed electrophysiological methods see (Manz, Siemann, McMahon, et al., 2021). In brief, PV$^{tdT}$ mice were killed under isoflurane anesthesia, brain dissected, and sagittal brain slices 250μm thick prepared using a Leica VT1200S Vibratome. Slices were prepared in oxygenated (95% O2; 5%CO2) ice-cold *N*-methyl-*D*-glucamine (NMDG)-based solution (in mM: 2.5 KCl, 20 HEPES, 1.2 $NaH_2PO_4$, 25 Glucose, 93 NMDG, 30 $NaHCO_3$, 5.0 sodium ascorbate, 3.0 sodium pyruvate, 10 $MgCl_2$, and 0.5 $CaCl_2$-$2H_2O$) and recovered for 10 minutes in the same solution at 34°C. Slices were then recovered for 1 hour in oxygenated artificial cerebrospinal fluid (ACSF) containing (in

mM: 119 NaCl, 2.5 KCl, 1.3 MgCl2-6H2O, 2.5 CaCl2-2H2O, 1.0 NaH2PO4-H2O, 26.2 NaHCO3, and 11 glucose; 287-295 mOsm). During experiments, slices were continuously perfused with oxygenated ACSF containing 50 μM picrotoxin ($GABA_AR$ antagonist to isolate excitatory postsynaptic currents; EPSCs) at a rate of 2 mL/min and a temperature of 32 ± 2°C. To isolate miniature (m) EPSCs, tetrodotoxin (1 μM) was added to the ACSF. $PV^{tdT}$ cells in the NAcc were visualized using Scientifica PatchVision software via 530 nm LED light. PV-FSIs were confirmed according to biophysical properties (capacitance, membrane resistance, and AMPAR decay kinetics) (Manz, Coleman, Grueter, et al., 2021; Manz et al., 2020).

Whole-cell patch-clamp electrophysiology was performed using a CV-7B headstage, Multiclamp 700B Amplifier, Axopatch Digidata 1550 digitizer, and 3-6 MΩ glass recording micropipettes (Sutter P1000 Micropipette Puller). Voltage clamp recordings were done at -70 mV using a $K^+$-based intracellular solution (in mM: 135 $K^+$-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 3 Na2ATP, 0.4 Na2GTP; 285-292 mOsm). For experiments using GDPβS trilithium salt, Na2GTP was excluded from the internal solution and 1 mM GDPβS added. BAPTA (10 mM), D15 (2 mM), S15 (2 mM) were added to the internal solution as indicated. In these subsets of experiments, cells were dialyzed for 30 minutes prior to the beginning of the recording.

A bipolar electrode was placed at the cortico-accumbens interface and stimulated at 0.1Hz to examine local neurotransmission. Paired-pulse ratio (PPR) was acquired within experiment by giving two 0.15 ms pulses with a 50 ms interstimulus interval and dividing the amplitude of the second EPSC by the first. For experiments examining mThal or PFC synapses onto NAcc PV-FSIs, 473-nm Cool LED stimulation was delivered at 0.1 Hz with a 0.3-ms pulse duration to slices

prepared from mice expressing ChR2 on terminals within the NAcc. Cells with a >15% change in series resistance (Rs) we excluded from analysis.

*Immobilization Stress*

For immobilization stress experiments, mice were constrained in a cylindrical tube for 1 hour, allowed to recover for 10 minutes, and then sacrificed for electrophysiology. KOR antagonist, nor-BNI (10 mg/kg), or saline was administered by intraperitoneal (IP) injection 24 hours prior to immobilization stress.

*Stereotaxic Injections*

To probe input specificity, mice underwent stereotaxic surgery for viral-mediated gene transfer of channel rhodopsin (ChR2). At ~4 weeks of age mice were given bilateral injections (400nL) of AAV5-CaMKII-ChR2-EYFP (Addgene, Watertown, MA) and killed 3-5 weeks after surgery for electrophysiological experiments. Coordinates were (relative to bregma): mThal (medial-lateral (ML): ±0.3, anterior-posterior (AP): −1.2, dorsal-ventral (DV): −3.0) or PFC (ML: ±0.3, AP: −1.75, DV: −2.75).

*Pharmacology*

1-Naphthyl acetyl spermine (NASPM) trihydrochloride, isoflurane, tetrodotoxin, (-)-U50488 hydrochloride, and nor-binaltorphimine dihydrochloride, were obtained from Tocris/Bio-Techne, Minneapolis, MN. Picrotoxin, BaCl2, and GDPβS trilithium salt were obtained from Sigma-Aldrich, St. Louis, MO. D15 (PPPQVPSRPNRAPPG) and S15 (ANVRRGPPPPPQPSP) were synthesized by Bio-Synthesis Inc. Lewisville, TX.

*Statistics*

Clampfit 10.4 and GraphPad Prism v7.0 were used to analyze all experiments. Changes in EPSC amplitude, EPSC frequency, coefficient of variance (CV), and PPR were calculated by comparing the mean values during the first 5 or 10 minutes (defined for each experiment) to the mean values in during the final 5 or 10 minutes of each experiment. Each data point represents the average of one cell, n = number of cells N = number of mice. Paired or unpaired *t*-tests were used. Errors bars represent SEM. For all analyses, $p < 0.05$ was considered significant.

## 2.5. Results

*The dynorphin/KOR system triggers LTD of excitatory drive onto NAcc PV-FSIs*

KORs depress excitatory synapses onto NAc MSNs by inhibiting glutamate release, however, their function at PV-FSIs is unknown (Hjelmstad & Fields, 2001, 2003; Mu et al., 2011). To determine the contribution of KORs to excitatory drive onto NAcc PV-FSIs, we tested KOR pharmacology on electrically evoked EPSCs (eEPSC). Application of the endogenous KOR agonist, dynorphin A, elicited a depression of eEPSCs (Fig. 2.1B, D, F; Dyn. A: 75.0 ± 5.8%, n=7, p=0.005, paired t-test). To gain KOR specificity, as dynorphin A exhibits activity at the mu and delta opioid receptors, we used KOR selective agonist (-)-U-50488. Consistent with dynorphin A, U-50488 triggered a decrease in eEPSC amplitude that persisted in the presence of KOR antagonist, nor-binaltorphimine (nor-BNI), indicating LTD (Fig. 2.1C, E, F U-50488: 71.4 ± 5.4%, n=7, p=0.002, paired t-test). To further ensure the specificity of the observed effect to KORs, we washed on the agonist U-50488 in the presence of nor-BNI and observed no depression (Fig. 2.1G; U-50488 in

nor-BNI: 95.7 ± 4.0%, n=8, p=0.31, paired t-test). Furthermore, KORs lack tonic regulation of excitatory drive onto PV-FSIs exemplified by no change in the eEPSC amplitude following the application of nor-BNI (Fig. 2.1H 105.5 ± 5.2%, n=8, p=0.327, paired t-test). These data demonstrate LTD of excitatory drive onto NAcc PV-FSIs by KORs.

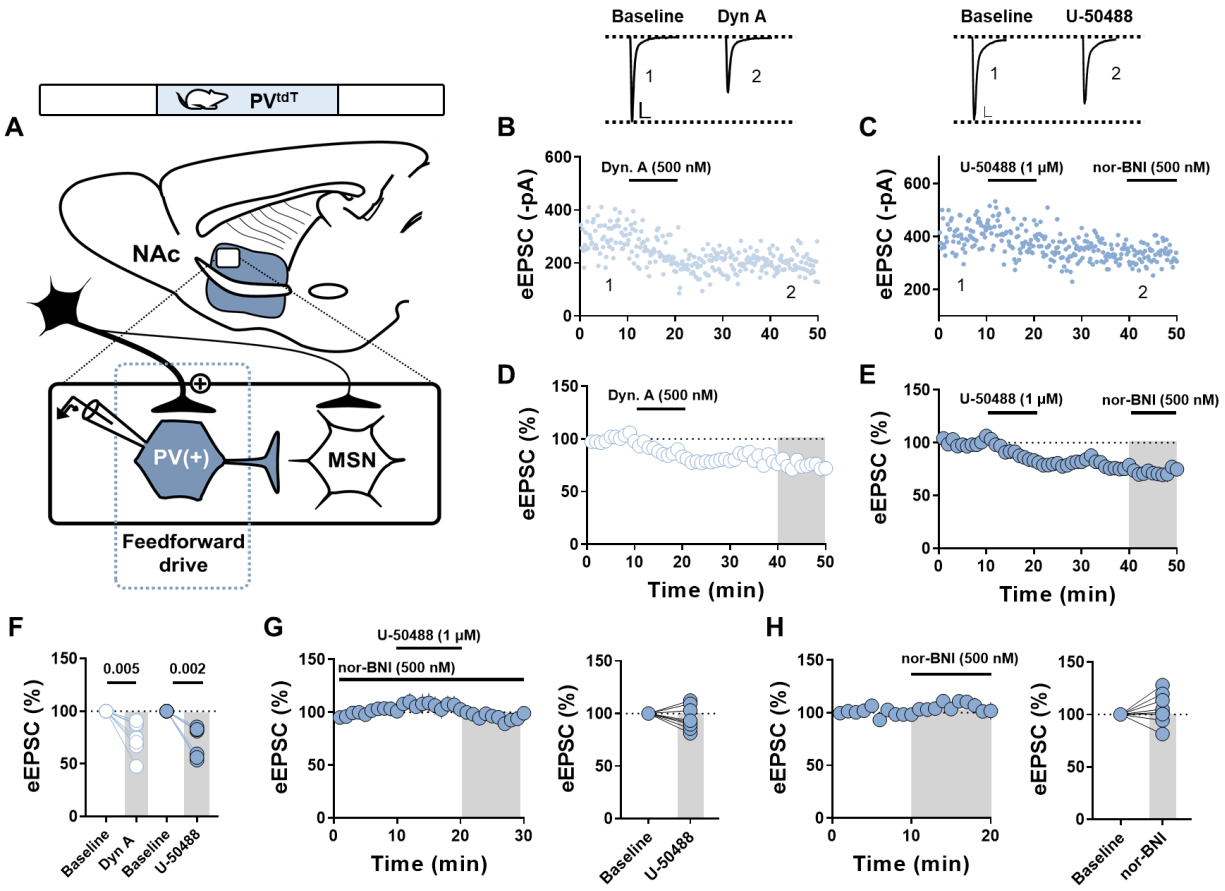
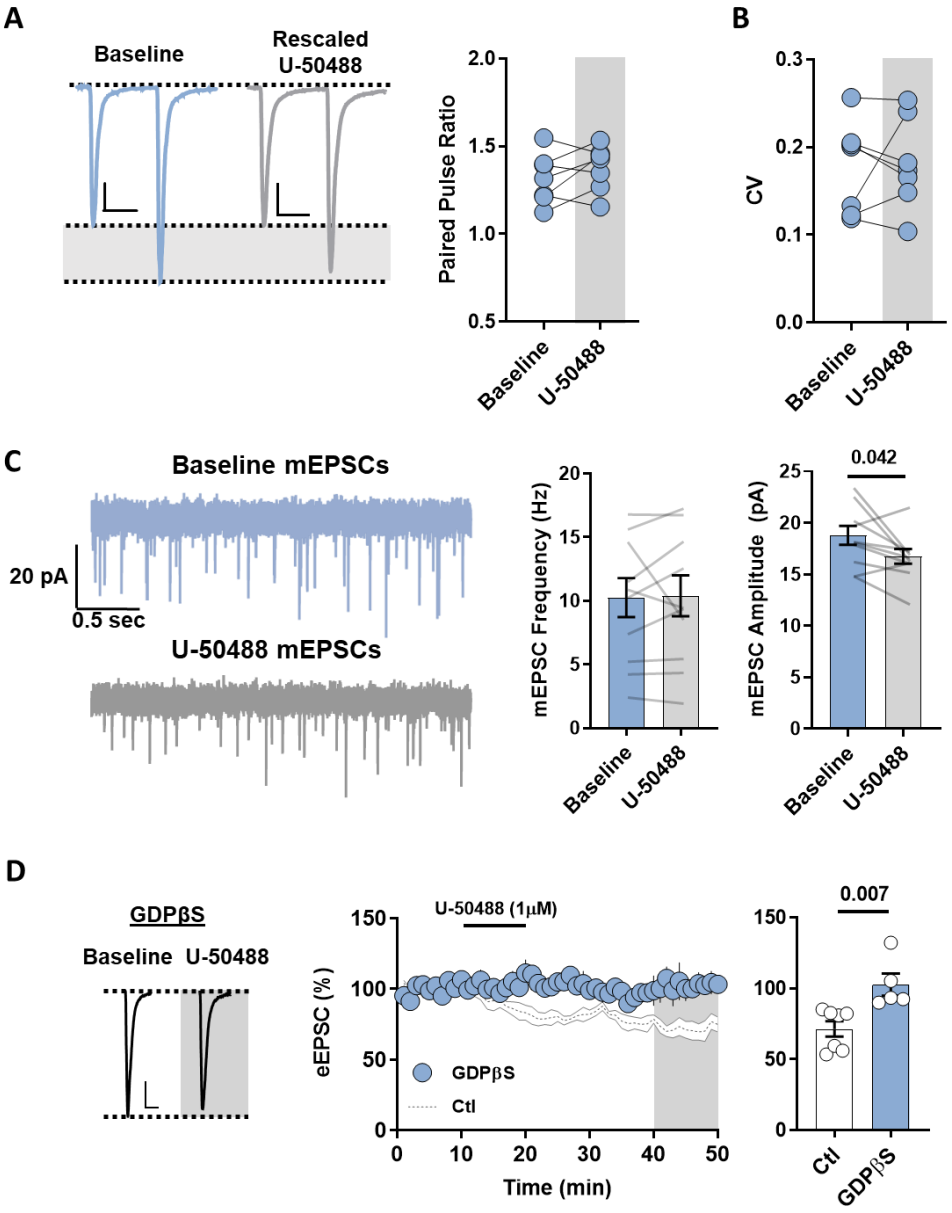



**Figure 2.1. Kappa opioid receptor stimulation triggers a long-term depression of excitatory transmission onto NAc core PV-FSIs.** **(A)** Sagittal mouse brain slice representing location of electrophysiological recordings in the dorsomedial NAc core. (**B)** Representative experiment of eEPSC amplitudes before, during, and post Dynorphin A (500 nM) drug wash from NAc core PV-FSIs, and representative current traces from the first and last ten minutes of the experiment. **(C)** Representative experiment of eEPSC amplitudes before, during, and post U-50488 (1 µM) drug wash and nor-BNI antagonist (500 nM) chase from NAc core PV-FSIs, and representative current traces from the first and last ten minutes of the experiment. **(D)** Time-course summary of normalized eEPSCs in PV-FSIs depicting a Dyn. A triggered depression that persists following drug washout. **(E)** Time-course summary of normalized eEPSCs in PV-FSIs depicting U-50488 triggered long-term depression. **(F)** Average eEPSC amplitudes taken from baseline and 40-50 min for Dyn. A and U-50488 drug washes (Dyn. A: 75.0 ± 5.8%, n=7; U-50488: 71.4 ± 5.4%, n=7). **(G)** Time-course summary of U-50488 (1 µM) washes in the presence of nor-BNI (500 nM) with average eEPSC amplitudes taken from baseline and 20-30 min (95.7 ± 4.0%, n=8, p=0.31). **(H)** Time-course summary of nor-BNI (500 nM) washes with average eEPSC amplitudes taken from baseline and 10-20min (105.5 ± 5.2%, n=8, p=0.327). Error bars indicate SEM. Baseline = 0-10 min. Scale bars = 50 pA/10 ms. Grey boxes indicate timepoint used to compare to baseline.

*KOR triggered LTD is expressed post-synaptically*

To determine whether KOR-induced LTD is expressed pre- or post-synaptically, we examined paired pulse ratio (PPR) and coefficient of variance (CV), metrics which are inversely correlated with release probability. Consistent with a postsynaptic locus of action, we observed no significant changes in either PPR (50 msec) or CV (Fig. 2.2A; PPR baseline: 1.32 ± 0.05; PPR 40-50min: 1.38 ± 0.05, n=7, p=0.25, paired t-test) (Fig 2.2B; CV baseline: 0.18 ± 0.02; CV 40-50min: 0.18 ± 0.02, n=7, p=0.84, paired t-test). However, presynaptic KOR mediated depression of EPSCs have been reported with no observable change in PPR (Tejeda et al., 2017). Therefore, we incorporated TTX-insensitive mEPSC analyses in response to pharmacological activation of KORs. mEPSCs were analyzed during the first 5 minutes and from minutes 40-45 of U-50488 application experiments. U-50488 elicited no change in mEPSC frequency while significantly decreasing amplitude, consistent with a decrease in post-synaptic response to neurotransmitter release (Fig. 2.2C; frequency 0-5 min: 10.26 ± 1.53 Hz; frequency 40-45 min: 10.40 ± 1.61 Hz, n=10, p= 0.87, paired t-test; amplitude 0-5 min: 18.78 ± 0.91 pA; amplitude 40-45 min: 16.75 ± 0.72 pA, n=10, p= 0.042, paired t-test). To further validate the postsynaptic action of KOR-mediated LTD of NAcc PV-FSIs EPSCs, we replaced GTP with non-hydrolysable GDPβS in the internal solution to inhibit G-protein signaling. In GDPβS loaded cells, U-50488-induced depression was lost and significantly different from control (containing GTP) (Fig. 2.2D; Ctl: 71.4 ± 5.4%; GDPβS: 102.6 ± 7.8%, n=7 and 5, p=0.007, unpaired t-test). Together, these data are consistent with a postsynaptic, G-protein dependent LTD initiated by KORs.




**<u>Figure 2.2. Kappa opioid receptor mediated long-term depression is expressed post-synaptically</u>. (A)** Representative PPR current traces taken from baseline and 40-50 min (scale bars =50 pA/25 ms). Average PPR taken from baseline and 40-50min (PPR baseline: 1.32 ± 0.05; PPR 40-50 min: 1.38 ± 0.05, n=7, p=0.25). **(B)** Average CV taken from baseline and 40-50 min timepoints (CV baseline: 0.18 ± 0.02; CV 40-50min: 0.18 ± 0.02, n=7, p=0.84). **(C)** Representative mEPSC traces taken from baseline and 40-45 min timepoints. Average mEPSC frequency taken from 0-5 min and 40-45 min timepoints (0-5 min: 10.26 ± 1.53Hz; 40-45 min: 10.4 ± 1.61 Hz, n=10, p= 0.87). Average mEPSC amplitude taken from 0-5 min and 40-45 min timepoints (0-5 min: 18.78 ± 0.91 pA; 40-45min: 16.75 ± 0.72 pA, n=10, p= 0.042). **(D)** Time course summary of U-50488 (1 μM) drug washes in the presence of internally loaded GDPβS or control internal solution, representative current traces from baseline and 40-50min timepoints for GDPβS loaded cells (scale bars = 50 pA/10 ms), and average eEPSC amplitudes taken from 40-50 min for control and GDPβS loaded cells (Ctl: 71.4 ± 5.4%; GDPβS: 102.6 ± 7.8%, n=7 and 5, p=0.007). Grey box represents timepoint used for GDPβS vs Ctl statistics. Error bars indicate SEM. Baseline = 0-10 min.

*KOR activation initiates the endocytosis of PV-FSI AMPARs*

A canonical signaling pathway for opioid receptors is the activation of GIRK channels, which leads to hyperpolarization of the cell and could explain the decrease in PV-FSI eEPSC amplitude mediated by KORs (Al-Hasani & Bruchas, 2011). To test this, we washed on U-50488 in the presence of $Ba^{2+}$ and recorded eEPSCs from NAcc PV-FSIs. U-50488 triggered LTD in the presence of $Ba^{2+}$, suggesting LTD is not mediated by $Ba^{2+}$ sensitive potassium channels, including GIRKs (Fig. 2.3A; U-50488 in $Ba^{2+}$: 75.0 ± 3.5%, n=4, p=0.006, paired t-test). Consistent with blockage of potassium channels, bath-application of $Ba^{2+}$ was accompanied by a decrease in $I_h$ (Fig. 2.3A inset). Given the established regulatory relationship between dopamine and the KOR in the midbrain dopaminergic system (Bruchas et al., 2010; Margolis et al., 2003), we tested if dopamine was required for KOR-induced LTD by applying U-50488 in the presence of D1- and D2-like dopamine receptor antagonists SCH 23390 and sulpiride, respectively. Inhibiting D1- and D2-like dopamine receptors had no effect on U-50488 triggered LTD of PV-FSI eEPSCs (Fig. 2.3B; U-50488 in SCH+Sulp: 68.8 ± 9.2%, n=4, p=0.043, paired t-test). GPCR signaling can recruit protein kinase A (PKA) via Gβγ. We determined that the mechanism was PKA dependent by the loss of LTD following application of U-50488 when the PKA antagonist H89 (10 μM) was included in the bath solution, implicating the Gβγ signaling arm of KORs (Fig. 2.3C; U-50488 in H89: 92.6 ± 3.6%, n=6, p=0.090, paired t-test). The loss of LTD in the presence of H89 was also significantly different from control LTD (Ctl: 71.4 ± 5.4%; H89: 92.6 ± 3.6%, n=7 and 6, p=0.009, unpaired t-test). We also report that H89 alone has no effect on the eEPSC amplitude of NAcc PV-FSIs (Fig. 2.3C inset; H89: 98.3 ± 4.9%, n=4, p=0.75, paired t-test, final 10 minutes compared to first 10 minutes).

To narrow the intracellular signaling mechanisms recruited by KORs to trigger LTD, we determined whether the effect was calcium dependent by incorporating the fast-acting calcium chelator, BAPTA, into the internal solution. Intracellular BAPTA prevented U-50488-LTD (Fig. 2.3D; U-50488 with BAPTA: 88.0 ± 10.1%, n=6, p=0.290, paired t-test). Given the requirement for calcium in U-50488-LTD of PV-FSI AMPARs, we tested whether the $Ca^{2+/}$calmodulin-dependent protein phosphatase calcineurin, a downstream effector of $Ca^{2+}$ that has been shown to initiate clathrin-mediated endocytosis of AMPARs (Chávez et al., 2010; Matsuda et al., 2013; Mulkey et al., 1994; Unoki et al., 2012), is responsible for KOR-LTD. We found that incubating slices in calcineurin inhibitor, cyclosporin A, for 1-2 hours blocked the ability of U-50488 to trigger LTD (Fig. 2.3E; U-50488 following cyclosporin A: 103.3 ± 11.3%, n=7, p=0.78, paired t-test). The loss of LTD following cyclosporin A incubation was also significantly different from control LTD (Ctl: 71.4 ± 5.4%; cyclosporin A: 103.3 ± 11.3%, n=7 and 7, p=0.026, unpaired t-test). Given this result, we wanted to determine if the activation of KORs was ultimately leading to the trafficking of AMPARs on PV-FSIs. To test this, we introduced the peptide inhibitor D15 (PPPQVPSRPNRAPPG), which blocks the interaction between dynamin and AP1/2 and inhibits the endocytosis of AMPARs, or the scrambled peptide S15 (ANVRRGPPPPPQPSP), into the internal patch solution (Grueter et al., 2010; Lüscher et al., 1999). U-50488 had no effect on cells loaded with D15, while it elicited a significant depression in cells loaded with S15 compared to those loaded with D15 (Fig. 2.3F; U-50488 with S15: 77.2 ± 4.8%; D15: 99.3 ± 8.1%, n=5 and 5, p=0.046, unpaired t-test). Previous work from our lab has established that ~50% of EPSC on NAc PV-FSIs is attributed to calcium permeable AMPARs (CP-AMPARs) with the remainder being resistant to the CP-AMPAR blocker NASPM (calcium impermeable AMPARs, CI-AMPARs) (Manz et al., 2020). To determine if KOR-LTD differentially targets the trafficking of CP- or CI-

AMPARs we bath applied NASPM following either time-matched control or dynorphin A triggered LTD. We found no difference in NASPM-induced depression of EPSCs, suggesting indiscriminate AMPAR targeting by KORs (Fig. 2.3G; control: 48.4 ± 5.1%; dyn. A: 41.4 ± 4.6, n=5 and 5, p=0.34, unpaired t-test). Taken together, these data suggest that KORs mediate the endocytosis of AMPARs on NAcc PV-FSIs in a PKA/calcineurin-dependent manner.

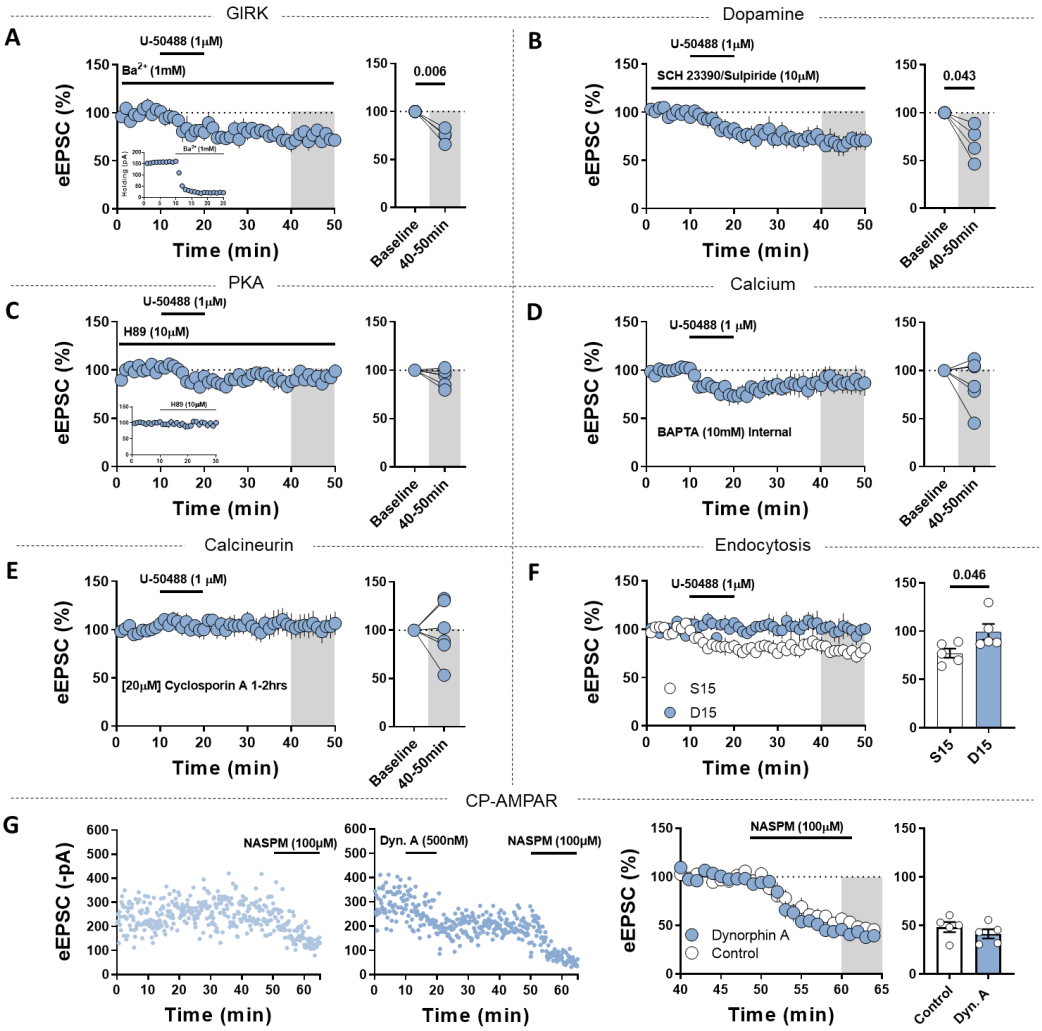



**Figure 2.3. Kappa opioid receptor activation triggers the endocytosis of PV-FSI AMPARs. (A)** Time-course summary of U-50488 (1 μM) washes in the presence of $Ba^{2+}$ (1 mM) and average eEPSC amplitudes from baseline and 40-50 min (75.0 ± 3.5%, n=4, p=0.006). Inset: representative experiment showing effect of $Ba^{2+}$ on holding current. **(B)** Time-course summary of U-50488 (1 μM) washes in the presence of SCH 23390/Sulpiride (10 μM) and average eEPSC amplitudes from baseline and 40-50 min (68.8 ± 9.2%, n=4, p=0.043). **(C)** Time-course summary of U-50488 (1 μM) washes in the presence of H89 (10 μM) and average eEPSC amplitudes from baseline and 40-50min (92.6 ± 3.6%, n=6, p=0.090). Inset: time-course summary of the effect of H89 (10 μM) on eEPSC amplitude **(**98.3 ± 4.9%, n=4, p=0.75, final 10 minutes compared to first 10 minutes) **(D)** Time-course summary of U-50488 (1 μM) washes in the presence of cellularly loaded BAPTA (10 mM) and average eEPSC amplitudes from baseline and 40-50 min (88.0 ± 10.1%, n=6, p=0.290). **(E)** Time-course summary of U-50488 (1 μM) washes in cyclosporin A incubated slices (1-2 hrs, 20 μM) and average eEPSC amplitudes from baseline and 40-50 min (103.3 ± 11.3%, n=7, p=0.78). **(F)** Time-course summary of U-50488 (1 μM) washes in cells loaded with either D15 or S15 (2 mM) and average eEPSC amplitudes taken from 40-50 min from both groups (S15: 77.2 ± 4.8%; D15: 99.3 ± 8.1%, n=5 and 5, p=0.046). Error bars indicate SEM. Baseline = 0-10 min. Grey boxes indicate timepoint used to compare to baseline in A-E, and between S15 and D15 in F. **(G)** Representative experiments of time-matched control or dynorphin A (500 nM) triggered LTD followed by NASPM (100 μM). Time course summary taken from minutes 40-65 to compare NASPM induced depression between control and dynorphin A (control: 48.4 ± 5.1%; dyn. A: 41.4 ± 4.6, n=5 and 5, p=0.34).

*KOR preferentially regulates mThal afferents*

The thalamus is implicated in drug seeking behaviors (Browning et al., 2014; James et al., 2010; Neumann et al., 2016), and the thalamic to NAc pathway is required for the expression of opiate withdrawal aversive symptoms (Zhu et al., 2016). Zhu et al also reports the recruitment of feedforward inhibitory currents in the thalamus to NAc pathway, making this afferent an attractive target for KOR regulation of feedforward inhibitory drive. To test this we injected an adeno-associated virus expressing channelrodopsin2 (AAV-ChR2) into the mThal and recorded optically evoked EPSCs (oEPSCs) in NAcc PV-FSIs (Fig. 2.4A). U-50488 elicited an LTD of mThal oEPSCs (Fig. 2.4B; U-50488: 72.6 ± 5.3%, n=6, p=0.003, paired t-test). We also note no change in CV following U-50488 application at this synapse (data not shown: CV baseline: 0.16 ± 0.016%; CV 40-50min: 0.20 ± 0.055%, n=6, p=0.33, paired t-test). To determine if KORs on PV-FSIs in the NAcc similarly regulate cortico-accumbens afferents, we injected AAV-ChR2 into the PFC and recorded oEPSCs (Fig. 4A). U-50488 had no effect on oEPSCs from PFC-ChR2 terminals (Fig. 2.4C; U-50488: 96.4 ± 9.6%, n=5, p=0.73, paired t-test). To determine if KOR-LTD of thalamo-accumbens synapses was due to trafficking of CI- or CP-AMPARs, we again washed on NASPM following the 30-minute U-50488 washout. There was no difference in depression elicited by NASPM at either the PFC or mThal afferent following U-50488 application (Fig. 2.4D; PFC: 40.1 ± 7.6%; mThal: 41.3 ± 4.1%, n=4 and 5, p=0.89, unpaired t-test), consistent with KOR-LTD of PV-FSIs AMPARs that does not involve the specific targeting of CI- or CP-AMPARs.

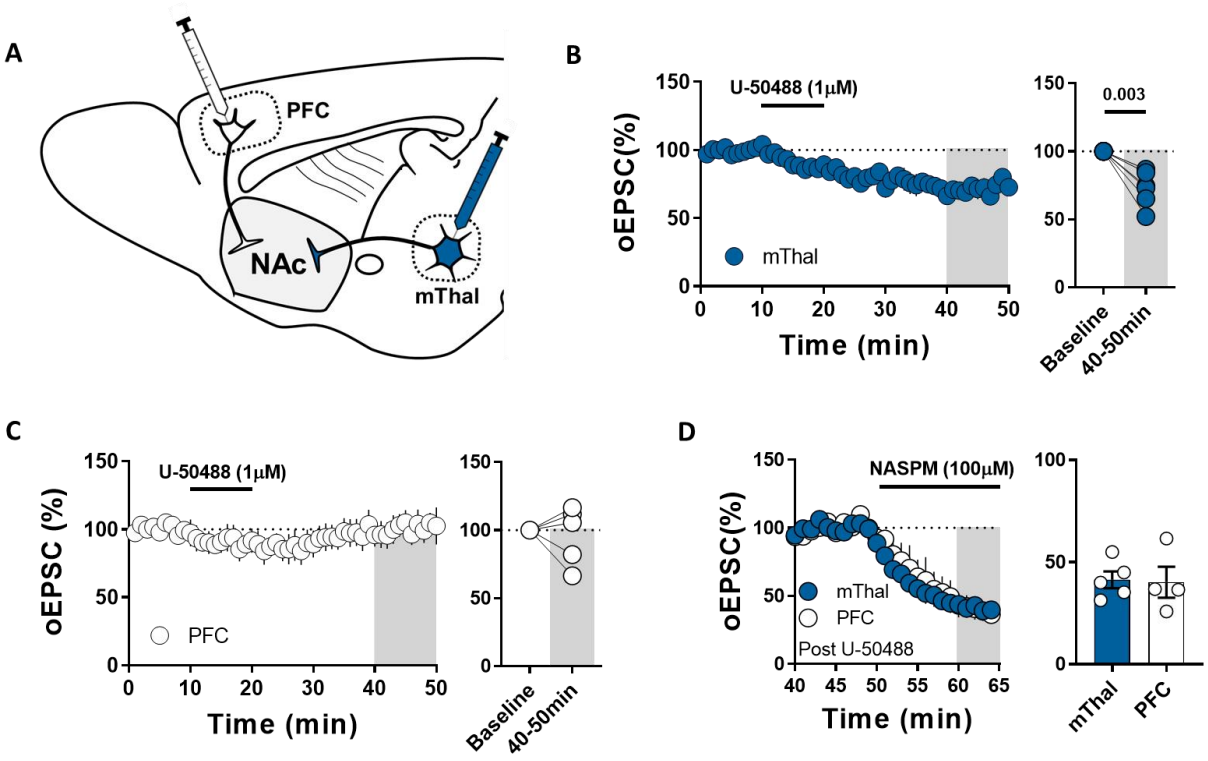



**Figure 2.4. Kappa opioid receptor preferentially regulates mThal afferents.** **(A)** Schematic depicting the optogenetic strategy used to isolate PFC or mThal synapses onto NAc core PV-FSIs. (**B)** Time-course summary of oEPSCs from mThal synapses onto PV-FSIs during U-50488 (1 μM) washes and average oEPSCs from baseline and 40-50min **(**72.6 ± 5.3%, n=6, p=0.003). **(C)** Time-course summary of oEPSCs from PFC synapses onto PV-FSIs during U-50488 (1 μM) washes and average oEPSCs from baseline and 40-50 min (96.4 ± 9.6%, n=5, p=0.73). **(D)** Time-course summary of oEPSCs from PFC or mThal synapses onto PV-FSIs beginning at the 40 min timepoint following U-50488 (1 μM) wash and through a 15 min wash of NASPM (100 μM) and average oEPSCs from 60-65 min compared between the two afferents (PFC: 40.1 ± 7.6%; mThal: 41.3 ± 4.1%, n=4 and 5, p=0.89). Error bars indicate SEM. Baseline = 0-10 min. Grey boxes indicate timepoint used for comparison to baseline in B and C and between mThal and PFC in D.

*KOR-LTD is absent following immobilization stress*

Dynorphin is increased in the NAc of persons diagnosed with cocaine use disorder and persons with suicidal ideologies, as well as in rodent models of addiction and depression (Carlezon et al., 1998; Hurd et al., 1997; Hurd & Herkenham, 1993; Pliakas et al., 2001). Immobilization stress has also been shown to increase dynorphin A immunoreactivity in the NAc (Shirayama et al., 2004a). To investigate the physiological relevance of KOR- LTD on NAcc PV-FSIs, we used an immobilization paradigm in which mice were restricted in a cylindrical tube for 1 hour, allowed to recover for 10 minutes prior to preparation for electrophysiology assays. U-50488 failed to induce LTD in stressed mice as compared to control mice (Fig. 2.5A; Stress: 106.8 ± 7.0%; Ctl: 71.4 ± 5.4%, n=5 and 7, p=0.002, unpaired t-test). To determine if the loss of U-50488 effect in stressed mice was due to activity at KORs *in vivo*, 24 hours prior to stress mice were given either an IP injection of nor-BNI or saline. Following immobilization stress, U-50488 elicited a significant depression in mice pretreated with nor-BNI compared to saline treated mice (Fig. 2.5B; Saline: 100.0 ± 4.6%, n=7; nor-BNI: 83.4 ± 2.7%, n=6, p=0.012, unpaired t-test). Together, these data suggest that PV-FSI KORs are involved in the response to immobilization stress.

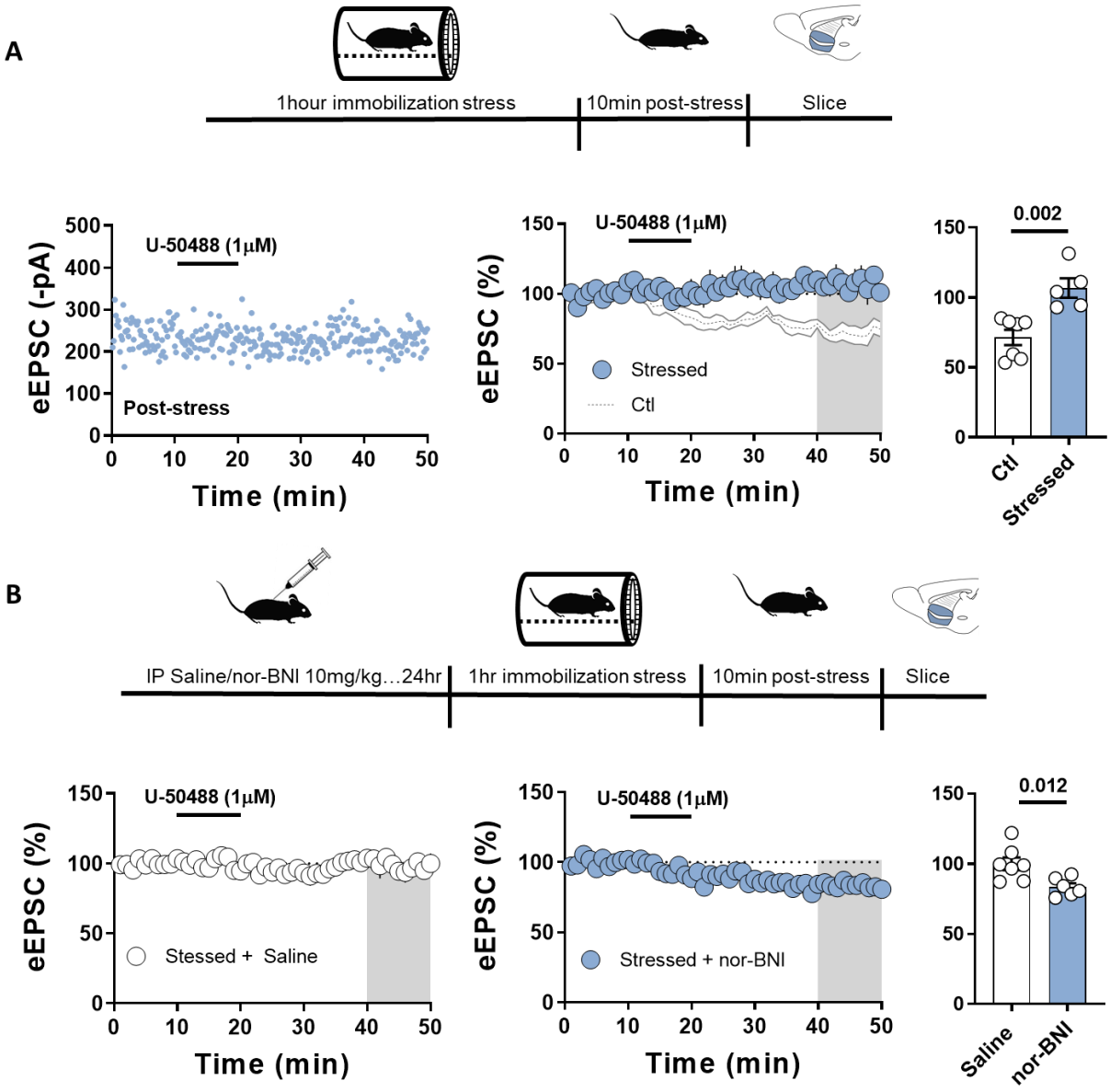



**Figure 2.5. Kappa opioid receptor agonist does not depress PV-FSI EPSCs following restraint stress and can be rescued by prior administration of the antagonist, nor-BNI. (A)** Above: Schematic depicting immobilization stress paradigm. Below: Representative experiment of eEPSCs collected during U-50488 (1 μM) wash from an immobilization stressed mouse, time course summary of eEPSCs collected during U-50488 (1 μM) washes from immobilization stressed or control (ghosted) mice, and average eEPSC amplitudes from 40-50min compared between stress and control mice (Stress: 106.8 ± 7.0%; Ctl: 71.4 ± 5.4%, n=5 and 7, p=0.002). **(B)** Above: Schematic depicting nor-BNI or saline administration prior to immobilization stress paradigm. Below: Time-course summaries of eEPSCs collected during U-50488 (1 μM) washes from IP saline or nor-BNI (10 mg/kg) administered mice prior to immobilization stress and average eEPSC amplitudes from 40-50 min compared between saline and nor-BNI groups (Saline: 100.0 ± 4.6%, n=7; nor-BNI: 83.4 ± 2.7%, n=6, p=0.012). Error bars indicate SEM. Grey boxes indicate timepoint used for comparison between treatment groups.

## 2.5. Discussion

Here we describe modulation of feedforward drive onto NAcc PV-FSIs by the dynorphin/KOR system. Using whole-cell patch clamp electrophysiology, pharmacology, and a PV reporter mouse, we find that activating postsynaptic KORs depresses excitatory transmission onto PV-FSIs by triggering the endocytosis of AMPARs via PKA/calcineurin-dependent mechanisms. We provide evidence for afferent-specific regulation of excitatory drive onto these cells, with mThal but not PFC synapses exhibiting KOR-LTD. Finally, we show that immobilization stress prevents U-50488 depression of glutamatergic transmission that is rescued by prior *in vivo* administration of the KOR antagonist, nor-BNI. Together, these findings provide evidence for the modulation of NAcc microcircuits via the dynorphin/KOR system, and the involvement of these mechanisms in the response to stress.

The predominant locus of KOR function at excitatory synapses onto MSNs is presynaptic, despite imaging data showing pre- and post-synaptic KOR expression (Hjelmstad & Fields, 2001, 2003; Svingos et al., 2001; Tejeda et al., 2017). This manuscript provides the first evidence for postsynaptic KOR mediated regulation of neurotransmission in the NAc. Work from the O'Donnell lab has shown presynaptic inhibition of PFC and hippocampal (HP) inputs onto NAc MSNs in rats (Brooks & O'Donnell, 2017). In contrast, Tejeda et al. show that BLA but not HP inputs onto NAc MSNs are inhibited by KORs in mice (Tejeda et al., 2017). Our studies address PFC and mThal afferents into the NAcc of mice and differs from the O'Donnell group in that we see little effect of KOR activation at the PFC input. These studies highlight possible species differences in KOR function as PFC and HP afferents are regulated in rats and BLA and mThal

afferents in mice. It is also important to note that the current study is the first to examine KOR regulation of excitatory drive onto PV-FSIs. While PV-FSIs in the NAc receive the same collateralizing afferents as MSNs, it is possible that the collateralizing axons express different molecular regulators. Further characterization of afferent specific regulation by KORs in the NAc across species and cell types will be important for the field.

Prior studies described presynaptic mechanisms by which the KOR inhibits glutamate release onto NAc shell MSNs downstream of $Ca^{2+}$ entry, an effect reversed by nor-BNI (Hjelmstad & Fields, 2001, 2003). These results differ significantly from the clear postsynaptic and non-reversible LTD shown here. Differences in these mechanisms may be ascribed to the different cell types being examined, PV-FSIs vs MSNs. Anatomical differences may also play a role as we exclusively recorded from cells in the rostral NAc core while Fields' group recorded in the NAc shell. Behavioral data suggests that KORs along the rostro-caudal axis can differentially regulate hedonic or aversive behaviors, providing behavioral precedent for differing KOR function depending upon the subregion of the NAc (Castro & Berridge, 2014; Pirino et al., 2020). Like the Fields' group we do see a range of depression percentages upon KOR activation. However, in contrast we see a depression in all cells recorded. It is possible that this range of depressions we see is due to a density difference of specific afferent synapses onto PV-FSIs.

The balance between PKA and calcineurin activity is vitally important for both long-term potentiation (LTP) and LTD of excitatory synapses, often both being required for plasticity to occur (Beattie et al., 2000; Esteban et al., 2003; Sanderson et al., 2016). KOR activation has been well characterized to lead to $PKC_{zeta}$ and $Ca^{2+}$ mobilization (Belcheva et al., 2005). Both PKC and

$Ca^{2+}$ can activate adenylyl cyclase subtypes to promote cAMP production and PKA activation (Halls & Cooper, 2011). We hypothesize this may be a mechanism behind KOR activation of PKA. PKA may then be required to phosphorylate an intermediate regulator of calcineurin, or leading to the release of ER stores of $Ca^{2+}$ and thus activating calcineurin. A well-documented mechanism of calcineurin-dependent LTD is the dephosphorylation of Ser845 on the GluA1 subunit of AMPARs (Derkach et al., 2007; Kam et al., 2010; Mulkey et al., 1993). As NAcc PV-FSIs express CI- and CP-AMPARs, both of which can contain GluA1, this would lead to the endocytosis of both receptors. This is supported by the NASPM experiments where no difference was found in NASPM induced depression following U-50488 at the PFC and mThal synapses, as well as no difference in the depression of eEPSCs using NASPM following dynorphin A (Fig. 2.3 and 2.4). In summary, our data support KOR-LTD depends on PKA, calcineurin and endocytosis of AMPARs.

The dorsal paraventricular and paratenial nuclei of the mThal project to the NAc and are implicated in affective states (Vertes et al., 2015; Zhu et al., 2016). Therefore these nuclei were targeted as in Joffe et al (Joffe & Grueter, 2016). The prelimbic region of the medial PFC has been shown to promote the expression of drug seeking behavior and fear learning, and projects to the NAc core (Corcoran & Quirk, 2007; Pietro et al., 2006). The mThal, but not PFC, input into the NAc is strengthened following chronic social defeat stress, and stimulating mThal – NAc afferents drives real-time place aversion while stimulating PFC – NAc afferents drives place preference (Britt et al., 2012; Christoffel et al., 2015; Zhu et al., 2016). Given the KOR's role in mediating stress and negative affective states, we wanted to determine if KORs differentially regulate these two afferents. KORs exhibited limited control over PFC inputs but triggered LTD at mThal synapses,

adding to previous work from our lab characterizing mThal – NAc specific plasticity mechanisms (Joffe et al., 2018; Joffe & Grueter, 2016; Manz, Becker, Grueter, et al., 2021; Turner, Rook, et al., 2018). Given the high level of expression of the KOR within the NAc, it is likely that KOR also regulates other NAc inputs, potentially in a stress-dependent manner. Given the robust loss of KOR-LTD following stress in all cells recorded, we believe it is likely that most inputs onto the NAcc PV-FSIs regulated by the KOR are affected by stress.

The NAc is highly implicated in stress-induced behavioral adaptations (Christoffel et al., 2015; Francis et al., 2015; Heshmati et al., 2020; Krishnan et al., 2007; LaPlant et al., 2010; Lim et al., 2012; Manz, Becker, Grueter, et al., 2021). Dynorphin levels are upregulated in the NAcc and shell of rodents following immobilization stress, and KOR expression is upregulated in the NAc 2 days following a single bout of immobilization stress (Lucas et al., 2011; Shirayama et al., 2004a). We found that restraint stress prevented U-50488-LTD of NAcc PV-FSI EPSCs and that nor-BNI given before the stress rescues the ability of U-50488 to trigger LTD. Similarly, acute stress has been shown to constitutively activate the KOR at a subset of synapses in the ventral tegmental area (Polter et al., 2017). These findings provide strong evidence for the involvement of KORs on NAc PV-FSIs in response to immobilization stress.

*Concluding Remarks*

The current hypothesis regarding NAc PV-FSIs functional contribution is that they orchestrate the required neuronal ensembles to support motivated behavioral activity (Schall et al., 2021). Inputs into the NAc have been shown to drive FFI, and in behavioral states where dynorphin is increased in the NAc, such as stress, this feedforward drive could be biased to ensure the proper neuronal

ensembles are recruited to initiate the appropriate behavioral response to the stress. An important future direction is to determine if KOR regulation of this feedforward circuit becomes dysregulated following chronic stress, and if so, what affect does this have on behavior. NAc PV-FSIs have been implicated in impulsivity, where chemogenetic and optogenetic silencing of PV-FSIs leads to loss of impulse control (Pisansky et al., 2019). Loss of impulse control is a hallmark of addiction and is evident in stress-induced relapse. KORs on NAc PV-FSIs represent an excellent candidate for the underlying molecular substrate for stress-induced loss of impulse control. Finally, to our knowledge this is the first description of postsynaptic KOR regulation of neurotransmission in the NAc and greatly advances our understanding of how the KOR regulates NAcc microcircuitry.

Funding and Conflicts of Interest

This study was supported by National Institute on Drug Abuse R01 DA040630. The authors have no conflicts of interest to declare.

Author Contributions

BCC, KMM and BAG designed experiments. Animal breeding, behavioral testing, data analysis, and the construction of the manuscript was performed by BCC, KMM, and BAG. BCC and KMM performed experiments. All authors contributed to editing and revisions of the manuscript.

# CHAPTER 3

## Kappa opioid receptor signaling biases nucleus accumbens feedforward inhibitory microcircuits

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## 3.1. Abstract

Complex microcircuit elements, such as FFI, dictate nucleus accumbens (NAc) input integration and output. FFI is the process by which parvalbumin fast-spiking interneurons (PV-FSIs) receive collateralizing glutamatergic input from corticolimbic and thalamic brain regions and exert robust inhibitory control over medium spiny neuron firing. Though integral for proper NAc signal transduction and behavior, the molecular mechanisms regulating NAc FFI remain largely understudied. Kappa opioid receptors (KORs) are widely expressed throughout the brain, with particularly high expression in the NAc. Predominately shown to presynaptically inhibit the release of neurotransmitters, KOR regulation of FFI remains unexplored. First, we found the KORs and mu opioid receptors (MORs) inhibit excitatory drive onto PV-FSIs through different mechanisms. Second, we show that KORs robustly decrease thalamic driven FFI onto both D1+ and D1- MSNs while only decreasing cortically driven FFI onto D1- MSNs. Third, we report a marked decrease in PV-FSI excitability following U69593 treatment. Finally, we provide evidence for the involvement of KORs expressed on PV-FSIs in anxiety. Together, these findings provide novel mechanistic insight into the contribution of KORs to the regulation of NAc microcircuit elements and the involvement of KOR expressing PV-FSIs in anxiety.

## 3.2. Introduction

Nucleus accumbens (NAc) output by D1- and D2-dopamine receptor-expressing medium spiny projections neurons (MSNs) orchestrates reward-related motivational output (Joffe et al., 2014; Turner, Kashima, et al., 2018). Complex circuit elements within the NAc integrate information from cortical, limbic, and thalamic glutamatergic afferents to govern MSN output. Drugs of abuse cause synaptic and cellular alterations that disrupt this integration, leading to maladaptive motivational disorders, such as addiction (Creed & Lüscher, 2013; Grueter et al., 2012; Koob & Volkow, 2016). A vital microcircuit element mediating input integration within the NAc is FFI (Burke et al., 2017; Winters et al., 2012). FFI is the process by which parvalbumin fast-spiking interneurons (PV-FSIs) receive collateralizing glutamatergic input and exert robust inhibitory control over MSN firing (Scudder et al., 2018; Wright et al., 2017). Studies suggest that NAc PV-FSIs support NAc dependent associative reward learning and restrain impulsivity (Pisansky et al., 2019; Wang et al., 2018; Yu et al., 2017). Though integral for proper NAc signal transduction and behavior, the molecular mechanisms regulating NAc FFI remain largely understudied.

The dynorphin/kappa opioid receptor (KOR) system contributes to NAc mediated negative affective states. Locally, the endogenous KOR ligand, dynorphin, is produced by D1+ MSNs and released upon aversive stimuli (Shirayama et al., 2004b). Repeated drug exposure leads to increased dynorphin immunoreactivity and heightened KOR sensitivity in the NAc, and dynorphin is increased in the NAc of individuals who suffered from cocaine use disorder and suicidal ideologies (Hurd et al., 1997; Hurd & Herkenham, 1993; Lindholm et al., 2000; Mu et al., 2011; Siciliano et al., 2015; Zan et al., 2015). Historically, recruitment of the dynorphin/KOR system in the NAc has been shown to be aversive (Bals-Kubik et al., 1993). However, this may be an

oversimplification for the roles KORs play in the NAc as multiple studies have identified distinct subregions within the NAc shell where KOR activation can lead to opposing (hedonic) behavioral outcomes (Al-Hasani et al., 2015; Castro & Berridge, 2014; Pirino et al., 2020). KORs are Gαi-coupled GPCRs that can signal via multiple downstream effectors. KORs canonically signal through Gβγ to activate potassium channels (Kir3 and Kv) and inhibit voltage gated calcium channels to hyperpolarize neurons and prevent signal transduction. KORs can also recruit intracellular signaling effectors such as beta-arrestin, G-protein coupled receptor kinases, and mitogen-activated protein kinases (Bruchas & Chavkin, 2010). Widely expressed throughout the brain, KORs exhibit particularly dense expression patterns in the ventral striatum (Meng et al., 1993). They have predominately been shown to presynaptically inhibit the release of glutamate, GABA, and dopamine (Brooks & O'Donnell, 2017; Ehrich et al., 2015; Hjelmstad & Fields, 2001, 2003; Tejeda et al., 2017). While much has been learned regarding the regulatory role of KORs on neurotransmission in the NAc, KOR regulation of FFI, a key computational microcircuit element of the NAc, remains unexplored.

We recently characterized postsynaptically expressed KORs on PV-FSIs in the NAc core (NAcc) and their role in modulating excitatory drive onto PV-FSIs (Coleman et al., 2021). Here we expand upon this work to examine KOR modulation of multiple synaptic and cellular loci within NAcc feedforward inhibitory circuits. Using a dynamic whole-cell patch clamp electrophysiology approach we found that KORs robustly decreased mThal driven FFI onto both D1+ and D1- MSNs. Interestingly, KORs only decreased PFC driven FFI onto D1- MSNs but not D1+ MSNs. We also show that KORs decreased locally stimulated GABA release (electrically stimulated GABA from PV-FSIs, somatostatin interneurons, and MSNs) onto both D1+ and D1- MSNs. The KOR agonist,

U69593, also exhibited a marked effect on PV-FSIs action potential firing that was reversible by the KOR antagonist, norBNI. Finally, we developed a PVcre/*OPRK1* knockout (KO) mouse and show that *OPRK1* heterologous deletion leads to an increase in basal anxiety. Together, these findings provide novel insight into mechanisms by which the endogenous dynorphin/KOR system differentially regulates FFI in the NAcc and implicates KORs in PV expressing cells in the regulation of anxiety.

## 3.3. Materials and Methods

### *Animals*

Mice were housed in the Vanderbilt University Medical Center animal care facility in accordance with Institutional Animal Care and Use Committee guidelines. Mice were group housed 2-5/cage with *ad lib* access to food and water and kept on a 12-hr light-dark cycle. Male mice ages 7-12 weeks were used for all experiments. To allow for fluorescent visualization of PV-FSIs in electrophysiology experiments, Cre-induced STOP$^{fl/fl}$-tdTomato mice (Ai9, *Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$) (Jackson Laboratory Stock No.: 007909) were crossed with parvalbumin (PV) PV-IRES-Cre mice (PV$^{Cre}$, *Pvalb*$^{tm1(cre)Arbr/J}$, Stock No.: 008069), generating PV$^{Cre}$-tdTomato$^{fl/fl}$ (PV$^{tdT}$) mice. For identification of MSN subtypes, C57BL/6J mice were bred to harbor a BAC carrying the tdTomato fluorophore under control of the Drd1a (D1 receptor) promoter (D1$^{tdT}$). For behavioral experiments, PV$^{Cre}$ mice were crossed with *OPRK1*$^{(fl/fl)}$ (Jax B6;129-*Oprk1*$^{tm2.1Kff}$/J, Stock No: 030076) to produce PV/OPRK1 KO mice. Female heterozygous KO and littermate controls expressing PV$^{cre}$ only were used for behavior.

*Electrophysiology*

For detailed electrophysiological methods see (Manz, Siemann, McMahon, et al., 2021). In brief, mice were killed under isoflurane anesthesia, brain dissected, and sagittal brain slices 250μm thick prepared using a Leica VT1200S Vibratome. Slices were prepared in oxygenated (95% O2; 5%CO2) ice-cold *N*-methyl-*D*-glucamine (NMDG)-based solution (in mM: 2.5 KCl, 20 HEPES, 1.2 $NaH_2PO_4$, 25 Glucose, 93 NMDG, 30 $NaHCO_3$, 5.0 sodium ascorbate, 3.0 sodium pyruvate, 10 $MgCl_2$, and 0.5 $CaCl_2$-$2H_2O$) and recovered for 10 minutes in the same solution at 34°C. Slices were then recovered for 1 hour in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM: 119 NaCl, 2.5 KCl, 1.3 MgCl2-6H2O, 2.5 CaCl2-2H2O, 1.0 NaH2PO4-H2O, 26.2 NaHCO3, and 11 glucose; 287-295 mOsm). During experiments, slices were continuously perfused with oxygenated ACSF at a rate of 2 mL/min and a temperature of 32 ± 2°C. $PV^{tdT}$ cells in the NAcc were visualized using Scientifica PatchVision software via 530 nm LED light. PV-FSIs were confirmed according to biophysical properties (capacitance, membrane resistance, and AMPAR decay kinetics) [(Manz, Coleman, Grueter, et al., 2021; Manz et al., 2020)].

Whole-cell patch-clamp electrophysiology was performed using a CV-7B headstage, Multiclamp 700B Amplifier, Axopatch Digidata 1550 digitizer, and 3-6 MΩ glass recording micropipettes (Sutter P1000 Micropipette Puller). To isolate AMPAR mediated excitatory postsynaptic currents (EPSCs) 50 μM picrotoxin was added to ACSF. Voltage clamp recordings of EPSCs were done at -70 mV using a $K^+$-based intracellular solution (in mM: 135 $K^+$-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 3 Na2ATP, 0.4 Na2GTP; 285-292 mOsm). For feedforward inhibitory recordings of inhibitory postsynaptic currents (IPSCs), interleaved electrically-evoked and optically-evoked IPSCs (e/oIPSCs) in MSNs were isolated electrochemically at 0 (±5) mV using

the $E_{Cl}$ and $E_{AMPAR/NMDAR}$ of the Cs+-based internal solution. Cs+-based intracellular solution contained (120 mm CsMeSO3, 15 mm CsCl, 8 mm NaCl, 10 mm HEPES, 0.2 mm EGTA, 10 mm TEA-Cl, 4.0 mm Mg-ATP, 0.3 mm Na-GTP, 0.1 mm spermine, and 5.0 mm QX 314 bromide). K+-based intracellular solution was used for current-clamp recordings. Cells were allowed to dialyze for 5 minutes following whole-cell configuration, after which a depolarizing plateau potential was established to maintain cells at −70 mV. To assess intrinsic membrane excitability, action potentials (APs) were elicited following 50-pA current steps increasing from −100 to 400 pA with an 800-ms step duration every 2 min.

For electrical stimulation, a bipolar electrode was placed at the cortico-accumbens interface and stimulated at 0.1Hz to examine local neurotransmission. Paired-pulse ratio (PPR) was acquired within experiment by giving two 0.15 ms pulses with a 50 ms interstimulus interval and dividing the amplitude of the second EPSC by the first. For experiments examining mThal or PFC driven FFI, 473-nm Cool LED stimulation was delivered at 0.1 Hz with a 0.3-ms pulse duration to slices prepared from mice expressing ChR2 on terminals within the NAcc. Cells with a >15% change in series resistance (Rs) we excluded from analysis.

*Stereotaxic Injections*

Mice underwent stereotaxic surgery for viral-mediated gene transfer of channel rhodopsin (ChR2). At ~4 weeks of age mice were given bilateral injections (400nL) of AAV5-CaMKII-ChR2-EYFP (Addgene, Watertown, MA) under ketamine (75 mg/kg, i.p.) and dexdomitidor (0.5 mg/kg, i.p.) anesthesia. Mice were recovered using antisedan (atipamezole, 0.5 mg/kg, i.p.) and treated with ketoprofen (5 mg/kg, i.p.) for 3 d postoperatively, and killed 3-5 weeks after surgery for electrophysiological experiments. Coordinates were (relative to bregma): mThal (medial-lateral

(ML): ±0.3, anterior-posterior (AP): −1.2, dorsal-ventral (DV): −3.0) or PFC (ML: ±0.3, AP: −1.75, DV: −2.75). Injection sites were located using Leica AngleTwo Stereotaxic software. The dorsal paraventricular and paratenial nuclei of the mThal and prelimbic region of the medial PFC were targeted. Coordinates were validated via dye injection.

*Behavior*

All behavior was recorded using Noldus Ethovision 10. Open field test (OFT) was performed in MedAssociates Activity Test Chambers where mice were allowed to freely roam for 60 minutes. Time spent in the center, edges, and the total locomotor activity were tracked. For elevated plus maze (EPM) mice were placed in the center of the apparatus and allowed to freely roam for 6 minutes. Time spent in open arm, closed arm, and center as well as total locomotor activity were tracked. For forced swim test (FST), 2-liter beakers were filled with 1700mL of water warmed to 30°C. Mice were placed in the water and their activity tracked for 6 minutes. Latency to immobility and total time spent immobile were scored by a blinded lab mate.

*Pharmacology*

Isoflurane, NBQX disodium salt, D-AP5 ,and nor-binaltorphimine dihydrochloride were obtained from Tocris/Bio-Techne, Minneapolis, MN. Picrotoxin and U69593 were obtained from Sigma-Aldrich, St. Louis, MO.

*Statistics*

Clampfit 10.4 and GraphPad Prism v7.0 were used to analyze all experiments. Changes in E/IPSC amplitude, coefficient of variance (CV), and PPR were calculated by comparing the mean values during the first 5 or 10 minutes (defined for each experiment) to the mean values in during the final

5 or 10 minutes of each experiment. Each data point represents the average of one cell, n = number of cells. Paired/unpaired *t*-tests were used where applicable with One-Way (Fig. 3.2 F, Fig. 3.5 C, D, E) and Two-Way ANOVAs (Fig. 3.5 B) with Tukey's multiple comparisons used where indicated. Errors bars represent SEM. For all analyses, $p < 0.05$ was considered significant.

## 3.4. Results

*Opioid receptors differentially modulate excitatory drive onto NAcc PV-FSIs*

Opioid receptors decrease glutamatergic transmission onto MSNs in the NAc primarily through presynaptic mechanisms (Brooks & O'Donnell, 2017; Chartoff & Connery, 2014; Hjelmstad & Fields, 2001; Tejeda et al., 2017). Few studies have examined the regulation of glutamatergic transmission onto NAc PV-FSIs (Manz, Coleman, Grueter, et al., 2021; Manz et al., 2019, 2020). Recently, we described the role of postsynaptic KORs in decreasing feedforward inhibitory drive in the NAcc (Coleman et al., 2021). Using a $PV^{tdT}$ reporter mouse, we recorded AMPAR mediated eEPSCs from PV-FSIs in the NAcc (Fig. 3.1A) and were able to reproduce the KOR mediated long-term depression with the KOR agonist U69593 (1 μM) (Fig. 3.1B; 80.0 ± 5.8%, n=5, p=0.027). This effect was not accompanied by a change in PPR (Fig. 3.1C; PPR baseline: 1.45 ± 0.04; PPR 35-40 min: 1.52 ± 0.11, n=5, p=0.52 ) or CV (Fig. 3.1D; CV baseline: 0.19 ± 0.02; CV 35-40 min: 0.22 ± 0.04, n=5, p=0.41), consistent with the characterization of postsynaptically located KORs. We next sought to determine if this effect on PV-FSI EPSCs is unique to KORs, or if all Gαi-coupled opioid receptors similarly decrease EPSC amplitude. The mu opioid receptor (MOR) agonist, DAMGO (1 μM), elicited a short-term depression of eEPSC amplitude (Fig. 3.1E; 79.2 ± 4.0%, n=6, p=0.004) that returned to baseline levels following washout. The depression was not accompanied by a change in PPR (Fig. 3.1F; PPR baseline: 1.41 ± 0.04; PPR 10-20 min: 1.43 ± 0.06, n=6, p=0.63) but was accompanied by an increase in CV (Fig. 3.1G; CV baseline: 0.16 ± 0.01; CV 10-20 min: 0.20 ± 0.01, n=6, p=0.03), suggesting the depression is expressed on the presynaptic axon. These data provide evidence for the opposing synaptic localization and function of KORs and MORs on glutamatergic synapses onto PV-FSIs.

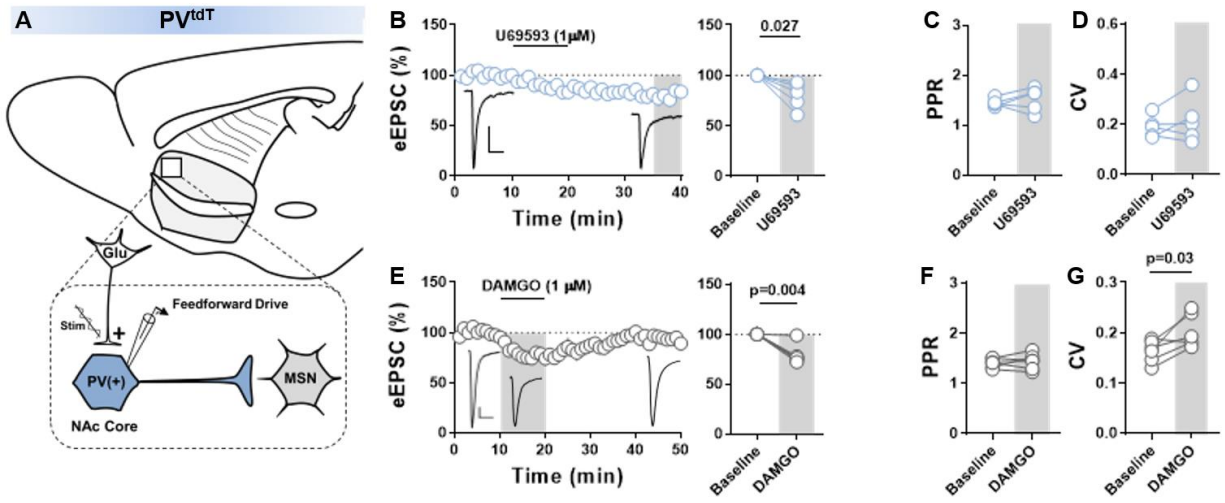


**Figure 3.1. Opioid receptor regulation of feedforward drive onto NAc PV-FSIs.** **(A)** Schematic depicting a sagittal mouse brain slice containing NAc core PV-FSI EPSC recording strategy. **(B)** Time-course summary of EPSCs onto PV-FSIs during U69593 (1 μM) drug washes and average % EPSC amplitudes taken from baseline and 35-40 min (80.0 ± 5.8%, n=5, p=0.027). Inset are representative current traces taken from baseline and 35-40 min (scale bars = 50pA/10ms). **(C)** Average PPR taken from baseline and 35-40 min of U69593 washes (PPR baseline: 1.45 ± 0.04; PPR 35-40 min: 1.52 ± 0.11, n=5, p=0.52). **(D)** Average CV taken from baseline and 35-40 min of U69593 washes (CV baseline: 0.19 ± 0.02; CV 35-40 min: 0.22 ± 0.04, n=5, p=0.41). **(E)** Time-course summary of EPSCs onto PV-FSIs during DAMGO (1 μM) drug washes and average % EPSC amplitudes taken from baseline and 10-20 min (79.2 ± 4.0%, n=6, p=0.004). Inset are representative current traces taken from baseline, 10-20 min, and 40-50 min (scale bars = 50pA/10ms). **(F)** Average PPR taken from baseline and 10-20 min of DAMGO washes (PPR baseline: 1.41 ± 0.04; PPR 10-20 min: 1.43 ± 0.06, n=6, p=0.63). **(G)** Average CV taken from baseline and 10-20 min of DAMGO washes (CV baseline: 0.16 ± 0.01; CV 10-20 min: 0.20 ± 0.01, n=6, p=0.03). Error bars indicate SEM. Error bars may not be visible when SEM is smaller than circle. Baseline = 0-10 min. Grey boxes indicate timepoint used to compare to baseline.

*Validation of feedforward inhibitory transmission recording strategy*

NAc dependent behavior is ultimately driven by MSN output. To better understand how KORs regulate feedforward inhibitory microcircuits, we developed a recording strategy that allowed for the examination of FFI onto MSNs (Manz, Coleman, Grueter, et al., 2021). By expressing channelrodopsin2 (AAV-ChR2) in the mThal or prefrontal cortex PFC of $D1^{tdT}$ mice, we were able to record oIPSCs from D1+ or D1- MSNs (Fig. 3.2A). oIPSCs were interleaved with eIPSCs (termed Local), and oIPSCs exhibited an increase in current latency (time from stimulation to IPSC onset), indicating the presence of a polysynaptic feedforward circuit (Fig. 3.2F; Local vs mThal: $p<0.0001$; Local vs PFC: $p<0.0001$; mThal vs PFC: $p=0.011$, One-Way ANOVA Tukey's). Interestingly, we also observed a difference in latency between mThal and PFC driven FFI, suggesting a difference in the timing of afferent specific FFI. First, to confirm that IPSCs obtained at 0 mV were mediated by $GABA_A$Rs we superfused picrotoxin (PTX) into the ACSF bath. PTX (50 μM) completely blocked e/oIPSC amplitude, confirming that feedforward and locally-evoked GABAergic transmission onto NAc MSNs is mediated by $GABA_A$Rs (Fig. 3.2B,C; Local: 4.5 ± 0.84%, n=6; mThal: 6.0 ± 0.51%, n=3; PFC: 6.5 ± 2.8%, n=3). If oIPSCs in this system are disynaptically mediated by FFI, then bath application of ionotropic glutamate receptor antagonists should block oIPSC amplitude while leaving eIPSC amplitude intact. Indeed, bath application of the AMPAR antagonist NBQX (10 μM) and NMDAR antagonist APV (50 μM) completely abolished oIPSC amplitudes while leaving eIPSCs unchanged (Fig. 3.2D,E; Local: 89.7 ± 4.6%, n=6, $p=0.08$; mThal: 8.6 ± 1.6%, n=3; PFC: 3.0 ± 0.93%, n=3). This confirms that mThal and PFC driven oIPSCs in MSNs require excitation of a GABAergic intermediate.

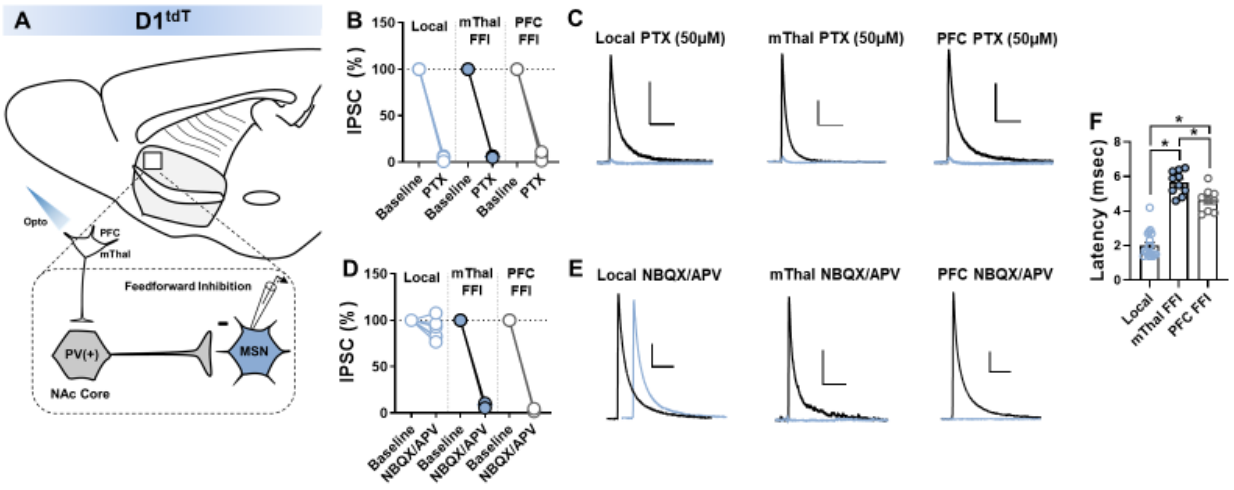


**Figure 3.2. Validation of mThal and PFC driven feedforward inhibition recording strategy. (A)** Schematic depicting optogenetically driven FFI onto NAc core MSNs and recording strategy in D1$^{tdT}$ BAC transgenic mice. **(B)** Average % IPSC amplitudes taken from baseline and following PTX (50 μM) drug washes (Local: 4.5 ± 0.84%, n=6; mThal: 6.0 ± 0.51%, n=3; PFC: 6.5 ± 2.8%, n=3). **(C)** Representative IPSC current traces for local, mThal, and PFC recordings from baseline (Black) and following PTX (50 μM) (Blue). Scale bars = 100pA/100ms. **(D)** Average % IPSC amplitudes taken from baseline and following NBQX (10 μM)/APV (50 μM) drug washes (Local: 89.7 ± 4.6%, n=6; mThal: 8.6 ± 1.6%, n=3; PFC: 3.0 ± 0.93%, n=3). **(E)** Representative IPSC current traces for local, mThal, and PFC recordings from baseline (Black) and following NBQX (10 μM)/APV (50 μM) (Blue). Scale bars = 100pA/100ms. **(F)** Latency from time of stimulation to initiation of local, mThal, or PFC driven IPSC (Local: 2.0 ± 0.17ms, n=19; mThal: 5.6 ± 0.21ms, n=10; PFC: 4.6 ± 0.22ms, n=9) (Local vs mThal: p<0.0001; Local vs PFC: p<0.0001; mThal vs PFC: p=0.011, One-Way ANOVA Tukey's). Error bars indicate SEM. Baseline = 0-5 min. Local=eIPSCs.

*KORs depress local and mThal driven FFI, but not PFC driven FFI, onto D1+ MSNs*

To examine the effect of selective KOR activity on signal transduction through the FFI circuit, U69593 was bath applied following a 10-minute baseline of interleaved eIPSCs and oIPSCs from D1+ MSNs. U69593 (1 μM) lead to a moderate decrease in locally evoked IPSCs from D1+ MSNs with no significant change in CV (Fig. 3.3A,B; 69.3 ± 4.5%, n=9, p=0.0001) (Fig. 3.3C; CV baseline: 0.24 ± 0.02; CV 20-30 min: 0.29 ± 0.03, n=9, p=0.10). U69593 elicited a robust depression in mThal-FFI IPSCs from D1+ MSNs (Fig. 3.3D,E; 38.9 ± 7.1%, n=5, p=0.001). This depression was accompanied by an increase in CV, suggesting the depression is mediated presynaptically (Fig. 3.3F; CV baseline: 0.15 ± 0.02; CV 20-30 min: 0.54 ± 0.13, n=5, p=0.048). Interestingly, the magnitude of the depression is several orders of magnitude larger than the effect U69593 has on excitatory drive onto PV-FSIs (Fig. 3.1B), suggesting KORs modulate mThal-FFI at multiple summating loci. In contrast, U69593 had no significant effect on PFC-FFI IPSCs from D1+ MSNs (Fig. 3.3G,H; 91.3 ± 6.0%, n=4, p=0.24) (Fig. 3.3I; CV baseline: 0.14 ± 0.008; CV 20-30 min: 0.15 ± 0.009, n=4, p=0.19).

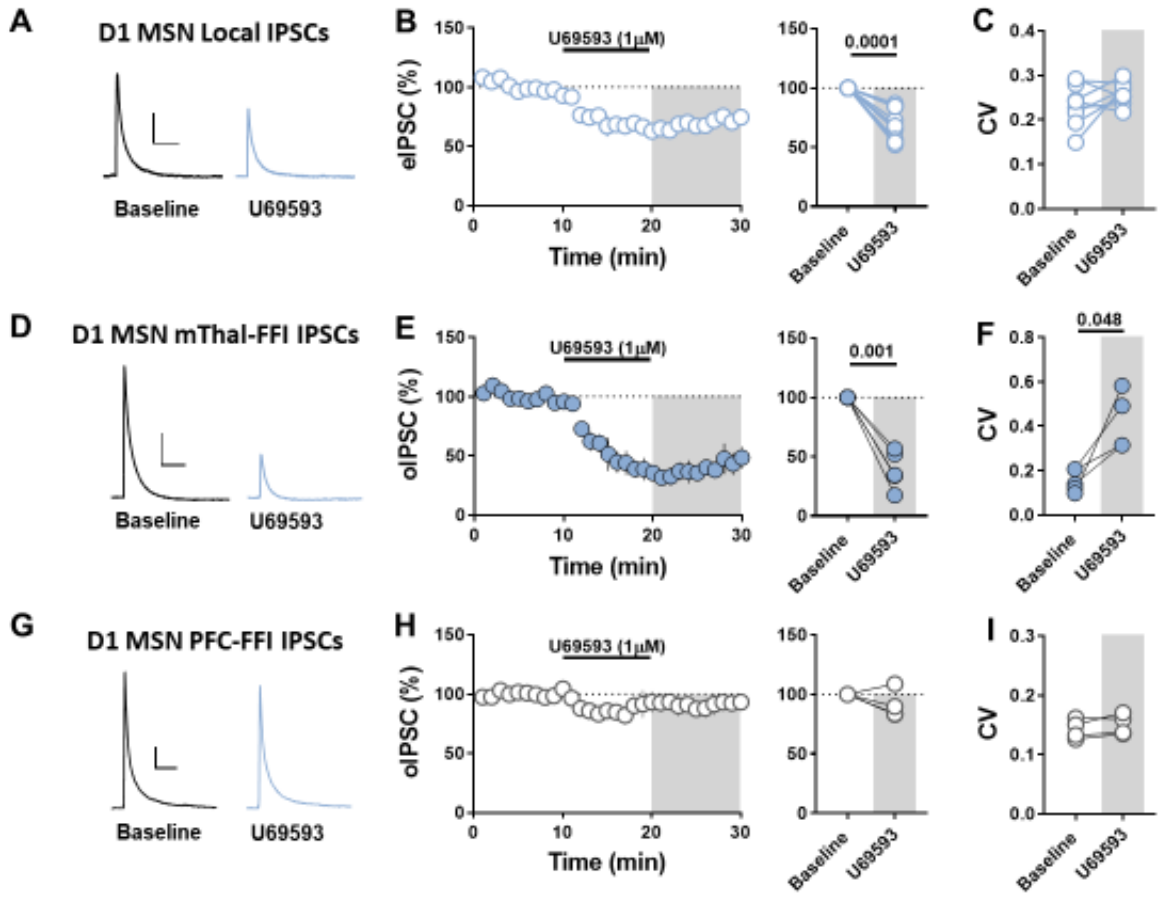


**Figure 3.3. Kappa opioid receptors differentially modulate feedforward inhibition onto NAc core D1+ MSNs.** **(A)** Representative eIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 50pA/100ms. **(B)** Time-course summary of local eIPSCs onto D1+ MSNs during U69593 (1 μM) drug washes and average % eIPSCs taken from baseline and 20-30 min (69.3 ± 4.5%, n=9, p=0.0001). **(C)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.24 ± 0.02; CV 20-30 min: 0.29 ± 0.03, n=9, p=0.10). **(D)** Representative mThal driven oIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 100pA/100ms. **(E)** Time-course summary of mThal oIPSCs onto D1+ MSNs during U69593 (1 μM) drug washes and average % oIPSCs taken from baseline and 20-30 min (38.9 ± 7.1%, n=5, p=0.001). **(F)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.15 ± 0.02; CV 20-30 min: 0.54 ± 0.13, n=5, p=0.048). **(G)** Representative PFC driven oIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 100pA/100ms. **(H)** Time-course summary of PFC oIPSCs onto D1+ MSNs during U69593 (1 μM) drug washes and average % oIPSCs taken from baseline and 20-30 min (91.3 ± 6.0%, n=4, p=0.24). **(I)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.14 ± 0.008; CV 20-30 min: 0.15 ± 0.009, n=4, p=0.19). Error bars indicate SEM. Error bars may not be visible when SEM is smaller than circle. Baseline = 0-10 min. Grey boxes indicate timepoint used to compare to baseline.

*KORs depress local, mThal, and PFC driven FFI onto D1- MSNs*

To determine if KORs differentially regulate FFI onto D1- vs D1+ MSNs, we also bath applied U69593 following a 10-minute baseline of interleaved eIPSCs and oIPSCs from D1- MSNs. Similar to in D1+ MSNs, U69593 (1 μM) led to a moderate decrease in locally evoked IPSCs from D1- MSNs with no significant change in CV (Fig. 3.4A,B; 73.7 ± 4.5%, n=10, p=0.0002) (Fig. 3.2C; CV baseline: 0.16 ± 0.02; CV 20-30 min: 0.19 ± 0.03, n=10, p=0.092). Again, U69593 elicited a robust depression in mThal-FFI IPSCs from D1- MSNs, however no significant change in CV was measured (Fig. 3.4D,E; 56.05 ± 11.4%, n=5, p=0.018) (Fig. 3.4F; CV baseline: 0.11 ± 0.02; CV 20-30 min: 0.19 ± 0.05, n=5, p=0.20). In contrast to PFC-FFI IPSCs recorded from D1+ MSNs, U69593 significantly decreased PFC-FFI onto D1- MSNs with no corresponding change in CV (Fig. 3.4G; 68.5 ± 9.5%, n=5, p=0.029) (Fig. 3.4H; CV baseline: 0.14 ± 0.02; CV 20-30 min: 0.18 ± 0.06, n=5, p=0.44). We would like to note that using CV to determine synaptic locus when examining a polysynaptic circuit has its caveats and may not reliably detect a presynaptic phenomenon (Faber & Korn, 1991). With that in mind, the larger effects on oIPSC amplitude in Figures 3.4E,H correspond to the larger increases in CV in Figures 3.4F,I. This suggests that KORs modulate FFI within these circuits at multiple synaptic or cellular loci, and depending on the locus can moderately inhibit FFI or lead to its near total inhibition.

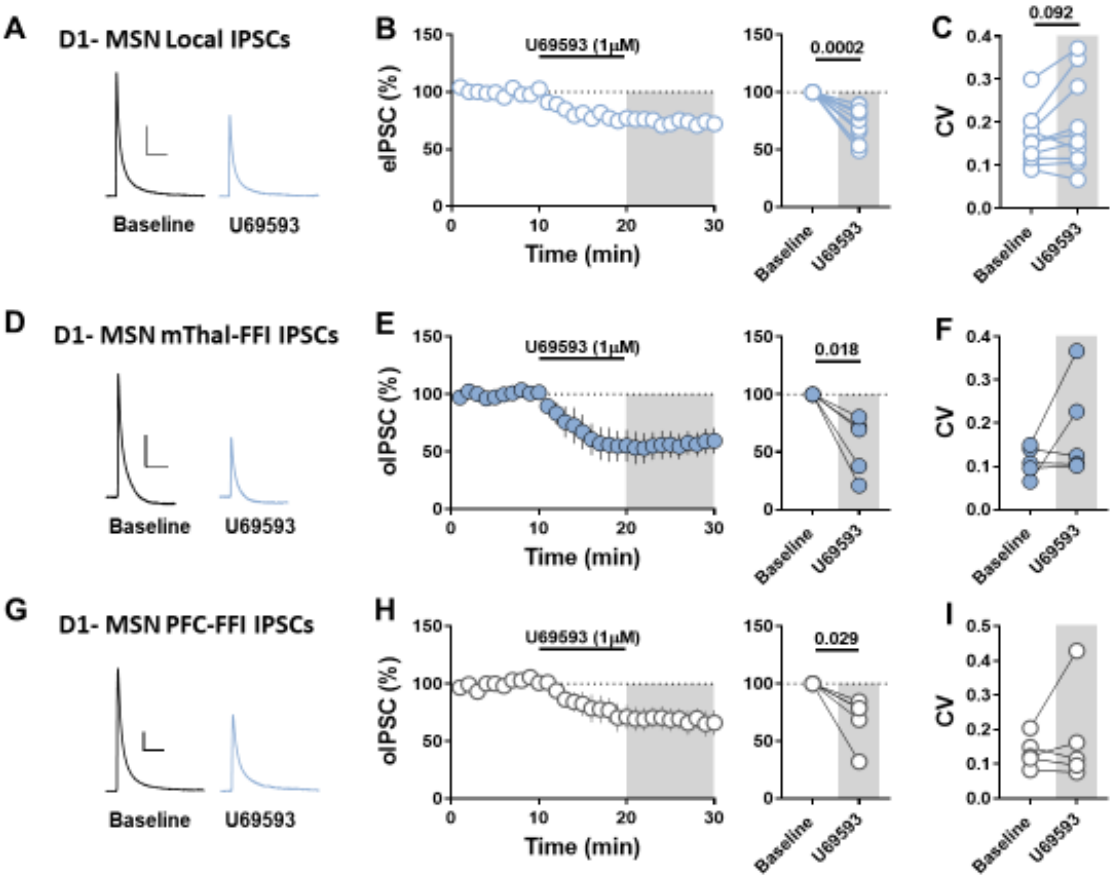


**Figure 3.4. Kappa opioid receptors decrease feedforward inhibition onto NAc core D1- MSNs.** **(A)** Representative eIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 100pA/100ms. **(B)** Time-course summary of local eIPSCs onto D1- MSNs during U69593 (1 μM) drug washes and average % eIPSCs taken from baseline and 20-30 min (73.7 ± 4.5%, n=10, p=0.0002). **(C)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.16 ± 0.02; CV 20-30 min: 0.19 ± 0.03, n=10, p=0.092). **(D)** Representative mThal driven oIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 100pA/100ms. **(E)** Time-course summary of mThal oIPSCs onto D1- MSNs during U69593 (1 μM) drug washes and average % oIPSCs taken from baseline and 20-30 min (56.05 ± 11.4%, n=5, p=0.018). **(F)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.11 ± 0.02; CV 20-30 min: 0.19 ± 0.05, n=5, p=0.20). **(G)** Representative PFC driven oIPSC current traces from baseline and 20-30 min of U69593 drug washes. Scale bars = 100pA/100ms. **(H)** Time-course summary of PFC oIPSCs onto D1- MSNs during U69593 (1 μM) drug washes and average % oIPSCs taken from baseline and 20-30 min (68.5 ± 9.5%, n=5, p=0.029). **(I)** Average CV taken from baseline and 20-30 min of U69593 washes (CV baseline: 0.14 ± 0.02; CV 20-30 min: 0.18 ± 0.06, n=5, p=0.44). Error bars indicate SEM. Error bars may not be visible when SEM is smaller than circle. Baseline = 0-10 min. Grey boxes indicate timepoint used to compare to baseline.

*KORs decrease PV-FSI action potential fidelity*

To investigate the role KORs may play in regulating the membrane excitability of PV-FSIs, we recorded PV-FSI action potential firing in response to a series of current step injections in the presence of U69593. We found that at higher current step plateaus, U69593 (1 μM) led to marked action potential accommodation and that this was KOR dependent by blocking the effect with the KOR antagonist, norBNI (500 nM) (Fig. 3.5A,B; $Ctl_{400pA}$: 92.5 ± 6.6Hz, n=7; $U69593_{400pA}$: 38.9 ± 7.6Hz, n=7; $norBNI_{400pA}$: 93.6 ± 19.8Hz, n=6.). The effect on action potential frequency was driven by a shortening of the action potential train and was quantified by measuring the time from the onset of the first action potential to the end of the last action potential during the 400-pA step (Fig. 3.5 C; Ctl: 737 ± 7ms, n=7; U69593: 302 ± 72ms, n=7; norBNI: 729 ± 20ms, n=6). No effect was found on the interstimulus interval (Fig. 3.5D; Ctl: 10.4 ± 0.62ms, n=7; U69593: 9.15 ± 0.76ms, n=7; norBNI: 15.8 ± 6.8ms, n=6), or on the minimal amount of current required to elicit action potential firing (Fig. 3.5E; Ctl: 242.9 ± 23.0pA, n=7; U69593: 207.1 ± 17.0pA, n=7; norBNI: 266.7 ± 38.0pA, n=6). It is unlikely that this effect contributes to the decrease in FFI in Figures 3.3 and 3.4 as the optical stimulation of mThal and PFC afferents used in this study elicits a rapid ~10ms EPSC onto PV-FSIs (Manz, Coleman, Jameson, et al., 2021) while these data suggest that KORs inhibit PV-FSIs sustained action potential firing in response to robust and prolonged excitation, perhaps by shifting the sensitization of sodium channels.

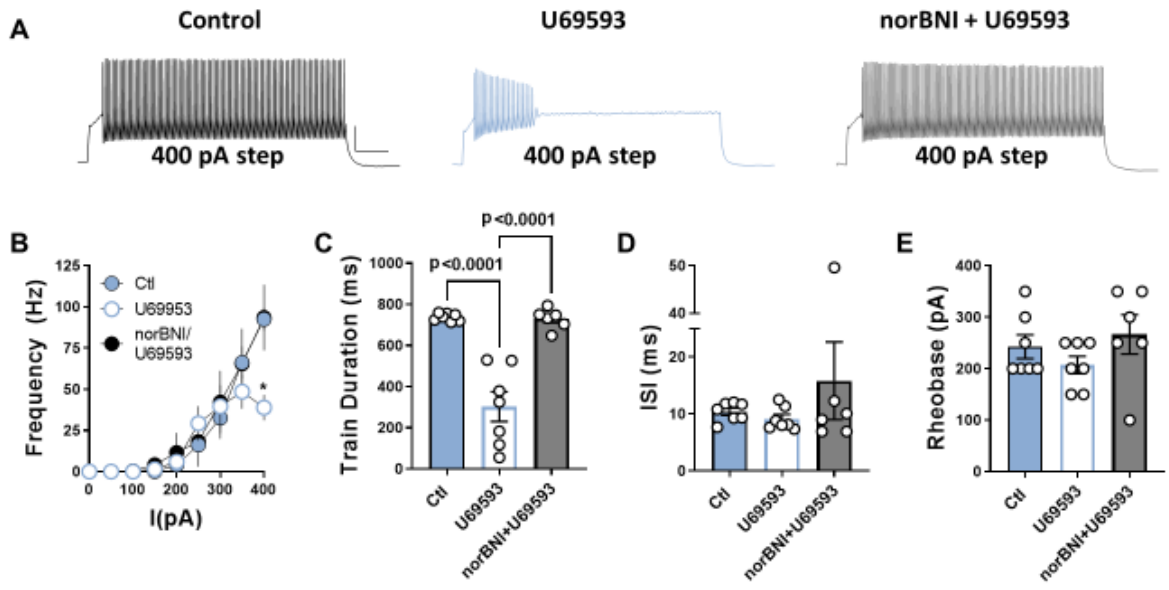


**Figure 3.5. Kappa opioid receptor activation leads to action potential accommodation.** **(A)** Representative voltage traces taken from PV-FSIs during an 800ms/400pA somatic current step injection under control, U69593 (1 µM), and norBNI (500nM) + U69593 (1 µM) conditions. Scale bar = 20mV/100ms. **(B)** Input-output function of PV-FSIs following 50-pA sequential increases in current injection under ACSF control (blue circles), U69593 (1 µM) (open circles), or norBNI (500nM) + U69593 (1 µM) (black circles) treatments. ($Ctl_{400pA}$: 92.5 ± 6.6Hz, n=7; $U69593_{400pA}$: 38.9 ± 7.6Hz, n=7; $norBNI_{400pA}$: 93.6 ± 19.8Hz, n=6.) (Ctl vs U6: p=0.0005; Ctl vs norBNI: p=0.99; U6 vs norBNI: p=0.09. Two-Way ANOVA Tukey's) **(C)** Average action potential train duration for the 400-pA current step across treatment groups (Ctl: 737 ± 7ms, n=7; U69593: 302 ± 72ms, n=7; norBNI: 729 ± 20ms, n=6) (Ctl vs U6: p<0.0001; Ctl vs norBNI: p=0.99; U6 vs norBNI: p<0.0001. One-way ANOVA Tukey's). **(D)** Average action potential interstimulus interval for the 400-pA current step across treatment groups (Ctl: 10.4 ± 0.62ms, n=7; U69593: 9.15 ± 0.76ms, n=7; norBNI: 15.8 ± 6.8ms, n=6) (Ctl vs U6: p=0.97; Ctl vs norBNI: p=0.55; U6 vs norBNI: p=0.41. One-way ANOVA Tukey's) **(E)** Rheobase obtained from PV-FSIs across treatment groups (Ctl: 242.9 ± 23.0pA, n=7; U69593: 207.1 ± 17.0pA, n=7; norBNI: 266.7 ± 38.0pA, n=6) (Ctl vs U6: p=0.59; Ctl vs norBNI: p=0.80; U6 vs norBNI: p=0.28. One-way ANOVA Tukey's).

*OPRK1 KO mice exhibit increased anxiety*

Finally, we developed a PV-FSIs constitutive *OPRK1* KO mouse to examine the behavioral role of KORs located on these cells. As partial loss of function is likely a better model for the human pathophysiological condition, we chose to examine heterozygous (+/-) KO mice. We focused our efforts on female mice as data pertaining to the role of KORs in females is particularly lacking (Chartoff & Mavrikaki, 2015). KOR agonists induce anxiety and depressive phenotypes in rodent models, so we ran a battery of anxiety and depression assays to determine if PV-FSI KORs are involved in these behaviors (Knoll & Carlezon, 2010; Van't Veer & Carlezon, 2013). We found that in the elevated plus maze $PV^{cre}$ x $OPRK1^{(+/-)}$ mice spent significantly less time in the open arms compared to their littermate controls ($PV^{cre}$) (Fig. 3.6A; Ctl: 13.6 ± 2.7%, n=5; $OPRK1^{(+/-)}$: 5.3 ± 1.5%, n=4, p=0.043). Importantly, this was not due to an effect of on general locomotor activity (Fig. 3.6B; Ctl: 1373 ± 72.2cm, n=5; $OPRK1^{(+/-)}$: 1264 ± 103cm, n=4, p=0.40). Similarly, in the open field test, $PV^{cre}$ x $OPRK1^{(+/-)}$ mice showed a trend towards spending less time in the center of the field compared to their littermate controls (Fig. 3.6C; Ctl: 340 ± 69.5s, n=5; $OPRK1^{(+/-)}$: 157 ± 58.8s, n=4, p=0.09). This trend was not driven by a change in locomotor activity during the assay (Fig. 3.6D; Ctl: 8462 ± 1063cm, n=5; $OPRK1^{(+/-)}$: 8548 ± 502cm, n=4, p=0.95). To examine the impact of the KOR KO on depressive behaviors, we put the mice through the forced swim test. $PV^{cre}$ x $OPRK1^{(+/-)}$ mice showed no differences in their latency to first immobility bout (Fig. 3.6E; Ctl: 42.8 ± 15.3s, n=5; $OPRK1^{(+/-)}$: 32.7 ± 26.21s, n=4, p=0.73) or total time spent immobile (Fig. 3.6F; Ctl: 165.5 ± 27.8s, n=5; $OPRK1^{(+/-)}$: 198.7 ± 17.7s, n=4, p=0.38). This data implicates PV-FSI KORs in anxiety but not depressive behaviors.

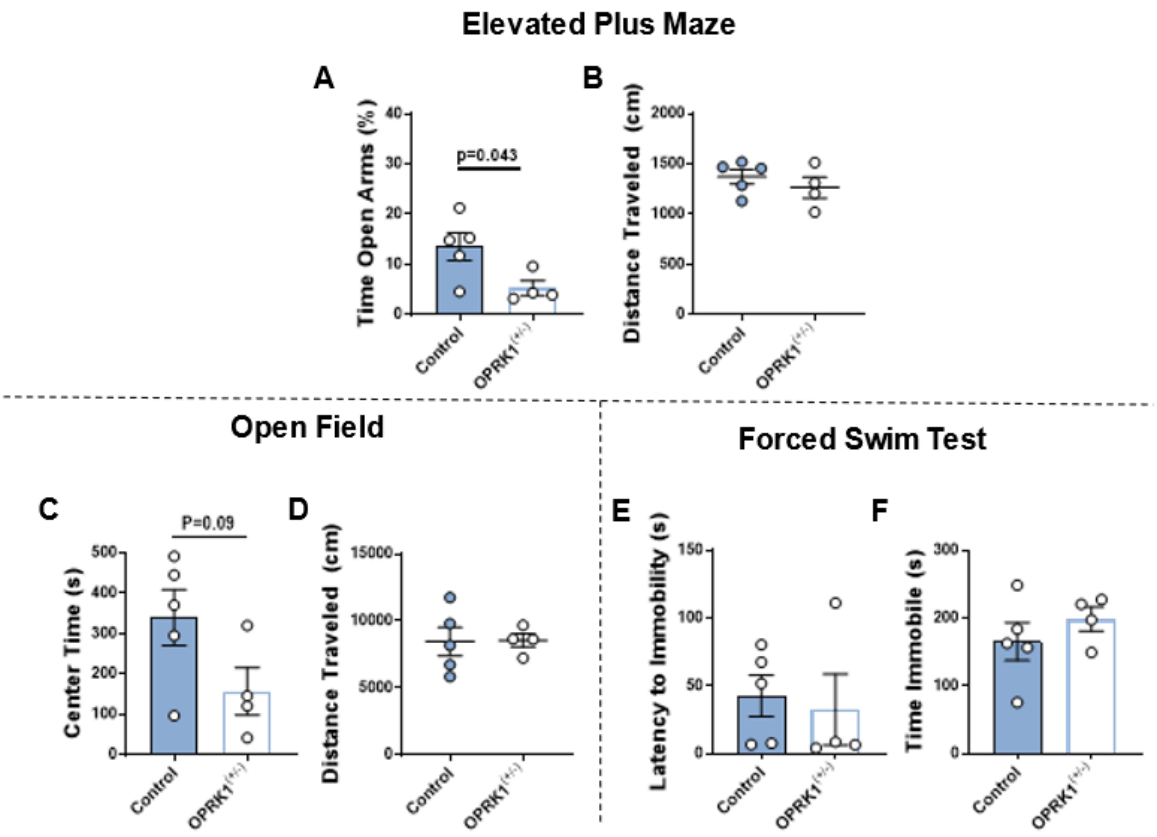


**Figure 3.6. *OPRK1* KO mice exhibit increased anxiety.** **(A)** Time spent in the open arms of the elevated plus maze (Ctl: 13.6 ± 2.7%, n=5; *OPRK1*$^{(+/-)}$: 5.3 ± 1.5%, n=4, p=0.043). **(B)** Total distance traveled during elevated plus maze (Ctl: 1373 ± 72.2cm, n=5; *OPRK1*$^{(+/-)}$: 1264 ± 103cm, n=4, p=0.40). **(C)** Time spent in the center of the open field (Ctl: 340 ± 69.5s, n=5; *OPRK1*$^{(+/-)}$: 157 ± 58.8s, n=4, p=0.09). **(D)** Total distance traveled in the open field (Ctl: 8462 ± 1063cm, n=5; *OPRK1*$^{(+/-)}$: 8548 ± 502cm, n=4, p=0.95). **(E)** Latency to immobility in forced swim test (Ctl: 42.8 ± 15.3s, n=5; *OPRK1*$^{(+/-)}$: 32.7 ± 26.21s, n=4, p=0.73). **(F)** Total time spent immobile in forced swim test (Ctl: 165.5 ± 27.8s, n=5; *OPRK1*$^{(+/-)}$: 198.7 ± 17.7s, n=4, p=0.38).

## 3.5. Discussion

NAc output is governed by PV-FSI embedded FFI microcircuits. FFI microcircuits have been shown to be involved in associative reward learning, locomotor sensitization to amphetamine, and impulsivity (Pisansky et al., 2019; Qi et al., 2016; Wang et al., 2018; Yu et al., 2017). However, the molecular mechanisms governing FFI microcircuits remain largely unexplored. Here we determined that MORs and KORs differentially decrease excitatory drive onto PV-FSIs, with MOR activation leading to a presynaptically mediated short-term depression and KOR activation leading to a postsynaptically mediated long-term depression of eEPSCs. With respect to FFI, we show that KORs preferentially decrease oIPSCs from mThal- and PFC-FFI onto D1- MSNs while only decreasing inhibition from mThal-FFI onto D1+ MSNs, leaving PFC-FFI onto D1+ MSNs intact. We also show that KORs have a strong effect on PV-FSI excitability, markedly decreasing AP fidelity during high frequency firing. Finally, we find that knocking out *OPRK1*, the gene encoding the KOR, specifically from PV-FSIs leads to heightened anxiety in mice.

The opposing synaptic expression and dampening of excitatory drive by MORs and KORs on glutamatergic synapses onto PV-FSIs represents an intriguing mechanism by which the two opioid receptors may differentially regulate NAc mediated behaviors. Given the presynaptic expression of the MOR effect on FFI, it is possible that MOR regulation of FFI is anatomically specific, only occurring during behaviors that activate specific terminals in the NAc while endorphins are being coincidently released. KOR activation leads to a persistent dampening of NAcc FFI, and therefore may have long lasting effects on behavior. These hypotheses align with the behaviors each receptor is known to mediate in the NAc. KOR signaling underlies persistent negative affective states while

MOR signaling mediates more transient behavioral states such as euphoria. It will be important to know if these mechanisms persist in the NAc shell, where opioid agonists in the rostrodorsal medial shell induce hedonic "liking" but suppress liking in the caudal medial shell (Al-Hasani et al., 2015; Castro & Berridge, 2014; Massaly et al., 2019). Interestingly, PV-FSI expression in the NAc exhibits a gradient similar to the opioid hedonic hot-coldspots, with greater expression rostrally and decreasing caudally (Trouche et al., 2019). Investigating this correlation may provide great insight into the local mechanisms that allow opioid receptors to mediate opposing behaviors in subregional compartments.

mThal and PFC projections were studied as their afferents into the NAc regulate opposing reinforcing properties of drugs of abuse (Calu et al., 2013; B. T. Chen et al., 2013; Neumann et al., 2016; Zhu et al., 2016). Given this difference we hypothesized that FFI microcircuits recruited by these two brain regions may undergo differential molecular regulation. Indeed, we found that while KORs dampened mThal-FFI microcircuits onto both D1+ and D1- MSNs, PFC-FFI microcircuits were only inhibited onto D1- MSNs. Functionally this would lead to the disinhibition of the PFC indirect pathway and disinhibition of both the mThal direct and indirect pathways. Recruitment of the dynorphin/KOR system in the NAc can be aversive and based on our data may bias NAc output to indirect pathway MSNs as well as mThal driven circuits (Al-Hasani et al., 2015; Bals-Kubik et al., 1993; Massaly et al., 2019). The biasing of activity to these pathways, in part by PV-FSI mediated FFI, may ultimately result in the aversive behaviors elicited by KOR/dynorphin signaling in the NAc. This idea is supported by the role of indirect pathway MSNs in mediating negative behaviors, as well as acute activation of thalamic afferents being aversive (Kravitz et al., 2012; Zhu et al., 2016). However, the dichotomy between direct and indirect pathway mediated

behaviors is unclear (G. Cui et al., 2013). This data provides support for the hypothesis that NAc FFI biases NAc circuit activity to ensure the recruitment of MSN ensembles necessary for proper behavioral responding (Schall et al., 2021).

An interesting result from this study was the difference in FFI latency between mThal and PFC driven circuits. Both FFI circuits exhibited significantly slower latency compared to locally stimulated IPSCs, indicative of a polysynaptic circuit. However, the significance of mThal FFI signal transduction occurring on a slower timescale than PFC FFI transduction is unknown. Changes in FFI timing can have a strong impact on MSN output as a primary function of FFI is to synchronize D1+ and D2+ activity (Damodaran et al., 2014; Gittis et al., 2010; Moyer et al., 2014). The difference in latency measured here may allow for the appropriate sequential selection of specific MSN ensembles downstream of mThal or PFC-NAc innervation. It would be interesting to know whether the difference in latency is due to conductance of the afferent, or if PV-FSIs themselves can magistrate FFI timing. As PV-FSIs are aspiny, it is difficult surmise complex computing processes such as this occurring at the afferent to PV-FSI synapse. More likely the difference in length of PFC vs mThal afferents explains the effect here. Nonetheless, this difference in timing through the FFI circuit could greatly alter MSN activity.

Behavioral studies utilizing cell-type specific knockouts of the KOR are limited. Of those that have been performed, much focus has been given to KOR mediated aversion through its signaling in dopamine neurons (Chefer et al., 2013; Ehrich et al., 2015). Global knockouts of *OPRK1* exhibit no changes affective behaviors, but do exhibit alterations in specific pain sensitivities (Filliol et al., 2000; Kieffer & Gavériaux-Ruff, 2002; Simonin & Kieffer, 1998). Other studies have

performed brain region specific knockouts of the KOR in order to validate presynaptic expression of the receptor in the NAc, but not the effect on behavior (Tejeda et al., 2017). Here we use a constitutive PV-*OPRK1* KO mouse to examine the role of PV-FSI KORs on anxiety and depressive like behaviors. In PV-*OPRK1*$^{(+/-)}$ females, we see an increase in basal anxiety suggesting PV-FSI KORs play a role in regulating anxiety. These findings differ from studies done by Filliol et al., where global KO of *OPRK1* had no effect on anxiety measures. These differences may be attributed to the heterozygous KO method used here. It is possible that decreasing KOR function may have a stronger impact on behavior than ablating it completely. Perhaps in the homozygous KO model compensatory mechanisms come into play, whereas in the heterozygous model those compensatory mechanisms do not fully develop. In agreement with previous studies, we find no effect of the genetic manipulation in the FST. There are multiple caveats to this approach. The first is that it is a constitutive KO which allows for developmental compensations to occur that could confound our results. Second is that *OPRK1* has been KO'd from all parvalbumin cells, not only PV-FSIs in the NAc. Finally, the PV-IRES-Cre mouse line used targets fewer PV+ cells in the NAc. Taking all of this into consideration, we have developed a more targeted and conditional approach utilizing a Pvalb-T2A-FlpO mouse crossed with *OPRK1*$^{(fl/fl)}$. A Flp-dependent cre virus (AAV-EF1a-fDIO-Cre) can then be injected directly into the NAc to specifically KO KORs from PV-FSIs. Plans for this mouse will be discussed in the "Works in Progress" and "Future Directions" sections of Ch. 4.

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Author Contributions

BCC, KMM and BAG designed experiments. Data analysis and construction and editing of the manuscript was performed by BCC, KMM, and BAG. BCC, KMM, and NGH performed experiments.

# CHAPTER 4

## Works in Progress, Future Directions, and Discussion

### 4.1. Works in Progress and Future Directions

*Electrophysiological characterization of a conditional NAc PV-FSI KOR KO mouse*

To better investigate the role of PV-FSI KORs in regulating NAcc FFI and to design more targeted behavioral experiments, I have developed a NAcc PV-FSI KOR conditional KO mouse line (Figure 4.1). This was accomplished by crossing *OPRK1*$^{(fl/fl)}$ (Jax B6;129-*Oprk1*$^{tm2.1Kff}$/J, Stock No: 030076) mice with PV$^{FlpO}$ (Pvalb-T2A-FlpO-D, Stock No: 022730) mice to create a *OPRK1*$^{(fl/fl)}$ x PV$^{FlpO}$ line. 4-5 week old mice homozygous for *OPRK1*$^{(fl/fl)}$ can then be injected with a FlpO dependent Cre virus (pAAV-EF1a-fDIO-Cre, addgene plasmid #121675) into the NAcc and the virus given approximately 4 weeks to express. This strategy has several advantages over the constitutive *OPRK1*$^{(fl/fl)}$ KO mouse characterized in Chapter 3. First, the conditional KO will avoid any compensatory mechanisms that may occur during development in the constitutive KO line that may be confounding the interpretation of the behavioral data in Figure 3.6. Indeed, paradoxical effects have been reported in constitutive prodynorphin KO models due to compensation of KOR expression (Chefer & Shippenberg, 2006). Second, The KOR will be regionally and cell type specifically excised due to the PV$^{FlpO}$ dependent viral strategy. This strategy will be extremely powerful compared to traditional pharmacology and genetic KO strategies as we will be able to link behavior directly to KORs on a distinct cell type within a restricted brain region. To date, breeding and acquisition of the required reagents has been completed.

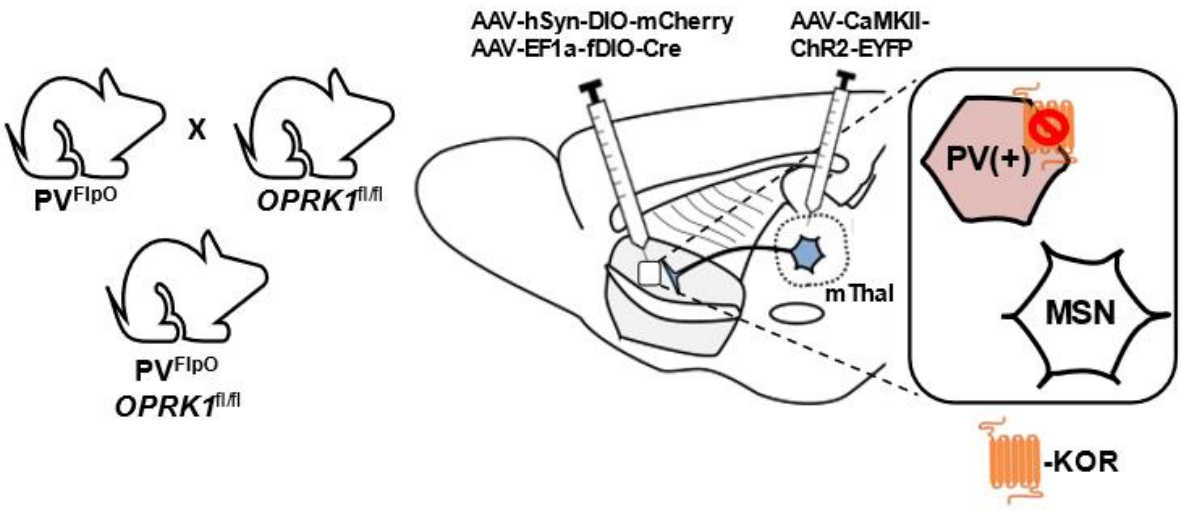


**Figure 4.1. Conditional and brain region specific knockout strategy for KORs from PV-FSIs.** Mice expressing FLP recombinase under the parvalbumin promotor will be crossed with floxed *OPRK1* mice to homozygosity. A FLP dependent cre virus will then be injected into the nucleus accumbens core along with a cre dependent mCherry virus. The mCherry florescence will mark PV-FSIs as well as serve as a proxy for successful *OPRK1* excision. ChR2 will be virally expressed in the mThal to allow for the recording of mThal driven FFI. To functionally validate the knockout, florescent PV-FSIs will be recorded. To examine the contribution of KORs on PV-FSIs to FFI, MSNs will be recorded. Once validated, this model can be used to investigate the role of nucleus accumbens PV-FSI KORs in maladaptive motivational states.

The first step in using this mouse line will be to functionally validate the KO model using electrophysiology. To identify PV-FSIs that have been transfected with the FlpO dependent Cre virus, a virus expressing Cre dependent mCherry (AAV-hSyn-DIO-mCherry, Plasmid #50459) will also be injected concurrently into the NAcc. mCherry will only express in cells that have successfully undergone FlpO dependent Cre transfection, putative PV-FSIs due to $PV^{FlpO}$ expression. This florescent strategy not only allows for the visualization of transfected PVs, but also serves as a proxy for functional FlpO dependent Cre viral expression that is required for *OPRK1* excision. To functionally validate that KORs have been KO'd from PV-FSIs, a series of

electrophysiology experiments will be performed. First, eEPSCs from PV-FSIs will be recorded and the KOR agonist, U69593, applied. I have extensively characterized that activating KORs at this synapse leads to a decrease in EPSC amplitude and that the effect is mediated by KORs on the postsynaptic cell (PV-FSIs) (Coleman et al., 2021). Therefore, in this experiment I hypothesize that washing on U69593 would have no effect on the EPSC amplitude. A null hypothesis in this experiment would most likely mean that the KO strategy was unsuccessful. However, it is also possible that there are presynaptic KORs working in concert to decrease excitatory drive onto PV-FSIs, though my data argues against this explanation. Second, the effect of U69593 on PV-FSI excitability will be investigated. I hypothesize that there will be no effect on the membrane excitability of PV-FSIs +/- U69593 in the KO mouse as opposed to the effect seen in Figure 3.5. This experiment also provides confirmatory evidence that the effect seen on excitability in Figure 3.5 is in fact due to U69593 activity at KORs, and not an off-target effect of the pharmacology. A null hypothesis in this experiment either means the KO strategy was not successful or that the effect on excitability seen with U69593 is not due to activity at the KOR.

Once the KO model has been functionally validated, the next step will be to determine the contribution of PV-FSI KORs to FFI in the NAcc. My data suggests that KORs on PV-FSIs are responsible for most of the depression KOR agonists elicit on FFI. However, FFI is a tripartite circuit and confirming the primary cellular loci to be on PV-FSIs will be extremely informative in understanding how KORs regulate FFI and the ability of this locus to modulate NAc dependent behavior. ChR2 will be virally expressed in the mThal of the KOR KO mice. I have chosen the mThal as the depression elicited by U69593 on FFI appears homogonous between D1+ and D1- MSNs while only FFI onto D1- MSNs is depressed in PFC driven FFI. There will not be an MSN

reporter for D1 or D2 in this strategy, which could lead to false positive or negative results based on population sampling in PFC driven FFI. I hypothesize that in KOR KO mice, mThal mediated FFI will be largely unaffected by U69593 compared to WT mice. A null hypothesis would suggest that KORs on either mThal projecting cells or MSNs are also contributing to the decrease in FFI. The mice required to perform this set of experiments are bred and the stereotaxic surgeries completed. Pending successful validation of the KO model, these experiments will be completed prior to the completion of my studies.

*Investigating the role of KORs on NAcc PV-FSIs in motivational behavior*

To determine the contribution of KORs on NAcc PV-FSIs to reward learning, KOR KO can be tested in cocaine conditioned place preference (CPP). Dynorphin is increased in the NAc following chronic cocaine exposure and has been implicated in the aversive properties of cocaine (Carlezon et al., 1998; Hurd & Herkenham, 1993). Therefore, the dynorphin/KOR system likely plays a role in mediating the negative reinforcing properties of cocaine. I hypothesize that KOR KO mice will exhibit no deficits in acquiring the conditioned place preference but that the persistence of the preference will be shorter. I posit that the rewarding effects of cocaine will remain intact in KOR KO mice and if anything, the formation of the place preference may even be accelerated. The persistence of the association following drug cessation is dependent, in part, on the negative reinforcing properties of cocaine. I propose that KOR KO mice will exhibit decreased negative reinforcement leading to a less persistent association.

There are several bigger picture future directions for this work that would be extremely insightful to the field of addiction and other affective behaviors such as stress or depression. Extending

behavioral studies to include negative affective states following chronic drug use, which is likely where KORs will play an important behavioral role. Negative affect increases drug seeking and craving and is a major driver of drug relapse (Hogarth, 2020), and KOR antagonists have been shown to block stress induced reinstatement to a variety of drugs of abuse (Graziane et al., 2013; Jackson et al., 2013; Mantsch et al., 2016; Polter et al., 2014). To investigate the role of KORs on PV-FSIs in negative affect, a CPA paradigm using morphine withdrawal as the aversive stimuli in PV-FSI KOR KO mice could be used. Somatic signs of opioid dependence such as rearing and tremors could be monitored as well as CPA scores to determine the contribution of KORs at this cellular locus to negative affect during morphine withdrawal. This set of experiments would provide clear evidence for KOR regulation of NAcc FFI being important for drug withdrawal induced negative affective states.

Loss of impulse control, especially during withdrawal and often triggered by stress, is a hallmark of addiction with lesion studies in mice and functional imaging studies in humans implicating the NAc as a key brain region important for impulse control (Basar et al., 2010; Kreek et al., 2005). Importantly, NAcc PV-FSIs have been shown to constrain impulsive behavior (Pisansky et al., 2019). Given what we know about KOR recruitment following chronic drug use and stressful stimuli, one possible scenario is that recruitment of KORs on PV-FSIs contributes to this loss of impulse control. Using the KOR PV-FSI KO model I have developed to examine the effect on stress induced reinstatement of drug seeking could be a highly translatable study to identify therapeutic targets for the prevention of relapse.

Exploring if KOR regulation of FFI is altered in maladaptive motivational states, including addiction, would provide key data for the underlying molecular mechanisms. Siciliano et al. provided evidence for drug induced changes in KOR activity by showing KOR sensitivity of dopamine inhibition in the NAcc is heightened in macaques following chronic ethanol exposure (Siciliano et al., 2015, 2016). By using the aforementioned behavioral paradigms in the PV-FSI KOR KO mice to provide information regarding the timing of the recruitment of this microcircuit regulatory element, electrophysiological experiments can be designed to determine any changes in KOR regulation of FFI at these time points. I have identified three separate loci for which KOR regulation of FFI could be disrupted: at excitatory synapses onto PV-FSIs, on PV-FSI cell bodies, and at PV-FSI – MSN synapses. Dysregulation of KOR activity at any of these loci could have a strong impact on FFI and downstream behaviors. Repeated KOR activation in PV-FSIs during chronic drug exposure could also lead to homeostatic changes in other signaling arms, such as PKA signaling (Muschamp & Carlezon, 2013). By using behavioral data from the KOR KO mice to identify relevant timepoints during the addiction cycle and then probing KOR regulation of FFI and its intracellular signaling mechanisms at these time points, very specific druggable targets for the treatment of addiction could be identified. It will also be important to know whether these regulatory mechanisms are limited to specific drugs or drug classes. I hypothesize that since dynorphin is increased in the NAc following various stressors, the therapeutic potential for these findings will be applicable for various NAc dependent pathological conditions.

Finally, I have gathered preliminary data for mu and delta opioid receptor (MOR, DOR) regulation of NAcc FFI. MOR activation led to a rapid and transient depression of EPSCs onto PV-FSIs while DOR activation had no effect (Fig. 4.1A,F). The effects from the DOR experiments should be

interpreted with caution however, as AR-M 1000390 is a low internalizing agonist and may activate all DOR signaling mechanisms. In contrast to the postsynaptically mediated depression by KORs, the MOR depression appears to be mediated presynaptically as indicated by an increase in CV (Fig. 4.1C). NAc MORs are involved in a number of behaviors including feeding, reward, and social attachment (Bakshi & Kelley, 1993; Y. Cui et al., 2014; Resendez et al., 2013). Future studies examining the role of MOR regulation of FFI in the NAc during these behaviors could provide further mechanistic insight into how MORs mediate signal transduction in the NAc to guide a range of behaviors.

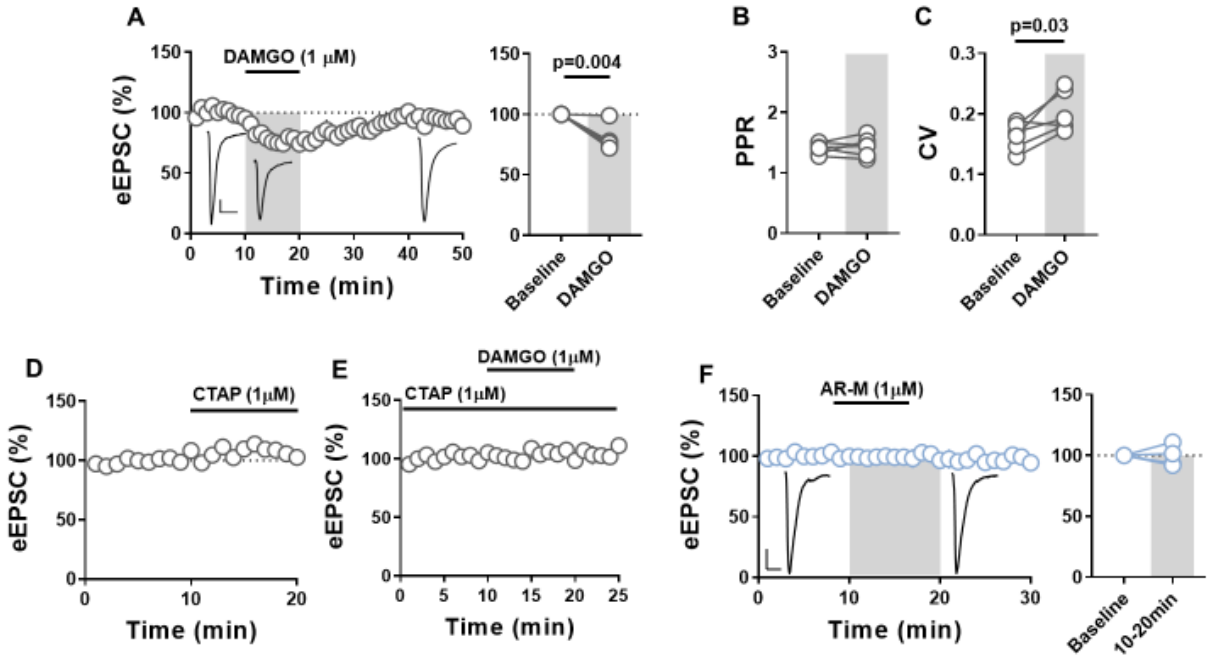


**Figure 4.2. Mu and Delta opioid receptor regulation of feedforward drive onto NAc PV-FSIs.** **(A)** Time-course summary of EPSCs onto PV-FSIs during DAMGO (1 μM) drug washes and average % EPSC amplitudes taken from baseline and 10-20 min (79.2 ± 4.0%, n=6, p=0.004). Inset are representative current traces taken from baseline, 10-20 min, and 40-50 min (scale bars = 50pA/10ms). **(B)** Average PPR taken from baseline and 10-20 min of DAMGO washes (PPR baseline: 1.41 ± 0.04; PPR 10-20 min: 1.43 ± 0.06, n=6, p=0.63). **(C)** Average CV taken from baseline and 10-20 min of DAMGO washes (CV baseline: 0.16 ± 0.01; CV 10-20 min: 0.20 ± 0.01, n=6, p=0.03). **(D)** Time-course summary of EPSCs onto

PV-FSIs during DOR antagonist, CTAP (1 µM), washes to determine tonic MOR activity. **(E)** Time-course summary of EPSCs onto PV-FSIs during CTAP (1 µM) block of DAMGO (1 µM) induced depression to test for MOR specificity. **(F)** Time-course summary of EPSCs onto PV-FSIs during AR-M (1 µM) washes (99.5 ± 4.4%, n=4, p=0.91). Inset are representative current traces taken from baseline, 10-20 min, and 40-50 min (scale bars = 50pA/5ms). Error bars indicate SEM. Error bars may not be visible when SEM is smaller than circle. Baseline = 0-10 min. Grey boxes indicate timepoint used to compare to baseline.

## 4.3. Discussion

*Conceptual advancements*

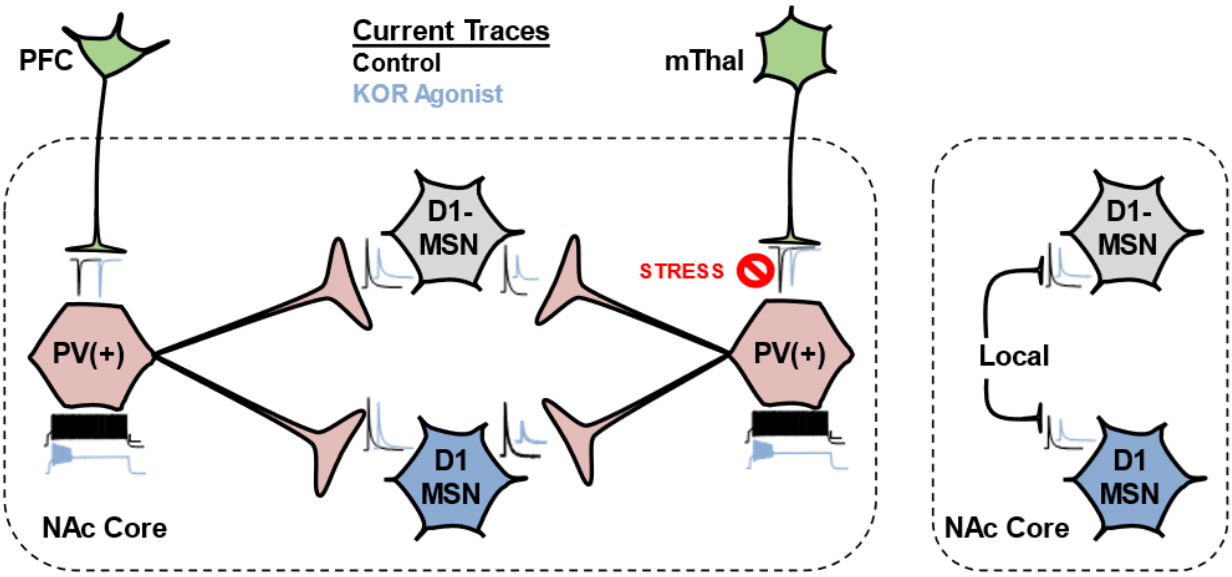


**Figure 4.3. Framework of KOR regulation of nucleus accumbens core feedforward inhibition.** The work from this dissertation is summarized here as a graphical abstract. Postsynaptic KOR signaling via a PKA/CaN mechanism differentially modulates PFC and mThal afferents onto PV-FSIs. Acute restraint stress blocks KOR mediated depression of EPSCs. Somatic KORs decrease PV-FSI excitability. KOR signaling decreases mThal driven FFI onto both D1+ and D1- MSNs while only decreasing PFC driven FFI onto D1- MSNs. Finally, KOR activation inhibits lateral inhibition between MSNs. This model provides the first framework for KOR mediated regulation of feedforward inhibitory microcircuits in the nucleus accumbens and increases our understanding of how signal transduction through the accumbens is altered during behavioral states that recruit the dynorphin/KOR system.

This body of work provides the first framework for how KORs modulate signal transduction and integration within the NAc through its actions on feedforward inhibitory microcircuits (Figure 4.3). Understanding how the endogenous dynorphin/KOR system influences NAc circuit activity gives us great insight into the mechanisms by which this system influences behavior. The dynorphin/KOR system may function as an anti-reward system to balance the rewarding properties of illicit drugs. However, following chronic drug use this system can become dysregulated, leading to the negative reinforcement of drug taking. The framework provided here may serve as a reference point for comparison of KOR activity during the cycle of addiciton to identify key loci involved in the development of maladaptive stress following drug abuse.

Prior to this work, KOR inhibition of neurotransmission and dopamine release in the NAc had exclusively been characterized as presynaptically mediated. We found that activation of KORs induced long-term depression (LTD) of excitatory drive onto PV-FSIs through postsynaptic PKA and calcium/calcineurin dependent endocytosis of AMPARs. This mechanism was determined to be KOR-G-protein signaling dependent, which opens an interesting line of inquiry regarding biased signaling. Beta-arrestin recruitment occurs following G-protein receptor kinase (GRK) phosphorylation of the receptor. GRKs are activated by direct interaction with the full length receptor, and occurs following G-protein subunit detachment from the receptor (Gurevich & Gurevich, 2019). Given this mechanism, the experiments performed in this study do not clearly differentiate between a G-protein signaling mediated effect or beta-arrestin as GDPβS likely blocks both pathways from occurring. However, the ultimate decrease in excitatory drive is due to AMPAR endocytosis, strongly implicating arrestin signaling. Repeating these experiments with biased agonists would give great insight into the signaling arms required, as well as the behavioral

state it supports. Work from the Roth and Bohn labs have shown that two distinct G-protein biased KOR agonists retain their analgesic properties while no longer inducing dysphoria or sedation that has caused KOR agonists to fail as therapeutic approaches for pain (Brust et al., 2016; White et al., 2015). This also suggests that beta-arrestin signaling, or at least non-G-protein signaling, is responsible for KOR mediated negative affective states. This evidence, as well as the obvious location of this circuit within the brain's reward circuitry, supports the involvement of the mechanism we describe as being involved in the regulation of affective states. It would be interesting to know if KORs located within areas such as the dorsal root ganglion signal primarily through G-protein mechanisms while KORs located in the brain favor arrestin signaling, explaining the effects seen with biased agonists. Indeed, G-protein biased KOR agonists do not alter dopamine dynamics in the NAc while unbiased agonists do (Brust et al., 2016). If this hypothesis holds true, then it would theoretically be possible to target KORs in the brain that regulate mood while having no effect on analgesia using beta-arrestin biased agonists. Unfortunately, very few beta-arrestin biased KOR agonists currently exist, and the ones that do are only slightly biased for beta-arrestin. The further development of these compounds will greatly enhance our understanding of the dynorphin/KOR system and open the door for novel therapeutic approaches to treat maladaptive responses to stress.

This work also highlights the ability of the KOR to bias signaling through the inhibition of inhibitory circuits, or more simply said, disinhibition of NAc output neurons. Interestingly, the circuits that we have identified as being disinhibited are known to regulate aversion, withdrawal induced negative affect, and social defeat stress (Christoffel et al., 2015; Kravitz et al., 2012; Zhu et al., 2016). This leads us to hypothesize that KOR signaling within NAc feedforward inhibitory

circuits supports these behaviors. Massaly et al. eloquently mapped out a disinhibition mechanism where KOR signaling within feedforward inhibitory circuits may serve this exact purpose. They found that negative affect following inflammatory pain is driven by the disinhibition of dynorphinergic neurons and increased dynorphin tone (Massaly et al., 2019). A likely explanation for this finding is that dynorphin release leads to a KOR dependent decrease in FFI onto dynorphin producing MSNs, serving as a positive feedback loop that increases dynorphin tone. In fact, our data shows that KOR signaling does disinhibit FFI onto D1- (dynorphinergic) MSNs in both PFC and mThal driven circuits. Our framework also provides a possible mechanistic link for the potentiation of the thalamic input into the ventral striatum following social defeat stress that underlies depressive states, as well as during opiate withdrawal that underlies withdrawal induced negative affective states (Christoffel et al., 2015; Zhu et al., 2016). Social defeat stress and morphine increase dynorphin in the NAc. It is plausible that this increase in dynorphin decreases FFI within the thalamic circuit as shown in Figure 4.3, supporting the potentiation of the thalamic-MSN circuit during these behaviors. Using the KOR KO mouse diagramed in Figure 4.1 would allow for the direct testing of these hypotheses and vastly increase our understanding for how these synaptic changes that mediate stress reactivity are governed.

The identification of postsynaptic KORs on PV-FSIs introduces many possible behavioral implications. PV-FSIs promote drug associative learning as evidenced by their ability to expedite cocaine self-administration (Yu et al., 2017). KORs have long been proposed to function as a homeostatic “anti-reward” system to counterbalance the rewarding properties of illicit drugs. This has been shown experimentally through the dose dependent increase in intracranial self-stimulation caused by the KOR agonist, U69593, which is interpreted as a decrease in the

rewarding impact of the stimulation (Todtenkopf et al., 2004). Since KORs are inhibitory, it follows that a logical locus for them to inhibit increased drug intake would be on a cell type shown to expedite drug intake, PV-FSIs. Therefore, KOR signaling on PV-FSIs may function to limit drug intake. This physiological "safety net" may then become disrupted following chronic drug use. KOR mRNA is downregulated in the NAc following chronic cocaine and ethanol consumption (Rosin et al., 1999). In this state, the KOR mediated "brake" on drug consumption may be overcome, leading to a loss of control over drug use.

In support of the loss of control hypothesis, PV-FSIs activity is highly correlated to impulsivity. Indeed, optogenetic and chemogenetic inhibition of NAcc PV-FSIs increases impulsivity (Pisansky et al., 2019). Multiple studies have shown the ability of KOR agonists to reinstate drug seeking, while KOR antagonists block stress induced reinstatement (Graziane et al., 2013; Jackson et al., 2013; Mantsch et al., 2016; Polter et al., 2014). None of these studies have identified the cellular or synaptic locus for this effect, however. Given these two lines of evidence, KORs located on NAc PV-FSIs represent an excellent candidate for mediating stress induced reinstatement of drug seeking. Currently, we are limited in our translational ability to administer drugs in such a specific manner. Experimentally, the only way to test this hypothesis is through the use of KO mouse models, as even locally infused KOR agonists will hit KORs on other cell types and terminals in the NAc. Even the KO model described in Figure 4.1 has temporal restrictions as the receptor will be absent throughout the entirety of the behavior. We also cannot rule out any unforeseen side effects caused by the removal of the KOR from the system, such as its role as a heterodimer.

Linking the behavioral effects of drugs to specific proteins in defined cells has been a long-standing problem in the field of neuropharmacology. Many of the drugs used to treat neuropsychiatric disorders have known molecular targets, though the exact circuits and cells targeted has been difficult to identify. Thankfully new tools are under development that may make this possible. Drugs acutely restricted by tethering (DART) is a technique that uses a bacterial enzyme called HaloTag to capture and tether drugs to the surface of defined cells (Shields et al., 2017). Our lab has had the opportunity to work with this technology to validate an AMPAR antagonist DART restricted to PV-FSIs in the NAc (Manz, Coleman, Grueter, et al., 2021). The HaloTag is driven by a cre-reporter line and sequesters designer ligands at approximately 100-fold enrichment on a seconds to minutes timescale. Currently, KOR DART ligands are not available, but may be in the near future. Using this technology would allow for cellular, molecular, and temporal specific pharmacology for the KOR to definitively test whether KORs on PV-FSIs in the NAc are necessary and sufficient for stress induced reinstatement of drug seeking.

Given that a wide range of illicit substances have been shown to increase dynorphin levels or KOR signaling in the NAc, it is likely that the relevance of KOR signaling in feedforward inhibitory circuits will be broadly applicable and not specific to a specific drug class. Similarly, KOR signaling been implicated in negative affective states following drug use, stress induced reinstatement, depression, and anxiety. To extrapolate to FFI, KOR activity within specific afferent driven feedforward inhibitory networks may influence distinct behaviors. For example, KOR signaling in BLA driven feedforward inhibitory circuits may contribute to anxiety disorders while signaling in mThal driven circuits may contribute to negative affect following during withdrawal.

Understanding how KOR signaling modulates other afferent driven feedforward inhibitory microcircuits is a key first step in identifying the behaviors that may be supported.

*Perspectives for the field*

PV-FSIs can sustain firing rates >50Hz. Given their role in temporally restricting and synchronizing MSN activity, it is likely that the firing rate of PV-FSIs is critical to maintaining balanced circuit activity and proper behavioral responding. In many studies investigating the contribution of PV-FSIs to behavior, 20Hz stimulation is used to drive PV-FSI activity (X. Chen et al., 2019; Yu et al., 2017). We caution the use of non-physiological firing rates when activating PV-FSI activity to drive behavior. It is possible that driving PV-FSI firing at non-physiological frequencies disrupts feedforward inhibitory circuit dynamics by altering timing. In this case, the manipulation would be performing the opposite function as intended. Similar logic can also be applied to the use of DREADDs. We must also not assume that decreasing PV-FSI activity leads to an increase in MSN firing. Lee et al. found that both activating and inhibiting PV-FSI activity in the dorsolateral striatum led to a decrease in MSN firing (Lee et al., 2017). This effect was attributed to the disinhibition of other inhibitory interneurons in the striatum following PV-FSI inhibition. Therefore, PV-FSIs may exert their effects through other disynaptic circuits and not directly through their activity on MSNs.

We also encourage the field to focus more attention towards KOR cellular location and signaling mechanisms. While these studies may not be the flashiest or easiest to perform, it is my strong belief that understanding the distinct cells types and signaling arms responsible for the effects of KOR pharmacology will be required for the successful development of KOR ligand based

therapeutics. The vast majority of drugs targeting the KOR have shown little efficacy in the clinic despite large amounts of preclinical data promoting their utility. While manipulating rodent behavior in a manner that suggests therapeutic potential may garner a high impact paper, understanding the mechanisms by which the behavior is changed will lead to better translational capabilities. The data strongly supports the use of KOR targeted pharmacology to treat a wide array of neuropsychiatric disorders and it is our responsibility as scientists to uncover how this system works. The devil is in the detail, and more pointed studies investigating KOR signaling events underlying behavior will pave the way for the development of more targeted therapeutic approaches.

*Conclusions*

As discussed in detail in this dissertation, PV-FSI embedded feedforward inhibitory circuits within the NAc are vital for the integration of cortical, limbic, and thalamic signals into goal-directed motivational behavior. As such, the molecular and electrophysiological profile of PV-FSIs are fine tuned to allow for the rapid coordination of circuit output. Their fast-gating AMPARs, rapid vesicular release properties, and fast-spiking capabilities all enable PV-FSIs to carry out microcircuit processing more efficiently than other NAc interneuron populations. It is likely that PV-FSIs use this highly specialized repertoire to support a range of NAc dependent behaviors. The ability of endogenous receptor systems to regulate FFI likely underlies shifts in NAc circuit activity required to support appropriate motivational responding. In the case of KORs, KOR signaling may be required for beneficial shifts in circuit activity that trigger negative affective states evolutionarily developed to discourage the continued use of illicit substances. However, upon repeated drug exposure these signaling mechanisms may become dysregulated leading to the

negative reinforcement of the same substances. It is my hope that the framework provided here serves as a starting point for future investigations into the behaviors mediated by KOR signaling within feedforward inhibitory networks, and how this signaling is altered during maladaptive motivational states. In my humble opinion, this work has the potential to bring to light novel therapeutic targets for the treatment of various neuropsychiatric disorders including addiction, PTSD, anxiety, and depression.

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