Characterizing the mechanisms of kappa opioid receptor signaling within mesolimbic dopamine circuitry

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Abstract & Public Summary

An essay is due, your rent check is late, the national guard has been deployed to your city, you are experiencing violence within your own home: humans experience stress in varying degrees and durations throughout their lives, and the severity and proportionality often align with societal inequities. As stress neurobiologists, we play a role in describing the ways this stress manifests in our bodies, how it forever alters the pathways in our brains and shifts our own reactions to the world around us. In part, we do this work to understand the basic science of stress and how it increases risk for diseases like heart disease, stroke, hypertension, anxiety, depression, and substance use disorders. We also seek to find treatments, so that while society inflicts unearned stressors on its most vulnerable citizens, we may use pharmacology to ameliorate a fraction of its harms.

Our bodies use neuropeptides to modulate our behavioral and emotional responding to stimuli, like stress. These small proteins act on receptors in our brain cells, or neurons, to promote certain behaviors and make long-lasting changes in neural connectivity. The opioid peptide dynorphin is produced in multiple brain regions, including those involved in decision making and reward learning, and plays an important role in the stress response. When released in response to drug-taking or stress, dynorphin (dyn) binds to a receptor called the kappa opioid receptor (KOR). Years of research from the Chavkin lab and others have shown that signaling at the kappa opioid receptor contributes to increased depression, anxiety, and substance use after the experience of behavioral stress. Researchers have also shown that molecules which can block dynorphin from binding to its receptor can reduce these negative psychological consequences. This dissertation digs into two big questions surrounding the effects of kappa opioid receptors: (1) How do the antagonists, or compounds which disrupt KOR signaling, work and how does

this knowledge help us better design therapeutic compounds in the future and (2) How do KORs signal within dopamine neurons, a population of neurons involved in decision making & reward that is altered by stress.

In Chapter 1, I review the literature evaluating the therapeutic potential for KOR antagonists in treating mood disorders and substance use disorders. The chapter describes studies that demonstrated the role of dyn/KOR in mood and substance use disorders, reviews the brain regions critical for these behaviors, and discusses the importance of biased agonism and development of functionally selective ligands.

In Chapter 2, I assess the role of c-Jun Kinase (JNK) signaling both by KOR antagonist norbinaltorphimine (norBNI) and Dopamine D2 Receptor agonist quinpirole. Both agonists and antagonists of the KOR can stimulate JNK signaling, producing reactive oxygen species and disrupting signaling. We found that Quinpirole can stimulate JNK-dependent ROS at both D2 receptor isoforms. We also found that NorBNI pretreatment shifts the dose response to Quinpirole at inhibiting dopamine release, indicating that JNK-ROS stimulated by the KOR can disrupt signaling at neighboring G_i-coupled receptors.

In Chapter 3, I present findings from two collaborations assessing the regulation of presynaptic dopamine release in the nucleus accumbens. We found that KOR-mediated inhibition of dopamine release is less potent in female mice, and that this effect is blocked by the GRK inhibitor CMPD101. In a collaboration with the Zweifel lab, I also helped demonstrate that different neuropeptide marker genes, CCK and CRHR1, segregate populations of dopamine

neurons which project to the NAc Shell and NAc Core, respectively.

In Chapter 4, I present findings which discuss the mechanism and limitations of KOR antagonist norBNI. We found that there are sex-differences in the long-lasting nature of norBNI, which are prevented by pretreatment with GRK inhibitor CMPD101. We also found that norBNI is not long-lasting at dopamine terminals, an effect that may be due to the lack of ROS production downstream of norBNI in NAc terminals.

In Chapter 5, results from a series of experiments assessing the role of potassium channels in the kappa opioid receptor regulation of dopamine neurons. We used a CRISPR-Cas9 based system to knockdown genes for $K_V1.2$, $K_V3.4$, and Kir3.2 or GIRK2 (KCNA2, KCNC4, KCNJ6). We then assess the effect of those knockdowns on basal dopamine physiology and KOR signaling in dopamine neurons.

Dedication

I dedicate this work, with gratitude & love, to my family: Mat, Jess, Sandell, Steve, and Fika.

This dissertation was written in the midst of the 2020 COVID-19 Pandemic. I also dedicate this work to the first responders, essential workers, medical professionals, basic scientists, virologists, epidemiologists, and vaccinologists fighting for humanity. To the souls we've lost and their families and those still battling this virus.

Chapter 1

The therapeutic potential of the targeting the kappa opioid receptor system in stress-associated mental health disorders

Introduction

The stress response evolved as an adaptive phenomenon that enhances our ability to respond to threats, redirecting resources from immune protection and metabolism to short-term survival (Chrousos & Gold 1992). A complement of signaling cascades activated by both the peripheral endocrine, sympathetic nervous system, and central neuropeptide systems occurs as a part of the physiological response to stress. Corticotropin-releasing factor (CRF) stimulates the peripheral stress response by inducing the release of adrenocorticotropic hormone from the pituitary which, in turn, activates the hypothalamic-pituitary-adrenal axis. CRF also binds to CRF receptors (CRFR1 and CRFR2) within the central nervous system, where it stimulates downstream effectors and behavioral responses to stress. In addition to its adaptive benefits such as increasing arousal, the acute experience of stress can elicit feelings of fear, anxiety, and a loss of cognitive flexibility; repeated or chronic stress is linked to an increased risk for depression, anxiety, and substance use disorder (Kessler 1997, Gold 2002, Gold 1998, Koob 2008). In order to understand this transition from acute, adaptive stress, to maladaptive stress and stress disorders, it is important to study the mechanism of its molecular effectors, including endogenous opioids.

Endogenous opioids are critical modulators of behavior: shaping responses to natural rewards, threats, and social interaction and contributing to maladaptive behavioral responses in disease states like mood and substance use disorders (Shippenberg 2007, Paredes 2014, Le Merrer 2009, Yeomans 2002, Lutz 2013). Dynorphin, the endogenous ligand for the kappa

opioid receptor, is functionally involved in analgesia, feeding, temperature regulation, and the psychological response to stress (Chavkin 1982, Morley 1983, Przewlocki 1983, Han 1982, Chavkin 2016). Both agonism and antagonism of its receptor, KOR, can reduce drug seeking behavior, depending on the timing of drug treatment and the signaling properties of the ligand (McLaughlin 2006b, Smith 2012, Kuzmin 1997, Mello & Negus 1998, Chartoff 2016). Characterizing the intricacies of KOR signaling and the neuroanatomical substrates for KOR action can further elucidate how the dyn/KOR system contributes to such a diverse array of stress-associated behaviors and psychological disorders.

Kappa opioid receptors are 7 transmembrane, G-protein coupled receptors (GPCRs) which, upon agonist binding, induce nucleotide exchange, dissociating the G-proteins from the receptor and initiating signaling (Childers & Snyder 1978, Minneman & Iverson 1976). Opioid receptors are generally inhibitory on the activity of neurons, which is attributed to their coupling to the $G\alpha_{i/o}$ family of G proteins (Taussig 1993). Endogenous opioids derived from prodynorphin specifically activate the kappa opioid receptor, and KORs are widely expressed throughout the central nervous system (Chavkin 1982, Schwarzer 2009). Upon ligand binding, KORs can activate multiple second messengers, including Gβɣ, ERK1/2, p38α MAP-Kinase, and c-Jun Kinase, depending on a dynamic set of possible conformations and signaling states (Bruchas & Chavkin 2010, Urban 2007). Dynorphin can activate each of these signaling cascades, but some synthetic agonists preferentially activate only one or several KOR second messengers, a phenomenon known as functional selectivity (Urban 2007, Kenakin & Miller 2010). The molecular basis for functional selectivity at KOR is not fully understood, but recent studies using nanobody stabilized structures of the KOR show that its conformation and ligand binding affinity change substantially depending on the structure of the molecule in the binding pocket and single

amino acid changes can dramatically shift the nanobody stabilized structure (Che 2018, Che 2020). These insights support the long-held hypothesis that different ligands induce different conformations of the receptor that result in different efficiency of G-protein activation. These conformational differences are what distinguish ligands with high intrinsic effciacies, partial agonists, and neutral antagonists. However, the functional selectivity concept posits that a ligand's intrinsic efficacy at GβɣɣGβɣ signaling is independent from its intrinsic efficacy at regulating effectors controlled by GRK3/β-arrestin activation. The medicinal chemistry required to establish the validity of this concept is still being explored (Ho 2018, Brust 2016, Bedini 2020; Dunn & Kreek 2019). Nalfurafine is an example of an efficacious KOR agonist that seems to show strong functionally selectivity for GβɣɣGβɣ over GRK3/β-arrestin signaling (Schattauer 2017). Further development of functionally selective KOR agonists holds promise that opioid medications might be generated that produce analgesia without adverse cognitive disruption (Mores 2019, Coussens 2019). Understanding which signaling cascades are activated by KOR ligands and where in the brain they act has been an area of active study to determine the therapeutic potential of kappa opioid receptor-targeting drugs in treating mood disorders and substance use disorders.

Since finding that dynorphin signaling generated the dysphoric component of stress, studies have demonstrated that there are multiple, redundant brain regions that contribute to the mood disturbances associated with KOR signaling. Much work has gone into parsing the mechanism by which dynorphin contributes to human and animal behavior, and this chapter will walk through the effect of KOR in multiple brain regions implicated in mood disorders and substance use disorders. Recent findings about functional selectivity of the kappa opioid receptor further inform our understanding of the mechanisms by which KOR signaling contributes to

depression, anxiety, and substance use disorder. This review will parse both where the dyn/KOR system acts to modulate behavior and how signaling cascades downstream of KOR shape behavior under acute stress, after chronic stress, and during drug withdrawal and relapse.

Activation of the dynorphin/kappa opioid receptor system is associated with dysphoria, cognitive disruption, and increased preference for drugs of abuse. Although kappa opioid agonists were first proposed as potential pain pharmaceutics due to their analgesic properties that were distinct from the addictive mu opioid analgesics, it quickly became clear that certain KOR agonists has striking psychotomimetic effects: causing hallucinations, dysphoria, and mood disturbance in humans (Pfeiffer 1986, Prisinzano 2005*)*. This clinical observation and the finding that KOR agonists reduce dopamine release (Di Chiara & Imperato 1988), led to the prediction that KOR agonists may suppress drug seeking by reducing drug-induced dopamine responses (Spanagel 1992). This prediction was borne out in studies that demonstrated that the KOR agonist U69,593 attenuated a drug primed cocaine reinstatment and decreased cocaine seeking behavior (Schenk 1999, Schenk 2000). Acute kappa agonist treatment can attenuate responding to reward, attenuate responding for cocaine reward, and acutely increase the threshold for intracranial self-stimulation (Kivell 2018, Chartoff 2016). Further, the KOR antagonist, norBNI, can increase ethanol consumption in high-ethanol drinking rats (Mitchell 2005, Walker & Koob 2008). However, this acute reduction in behavioral responding for drugs of abuse caused by KOR agonists is substantially counterbalanced by a prolonged increase in depressive and anxiogenic behaviors by the kappa opioid receptor (Chavkin & Koob 2016). Because drug-seeking behavior is increased in a KOR-dependent manner, KOR antagonists are a more promising therapeutic than KOR agonists.

Chronic behavioral stress, like KOR activation, can acutely reduce behavioral responding for reward, induce mood disturbance, and disrupt cognition (Kivell 2018, Abraham 2017, Pfieffer 1986, Fellinger 2020, Paris 2011*)*. Substantial literature now demonstrates that the dyn/KOR system is engaged in encoding the dysphoric component of stress: dynorphin is released in multiple corticolimbic brain structures downstream of CRF after stressors such as swim stress, social defeat stress, and foot shock (Land 2008). Antagonism of KOR with norBNI or other antagonists disrupts swim immobility in a forced swim test, blocks conditioned place aversion to stressful stimuli, and reduces social defeat (Land 2008, McLaughlin 2006a). This dysphoric response to KOR agonists can also increase the negative affect associated with acute pain despite its analgesic effects. For example, KOR agonist U69-593 increases acid-induced reductions in iCSS while also reducing paw responses to pain (Negus 2010).

Many early studies deciphering the role of the dyn/KOR system in substance use disorder indicated it could work to attenuate drug taking: data show that cocaine consumption increases the dynorphin message in the striatum and predicted that it could act as an inhibitory brake to oppose the excitatory, pro-dopamine release effects of cocaine, but also predicted that its dysphoric effects would persist, perhaps contributing to the experience of withdrawal (Hurd & Herkenham 1993, Carlezon 1998, Muschamp & Carlezon 2013) . An abundance of studies suggest that chronic stress exacerbates compulsive drug taking and seeking: inducing relapse, increasing escalation, and modulating the mesocorticolimbic dopamine system implicated in the physiological adaptations to drug taking (Breese 2005, Sinha 2007, Norman 2015, Tye 2012, Holly 2015). Both repeated forced swim stress and treatment with KOR agonists 60 minutes prior to cocaine conditioned place preference (CPP) increase a mouse's preference for cocaine, a phenomenon known as cocaine CPP potentiation (McLaughlin 2006b). This CPP potentiation

phenotype has been recapitulated with other drugs of abuse including nicotine and alcohol (Sperling 2010, Smith 2012). KOR agonists like U50,488 can also induce this potentiation of drug preference. KORs are necessary for stress-induced potentiation of drug preference, as shown by the fact that pretreatment with norBNI, the KOR antagonist, prior to behavioral stress blocks the effect (Sperling 2010). Although conditioned place preference models components of drug salience and drug seeking behavior, these studies do not, alone, indicate that KOR activation is sufficient to drive increased drug taking and seeking after stress. They did, however, provide evidence that KOR antagonists may be a more effective treatment in reducing compulsive drug seeking behavior, particularly in dependent individuals or after a stressor.

Contribution of the dyn/KOR system to substance use disorder, anxiety, and depression

KOR antagonists can reduce drug-taking in preclinical models of addiction and are in clinical studies for use in treating substance use disorders**.** Systemic KOR antagonism by norBNI can reduce the excessive alcohol sef-administration detected in ethanol dependent rats (Walker 2010). Deletion of the kappa opioid receptor decreases ethanol preference in a two-bottle choice paradigm and subcutaneous treatment with the KOR antagonist JDTic reduced ethanol drinking in ethanol preferring rats (Van't Veer 2016, Uhari-äänanen 2018). This phenotype appears to be specific to dependent animals. In a study comparing norBNI (a KOR antagonist), nalmefene (a KOR partial agonist/antagonist and a MOR antagonist with higher affinity for KOR), and naloxone: naloxone reduced ethanol drinking in all rats, norBNI reduced ethanol drinking only in dependent mice, and nalmefene reduced ethanol drinking in both populations (Nealey 2011). Inhibition of KOR can also reduce cocaine intake after extended access, block stress-induced relapse in rats, and inhibit amphetamine-abstinence induced withdrawal (Wee 2009,

Graziane 2013, Raffa 2008). Repeated stress exposure can increase the behavioral economics measure of maximum price value for cocaine, P_{max}, and this stress-induced increase in P_{max} be blocked by KOR antagonist norBNI (Groblewski 2014). This literature illuminates how KOR activation can enhance compulsive drug seeking (Koob 2013). In addition to its impact on drug use, behavioral stress KOR activation can contribute to anxiety and depressive-like phenotypes (Lalanne 2014).

The dyn/KOR system also contributes to depression and anxiety-like behavior. Dynorphin mRNA and KOR receptors are found in brain regions related to stress and anxiety in rodents, such as the paraventricular neucleus of the thalamus (PVN), the bed nucleus of the stria terminals (BNST), and the amygdala (AMY) (Lin 2006, Morris 1986). Recreational users of the KOR agonist salvinorin A reported increased anxiety in a self-rated scale and dynorphin expression is necessary for the generation of stress-induced anxiety- and depression-like behavioral responses (González 2006, McLaughlin 2003). KOR agonists can produce anxiety-like behaviors on the elevated plus maze, and deletion of KOR or dynorphin reduces anxiety like behaviors in the open field and light-dark box (Gillett 2013). Despite this, deletion of prodynorphin can also increase anxiety-like behavior in some tasks and in the hands of some experimenters (Kudryavtseva 2004, Kudryavtseva 2005). These findings indicate that KOR signaling likely plays a role in modulating anxiety, which may depend on *where* in the brain dynorphin is released and the duration or amount of release. Systemic KOR antagonists are able to block traits associated with depression including immobility in the forced swim test, social defeat postures, and learned helplessness in escaping footshock (Carr 2010, Mclaughlin 2006a, McLaughlin 2003). A study of buprenorphine, a μ-opioid receptor agonist and KOR antagonist, demonstrated that the antidepressant-like effects of buprenorphine are mediated by kappa opioid

receptors (Falcon 2016), and several compounds have been assayed for anti-depressant efficacy in humans (Li 2016), showing mixed results depending on study design (Krystal 2020, Karp 2014). In order to improve drug design and understand the contradictions in the behavioral effects of KOR agonists and antagonists, its important to look at how differences in dose, study design, and the type of agonist or antagonist used contributes to this variable.

As its been clearly established that the KOR plays an important role in encoding some of the psychologically harmful aspects of chronic stress, it is also critical to determine where in the brain dynorphin is released and KOR is expressed to understand how dyn/KOR modulates physiology in these brain regions to contribute to the behavioral phenotpyes of stress. Research into the neural substrates of stress and the dyn/KOR system has uncovered multiple, possibly redundant loci contributing to behavioral phenomenon like swim immobility, stress-induced relapse, reduced social interaction, and other depressive, compulsive, and anxiogenic behaviors. Within the next section, we will review the mechanisms by which dorsal raphe serotonergic- and mesolimbic dopaminergic circuitry contribute to the behavioral effects of KOR activation. We will then describe how KOR receptors in their projection targets in the nucleus accumbens, prefrontal cortex, and amygdala, both on presynaptic serotonergic and dopaminergic terminals and local neurons, also contribute to the modulatory effects of KOR on mood.

KORs are expressed on dorsal raphe serotonin neurons and contribute to stress-induced plasticity within serotonin circuitry. Serotonin levels in the brain have long been associated with mood disturbance and depression, and the dorsal raphe serotonin neurons are vulnerable to physiological changes after behavioral stress (Mahar 2014, Bambico 2009, Jans 2006, Grahn 1999). Swim stress alters the physiology of serotonin neurons in the DRN, elevating the

frequency of and reducing the amplitude of spontaneous extracellular post-synaptic potentials (EPSPs) (Kirby 2007). Chronic stress can increase serotonin in the rat brain (Adell 1988, Mo 2008), but acute stressors can also reduce serotonin levels by increasing reuptake (Schindler 2012). Due to its role in stress and depression as well as its ability to modulate signaling through acute, g-protein mediated events and prolonged p38-MAPK dependent changes, the KOR was proposed to be a critical modulator of serotonin neurons.

Local KOR antagonist, norBNI in the dorsal raphe nucleus blocks place aversion to U50,488, indicating that the receptors in the DRN are critical to the formation of an aversion to KOR agonists (Land 2009). Further, local norBNI in the DRN prevents the social defeat stress-mediated reinstatement of cocaine preference, demonstrating the KOR in the DRN are necessary for this behavioral response to stress (Land 2009). Interestingly, these effects may depend on prior experience of stress. One study in California mice found that directly injecting the KOR agonist, U50,488, into the DRN induces anxiety-like behaviors in unstressed mice but alleviates social defeat behaviors in defeated mice (Wright 2018). Acutely, KOR agonists may be able to increase extracellular serotonin and attenuate cocaine primed drug reinstatement (Rüedi-Bettschen 2010), but repeated forced swim stress and U50,488 treatment can also increase the surface expression and reuptake of serotonin, reducing extracellular serotonin (Schindler 2012). These seeming contradictory results make clear that KOR modulation of serotonin neurons is variable depending on the stressor, the duration of the stressor, or the duration/dose of agonist treatment.

KOR activation modulates the DRN to nucleus accumbens (NAc) projection in a p38-MAPK dependent manner, shaping behavioral responding after stress. Application of U69,593 to serotonin neurons leads to an increase in Ba+-sensitive potassium conductance attributed to a G-

protein coupled inwardly rectifying potassium channel (GIRK), a decrease in the amplitude of evoked EPSCs, and a decrease in the frequency, but not amplitude of mini-EPSCs (Lemos 2012). Exposing animals to repeated forced swim stress generates dynorphin release and phosphorylation of the kappa opioid receptor and GIRK subunit in the dorsal raphe nucleus. This phosphorylation of the Kir3.1 subunit of GIRK leads to a p38-MAP Kinase dependent reduced U69,593 activation of GIRK conductance. This study demonstrated that repeated forced swim stress changes the physiological influence of KOR on DRN serotonin neurons: after stress presynaptic glutamatergic inputs onto serotonin neurons were still inhibited by U69,593, but GIRK channels no longer increased their conductance (Lemos 2012). This leads to the prediction that prior stress experience primes the serotonin system to be more active and less inhibited by KOR agonists. In the NAc, however, repeated forced swim stress increases the surface expression of SERT, in a KOR- and p38 MAPK-dependent manner, reducing the amount of serotonin in the postsynaptic space (Schindler 2012). More work is needed to understand how KOR signaling modulates serotonin release both during and after a stressor.

Kappa opioid receptor expression in the VTA contributes to the behavioral response to stress. The ventral tegmental area (VTA) dopamine system consists of a dense population of dopamine neurons and is regulated by local GABAergic neurons and inputs from many brain nuclei engaged in motivation, fear responding, and reward learning (Berke 2018, Pignatelli 2017, Zweifel 2011, Schultz 1998*).* Dopamine neurons are important for reward learning, and have been modeled to compute reward prediction errors, encoding the difference between an expected reward and that received after a known cue (Schultz 1997, Cohen 2012, Bayer 2005). Despite this long-understood relation to reward, there is substantial literature demonstrating that

dopamine neurons encode general salience and can respond to aversive stimuli such as airpuff, footshock, and tail pinch (Budygin 2012, Brischoux 2009, Zweifel 2011), and footshock can increase FOS in dopamine neurons (Morrow 2000). Dopamine neurons are engaged in aversive learning, or processing of salient stimuli that cause animals to engage in unconditioned escape or avoidance responses (Fuchs & McKenna 2008), and a subpopulation of them are stimulated by footshock cues and encode uncertainty in fear generalization to shock (Jo 2018). In addition to increases in dopamine in response to aversive stimuli and cues, an absence of dopamine can signal an aversive event or act as a "negative reward signal" (Danjo 2014, Saunders 2018). After chronic mild stress, stimulation of dopamine neurons is sufficient to rescue depression-like behaviors, including reducing immobility in forced swim stress and rescuing sucrose preference in stressed mice (Tye 2013). These data lead to the prediction that selectively activating a subset of dopamine neurons involved in aversive learning may itself induce aversive learning, but that inhibiting all dopamine neurons or reducing dopamine release can also be an aversive stimulus associated with negative mood. It is possible that through direct action on dopamine neurons or action on presynaptic inputs to VTA dopamine neurons, KOR may play a role in modulating both the increases and decreases in DA associated with stress and fearful or aversive events.

Kappa opioid receptors are expressed on the presynaptic terminals of dopamine neurons and inhibit the release of dopamine (Di Chiara & Imperato 1988, Svingos 2001). This acute inhibition of dopamine release by KOR agonists has been recapitulated both in vivo and in ex vivo slices (Ehrich 2014, Ehrich 2015, Rose 2016). Elevated dopamine release during drug-taking increases activity of D1 medium spiny neurons, which release the KOR agonist, dynorphin. It is hypothesized that the potentiated dynorphin release during drug seeking leads to activation of the kappa opioid receptor on DA terminals, reducing DA release and generating the

negative affective state associated with withdrawal (Nestler 2004, Koob 2008, Trifilieff 2013). Dyn/KOR signaling on DA neurons is likely involved in predisposing stressed individuals to substance use disorder *and* in facilitating the development of a substance use disorder through nucleus accumbens (NAc) dynorphin signaling. In a model of alcohol use disorder, KORs are more sensitive to agonist treatment in dopamine terminals after chronic intermittent ethanol exposure. This augments the dyn/KOR system and helps to drive a reduction of dopamine release in the NAc, generating a sign change in dependent animals: switching ethanol from being dopamine enhancing to dopamine inhibiting after chronic ethanol exposure (Karkhanis 2016). Despite this robust inhibitory effect, much of the evidence points to somatic kappa opioid receptors within the VTA as essential for behavioral responses like conditioned place aversion to KOR agonists and cocaine place preference potentiation.

Conditional knockout of kappa opioid receptors from the ventral tegmental area reduces anxiety-like behavior and prevents conditioned place aversion to U50,488 treatment (Van't Veer 2013, Chefer 2013, Ehrich 2015). Lentiviral expression of KOR in the VTA is sufficient to reinstate a CPA to U50,488: indicating that although VTA is not sufficient to cause CPA in a wild type animal, it is in a knockout mouse. norBNI injected directly into the VTA blocks U50,488 CPA and compulsive behaviors such as increased marble burying and burst error responding in the differential-reinforcement-of-low-rate-schedule (DRL) task (Ehrich 2015, Abraham 2017). This indicates that KOR activation in DA neurons disrupts behavioral inhibition. Related to this, KOR activation appears to potentiate compulsive checking behavior in the quinpirole model of obsessive-compulsive disorder (Perreault 2007). Repeated KOR activation can facilitate dopamine neurotransmission and dopamine-mediated compulsive behaviors (Escobar 2020). These effects on compulsive behavior after chronic treatment indicate

there is a “switch” in the directionality of KORs effect on dopamine neurons after repeated treatment.

In order to understand the mechanisms by which KOR may switch from being primarily inhibitory at dopamine terminals and hypolocomotive, to increasing compulsive behaviors and drug seeking after prolonged or repeated activation, it is important to look at how KORs change DA physiology. KOR agonists hyperpolarize a subset of dopamine neurons within the VTA, an effect that appears to be species dependent: U69,593 hyperpolarizes mPFC projecting dopamine neurons in Sprague-Dawley rats (Margolis 2006) and inhibits NAc-projecting and BLA-projecting dopamine neurons in C57BL6 mice (Ford 2006). When administered to mice in vivo, a single injection of U50,488 does not change firing frequency or interspike interval of dopamine neurons, however a second U50,488 injection the following day reduced firing frequency in the same mice (Ehrich 2015). In wild type mice, this effect did not persist in response to a third U50,488 agonist, but in mice lacking p38-MAP Kinase in dopamine neurons, the reduction of firing frequency after U50,488 persisted. An acute cold swim stress ablates long term potentiation (LTP) of presynaptic GABAergic inputs to VTA DA neurons, an effect that can be blocked by norBNI (Polter 2014, Polter 2017). Treatment with U50,488 can also ablate the LTP GABA effect similarly to stress, indicating that KOR on presynaptic GABAergic inputs onto VTA dopamine neurons may attenuate inhibitory signaling onto dopamine neurons after stress (Polter 2017). These data indicate that KOR works through multiple mechanisms within the VTA both on presynaptic terminals and on dopamine neurons, to modulate activity after stress or repeated KOR treatment.

KOR activation also modulates excitability and dopamine circuit dynamics in the nucleus accumbens. There are multiple mechanisms for presynaptic inhibition including blockade of

voltage-gated potassium channels, regulation of synaptic vesicle release machinery, and increased potassium channel conductance but the primary mechanism(s) responsible for the regulation of DA release by KOR are not known (Berecki 2016, Miller 1998, Martel 2011). KOR activation by Salvinorin A can increase the rate of DA uptake through the dopamine transporter, DAT (Kivell 2014). Nalmefene, a mixed agonist/antagonist of KOR and MOR receptors, can reduce DA uptake through DAT, but in both ex vivo and in vivo dopamine voltammetry studies, U50,488 and U69,593 had no impact V_{Max}, indicating no change to DAT reuptake of dopamine in the NAc (Rose 2016, Ehrich 2014, Ehrich 2015). Partial KOR agonists nalfurafine and nalmefene inhibit dopamine release without causing aversion, demonstrating the separability of these two phenomena (Browne 2020, see Chapter 4). Increased dynorphin in the nucleus accumbens is associated with depression and drug-use: viral-delivered shRNA against pDYN in the NAc attenuates depression-like behavior and acute nicotine enhances striatal prodynorphin mRNA (Cohen 2013, Isola 2009). NorBNI in the NAc can reduce drug-taking and escalation of drug use (Rose 2016, Whitfield 2015, Schlosburg 2013), an effect potentially caused by KOR on serotonin terminals, dopamine terminals, and medium spiny neurons.

There is substantial evidence of plasticity in dopamine circuits mediated by the kappa opioid receptor due to stress or escalating drug use. Somatic KORs on dopamine neurons hyperpolarize some DA neurons at baseline and reduce firing frequency in vivo after multiple injections (Ehrich 2015).

Other brain regions contributing to the KOR-dependent behavioral response to stress. Although dopaminergic and serotonergic circuitry appear critical for the KOR effects on mood, kappa opioid receptor signaling has been shown to modulate behavior through action in other

brain regions as well. KOR agonists in the intra-paraventricular thalamus (PVT) can inhibit drug-seeking behavior. This PVT effect is dependent on dynorphin inhibition of postsynaptic PVT neurons (Chen 2015). The basal forebrain cholinergic neurons, important for arousal, are also modulated by dynorphin neurons, indicating a role for KOR signaling in tempering arousal (Ferrari 2016). NorBNI injected into the locus coeruleus (LC) attenuates, but does not completely block stress reinstatement of cocaine CPP, indicating that LC KORs contribute to this behavior (Al Hasani 2013). Neurons in both the central amygdala (CeA) and basolateral amygdala (BLA) are modulated by kappa opioid receptors. KOR inhibits BLA terminals in the bed nucleus of the stria terminalis (BNST), dampening the anxiolytic effects of this BLA to BNST circuit (Crowley 2016). Dynorphin decreased excitatory IPSCs onto CeA neurons, opposing the action of ethanol and its anxiolytic effects (Gilpin 2013). Kappa opioid receptors in the prefrontal cortex can also affect aversive learning. Polymorphisms of the pDYN gene can lead to differences in reversal learning, and CPA to KOR agonist, U69,593 can be reversed by intra-mPFC norBNI (Votinov 2015, Tejeda 2013).

Although it is clear that kappa opioid receptors are widely expressed throughout the brain and play an important mode in the behavioral consequences of acute and chronic stress, the multiple, redundant loci of action leave many remaining questions about how the endogenous dyn/KOR system facilitates behavioral plasticity in response to stressors and drugs of abuse. Better tools to measure endogenous dynorphin release and KOR activation will provide insight into where and when KOR is modulating circuitry. The kappa opioid receptor, like many GPCRs, can signal through multiple second messenger cascades, and KOR ligands preferentially activate some or all of these cascades depending on the agonist, duration of action, and location of the receptor. In this next section, we will discuss how expanded knowledge of KOR signaling

cascades can inform sensor development and our understanding of where and when dynorphin acts to cause the behavioral consequences of stress.

G Protein signaling at the KOR

Endogenous dynorphins, classical KOR agonists like U50,488 and U69,593, and biased ligands like nalfurafine all cause activation of Gα and Gβγ, triggering G-protein signaling. In neurons, Gβγ rapidly couples to G-protein coupled inwardly rectifying potassium channels, GIRKs, which can hyperpolarize neurons, modulating excitability. GβɣV signaling downstream of the KOR also causes phosphorylation of extracellular related kinase (ERK) (Belcheva 2005, Lovell 2015*)*. The behavioral consequences of KOR activation are attributed in part to both increased conductance through GIRKs and increased phosphorylation of ERK. Further, the inhibition of presynaptic terminals by the kappa opioid receptor is G-protein dependent, and independent of signaling via p38 MAP-Kinases (Ehrich 2015*)*.

Biased agonists of the KOR, like 6-GNTI, RB-65, and HS666, cause less internalization than traditional agonists like U50,488 and have a bias toward GTPɣs activation over β-arrestin recruitment (Ho 2018). These "G-biased" agonists can suppress pain and itch without causing dysphoria or sedation, as measured by suppression of intraccranial self stimulation (iCSS) or suppression of locomotion (Brust 2016). Although the KOR agonist nalfurafine inhibits dopamine release (see Chapter 4), it does not cause significant dysphoria when taken as an anti-pruritic in human patients (Inui 2015*).* This is likely due to it being 250-fold more potent at ERK1/2 phosphorylation than at p38 MAPK phosphorylation (Schattauer 2017). Behavioral and physiological effects exerted by the KOR which depend upon G-protein activation but not arrestin-recruitment and activation of p38 MAP Kinase include the inhibition of neurotransmitter

release, the increased excitability of dentate gyrus granule cells, swim-stress mediated increases in pERK1/2, and increased marble burying and compulsive behavior in a mouse DRL task (Ehrich 2015, Li 2012, McDermott & Schrader 2011, Kivell 2014, Abraham 2017).

Modulation and opening of GIRKs likely play a role in both the acute and chronic physiological effects of the kappa opioid receptor. Although the opening of GIRKs is associated with KOR Gβɣ signaling, the phosphorylation of these channels at Tyrosine-12 (Y12) is also linked to KOR activation of p38 MAP Kinase; this transition from increased conductance through GIRKs to reduced GIRK conductance after Y12 phosphorylation is critical to understanding how DRN serotonin and VTA dopamine neurons are modulated by stress.

Kir3 channels are heterotetrameric, inwardly rectifying potassium channels. There are three subunits commonly expressed in the central nervous system: GIRK1, GIRK2, and GIRK3, also known as Kir3.1, Kir3.2, and Kir3.3 and encoded by the genes KCNJ3, KCNJ6, and KCNJ9. These channels are members of the K_{IR} family, channels that conduct significantly greater amounts of inward current than outward current (Rifkin 2018, Hille 2007). However, at voltages near V_{REST}, the small amount of outward potassium current is sufficient to hyperpolarize the neuron, reducing spike probability by increasing the depolarization required to reach action potential threshold. At hyperpolarized potentials more negative than the reversal potential for potassium, however, these channels can conduct large amounts of inward current, restoring V_{REST}.

GIRKs are stimulated by $G_{i/o}$-coupled receptors like $GABA_B$, dopamine D_2, and kappa opioid receptors (Gahwiler 1985, Lacey 1988), couple through Gβɣ subunits, and provide sustained inhibition on neuronal activity (Wickman 1994). GIRK1, GIRK2, and GIRK3 channels can form heterotetramers and are expressed widely throughout the brain, however,

GIRK1 expression is limited in VTA dopamine neurons, but abundant in VTA GABA neurons (Karschin 1996, Cruz 2004). Aside from acute activation, Kir3 channels can also be regulated via tyrosine phosphorylation. KOR activation of p38 MAPK causes heterologous desensitization of Kir3 and tyrosine phosphorylation in the N-terminal, cytoplasmic domain of Kir3.1 (pY12-Kir3.1) that reduces the evoked currents through Kir3 channels (Clayton 2009). This pY12-Kir3.1 phosphorylation is seen in the dorsal horn of the spinal cord after sciatic nerve ligation, can be blocked by a p38 inhibitor, and does not occur in KOR knockouts (Clayton 2009). The Chavkin lab has developed an antibody technique to detect pY12-Kir3.1, which will be referred to as "p-GIRK-IR" (Lemos 2011). p-GIRK-IR was detected after U50,488 treatment in the dorsal raphe nucleus and ventral tegmental area (Lemos 2012, Ehrich 2015).

KOR activation of GIRK currents has been recorded in multiple brain regions implicated in the behavioral response to stress, including in the serotonin neurons of the dorsal raphe nucleus, basal forebrain cholinergic neurons, the paraventricular nucleus of the thalamus, and dopamine neurons in the ventral tegmental area (Lemos 2012, Ferrari 2016, Chen 2015, Ford 2006, Margolis 2005). GIRK channel inhibitor Tertiapin-Q has been shown to have antidepressant action when directly injected into the VTA or the DRN, an effect presumed to relate to reduced 5-HT1, $GABA_B$, and D2 Receptor signaling (Llamosas 2017, Llamosas 2015). However, it is possible that the effect of Tertiapin-Q in disrupting KOR-mediated GIRK signaling is critical for its anti-depressant effects.

A neuron could change the impact of KORs on its physiology through multiple mechanisms: changing the physical proximity between the receptor and GIRK channel, changing the subunit composition, or changing its phosphorylation state. BRET and FRET imaging have shown that G-proteins may precouple to GIRK channels, potentially through Gβɣ (Nagi &

Pineyro 2014, Riven 2006, Richard-Lalonde 2013). There is evidence that GIRKs and opioid receptors exist in these precoupled complexes, and also evidence that activation of these channels can happen due to collision (Nagi & Pineyro 2014). As previously mentioned, GIRK1 channels have not been detected using in situ hybridization in dopamine neurons, but p-GIRK detection of pY12-Kir3.1 was found in dopamine neurons after repeated U50,488 treatment (Ehrich 2015). This p38-MAPK dependent effect in dopamine neurons is likely related to the p38 MAPK dependent shift from U50,488 being inhibitory to it losing its effect after 3 days of treatment found in the same study (Ehrich 2015). In serotonin neurons, forced swim stress leads to a p38-MAPK and KOR-dependent increase in p-GIRK, which causes a reduction in the conductance through GIRK current (Lemos 2012). This phosphorylation and change in GIRK conductance require the GIRK1 (Kir3.1) subunit, whose expression is not found in abundance in dopamine neurons. Further study is needed into how KORs couple to GIRK channels in dopamine neurons and if GIRK1/GIRK2 heteromers exist in dopamine neurons, as opposed to the GIRK2/GIRK2 homomers and GIRK2/GIRK3 heteromers coupled to D2R and $GABA_B$ receptors.

Another signal transduction cascade important for mechanisms of KOR action is the ERK MAP kinase pathway. ERK is rapidly phosphorylated downstream of the kappa opioid receptor in a PTX-sensitive manner; there is a second KOR-mediated ERK phosphorylation event that occurs 30-60 minutes after agonist treatment that is arrestin-dependent. Recent proteomics work has shown that although this rapid ERK phosphorylation is tied to KOR activation, its activation after U50,488 treatment in mice varies by brain region (Liu 2018), and at a 30-minute time point can only be detected in the CA3 region of the hippocampus. The pERK response is associated with presynaptic inhibition of BNST terminals, salvinorin-A regulation of the dopamine

transporter, and is seen after swim stress. Future research should investigate the molecules downstream of ERK, how they facilitate the above effects, and if this signaling varies between cell types and neuronal compartment. Further, clarifying the role of the "late pERK" response, activated downstream of arrestin recruitment, may explain some effects associated with receptor desensitization and signaling after prolonged KOR activation.

It is clear that the transition from rapid G-protein mediated signaling to arrestin recruitment and p38 MAPK signaling cascades is critical for the development of outcomes like conditioned place aversion and reinstatement of drug seeking. p38 MAP Kinase activation occurs downstream of GRK3 phosphorylation of the kappa opioid receptor and arrestin recruitment (Bruchas 2006). p38 MAPK phosphorylation is necessary for the SRC kinase dependent phosphorylation of Kir3.1 (pY12). Swim stress promotes both GRK3-mediated phosphorylation of the KOR and p38 MAPK phosphorylation in the NAc, prefrontal cortex, and hippocampus, implicating it in the behavioral response to stress (Bruchas 2007). Animals with a conditional knockout of p38 MAPK from serotonin neurons in the DRN are stress resilient (Bruchas 2011). These p38 MAPK-mediated effects can be ablated by genetically disrupting GRK3 or p38 MAPK, however most biased ligands preferentially activate G-proteins and their downstream effectors. As such, it is challenging to develop a model that looks at the effects of KOR agonists that preferentially activate p38 MAP Kinase pathways. Endogenous allosteric modulators that increase arrestin-mediated signaling would help characterize this pathway independently from G-protein and ERK-mediated effects.

"Non-biased" KOR agonists can become functionally selective when "regulator of G-protein signaling" or RGS molecules allosterically modulate KOR (Liu 2018, Gross 2019). RGS12 increases the activation of p38 MAP Kinase after U50,488. It also reduces the potency of

U50,488 in G-protein assays, but increases the recruitment of arrestin to the KOR (Gross 2019). RGS12 null mice have heightened sensitivity to the analgesic effects of U50,488 and attenuated conditioned place aversion to U50,488; fruther indicating that RGS12 works endogenously to bias KOR signaling toward arrestin/p38 MAPk signaling (Gross 2019). It is presumed that this biased agonism, whether through endogenous RGS molecules or due to functionally selective ligands, is due to the stabilization of a receptor conformation that stimulates one set of signaling partners over another, and further knowledge of these stabilized structures may inform later drug development (Kenakin & Miller 2010, Urban 2007).

Although most of the known outcomes of KOR agonists relate to ERK activity, p38 MAP Kinase activity, or membrane-delimited changes to ion-channel conductance by G-proteins, a third MAPK can also be activated by KOR. Receptor binding by both agonists and antagonists of KOR can activate c-Jun N-terminal kinase (JNK) (Bruchas & Chavkin 2010). Both norBNI and JDTic produce their long-lasting inhibition of KOR signaling with JNK signaling. Phosphorylated JNK stimulates peroxiredoxin 6 (PRDX6), which can stimulate NADPH oxygen and generate reactive oxygen species (ROS) (Schattauer 2017). These ROS lead to the oxidation of a cysteine sulfhydryl in Gαi and prevent reversible palmitoylation of the Gαi. Depalmitoylated Gαi binds more tightly to the KOR, preventing nucleotide exchange and signaling (Schattauer 2017). Despite this known inactivation by JNK, recent findings indicate that KOR agonists can also stimulate JNK and reactive oxygen species (Schattauer 2017). It is worth considerable future study to determine if JNK-stimulated reactive oxygen species can modulate physiology, contributing to the behavioral response to stress. Further characterizing this JNK mechanism is also important for understanding KOR antagonists and their therapeutic potential.

Although the dynorphin/KOR system is not the only means by which circuit plasticity occurs in response to stress and drug-taking, it is clearly important in developing maladaptive behaviors and mood disorders after stress. If we are to target the kappa opioid system with novel therapeutics, however, we have to be able to understand why initial clinical trials of KOR antagonists have not met success criteria. It is important to note that, as described within this chapter, KOR antagonists are typically only effective at reducing stress-induced reinstatement, and that they only reduce drug-taking in dependent animals. Additionally, the behavioral responses to KOR agonists vary widely by dose, where in the brain they are administered, and the degree of functional selectivity of the agonist. If we can better parse which second messengers are necessary to mediate important, adaptive component aspects of KOR signaling and which facilitate harmful consequences, we may be able to better utilize this endogenous opioid system to reduce the harm of chronic stress and treat substance use disorder.

Chapter 2

D2 Receptors are regulated by JNK-stimulated reactive oxygen species

Data presented in this section were collected by Katie Reichard for the following manuscript:

Schattauer, S.S., Land, B.B., **Reichard, K.L**., Abraham, A.D., Burgeno, L.M. Kuhar, J.R., Phillips, P.E.M., Ong, S.E., Chavkin, C. (2017) Peroxiredoxin 6 mediates Gαi protein-coupled receptor inactivation by cJun Kinase. *Nature Communications*, 8:743. [PMC5622097]

Graphs & results are presented from the published paper with additional background and discussion written for this dissertation.

Introduction

Long-acting antagonists of the kappa opioid receptor like norbinaltorphimine (norBNI) and JDTic require c-Jun Kinase (JNK) in order to be effective (Bruchas 2007, Melief 2011). However, prior to this 2017 study, the mechanism of receptor inactivation was not clear. Schattauer and colleagues demonstrated, using a SILAC proteomics & mass-spectrometry approach, that after norBNI treatment, the KOR associates more with Gαi protein, indicating that the G-protein is being "locked" to the receptor to facilitate inactivation (Schattauer 2017). Further, they demonstrated that the Gαi increased its association with Peroxiredoxin 6 (PRDX6), a bifunctional enzyme with glutathione peroxidase activity and phospholipase A2 (PLA2) activity (Fisher 2011). Schattauer, et al, demonstrated that norBNI produces a JNK-dependent, PRDX6 activation that stimulates production of reactive oxygen species that can be measured with ROS indicators like CellROX green (Schattauer 2017). This characterization of a novel signaling pathway downstream of GPCR activation led to the following questions: can other $G\alpha_i$-coupled receptors and agonists stimulate ROS production and is local norBNI stimulated ROS promiscuous, meaning can it inactivate other local GPCRs.

The D2 dopamine receptor (D2DR), like the kappa opioid receptor, is highly expressed in dopamine neurons, couples to $G\alpha_i$, and hyperpolarizes neurons through G-protein coupled inwardly rectifying potassium channels (GIRKs). In dopamine terminals, D2 receptors act as autoreceptors, inhibiting neurotransmitter release. Repeated activation of the D2 receptor with its agonist, quinpirole, can cause compulsive behaviors and is used as a model of obsessive-compulsive disorder in mice. Co-administration or pretreatment of mice with a KOR agonist can potentiate this compulsive behavior, indicating that the actions of KOR agonists on dopamine neurons can 'crosstalk' or affect the actions of D2 receptors on behavior. This closely linked signaling, anatomical colocalization in dopamine terminals, and behavioral outcome make D2 receptors a good model to test if activation of JNK-ROS downstream of the kappa opioid receptor is able to shift signaling at the D2 receptor in a PRDX6 dependent manner.

In addition to the possibility that norBNI-stimulated ROS may act upon non-KOR receptors in spatial proximity to the ROS signal, it is possible that other $G\alpha_i$-coupled receptors that stimulate JNK may generate PRDX6 signaling and functionally significant ROS production as well. The D2DR receptor exists in short (D2DR(S)) and long (D2DR(L)) splice variants that differ in their 3rd intracellular loop. The two splice variants have been shown to differ in their signaling, function and desensitization (Picetti 1997). In order to determine if D2 receptors can stimulate ROS through a JNK-PRDX6 dependent mechanism, we tested if the agonist quinpirole was able to generate ROS in HEK293 cells expressing the D2DR. Because the variance in the signaling in the two splice variants, we also tested if both D2DR(L) and D2DR(S) were able to stimulate ROS after treatment with quinpirole.

In this study, we predicted that the KOR antagonist would shift the potency of quinpirole at inhibiting dopamine terminals, via a JNK-PRDX6 dependent mechanism. We tested this by

measuring the dose-response curve to quinpirole at inhibiting electrically stimulated dopamine release, as measured by fast-scan cyclic voltammetry. To test the prediction the D2 agonists would also stimulate reactive oxygen species, we transfected HEK293 cells with D2DR receptors, treated with D2DR agonist quinpirole, and measured ROS levels using cellular ROS indicator CellROX Green.

Materials & Methods

Cloning HA-D2DR(L) plasmid was purchased from Sino Biological. HA-D2DR(S) was made by amplifying the D2s coding region from a pcDNA-D2s-L-Venus (Addgene Plasmid #19966) with primers that incorporated 5′ HindIII and 3′ NotI restriction sites and an HA tag. The HA-D2DR(L) coding sequence was excised using HindIII and NotI restriction sites and the amplified HA-D2DR(S) was subcloned into the cassette at the HindIII and NotI sites.

CellROX Green Experiments HEK293 cells were maintained in Dulbeccos' modified medium/F12 with 10% fetal bovine serum and penicillin-streptomycin-L-glutamine. For D2DR experiments, HEK293 cells were plated on coverslips 48 h prior to the experiment and transiently transfected with HA-D2DR(L) or HA-D2DR(S) 24 h prior. Cells were serum starved 5 h, then treated with MJ33 (10 μM) or vehicle 30 min prior to 1 h treatment with quinpirole (100 nM). CellROX Green (10 μM, Molecular Probes) was added during the last 30 min of treatment. Cells were rinsed in PBS and fixed 15 min with 4% PFA. Cells were mounted on glass slides with VectaShield HardSet with DAPI (Vector Laboratories), and imaged within 12 h. Coverslips were imaged on a Nikon upright fluorescent microscope with Nikon Elements AR v3.1 software (Nikon Instruments). To prevent photoactivation, exposure to light during sample generation and imaging was minimized. Two representative fields from each coverslip were imaged for

CellROX (488 nm) and DAPI. Exposure times were held constant for every fluorophore throughout the entire experiment. Image intensities for between 7 and 20 cells per image were quantified using ImageJ v 1.42q (National Institute of Health), and these were averaged to give an average field intensity value. This was done for the two cover slip images and averaged to make one sample (n).

Voltammetry Fast-scan cyclic voltammetry was performed on nucleus accumbens slices from C57BLJ/6 male wild-type mice as previously described. Mice were treated with vehicle or norBNI one hour prior to decap and all recordings were completed within seven hours of norBNI injection. MJ33 pretreatment was injected thirty minutes prior to norBNI, or 1.5 hours prior to decap. The last 8 min (four recordings) were used for calculating a quinpirole concentration response curve in GraphPad Prism, using a three parameter least squares nonlinear regression with top constrained to 100%.

Drugs For cell culture studies, (−)-quinpirole hydrochloride (Sigma-Aldrich) was dissolved in H_2O. For in vivo studies, norBNI and MJ33 were dissolved in 0.9% saline.

Results

Pretreatment with norBNI significantly reduces the potency of quinpirole at inhibiting dopamine release as measured in NAc slices. To determine if norBNI could inhibit D2DR function in vivo, slice voltammetry was used to measure quinpirole inhibition of stimulated dopamine release in the nucleus accumbens. Mice were injected with norBNI (10 mg/kg) or vehicle 1 h prior to harvesting brain slices (Figure 2.1a). Dopamine release was electrically evoked and measured within 7 hours of drug administration. Dopamine was detected with fast-scan cyclic voltammetry. The relative concentration was then averaged for the final four

measurements at each dose and a dose response curve was generated (Figure 2.1b). NorBNI pretreatment resulted in a significant, 2-fold rightward-shift in the quinpirole concentration response curve, with an EC_{50} of 53 nM (95% confidence interval 46–61 nM), relative to a vehicle-treated EC_{50} for quinpirole of 28 nM (95% confidence interval 21–34 nM) (Figure 2.1c). This shift in potency was prevented by pretreatment with MJ33 (EC_{50} of 40 nM, 95% confidence interval 34–45 nM). These data indicate that KOR activation of JNK/PRDX6 also results in cross-inhibition of D2DR in vivo.

Quinpirole, a DRD2 agonist, can stimulate production of reactive oxygen species in HEK293 cells expressing DRD2(S) and DRD2(L) isoforms. HEK293 cells stably expressing D2DR(L) isoform were treated with the D2 agonist Quinpirole (200 nM). After 30 min, they were treated with CellROX green, a ROS indicator that fluoresces green and translocates to the nucleus after exposure to H_2O_2. Pretreatment with Quinpirole significantly increased the measured CellROX fluorescence one hour after treatment. Pretreatment of slices thirty minutes prior to Quinpirole with MJ33, an inhibitor of the PLA2 activity of PRDX6, prevented this increase in ROS, as measured with CellROX Green (Figure 2.2a,b). These data, primarily collected by Selena Schattauer in the Chavkin lab, with support from Ben Land and myself, answered if the D2 receptor short isoform (D2DR(L)) was able to stimulate ROS after agonist treatment, however, it did not confirm if the D2DR(S) isoform, which is more vulnerable to Ca^{2+}-dependent desensitization, was able to stimulate ROS (Gantz 2015).

Using Gibson Assembly, we subcloned the D2DR(S) coding sequence into the plasmid that was coding for D2DR(L) and made a successful insertion, as measured by a loss in the HindIII restriction enzyme cutting site. HEK293 cells were then plated 48 hours prior to an

experiment, transfected with either D2DR(L) or D2DR(S) plasmid 24 hours prior to drug treatment, and tested for Quinpirole-dependent ROS production. Quinpirole led to an increase in CellROX fluorescence in cells transiently transfected with the D2DR(L) and D2DR(S) isoforms (Figure 2.2c,d). This indicated that both isoforms of the D2DR can effectively stimulate JNK and downstream signaling, but does not inform if either isoform can be inactivated by ROS signaling, because the ROS is upstream of receptor inactivation.

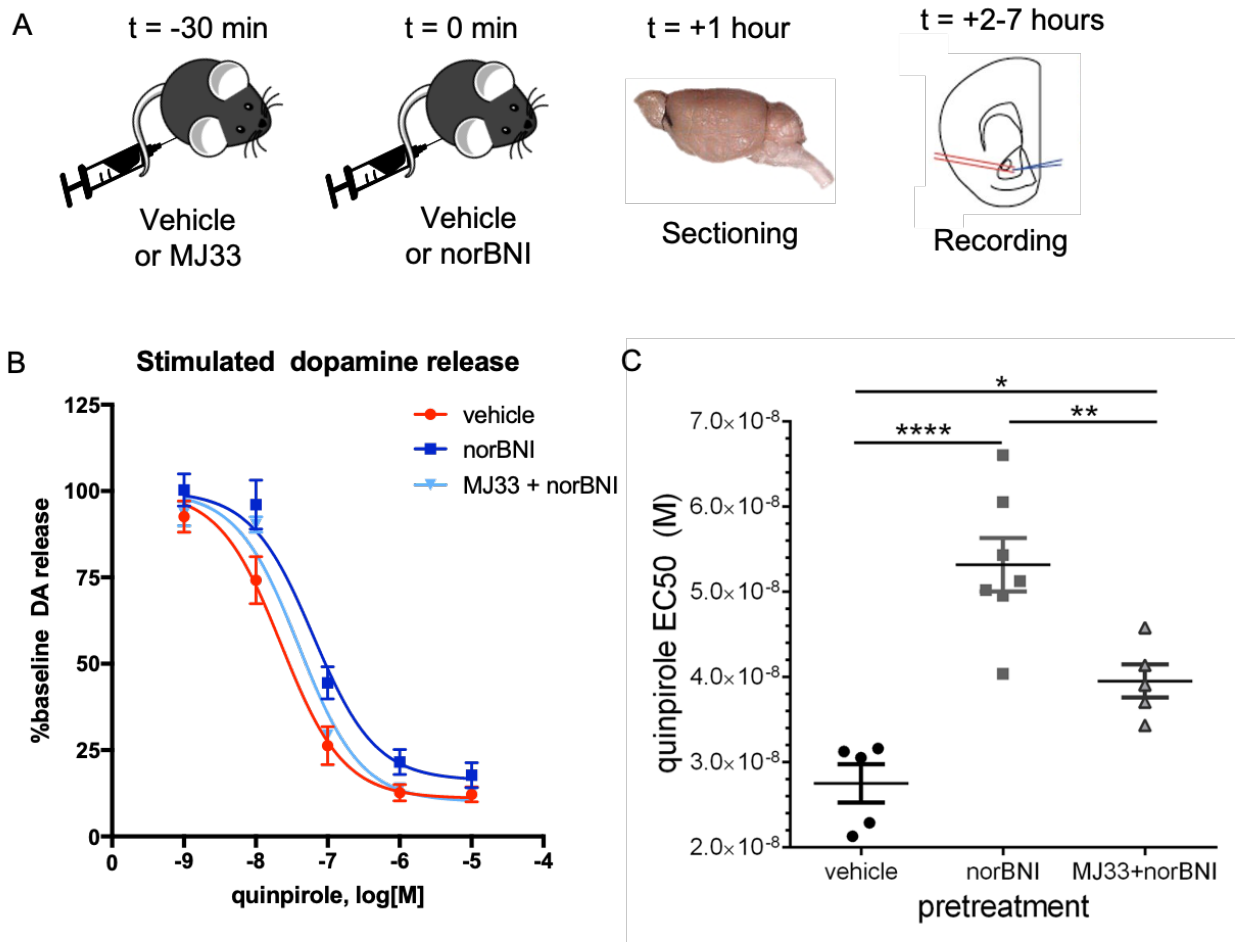

Figure 2.1 NorBNI pretreatment shifts potency of quinpirole at inhibition of dopamine release: A. Schematic of quinpirole experiment Mice were injected with MJ33 or vehicle 30 minutes prior to norBNI injection. Mice were treated with norBNI and this was considered "t=0"

for the experiment, as only experiments done within 7 hours of norBNI treatment seemed to shift the dose response. One hour after norBNI injection, mice were decapitated and slices of the Nucleus Accumbens (NAc) were prepared for voltammetry. Quinpirole dose-response recordings were taken from NAc slices, within seven hours of norBNI treatment. **B. Dose response to quinpirole for each group.** A dose response was curve was calculated for quinpirole using least squares regression. The maximum was fixed to 100% of baseline for each, and the Hill slope was fixed to -1. An extra sum-of-squares F test determined that the curves were significantly different from each other and the EC50s were extracted for statistical comparison. **C. EC_{50} comparisons between treatment groups.** ***(****graph from Schattauer et al 2017)* EC50 values were calculated from concentration-response curves for quinpirole inhibition of dopamine release 5–7 h after vehicle, norBNI (10 mg kg−1, i.p.), or MJ33 (1.25 mg kg−1 i.p.) prior to norBNI. NorBNI treatment resulted in a significant increase in quinpirole EC50, which was reduced by MJ33 pretreatment (one-way ANOVA, $P < 0.0001$; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, Holm–Sidak post hoc). Error bars represent mean ± SEM

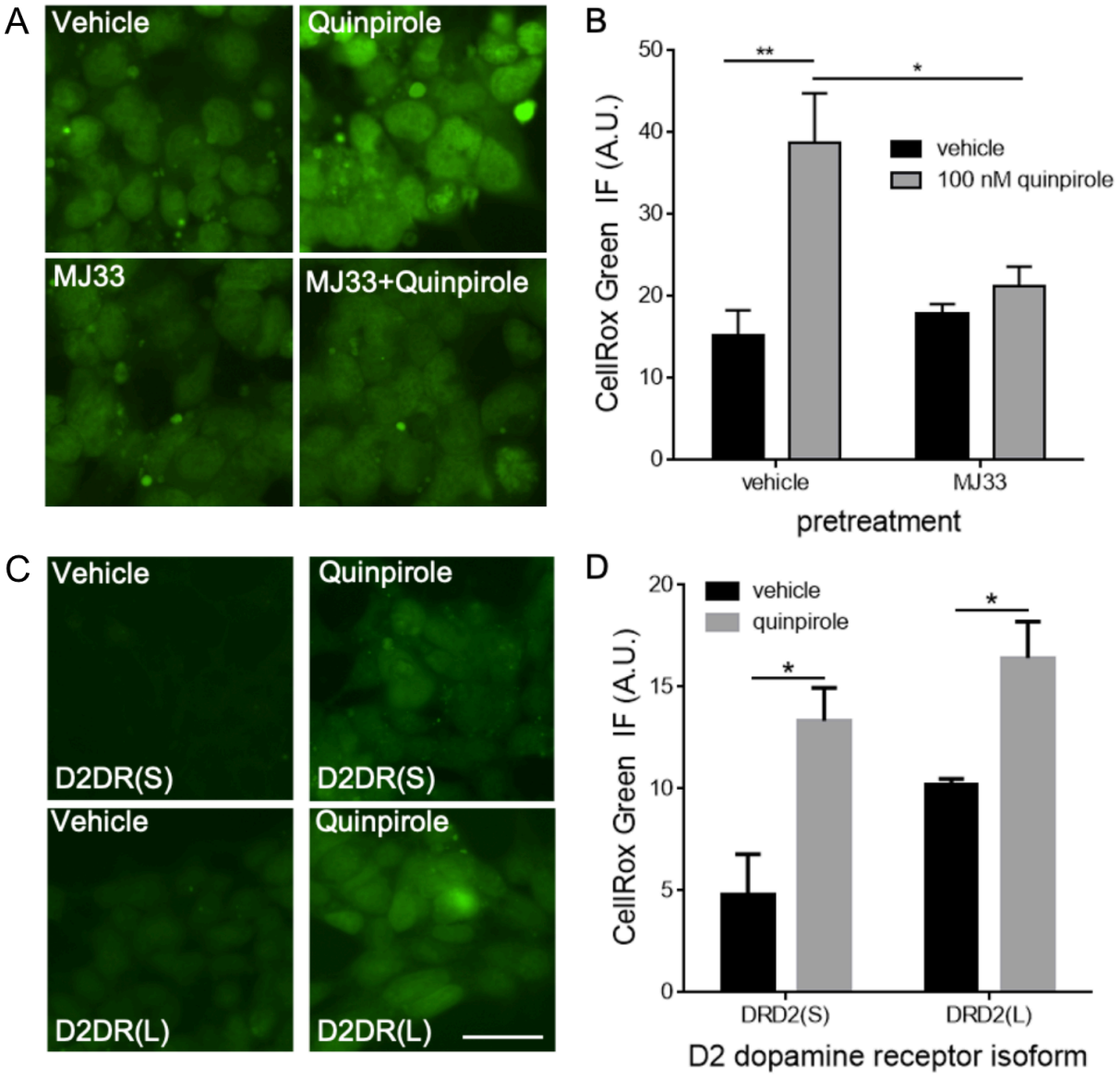

Figure 2.2: D2 Receptor stimulation by quinpirole results in MJ33-dependent ROS production in HEK293 cells expression both the short and long isoform of DRD2. A. HEK293 cells transiently expressing D2DR(L) were pretreated with MJ33 (10 µM) or Vehicle (30 min prior to drug treatment). They were then treated with Vehicle or Quinpirole (100 nM), 30 min prior to CellROX green treatment and 60 min prior to fixation in formalin. Cells were imaged for ROS using CellROX Green. **B**. Fluorescence was quantified by circling and

averaging fluorescence in 10-15 cells per cover slip Quinpirole increased CellROX Green fluorescence; this was blocked by pretreatment with MJ33 (two-way ANOVA, significant effect of quinpirole ($P < 0.01$) and significant interaction ($P < 0.05$), $n = 4–5$; *$P < 0.05$, **$P < 0.01$, Holm–Sidak post hoc analysis of MJ33 vs. vehicle pretreatment). **C.** HEK293 cells transiently expressing either DRD2(S) or DRD2(L) isoform were treated with either vehicle or quinpirole (100 nM) thirty minutes prior to CellROX green treatment and one hour prior to fixation. Cells were then imaged for ROS using CellROX Green. Fluorescence was quantified, quinpirole increased CellROX Green fluorescence in both DRD2(L) and DRD2(S) expressing cell lines (two-way ANOVA, significant effect of quinpirole ($P < 0.05$).

Discussion

Through this study, we demonstrated that $G\alpha_i$-coupled receptors besides KOR can signal to stimulate PRDX6-dependent ROS. Data included in the manuscript, but not shown here, demonstrated that morphine, a μ-opioid receptor (MOR) agonist, can also stimulate the production of ROS, and that this ROS production can be disrupted by MJ33 pretreatment or pretreatment with the JNK inhibitor SP6. Further study would be needed to confirm DRD2 activation of JNK, as well as the dose response relationship between quinpirole dose and ROS activation. Additionally, although the Schattuaer, et al 2017 manuscript describes reduced palmitoylation and increased G-protein and GPCR association after norBNI treatment that is the likely consequence of PRDX6-ROS signaling, these molecular signatures of receptor inactivation have not been shown for D2DR or MOR.

One functional ramification of quinpirole-stimulated ROS in vivo is acute desensitization of the receptor. Within this same study, colleagues demonstrated that pretreatment with a

quinpirole agonist two hours prior to a second quinpirole injection produces a ~20% reduction in quinpirole-induced hypocomotion. However, if animals were given either saline or MJ33+quinpirole at this -2 hour timepoint, they showed a larger response to the test dose of quinpirole, indicating that the mild, acute desensitization was dependent on PRDX6. This short duration of action, two hours, is similar to the time frame within which effects were seen on FSCV. This leads to a prediction that desensitization effects on D2DR from PRDX6-ROS may be small and significant only within a short time frame after treatment. Future studies should look at a time course of these effects and mechanisms by which presynaptic JNK-PRDX6 may be reduced as compared to somatic. Later in this dissertation, the duration of action of norBNI in dopamine terminals and its ability to stimulate measurable reactive oxygen species is discussed.

We also saw, both through the quinpirole data and morphine data, that agonists can activate JNK and, in turn, stimulate ROS. In later work, it was shown that KOR agonists can also stimulate ROS. JNK-stimulation occurs at lower doses of agonists like U50,488 because higher doses preferentially activate GRK-Arrestin mediated signaling and p38-MAP Kinases, reducing overall ROS (Schattauer 2019). This leads to a prediction that a KOR-agonist that preferentially activates G-protein signaling, without activating the arrestin pathway, would stimulate ROS. This will be addressed at a later point in this dissertation, interrogating the ability of KOR agonist, nalfurafine, to stimulate reactive oxygen species in dopamine neurons. Further study should investigate the ability of G_s and G_q-coupled GPCRs to stimulate JNK & reactive oxygen species.

Ultimately, to more clearly study the effects of receptor-stimulated reactive oxygen species, it will be critical to both detect and monitor the stimulation of ROS in living tissue. The ROS sensor, CellROX Green, used in this study is effective in fixed tissue. The Chavkin lab has

since utilized a genetically encoded in vivo sensor, HyPerRed, which will more thoroughly and clearly establish if this effect is occurring in brain tissue, in awake-behaving mice, and where.

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Chapter 3

Dopamine neurons of the VTA are heterogenous and there is a sex difference in their responses to KOR agonists

Data presented in this section were collected by Katie Reichard for the following manuscripts:

Abraham, A.D., Schattauer, S.S., **Reichard, K.L**., Cohen, J.H., Fontaine, H.M., Song, A.J., Johnson, S.D., Land, B.B, Chavkin, C (2018) Estrogen regulation of GRK2 in female mice inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. *Journal of Neuroscience*, 38:8031-8043. [PMC6136151].

Heymann G, Jo YS, **Reichard KL**, McFarland N, Chavkin C, Palmiter RD, Soden ME, Zweifel LS. (2019) Synergy of Distinct Dopamine Projection Populations in Behavioral Reinforcement. *Neuron*. pii: S0896-6273(19)31010-4.

Graphs & results are presented from the published papers with additional background and discussion written for this dissertation.

A population of neurons in the midbrain, ventral tegmental area (VTA) dopamine neurons, can control depressive-like symptoms, change their firing after stress, and regulate aberrant reward seeking in motivational circuits (Tye 2012, Chaudhury 2013, Pascoli 2015). Dopamine neurons in the VTA fire in response to unexpected rewards and reward-predictive cues, including both natural rewards and drugs of abuse (Schultz 1997, Stuber 2005). The absence of reward can produce a pause in dopamine neurons, although its contribution to encoding negative reward prediction error is unresolved (Glimcher 2011, Schultz 1997*,* Chang 2016*)*. Dopamine neurons are also known to promote behavioral responding through incentive salience signaling (Berridge

& Robinson 1998). Despite this known role in reward encoding, dopamine neurons can also fire in response to neutral, but salient stimuli like tones or novel objects, or fire in response to aversive cues like toe pinch, footshock, or bitter tastes (Young 1998, Stelly 2019, Jo 2018, Budygin 2012, Bromberg-Martin 2010*)*. Like their heterogeneity in behavioral responding, dopamine neurons have heterogeneous physiological responses and genetic expression (Barker 2016, Trudeau 2014). Sub-categorizing and classifying VTA dopamine neurons will be important to understanding the plasticity that occurs during stress, or after compulsive, escalating drug use. Although kappa opioid receptors are expressed on all dopamine neurons, the role of KOR activation and its ability to signal likely depends on the context in which these subpopulations of DA neuron fire, their dynorphin inputs, and the coexpression of other GPCRs.

In addition to differences in neurotransmitter content and physiological properties, dopamine neurons express and are regulated by a variety of neuropeptides (Kalivas 1985, Tyree and de Lecea 2017). To further investigate the functional segregation of dopamine neuron subpopulations within the VTA, the Zweifel lab utilized mouse lines in which Cre expression was under the control of different neuropeptide-associated genes and studied their physiology and contribution to reward learning (Heymann 2020). Neurons isolated by the *Crhr1*-Cre line primarily projected from the VTA to the NAc core, and neurons isolated by the *Cck*-Cre line primarily projected from the VTA to the NAc shell. *DAT*-Cre mice were used as a control population, in which all dopamine neurons in the VTA expressed Cre and drove expression of injected viruses. *TacR3*-Cre mice also had a population of dopamine neurons whose expression was more sparse than *DAT*-Cre mice, but that projected throughout the NAc. The data presented in this section demonstrate that ChR2 expression in all four of these driver lines leads to optical stimulation of dopamine release in the nucleus accumbens, but that the location of dopamine

release varies by driver line and is fairly consistent with visualized fibers in the NAc.

Although there are very few gene expression differences between dopamine neurons in male and female mice (Chung 2017), sex hormones can modulate the signaling and trafficking of GPCRs (Valentino 2013, Rhinehart 2018). Further, both chronic pain disorders and depression are more prevalent in women (Berkley 1997, Cyranowski 2000, Ford 2004). KOR agonists have been reported to differ in efficacy between males and females (Laman-Maharg 2018), and after persistent observation that the kappa opioid receptor system was less responsive in female mice, the Chavkin lab systematically reviewed KOR-mediated signalling cascades and behavioral responses to determine if they were intact in male and female mice. Although KOR agonists led to p38 MAPK phosphorylation in samples from both male and female mice, significant phosphorylation of ERK1/2 MAP Kinase was not observed in tissue taken from female mice (Abraham 2018). Behavioral analysis showed that although conditioned place aversion to U50,488 and stress-potentiation of cocaine CPP were intact in female mice, tail-flick and hot-plate analgesic effects of U50,488 were attenuated in female mice (Abraham 2018). These pharmacological and behavioral data points indicated that GRK3/Arrestin dependent effects were intact in female mice, but G-protein and ERK1/2 dependent effects were disrupted.

There were no differences in expression of KOR mRNA, but OVX restored U50,488 tail flick in female mice, indicating that the effect was sex hormone dependent. Previous literature indicating that estradiol can lead to G-protein independent phosphorylation of GRK2, and GRK2 sequestration of Gβγ, reducing Gβγ-mediated signaling cascades (Dominguez 2009, Daaka 1997). In female mice, it was shown that treating ovariectomized female mice with estradiol increased co-immunoprecipitaiton of GRK2 and Gβγ, and that GRK2 phosphorylation was increased in female mice. This leads to the prediction that an inhibitor of GRK2 phosphorylation

should prevent Gβγ sequestration and restore typical GPCR signaling through Gβγ in the presence of high estradiol. Treatment with CMPD101, the GRK2/3 inhibitor restored U50,488-mediated ERK1/2 phosphorylation in female mice. In this study, we investigated if there was a sex difference in the ability of KOR agonists to inhibit dopaine release, and if so, if this was attenuated by preatreatment of tissue with CMPD101. As predicted based on previous research showing that presynaptic inhibition by KOR agonists was independent of p38 MAP Kinase (Ehrich 2015), we found that there was a shift in the dose response of dopamine inhibition by U69,593 in female mice. Pretreatment of nucleus accumbens slices from female mice with CMPD101 generated U50,488 dose response that more closely matched those of male mice.

Both of the studies presented in this chapter inform how dopamine signaling can be modulated by neuropeptides in a heterogenous manner, based on genetic expression, projection target, and sex hormones. This divergence in responding is likely important for our understanding of how dopamine responses are shaped in motivational tasks and how behavioral stress modulates these behaviors. The data presented here was previously published in two manuscripts listed above, on which I was a contributing author.

Methods

Dopamine Voltammetry with Optical Stimulation (*From Heymann, et al 2019)* Crhr1-Cre, Cck-Cre, Tacr3-Cre, and DAT-Cre mice (4 - 5 wk old) were injected bilaterally in the VTA with a Cre-conditional Channelrhodopsin virus (AAV1-FLEX-ChR2-mCherry). Approximately 3 wks post-surgery, mice were euthanized and decapitated. Brains were immediately submerged in ice-cold aCSF solution in which sucrose replaced NaCl. Coronal slices (250 μm) through the striatum were kept in oxygenated aCSF (in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, and 26 $NaHCO_3$) at 37°C for 1-h prior to recording. Slices were perfused

with oxygenated aCSF at 31-33°C at 1.5-2.0 mL/min.

Dopamine release was recorded with a carbon fiber microelectrode as described previously in manuscript (Clark et al., 2010). Recordings were performed in three locations per slice: nucleus accumbens medial shell, lateral shell, and core. Fluorescence was verified and imaged after recordings. Representative recordings were taken across the A-P axis from +1.5 to +0.7 from bregma. Dopamine release was evoked from ChR2-expressing terminals with a 3 s train (20 Hz, 5-ms pulse width) at 485 nm using the Spectra 4-LCR-XA (Lumencor) light source. Three stimulations were averaged to determine release from each subregion. For each section, measurements were normalized to the largest current amplitude evoked in the slice (from any of the 3 subregions).

Drugs U69,593 was purchased from Santa Cruz Biotechnology. The GRK2/3 inhibitor CMPD101 (Tocris Bioscience) was dissolved in 10% ethanol/10% Cremaphor EL (Sigma-Aldrich)/80% water to 30 μM. For CMPD101 voltammetry experiments, aCSF with 30 μM CMPD101 was washed onto the slice for 10 min before washing on both 30 μM CMPD101 and increasing doses of U69,593.

Results

VTA neurons expressing the CRHR1 receptor primarily release dopamine in the NAc core, whereas CCK-expressing dopamine neurons primarily release dopamine in the shell.

Optical stimulation of dopamine release in the NAc medial shell, lateral shell, and core varied between the four transgenic lines: DAT-cre, CRHR1-cre, CCK-Cre, and TACR3-cre. CRHR1-expressing neurons that project from the VTA to the NAc primarily ennervate the NAc core (Heymann 2019). However, even with a 3 second, 10 Hz train of light stimulation, ChR2 stimulation in CRHR1-Cre sections resulted in the lowest amount of dopamine of the four

transgenic lines (Figure 3.1, top). Stimulation of ChR2 in the CRHR1-Cre line led to release only in the NAc core, with a much lower absolute value, indicating that this population of neurons may co-release another neurotransmitter in addition to dopamine. Stimulation of ChR2 in the CCK-Cre line led to substantial dopamine release volume in the NAc medial shell, consistent moderate release in the NAc lateral shell, and little to no release in the NAc core, consistent with the lack of visible fibers in this region (Figure 3.1, middle). Stimulation of ChR2 fibers in both the TacR3 and DAT-Cre animals led to release of dopamine throughout the nucleus accumbens (Figure 3.1, bottom). The amount of stimulated dopamine current in the DAT-Cre mice was larger, which is consistent with this mouse line including all, rather than a subpopulation, of VTA dopamine neurons. The TacR3 population led to fairly even release thoughout the NAc, whereas the DAT-Cre stimulation had the most release in the core. This indicates that the densest or most dopaminergic projection from the VTA is to the NAc, but that the TacR3+ population of dopamine neurons is more balanced throughout.

Pharmacological inhibition of dopamine release by KOR agonist U,69-593 is less potent in female mice. In a collaborative study of sex differences in KOR signaling (Abraham 2018), I tested whether presynaptic inhibition of transmitter release by KOR activation (Chefer et al., 2005) was different in males and females using dopamine voltammetry in the striatum. Concentration–response curves were generated for the KOR agonist U69,593 as described previously (Ehrich et al., 2015). Treatment with U69593 inhibited evoked dopamine release with an EC50 of 17 nM (95% CI: 11–27 nM) in males and 63 nM (95% CI: 27–148 nM) in females. U69,593 was significantly more potent in males than females across multiple doses (nonlinear regression, F(4,81) 12.9, p 0.0001) (Figure 3.2a,b). There was a significant difference (F(2,19)

5.199, p=0.0158) in the logEC_{50} between groups and post hoc analysis showed that there was a nonsignificant trend (p=0.087) toward a difference between males and females. Slices prepared from females incubated with 30 μM CMPD101 showed a significantly (p = 0.0013) lower U,69-593 logEC_{50} (EC_{50}: 10 nM; 95% CI: 6 –16 nM) compared with female slices in the absence of CMPD101. There was no effect of CMPD101 treatment alone on dopamine release. To further assess dose effects of CMPD101, we examined a U69,593 dose close to the EC_{50} of all three groups at a 100 nM concentration (Figure 3.2b) and found that there was a significant effect of group (F(2,19) 9.69, p=0.0013). Post hoc analysis confirmed the potency of U,69-593 was significantly lower in female brain slices than in either slices of male brain or slices of female brain in the presence of CPMD101 (p=0.011).

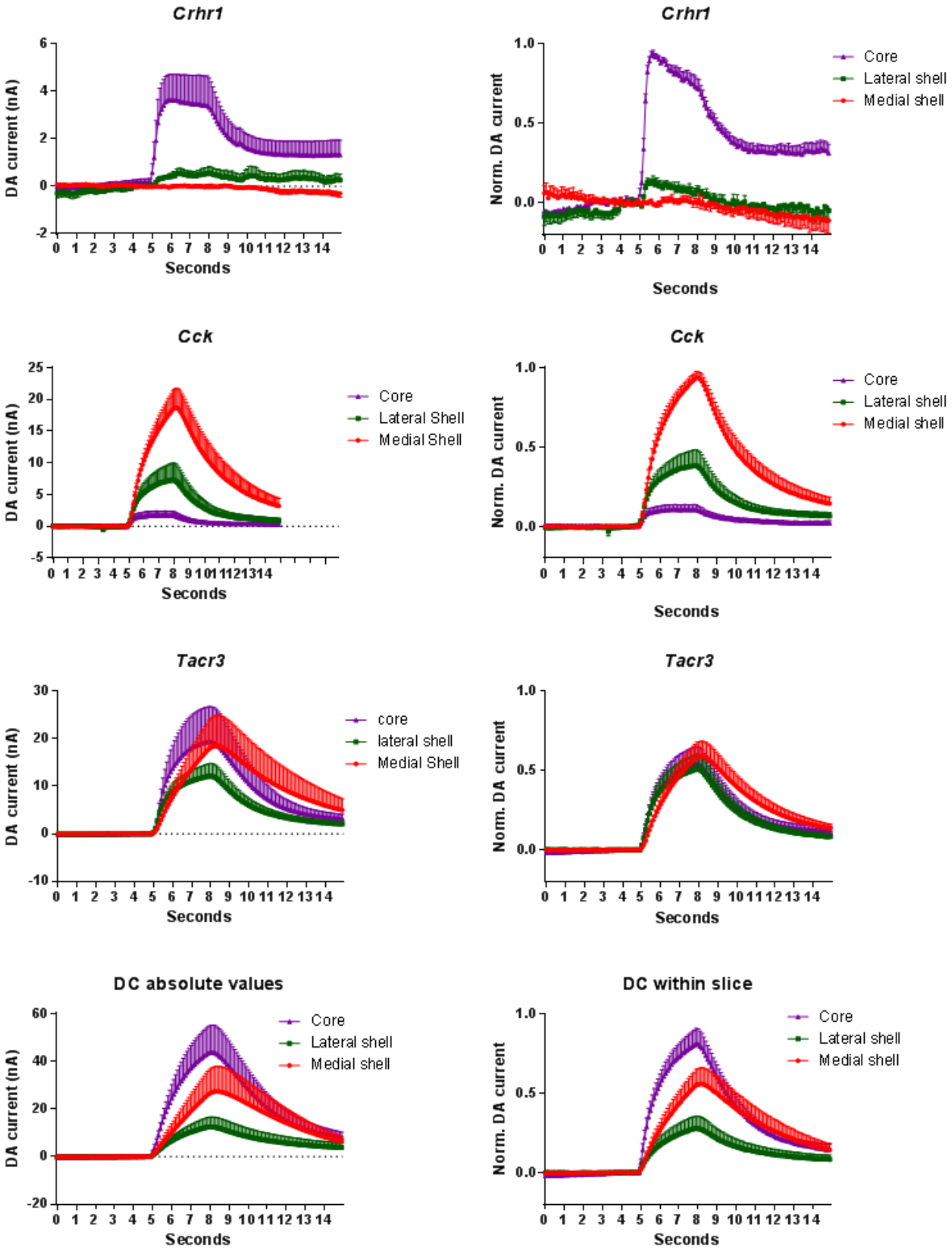


Figure 3.1: Characterization of optically evoked dopamine release from subpopulations of

dopamine neurons genetically defined by neuropeptide genes. Averaged dopamine traces in response to a 10 Hz, 3 second train of blue light, stimulating ChR2-mediated release of dopamine into the nucleus accumbens and as recorded by a carbon fiber microelectrode using fast-scan cyclic voltammetry. Traces on the left demonstrate absolute values, and traces on the right are normalized to the peak of dopamine release in each slice: core for Crhr1-Cre, Medial shell for Cck-Cre, Medial shell for TacR3-Cre, and Core for DAT-Cre.

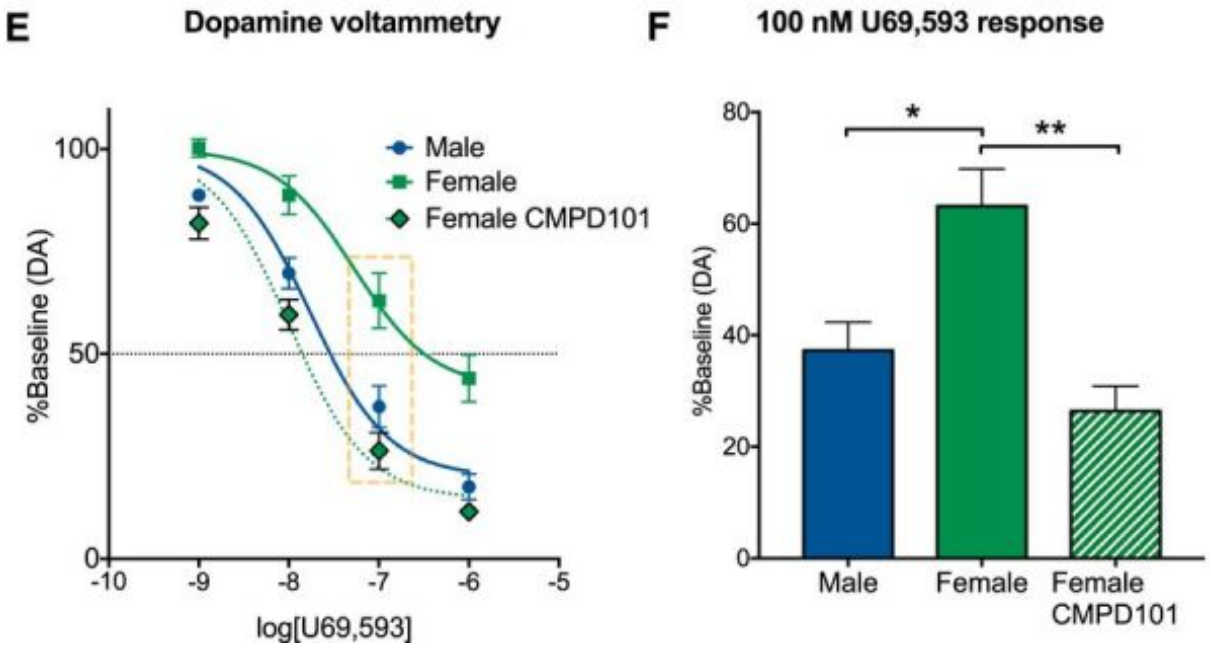


Figure 3.2: Sex Differences in U69,593 inhibition of dopamine release. E. Concentration–response curves for U69,593 inhibition of evoked dopamine were determined using FSCV in slices (n = 5–9 per group) from male & female mice. Nonlinear regression showed that KOR-mediated dopamine inhibition was significantly different between control and CMPD101-treated females. **F.** U69,593 at 100 nM produced significantly less inhibition of dopamine release in slices from females than from males or females in the presence of 30 μM CMPD101. Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$.

Discussion

Work in this chapter demonstrates that VTA dopamine neurons are heterogenous in their peptide expression, and that these populations release dopamine in distinct portions of the NAc. This peptide expression indicates that regulation of this population & mesolimbic circuitry by peptides plays an important role in behavioral regulation. Our lab studies the kappa opioid receptor, so to better understand the KOR regulation of DA neurons, we must study sex

differences in the regulation of dopamine and the mechanism of inhibition. This study demonstrated that KOR inhibition is shifted rightward in female mice, and this is modulated by GRK inhibition (CMPD101), indicating that it is a G-protein dependent event.

The dopamine voltammetry from the CCK-Cre and CRHR1-Cre mice was part of a larger study which showed that the CRHR1-Cre neurons were important for acquiring lever pressing for light, whereas the CCK-Cre neurons were important for maintaining responding. In mice expressing ChR2 in CRHR1+ dopamine neurons, mice were able to acquire lever pressing for light. On the other hand, mice did not acquire ChR2 stimulation in CCK+ neurons, but after learning to press the lever for food, they maintained the lever pressing behavior for CCK+ stimulation. When ChR2 is expressed in all dopamine neurons, however, both behaviors are more robust, indicating that both the CCK-Cre and CRHR1-Cre neurons are subpopulationst that together do not comprise the totality of VTA dopamine neurons. As observed in the voltammetry reported here, the CRHR1-Cre population did not release a large amount of dopamine. However, Heymann, et al, found that dopamine was necessary for the reward-learning lever for light behavior this population exhibited. Both ChR2 stimulation of CCK-Cre and CRHR1-Cre terminals produced picrotoxin-sensitive inhibitory currents and CNQX-sensitive excitatory currents in medium spiny neurons (Heymann 2019 – supplemental). This finding demonstrates that these populations may co-release glutamate and/or GABA and follow up studies can investigate the role of fast-acting neurotransmitters in this circuit. This initial characterization used the expression of neuropeptide-associated genes to anatomically segregate a VTA to NAc Shell and VTA to NAc Core circuit, but future work can clarify if peptides differentially modulate the release of dopamine from these populations and any interaction between CRHR1, CCK, and other peptide receptors like KOR.

Unlike some neuropeptide receptors, the kappa opioid receptor is expressed uniformly throughout the ventral tegmental area and on terminals throughout the nucleus accumbens. We found that despite this expression pattern and consistent mRNA and protein expression of KOR between male and female mice, there was a difference in the potency of U69,593 at inhibiting dopamine release (Abraham 2018). One interesting aspect of this finding is that the sex difference in dopamine inhibition was merely a shift in potency. However, effects like the detection of ERK phosphorylation were completely absent in female mice. This suggests that the ability of KOR agonists to inhibit dopamine release is likely due to overlapping or redundant mechanisms, only some of which may be sensitive to sex hormones and Gβγ sequestration. Follow up studies should more clearly characterize the mechanism by which KORs inhibit dopamine terminals.

Chapter 4

Regulation of kappa opioid receptor inactivation depends on sex and cellular site of antagonist action

The data and content of this section are from a first-author manuscript, accepted, pending minor revision at the journal Molecular Pharmacology. "Regulation of kappa opioid receptor inactivation depends on sex and cellular site of antagonist action." *Co-Authors: Kathryn L. Reichard, Keionna A. Newton, Zeena M.G. Rivera, Paulo Sotero de Menezes, Selena S. Schattauer, Benjamin B. Land, Charles Chavkin.*

ABSTRACT The prototypical member of the receptor-inactivating kappa opioid receptor (KOR) antagonists norbinaltorphimine (norBNI) produces prolonged receptor inactivation by a cJun kinase mechanism. These antagonists have potential therapeutic utility in the treatment of stressdisorders, however additional preclinical characterization is necessary to understand important aspects of their action. In this study, we report that norBNI does not work as effectively in female mice as in males because of estrogen regulation of G-protein receptor kinase (GRK); pretreatment of ovary-intact female mice with the selective GRK2/3 inhibitor, CMPD101, made females equally sensitive to norBNI as males. Prior observations suggested that in vivo treatment with norBNI does did not produce long-lasting inhibition of KOR regulation of dopamine release in the nucleus accumbens. We assessed the persistence of norBNI receptor inactivation in subcellular compartments. Fast-scan cyclic voltammetry recordings confirmed that presynaptic inhibition of dopamine release by the KOR agonist U69,593 was not blocked by in vivo pretreatment with norBNI under conditions that prevented KOR mediated aversion and analgesia. We employed a novel in vivo proxy sensor of KOR activation, AAV-DIO-HyPerRed and demonstrated that KOR activation stimulates JNK-dependent ROS

production in somatic regions of VTA dopamine neurons, but did not activate ROS production in dopamine terminals. The compartment selective action helps explain how dopamine somatic, but not terminally expressed KORs are inactivated by norBNI. These results further elucidate molecular signaling mechanisms mediating receptor-inactivating KOR antagonist action and advance medication development for this novel class of stress-resilience medications.

INTRODUCTION

Prolonged or severe behavioral stress exposure increases risk of mood and substance use disorders (Kessler, 1997; Gold, 2002; Gold, 1998; Koob, 2008). The release of endogenous opioid dynorphin peptides throughout the brain contributes to these adverse behavioral effects (Ehrich, 2015; Abraham, 2018a). For this reason, researchers have explored using kappa opioid antagonists as antidepressants and anti-addiction therapeutics in both pre-clinical research and human clinical trials (Buda, 2015; Carroll & Carlezon, 2013; Lowe, 2014; Chavkin, 2019). Three different forms of KOR antagonists have been identified: nonselective (e.g. buprenorphine and naltrexone) (Leander, 1988), selective long-acting receptor inactivating compounds (e.g. norBNI and JDtic) (Bruchas, 2007; Melief 2011), and selective competitive antagonists (e.g. LY2456302 and BTRX-140) (Rorick-Kehn, 2014; Blackthorn Therapeutics, 2019). There are theoretical advantages and disadvantages to each antagonist type, and it has yet to be determined which avenue is best to pursue for clinical development. The receptor-inactivating compounds can produce a stable effect with less concerns about patient compliance, however, several observations about their mechanism must be resolved before moving forward with the development of this class of medications.

A recent study showed that there is a sex difference in JNK activation by norBNI in

C57BL6-J mice (Laman-Maharg, 2018). This work also demonstrated that in female C57BL6-J mice, pretreatment with norBNI does not reduce immobility in the forced swim test, in contrast to its effects in males. In addition to published concerns about effectiveness in female mice, we also noted that norBNI was not persistently antagonizing KOR inhibition of dopamine release when used in in vitro voltammetry assays of dopamine release (unpublished observations). In order to understand and resolve these issues, we measured sex differences in response to norBNI and clarified the mechanisms responsible for its long-lasting effects on KOR function (Abraham, 2018b; Becker, 2019).

The prototypical receptor-inactivating KOR antagonist norBNI has a long duration of action in vivo with effects lasting 14-21 days, but the drug does not persistently or covalently bind to the receptor (Smith, 1990; Butelman, 1993; Horan, 1992). Instead, norBNI has been demonstrated to disrupt KOR-signaling by selectively activating a c-Jun Kinase (JNK) signaling cascade which activates the phospholipase PRDX6 and subsequently stimulates the generation of reactive oxygen species (ROS) through an NADPH-oxidase mechanism (Bruchas, 2007; Schattauer, 2017a; Fisher, 2011). Local ROS production oxidizes the Gαi subunit at a critical cysteine palmitoylation site, disrupts G protein-receptor interaction, and thus prevents agonist-dependent guanine nucleotide exchange (Schattauer 2017a). This KOR-inactivating process can be blocked by inhibitors of JNK or the PLA2 activity of PRDX6 and can be detected with ROS sensors, including the genetically encoded indicator HyPerRed fluorescent protein indicator which sensitively reports production of hydrogen peroxide (Schattauer, 2017a; Schattauer 2019). ROS-dependent inactivation of KOR can be produced by administering a single high dose of norBNI, or by administering repeated low doses of norBNI (Chavkin, 2019). This action is due to collateral agonist activity, an effect wherein a compound acts as an antagonist by selectively

activating a subcomponent of receptor signaling, in turn inactivating subsequent signaling at that receptor (Kenakin, 2005; Bruchas, 2007). NorBNI's collateral agonist activity led us to hypothesize that the long-lasting effect is likely sensitive to sex hormone regulation, and may be subject to signaling differences between different cell-types and subcellular compartments.

In this study, we confirmed that norBNI is not a consistently effective long-acting antagonist in female mice. We found that its antagonist activity can be restored with the GRK2/3 inhibitor Compound101 (CMPD101) or by treating female mice with repeated, low-doses of norBNI. We also demonstrated that norBNI was not an effective long-acting antagonist at VTA dopamine terminals, but did block KOR-activation in VTA cell bodies. KOR activated potassium (GIRK) currents in VTA dopamine neurons were inhibited one-week post norBNI-injection. Consistent with prior mechanistic studies of receptor inactivation, we found KOR activation of ROS was evident in VTA dopamine cell bodies but not in the axons and terminals of dopamine neurons. These findings indicate that the pharmacological actions of norBNI depend on sex and cellular site of action.

METHODS

Animals Drug- and procedure-naïve C57BL/6-J mice (adult, male 20–30 g, female 17-25g) were group housed on a 12 h light/dark cycle with food and water ad libitum. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington and conform to the guidelines on the care and use of animals promulgated by the National Institutes of Health. Mice expressing Cre-recombinase under the control of the endogenous dopamine transporter locus (Slc6a3IRES-Cre or DAT-IRES-Cre) were used for viral injections (Zhuang, 2005).

Drugs U69,593 ((+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) (NIDA Drug Supply) was dissolved in solvent (DMSO) to 1 mM stocks and stored at -20˚C for up to one month. β-chlornaltrexamine dihydrochloride (β-CNA) (SigmaAldrich) was dissolved 1 μg/μl in saline and stored at -20˚C for up to one month. Norbinaltorphimine (norBNI, NIDA Drug Supply) was dissolved 1 mg/mL in saline prior to IP injection unless stated otherwise. CMPD101 (Tocris) was dissolved at 1.5 mg/mL in 10% ethanol/10% cremaphor/80% saline prior to IP injections. CMPD101 and norBNI were delivered in IP injections of 10 ml/1 kg of body weight to doses of 15 mg/kg and 10 mg/kg, respectively. Nalfurafine hydrochloride ((2E)-N-[(5α,6β)-17-(cyclopropylmethyl)-3,14- dihydroxy-4,5-epoxymorphinan-6-yl]-3-(3-furyl)-N methylacrylamide) (NIDA Drug Supply) was dissolved in sterile water at 100 μM and stored at -20˚C.

Behavioral Assays Female mice were tested for latency (in seconds) to withdraw tail (flick) from 52.5°C water to assess anti-nociceptive effects of KOR activation (Bruchas 2007). Mice were tested for basal latency to flick and treated with CMPD101 (15 mg/kg, i.p.), then tested 30 min later and treated with U50,488 (10 mg/kg, i.p.) and retested for latency to flick 30 min later. For repeated lowdose norBNI pre-treatment, female mice received one injection of norBNI (1 mg/kg, i.p.) or an equivalent volume of saline daily for seven days prior to the tail withdrawal assay. For CMPD101 and norBNI pre-treatment, female mice received CMPD101 (15 mg/kg, i.p.) and thirty minutes later, received norBNI (10 mg/kg, i.p.) or an equivalent volume of vehicle or saline, respectively, seven days prior to the tail withdrawal assay.

Stereotaxic Surgery (ICV) Mice were anesthetized with isoflurane and mounted on a model

1900 stereotaxic alignment system (David Kopf Instruments). β-CNA was loaded into a Hamilton Syringe and delivered at a rate of 200 nl/min into the lateral ventricles (Coordinates: A/P +0.3, M/L +0.8, D/V -3.0 or A/P -0.5, M/L +1.0, D/V -2.5) (Paxinos & Franklin, 2008; Devos & Miller, 2013). Two coordinates were used to avoid excess bleeding because of divergent location of skull sutures. An adenovirus associated double floxed inverted (AAV-DIO) virus was generated containing the genetically encoded ROS sensor HyPerRed (Schattauer, 2019; Ermakova, 2014). AAV-DIO HyPerRed virus was injected into the VTA of DAT-IRES-Cre heterozygous mouse (Coordinates: A/P -3.3, M/L +/- 0.5, D/V -4.4) so that the HyPerRed sensor would be expressed exclusively in dopamine neurons expressing Cre recombinase.

Fast-Scan Cyclic Voltammetry Mice were decapitated and the head placed in pre-oxygenated, cold modified artificial cerebrospinal fluid (aCSF) (248 mM sucrose was substituted for NaCl). The brain was removed, and 250 μm coronal slices containing nucleus accumbens were prepared (Leica, model VT1000X). Nucleus accumbens slices were transferred to standard oxygenated aCSF (124 mM NaCl, 2.5 mM KCl, 1.25 mM 12 NaH2PO4, 1.25 mM MgSO4, 2 mM CaCl2, 10 mM dextrose, 25 mM NaHCO3) and incubated for 1 hr at 37˚ C, then held at room temperature before recordings. Slices were placed in a recording chamber, and perfused with oxygenated aCSF at 31-33°C throughout recording. Carbon-fiber working electrodes were fabricated as described (Clark, 2010; Lemos, 2012), hand-cut to 150-200 μm past the capillary tip. A single electric pulse (120- 200 μA, 2 ms per phase) was applied to a parallel bipolar stimulating electrode to evoke dopamine release, and the potential at the working electrode was held at -0.4V versus Ag/AgCl, ramped to +1.3V and back to -0.4V at a rate of 10 Hz. Baseline dopamine release was measured every 2 min for 20 min prior to measuring release in response to 100 nM

U69,593. Waveform generation, data acquisition and analysis were carried out using two PCI multifunction data acquisition cards and software written in LabVIEW v7.1 (National Instruments, Austin, TX) as described (Clark, 2010; Lemos, 2012). Responses in the last 8 min (4 recordings) were averaged to calculate the U69,593 response.

Electrophysiology Horizontal VTA slices were prepared using the N-Methyl D-Gluconate (NMDG) GaCSF and HEPES solutions previously described (Ting, 2014). To record VTA G protein gated inwardly rectifying potassium channel (Kir3, GIRK) currents, the resistance of the electrodes was 3–7 MΩ when filled with an internal solution of (mM) K-gluconate 130, NaCl 5, Na phosphocreatine 10, MgCl2 1, EGTA 0.02, HEPES 10, MgATP 2, Na2GTP 0.5, 0.1% Biocytin, pH 7.3. Whole cell voltage clamp recordings were performed on neurons in the VTA expressing tdTomato reporter expressed only in DAT-expressing neurons. The cell was voltage clamped at −60 mV using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA). Signals were digitized by a Digidata 1440 A/D converter (Molecular Devices) and stored using pClamp 10.2 software (Molecular Devices). To record GIRK currents, once a cell was stably patched and basal physiology assessed, the slice was bathed in high potassium (5.5 mM K+) ACSF to enhance the currents in the cells at hyperpolarized potentials. As expected, when the slice was incubated in high [K+], the holding current increased and inward rectification was more apparent. The membrane potentials of VTA neurons were subjected to a voltage ramp protocol in which they were brought from −120 mV to −50 mV over 10 sec. After three stable ramp measurements (the average of three sweeps) were obtained, U69,593 (1 μM) was bath applied and the slice was allowed to equilibrate for 3 min. Subsequently, a ramp measurement was taken after 12 min of U69,593 application and the three sweeps were averaged. Following

U69,593 application and stable current responses, BaCl2 (100 μM) was added to distinguish Ba2+- sensitive GIRK currents. Conductance was calculated as the slope of the I-V curve in response to a voltage ramp from -120 mV to -50 mV. Maximal inward current was calculated as the average current in response to a -115 mV applied voltage.

HyPerRed Imaging and Analysis VTA slices were prepared as described above for the slice recordings. Modified aCSF solution included 800 μM thiourea and 400 μM sodium ascorbate (Rice, 1999) to reduce cellular stress and keep basal ROS levels low. Agonist-induced ROS HyPerRed fluorescence was recorded on an upright fluorescent microscope (Olympus BX51WI), with the fluorophore excited by CoolLED pE300 through the 20x objective at 555 nm and collected using an Hammamatsu ORCA-Flash4.0 V2 camera and HC Image Live software. Images were collected every 2 min with an exposure time of 100 ms to 500 ms and LED intensity of 50%. For nalfurafine experiments, 35 images were taken over 70 minutes. aCSF was washed on for 15 min, aCSF containing nalfurafine (100 nM) was washed on for 45 minutes, 200 μM H_2O_2 was added for the final 10 min (images 31-35). For naloxone-nalfurafine experiments, 40 images were taken over 80 min to establish a secondary baseline of aCSF plus naloxone (1 μM), prior to wash-on of aCSF with both naloxone and nalfurafine. ΔF/F was calculated for the VTA region. The region was circled in ImageJ and the fluorescent was measured over time as a raw value. ΔF/F was measured as the current fluoresence minus the average fluorescence of the four images prior to nalfurafine ("baseline fluoresence"), divided by the baseline fluorescence. Inclusion criteria required that the VTA region significantly increase ΔF/F at the end of experiment in response to H_2O_2 (100 μM) application. Due to mild bleaching over the course of the experiment, a "Baseline2" was calculated as the average of the 2 images prior to H_2O_2 wash

on. Any image that did not show a fluorescence increase of at least 5% in response to H_2O_2 was excluded from this study, as in these cases we could not confirm functional protein expression or tissue viability.

Experimental Design and Statistical Analysis Animal numbers were as follows: tail flick, male n = 7 per group; CMD101-norBNI n = 4-5 female mice per group; repeated n = 7 female mice per group; norBNI voltammetry n = 5-6 animals per group; b-CNA voltammetry n = 4-6 animals per group; electrophysiology n = 7-8 animals, 7-10 cells per group; HyPerRed (4 animals per group). Due to sex-dependent effects in norBNI action (described in Figure 4.1), experimental animals were all male in Figures 4.2-4. Data are expressed as mean ± SEM and were analyzed with Prism 7 software (GraphPad). Group differences were determined using t tests, ANOVA, or repeated-measures ANOVA as described in the Results. Post hoc comparisons were analyzed with Tukey's or Sidak's test. For all statistical tests, α was set to 0.05.

RESULTS

Duration of antinociceptive action of norBNI in the tail-withdrawal assay. Male mice treated with 10 mg/kg U50,488 show a significant increase in latency to withdraw their tails from 52.5˚C water, which was normalized to 100% of maximum possible effect (MPE). As previously reported, a single (10 mg/kg, i.p.) injection of norBNI seven days prior to analgesic testing blocked U50,488-mediated increase in tail flick latency in male mice (Figure 4.1A) (Unpaired ttest: t2.916,18 p=0.0092). In contrast to male mice, U50,488 alone did not significantly increase tail-flick latency in female mice (Figure 4.1B). As previously reported, the estrous state-dependent insensitivity to U50,488 effect is caused by estradiol-stimulated GRK2/3 signaling (Abraham, 2018b). Pretreatment with the GRK2/3 inhibitor CMPD101 restored sensitivity to

U50,488 in female mice (Figure 4.1B). Although CMPD101 given just prior to U50,488 restores the effectiveness of the agonist, norBNI administered one week prior to CMPD101-U50,488 treatment did not block KOR-mediated analgesia in female mice, indicating that there is also a sex difference in norBNI sensitivity (Figure 4.1C). To test if estradiol-GRK2/3 signaling was also responsible for this sex difference, we administered CMPD101 prior to norBNI antagonist pretreatment seven days before the tail flick assay. The analgesic effects of U50,488 were blocked in mice treated with CMPD101 and norBNI, but not in those female mice treated with norBNI alone (Figure 4.1C). Neither CMPD101 alone nor norBNI alone blocked CMPD101-U50488 analgesia evident in female mice (One-Way ANOVA $F = 5.316$, $p=0.0242$; Tukey's post-hoc CMPD101-vehicle vs. CMPD101-norBNI $p=0.0215$). These data suggest that the estrogen-GRK2 mechanism responsible for sex differences in KOR agonist action previously identified (Abraham, 2018b) also contributes to the sex differences in norBNI duration of action. Thus, estradiol-stimulated GRK2 sequestration of Gbg likely disrupts norBNI activation of JNK, thereby disrupting receptor inactivation of KOR by norBNI stimulated JNK production of ROS (Abraham, 2018b, Schattauer, 2017a).

Repeated low-doses of norBNI produce a long-lasting antagonist effect in female mice. The utility of a long-acting KOR antagonist in women that requires concomitant GRK2/3 inhibition is not practical from a compliance perspective. An alternative dosing strategy might circumvent that constraint. We hypothesized that if female mice were treated repeatedly with a low-dose of norBNI, the cumulative effect of dosing over multiple days of their estrous cycle including during low-estradiol phases, might produce a long-acting block of U50,488 mediated tail flick. This potentially safer low-dosing strategy was previously demonstrated to be effective in males

(Chavkin, 2019).

Female mice were injected with norBNI (1 mg/kg) or saline once per day for seven days. We subjected these mice to CMPD101-U50,488 tail flick 48 hours after the end of the week-long pretreatment. U50,488-induced analgesia was blocked by repeated norBNI, but not affected by repeated saline pretreatment (Figure 4.1D). There was no significant interaction, but there was a trend toward an interaction and a significant effect of pretreatment and time (Unpaired t-test: t2.607,11 p=0.0204). Collectively, these data demonstrate that norBNI acts effectively as an antagonist against KOR-mediated analgesia in both male and female mice. However, in female mice, because high estradiol can make norBNI less effective, KOR inactivation is only evident either after pretreatment with CMPD101 or in mice repeatedly treated with low doses of norBNI. In conjunction with recently published data (Chavkin, 2019), these results indicate that repeated, low-dose norBNI treatment may be effective in both male and females.

A significant increase in phospho-JNK is observed in CMPD101 pretreated female mice after norBNI. To test if the sex difference we see in norBNI action is due to estradiol-dependent shifts in GβɣGβɣ sequestration, we measured the phospho-JNK immunoreactivity with Western blot in samples taken from mouse striata given norBNI or saline with either a vehicle or CMPD101 pretreatment. Mice were given vehicle (80% saline, 10% cremophor, 10% ethanol) thirty minutes prior to norBNI (10 mg/kg IP in saline) or saline, after an hour, we dissected their striata and measured phospho-JNK. The norBNI alone did not cause a consistent increase over vehicle in our preliminary data (Figure 4.1B,C). However, pretreatment with CMPD101 (15 mg/kg in 80% saline, 10% cremophor, 10% ethanol), prior to norBNI led to a significant increase over the CMPD101/saline controls (Figure 4.1B,C). These data are preliminary, and this data will be

finalized in a manuscript resubmission (Reichard 2020).

NorBNI antagonism of KOR inhibition does not persist in dopamine terminals. KOR expression has been shown to be required on VTA dopamine neurons for conditioned place aversion to U50,488 (Ehrich, 2015). However, while studying the underlying mechanisms involved, we consistently found that in vivo pretreatment with norBNI did not have long-lasting effects on KOR regulated dopamine release as measured using slice ex vivo voltammetry. The discrepancy between norBNI's long-lasting effects on KOR-dependent aversion and its lack of long-lasting effect in vitro is surprising because the dominant theory rationalizes the KOR aversion as a consequence of presynaptic inhibition of dopamine release (Chefer, 2013).

Traditional kappa opioid receptor agonists are known to generate a robust inhibition of dopamine terminals in the nucleus accumbens (Di Chiaro & Imperato, 1988). To systematically establish whether presynaptic inhibition can be blocked by norBNI, C57BL6-J mice were injected with 10 mg/kg norBNI or an equivalent volume of saline one week, 24 hours, or 1 hour prior to brain dissection and tissue sectioning (Figure 4.2A-D). NorBNI acted as an effective antagonist in animals sectioned 1 hour post IP injection of norBNI, but neither 24 hour nor one-week pretreatment with norBNI was effective at blocking the U50,488 effects on evoked dopamine release (One-way ANOVA $F = 8.17$, $p = 0.0012$, Tukey's post-hoc Saline vs 1-hour $p = 0.0031$, Saline vs. 24 hr $p = 0.8396$, Saline vs. 1 week $p = 0.9381$). These results confirm that norBNI could penetrate to the ventral striatum and acutely block KOR but did not produce long-lasting receptor inactivation at VTA dopamine terminals.

Covalent inactivation of KOR by β-chlornaltrexamine effectively blocked KOR signaling for up to a week. To test if a higher rate of receptor recycling in the terminals as

compared to cell bodies explained the shortened duration of action of norBNI in dopamine terminals, we used beta-chlornaltrexamine (β-CNA) to block opioid signaling. β-CNA is a nitrogen mustard analog of naltrexone that covalently binds to the ligand binding site in the opioid receptor to irreversibly prevent agonist occupation (Schoenecker, 1987). ICV injection of 1 μg β-CNA significantly blocked KOR agonist-mediated inhibition of dopamine release from dopamine terminals after either 48 hours or one-week post injection (Figure 4.2E,F) (One-way ANOVA $F = 7.545$, $p = 0.0067$, Tukey's post-hoc Sham vs 48-hour $p = 0.009$, Sham vs. 1 week $p = 0.0129$). Because β-CNA effectively blocked KOR signaling a week post-treatment, faster receptor turnover in dopamine nerve terminals cannot explain the lack of long-lasting norBNI antagonism.

NorBNI antagonism of U69,593 mediated activation of GIRK currents persists up to a week in dopamine cell bodies. The lack of long-lasting norBNI effects in dopamine terminals raises the possibility that the cellular machinery for long-lasting inactivation by norBNI is not expressed by dopamine neurons. To address this, as well as any concerns that slice preparation itself disrupts the norBNI receptor inactivation, we tested whether norBNI produced a longlasting inhibition of the physiological responses to KOR activation evident in the cell bodies of dopamine neurons. KOR agonists, like other Gi/o-coupled receptors can acutely reduce the excitability in the somatodendritic compartment by increasing conductance of GIRK channels, and norBNI has been previously shown to acutely block this response in VTA dopamine neurons (Margolis, 2006).

We injected mice with 10 mg/kg norBNI or saline one week prior to in vitro electrophysiological recording. Dopamine neurons, identified using tdTomato reporter expression in DAT-IRES-cre mice (Figure 4.3A), consistently showed an increase in the BaCl-

sensitive GIRK current and membrane conductance following in vitro application of 1 μM U69,593 (Figure 4.3B) (Paired ttest, current at -115 mV: t2.4,9, p = 0.0397, conductance: t3,9, p = 0.015; n = 8 animals, 10 cells). However, cells from mice pretreated with norBNI 1 week prior to brain dissection showed no significant increase in GIRK current or conductance in response to 1 μM U69,593 (Figure 4.3C) (Paired t-test, current at -115 mV: t0.962,6, p = 0.374, conductance: t0.242,6, p = 0.817; n = 7 animals, 7 cells). These data show that norBNI does block KOR-mediated facilitation of somatic GIRK currents in VTA dopamine neurons for at least a week post-injection. In most VTA dopamine neurons we patched (primarily in the lateral aspect of the VTA and labeled with DATCre TdTomato fluorescent reporter), we showed potentiated GIRK current in response to KOR agonists. Because long-lasting KOR inactivation is a consequence of JNK/PRDX6-mediated ROS generation (Schattauer, 2017a), these results suggest that norBNI may effectively activate this signaling cascade in the cell bodies but not in terminals of VTA dopamine neurons. The local effects of norBNI have not previously been reported and suggest that neither the kappa receptors nor the signal transduction mediators can effectively migrate between somatic and terminal cellular compartments.

We recently developed a virus containing a sensor for H2O2 that acts as a proxy for JNK-ROS activation by KOR (Schattauer, 2017a, 2019). To further delineate the differences in KOR signal transduction in dopamine neuron cell bodies and terminals, we measured the ROS generation stimulated by KOR agonist activity. We used the functionally selective KOR agonist nalfurafine (Schattauer, 2017b). Nalfurafine is a better reagent to address these mechanistic questions because it is a KOR agonist that does not effectively activate p38 MAPK, which inhibits ROS generation (Schattauer, 2019). We have observed that it stimulates ROS to a greater extent than U69,593 and more rapidly than norBNI. As shown in Figure 4.4A, nalfurafine

potently inhibited the release of dopamine with a logEC50 of -8.54 (95% CI -8.72 to -8.33) (equivalent to 2.9 nM). Nalfurafine was approximately 10-fold more potent than U69,593, with comparable efficacy (Figure 4.4A). To visualize neuronal ROS dynamics (Figure 4.4B), we recorded fluorescence emitted from the virally encoded ROS indicator, AAV-DIO-HyPerRed selectively expressed in VTA dopamine neurons. The HyPerRed protein, when oxidized in the presence of H2O2, forms a reversible disulfide bond that increased a ratiometric increase in fluorescence (Ermakova, 2014).

VTA dopamine neurons in DAT-IRES-Cre mice express the HyPerRed protein (Figure 4.4C). Fluorescence intensity was significantly increased by 100 nM nalfurafine as shown in these representative images (Figure 4.4C). To confirm HyPerRed sensitivity, slices were treated with 200 μM H_2O_2 after opioid exposure (Figure 4.4C&E, right panels). Nalfurafine caused a consistent, slow onset ~15% increase in the HyPerRed signal in VTA dopamine neurons, with a peak about 25 minutes after the onset of drug perfusion (Figure 4.4D). The nalfurafine-induced increase in HyPerRed fluorescence in VTA cell bodies was not evident in slices pretreated with the non-selective opioid receptor antagonist naloxone (10 μM) (Figure 4.4C,D). Both naloxone-nalfurafine and vehicle-nalfurafine samples showed an increased HyPerRed fluorescence signal in response to 200 μM H2O2 (Panel 3 of Figure 4.4 C,E). Dopamine terminals in the nucleus accumbens of in vitro midbrain slices also expressed the HyPerRed construct and responded to 200 μM H2O2 treatment (Figure 4.4 E); however, no significant increase in HyPerRed fluorescence in response to nalfurafine was observed in dopamine terminals (Figure 4.4 E,F).

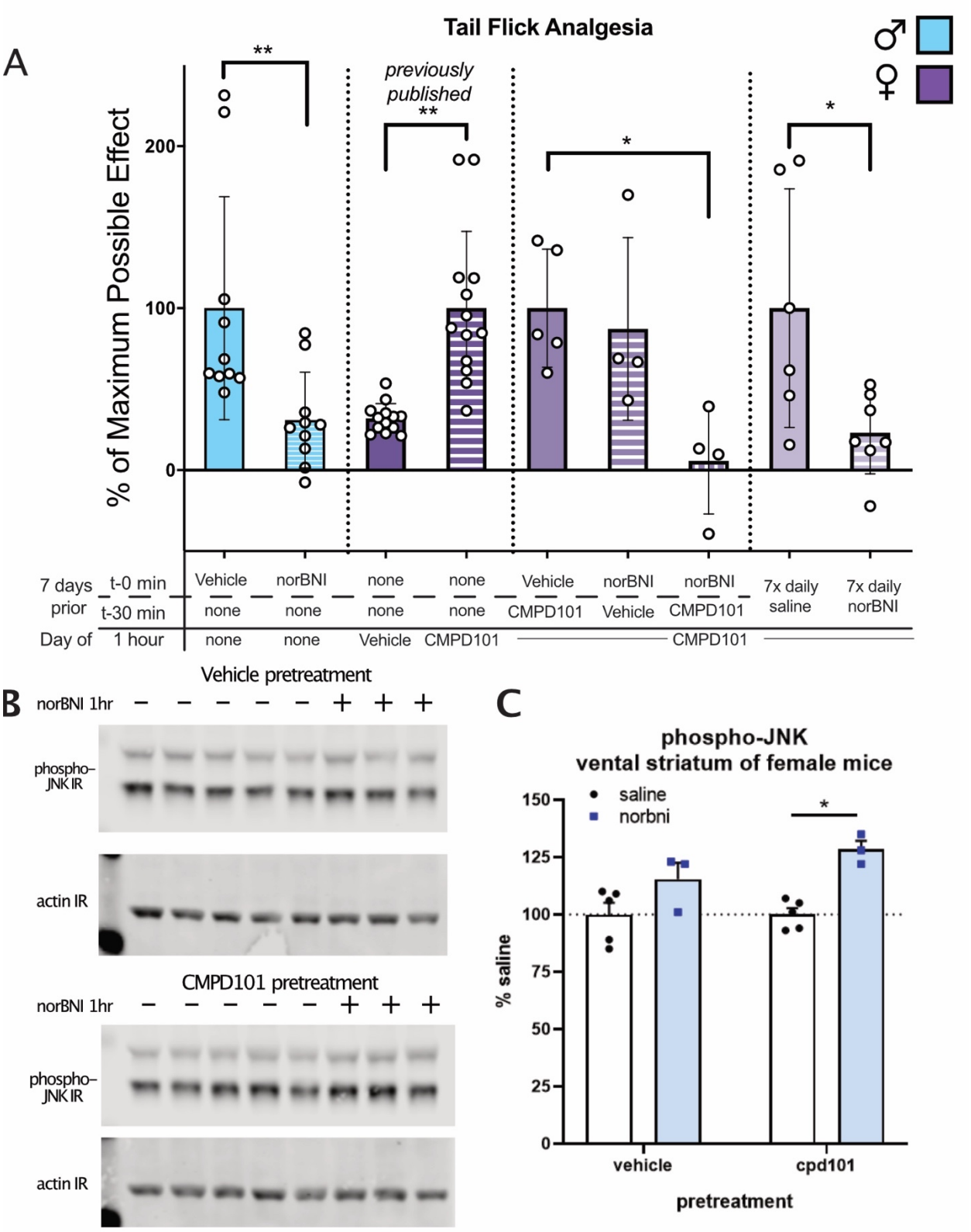


Figure 4.1 – Long-acting norBNI antagonism in females is effective if given in repeated

doses or after CMPD101. *Note: all groups are independent studies with unique control group, each experiment is separated by a dashed line. They are shown as maximum possible effect (%MPE), to compare across groups.* (A) First panel: Male mice were injected with either saline or 10 mg/kg norBNI (i.p.). One week later they were injected with 10 mg/kg U50,488 and tested for a shift in tail withdrawal latency after immersion in 52.5^{o}C water. Male mice pretreated with norBNI did not show a significant increase in tailflick latency, but those pretreated with saline did. (Unpaired t- test: $t_{2.916,18}$ p=0.0092). Second panel: *F*emale mice did not show an increased latency to flick after U50,488 treatment (data reprinted from Abraham et al 2019), but did after pretreatment with CMPD101(15 mg/kg), U50,488. Third panel: One-week pretreatment with either CMPD101 alone or norBNI alone did not affect U50,488 increase in tail-flick latency, but treating mice with CMPD101 prior to norBNI led to a long-acting block of the U50,488 increase in tail-flick latency (One-Way ANOVA F = 5.316, p=0.0242; Tukey's post-hoc CMPD101-vehicle vs. CMPD101- norBNI p=0.0215). Fourth panel: Female mice treated with repeated saline injections showed an increase in tail-flick latency to CMPD101 plus U50,488, but those treated with repeated norBNI doses did not. (Unpaired t-test: $t_{2.607,11}$ p=0.0204) (Each group separated by dotted lines was normalized to the max possible effect within the group. All groups were compared using a one way ANOVA $p < 0.05$; post-hoc Bonferonni *,** indicates $p<0.05$, $p<0.01$). (B) Western blots showing immunoreactivity (IR) for Phospho-JNK and Actin in samples taken from the striata of female mice injected with CMPD101 or Vehicle pretreatment and then treated with either Saline or NorBNI. (C) Quantitation of Phospho-JNK demonstrating that NorBNI causes a significant increase in JNK-IR in striata from female mice with CMPD101 pretreatment, but not in mice without.

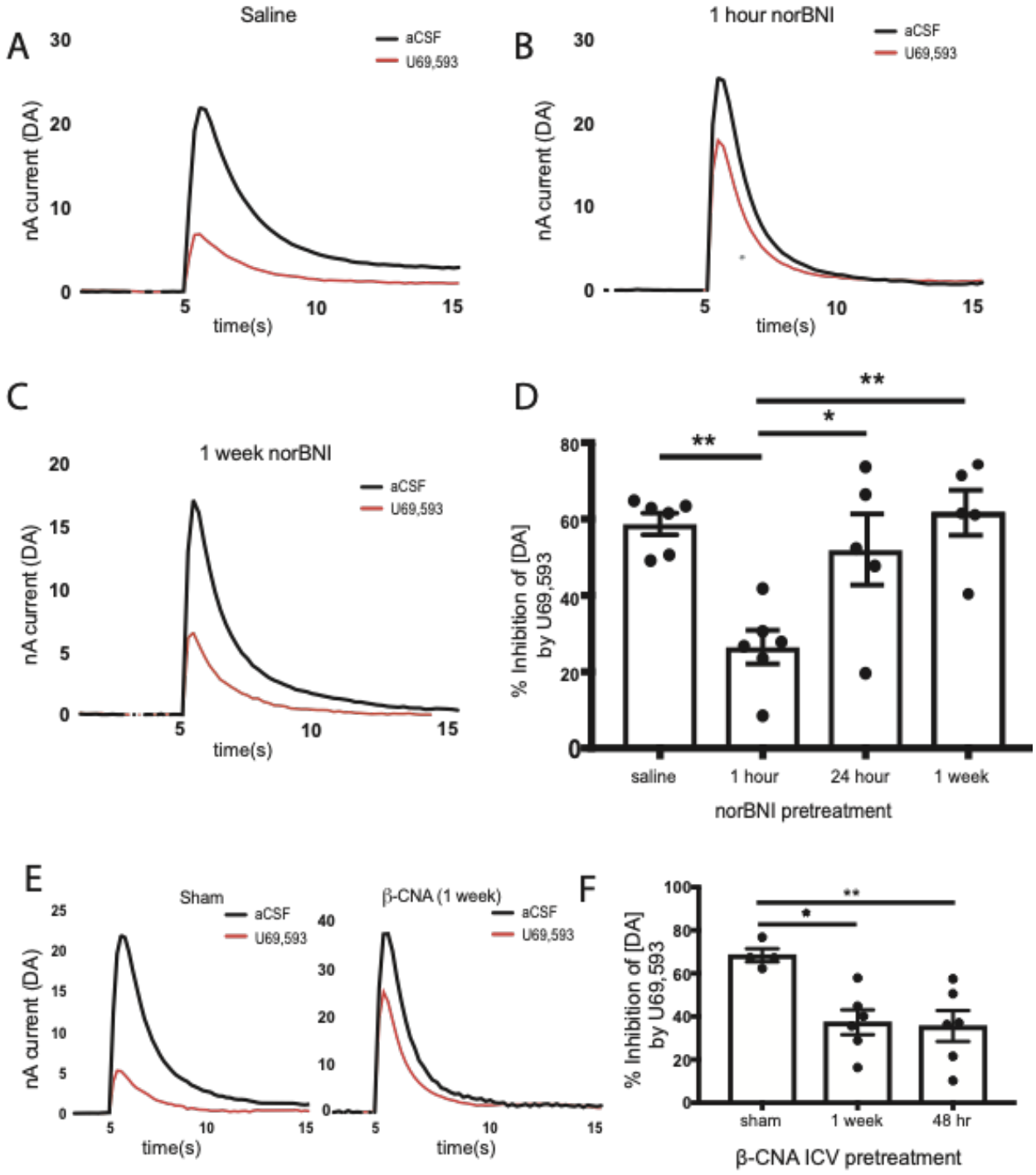


Figure 4.2 – norBNI does not cause a long-lasting block of KOR inhibition of dopamine release in the nucleus accumbens (NAc) core, whereas b-CNA receptor inactivation persists

at least a week in NAc terminals. (A,B,C) Example traces demonstrating evoked dopamine release in response to a 120-180 μA electrical stimulation before (black) and after (grey) washing on KOR agonist U69,593 (1 μM) in animals who had either been pre-treated with an IP injection of saline or norBNI prior to brain dissection and tissue sectioning. Inset showing Current-Voltage traces demonstrating that peak dopamine current occurred around 0.7 V. D. U69,593 inhibits dopamine release in NAc core slices, and this effect was significantly attenuated by pretreatment with norBNI one hour prior to slicing, but not by 24 hour or 1-week pretreatment with norBNI (One Way ANOVA $p < 0.05$, post-hoc saline vs 1 hour $p < 0.01$). E. Example traces of evoked dopamine before and after U69,593 in sham pretreated animals. Inset showing Current-Voltage traces demonstrating that peak dopamine current occurred around 0.7 V F. Intracerebro-ventricular (IVC) injection of 1 μg β-Chlornaltrexamine significantly attenuated the effect of U69,593 on inhibition of dopamine release (One-Way ANOVA $p< 0.01$; Sham vs 1 week $p< 0.05$, Sham vs 48 hr $p < 0.01$)

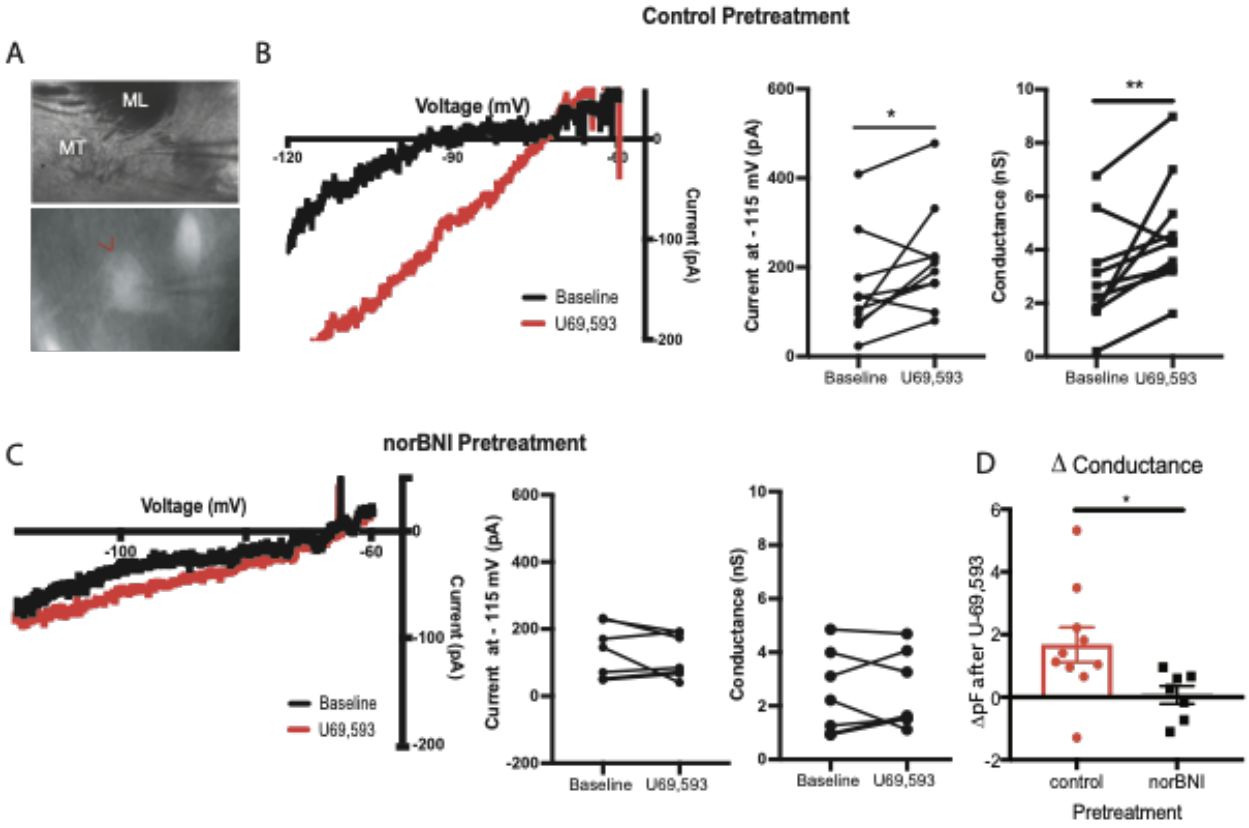


Figure 4.3 – KOR Agonist U69,593 Activates a GIRK current in VTA dopamine neurons that can be blocked by norBNI pretreatment **A.** Fluorescent cells in the VTA DAT-ires-cre Flox-tdTomato mice were patched and held at -60 mV. GIRK currents were elicited by subjecting the cells to a voltage ramp from -120 mV to -50 mV. B. GIRK current at baseline and in response to 1 μM U69,593: example trace, current at -115 mV, and conductance. Treatment with the KOR agonist significantly increased GIRK currents (paired t-test, $p<0.05$) and conductance as calculated by the slope of the line from -120 mV to -50 mV (paired t-test, $p<0.05$). C. GIRK currents as measured at baseline and after U69,593 from animals pretreated with: example trace, current at -115 mV, and conductance. D. Peak current after U69,593 minus peak current at baseline denoted as "DpF after U69,593" is significantly different in controls as compared to animals pretreated with norBNI. (* indicates $p<0.05$).

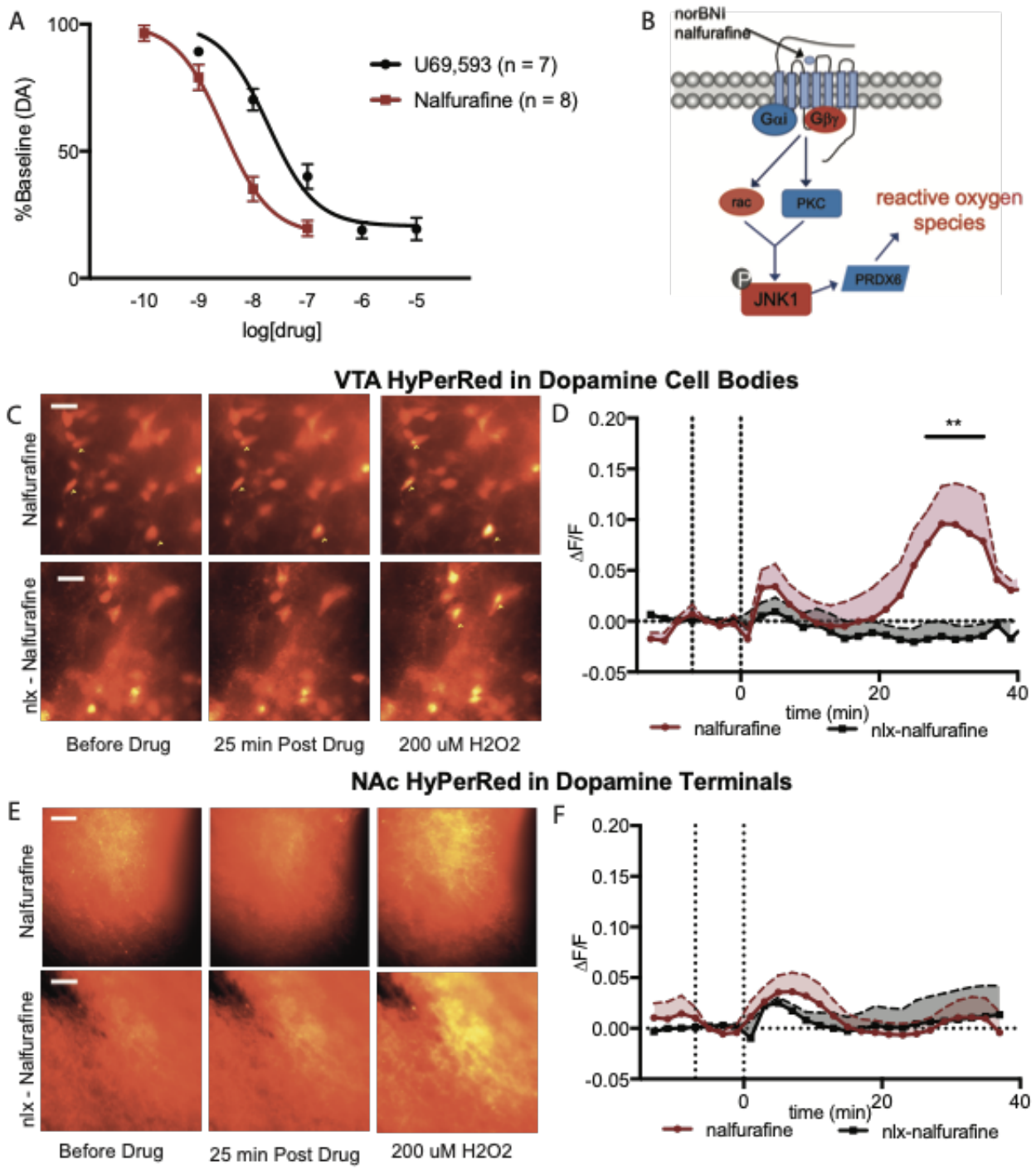


Figure 4.4 KOR agonist, nalfurafine, generates reactive oxygen species in VTA Dopamine cell bodies, but not dopamine terminals in the nucleus accumbens, as measured by HyPerRed. A. Nalfurafine inhibits electrically evoked dopamine release in the nucleus accumbens. It is more potent than the agonist U69,593 ($logEC_{50}$-Nalfurafine = -8.54 vs. $logEC_{50}$-

U69,593 = -7.72). In all groups tested, 200 μM H_2O_2 was applied after agonist treatment. B. The agonist nalfurafine preferentially activates the G-protein mediated signaling at the KOR receptor, including dopamine inhibition *and* JNK signaling, allowing us to confirm if the JNK-ROS signaling also activated by norBNI is occurring in dopamine neurons. C. VTA dopamine neurons HyPerRed fluorescence response pseudocolored in "Red Hot" in FIJI to represent intensity. Cells with clear nalfurafine and H_2O_2 responses indicated with carat (scale = 100 μm) D. Oxygenated aCSF was perfused at 2 ml/min over sections, with images every two minutes. At t = -7 naloxone was washed onto naloxone-nalfurafine slices. At t = 0, aCSF with 100 nM nalfurafine was washed over slices. At ~20 min post-nalfurafine wash-on, VTA HyPerRed fluorescence begins to increase. This persists for ~15 minutes. Images were taken every two minutes. The nalfurafine group was significantly different from the naloxone-nalfurafine group at t = 28-34 (Two-Way ANOVA $F(23, 69) = 4.815$, $p < 0.0001$. Multiple comparisons, time of 27,35 $p<0.05$, time of 29-33 $p<0.01$). E. Comparable recordings of HyPerRed expressing fibers in the NAc showed clear H_2O_2 response (scale = 100 μm). F. Neither nalfurafine treated slices or naloxone-nalfurafine treated slices showed a significant response to nalfurafine (Two-Way ANOVA $F(25,150) = 0.3309$, $p >0.9$).

Discussion

In this study, we found that norBNI is an effective long-acting antagonist in female mice, when given in repeated, low-doses. However, like unbiased KOR agonists, norBNI signaling is disrupted by GRK2-mediated phosphorylation in female mice, likely due to high levels of estradiol. We also found that norBNI effectively blocks KOR activation of GIRK currents in dopamine cell bodies for a week after pre-treatment. However, norBNI does not produce a

longlasting block of KOR-mediated inhibition of presynaptic dopamine terminals. This is in contrast to the long-lasting effects of norBNI in serotonergic terminals (Schindler, 2012). Together these data emphasize the importance of somatic KOR on VTA dopamine in mediating the behavioral effects of stress. We also found that nalfurafine, a KOR-agonist used to treat pain and itch in humans without psychotomimetic effects or dysphoria (Endoh, 2000; Inui, 2015) inhibits dopamine release with higher potency than U50,488, despite its inability to generate aversion or dysphoria. These data further emphasize that presynaptic inhibition of dopamine alone is not sufficient to generate conditioned place aversion or dysphoria in response to KOR agonists.

We found that norBNI is not long-lasting in female mice, unless they are pretreated with CMPD101. This indicates that, as with KOR agonists, interaction between increased estradiol level and GRK2/3 signaling may disrupt the collateral-agonist activity of norBNI in activating JNK. Animals pretreated with norBNI alone had more variance in their tail-flick response, indicating this likely is an estrous dependent effect as was found for agonist active KOR responses in female mice (Abraham, 2018b). We also would expect, due to this sex difference, that all long-acting JNK-dependent antagonists, including JDTic would not work in females during high-estradiol periods of their cycles. We would predict that in humans, JNK-dependent antagonists would potentially be more effective in people taking estradiol-suppressing medications, in menopause, or if taken during low-estrogen points in cycle, which are easier to determine in humans than mice. Regardless, repeated low-dose is a more effective dosing regimen because it reduces compliance issues of missing a single dose without as many potential off target effects of a single high-dose of norBNI (Chavkin, 2019). This repeated dosing attenuates the sex difference in norBNI's action. It will be important to further assess the

effectiveness of low-dose repeated norBNI at blocking behavioral effects of stress such as immobility in forced swim test, especially in females (Laman-Maharg, 2019).

Our finding that norBNI acts as a long acting antagonist blocking the analgesic effects of KOR recapitulate previous results. However, the finding that norBNI does not block KOR-inhibition of dopamine terminals is novel and contributes to both our understanding of collateral agonism by compounds like norBNI and subcellular signaling differences. We believe this effect may be restricted not just to terminals, but specifically dopamine neurons. Previous work demonstrated that pretreatment with norBNI 5 days prior blocks the KOR-mediated increase in SERT translocation to the membrane in serotonin terminals in the nucleus accumbens (Schindler, 2012). We originally predicted that the balance of receptors recycling to the membrane after endocytosis and proteolysis may differ in terminals, leading to sufficient degradation and replacement of KOR in presynaptic terminals that after a week, there were no longer enough "locked" receptors to block inhibition. However, the finding that β-Chlornaltrexamine effectively attenuates KOR inhibition of dopamine release for a week disproves this hypothesis. As such, we predict that this indicates there are differences in the signaling effects of norBNI's collateral agonist activity specifically in dopamine presynaptic terminals. This prediction is further confirmed by the lack of ROS generation in response to nalfurafine in dopamine presynaptic terminals, as compared to the response within the ventral tegmental area.

One potential explanation for the inability of norBNI to be long-acting at dopamine terminals is that the biochemical environment is less conducive to ROS-mediated signaling (Smythies, 2000; Stokes, 1999). The metabolism of dopamine by monoamine oxidase produces reactive hydrogen peroxide as a byproduct and dopamine can become oxidative itself by generation of dopamine-oquinone (Stokes, 1999). Additionally, dopamine neurons have been shown to have

more active innate antioxidant activity to counteract ROS generation by catecholamine metabolism (Smythies, 2000). Dopamine terminals may be either too inundated by ROS, or may too tightly regulate ROS to facilitate JNK-dependent ROS signaling. In a small subcompartment with higher ROS production, the additional ROS generation from norBNI activation of JNK may not be effective to cause depalmitoylation of G proteins and would not be detectable above baseline values, as measured by HyPerRed. Although we hoped that treatment of slices with sodium ascorbate and thiourea would abrogate these concerns, it is still possible that the ROS levels in dopamine terminals are already too efficiently suppressed to be detected by a cytosolic sensor. Further development of the HyPerRed or related sensors to be membrane targeted may improve our signal to noise in such ROS-sensitive neurons.

In addition to our findings demonstrating the heterogeneity of signaling between cell compartments, the finding that norBNI does not have a long-lasting effect on presynaptic inhibition of dopamine release by KOR generates several key predictions about animal behavior. It has been previously shown that dysphoric components of stress that lead to conditioned avoidance and potentiation of preference for a drug reward depend on KOR on dopamine neurons. norBNI blocks conditioned place aversion to a KOR agonist (McLaughlin 2006b), and the data presented here indicate that this effect cannot be attributed to inhibition of dopamine release. This is further supported by the fact that p38 MAPK is necessary for the aversive effects of KOR, but not necessary for presynaptic inhibition of KOR. Pretreatment with norBNI produces a long-lasting block on increased swim immobility and stress-enhanced cocaine CPP (Laman-Maharg, 2018; Chartoff, 2012), indicating that presynaptic inhibition of dopamine release is not sufficient to cause these effects. As such, we predict that blockade of kappa opioid receptors in NAc terminals alone would not be sufficient to block behavioral effects of KOR on

mood and drug seeking. These findings lead us to predict that G-biased agonists would not be able to produce conditioned place aversion or potentiate drug reward, but would still inhibit dopamine release. Further studies should try to delineate the molecular mechanisms of presynaptic inhibition by KOR to better separate the functional role of this robust physiological effect.

Chapter 5

The contribution of potassium channels to KOR regulation of dopamine neurons

The data presented in this section are part of an ongoing project utilizing the conditional CRISPR-Cas9 gene editing system developed in Hunker, et al 2020. These studies were designed by, conducted by, and analyzed by me (Katie Reichard), unless otherwise noted.

Introduction

Elevated dopamine release during drug-taking increases activity of D1 medium spiny neurons, which release the KOR agonist, dynorphin (Cole 1995, Nestler & Malenka 2004). It is hypothesized that escalation of drug taking and the acute dysphoric effects of withdrawal depend on dynorphin-mediated activation of KORs on dopamine terminals (Wee and Koob 2010), however it is challenging to test this hypothesis because KOR conditional knockouts remove the receptor in both VTA cell bodies and DA terminals and local norBNI experiments block KOR on all cell types in the NAc. Presynaptic inhibition by Gαi-coupled receptors can occur through multiple mechanisms, including blockade of Ca^{2+} currents and facilitation of K^{+} currents (Miller 1998). Prior work shows that potassium channels contribute to presynaptic inhibition by KOR in the BNST and hippocampus (Li 2012, Simmons & Chavkin 1996). By determining the contribution of potassium channels to presynaptic inhibition, this study will present new druggable targets in preventing and treating addiction. If the mechanism of KOR inhibition of presynaptic DA release is determined, it may also be possible to isolate KOR inhibition of terminals and measure its unique contribution to drug-seeking behaviors.

KOR-mediated presynaptic inhibition of GABAergic neurons in the BNST requires ERK signaling (Li 2012). Additionally, in the hippocampus cannabinoid receptor 1 (CB1) and metabotropic glutamate receptors (mGluR), other Gαi/o- coupled receptors, reduce neurotransmitter release via ERK phosphorylation of Munc18-1, a protein involved in vesicle fusion (Schmitz 2016). KORs can also activate a dendrotoxin sensitive K^+-channel in mossy fiber terminals in the hippocampus, which contributes to presynaptic inhibition (Simmons 1996). The blockade of voltage-gated calcium channels (VGCC) is another common mechanism for presynaptic inhibition (Berecki 2016). There are indications that some of these mechanisms may be redundant, but the complement of ways by which KOR inhibits presynaptic terminals likely varies based on cell type, synapse type, and gene expression.

In order to study presynaptic inhibition of dopamine release by the KOR and its contribution to animal behavior, we sought to determine the mechanism(s) by which KOR inhibits dopamine release in NAc terminals. Opioid receptors and dopamine D2 receptors have both been shown to act through the delayed rectifier, shaker family of potassium channels, Kv1 (Faber & Sah 2004, Simmons & Chavkin 1996, Wimpey & Chavkin 1991, Fulton 2011, Martel 2011). In addition to Kv1 family channels, Kv3.4 channels have been identified presynaptically at corticostriatal synapses and their expression has been detected in dopamine neurons (Meneses 2016, Chung 2017). Kv3.4 channels fast-inactivation kinetics can be modulated by GPCR agonists, increasing K^+ conductance and inhibition of neurotransmitter release (Ritter 2012). Although axonally-expressed voltage-gated potassium channels may play an important functional role in KOR inhibition of dopamine release, G-Protein Coupled Inwardly Rectifying Potassium Channels (GIRKs) play a known role in KOR regulation of somatic excitability.

Disruption of GIRKs should reduce KOR regulation of cell bodies without disrupting presynaptic inhibition and serves as a negative control.

Despite not being important for presynaptic inhibition, GIRKs are an important mechanism by which KOR can regulate excitability in the somatodendritic compartment. Prior work from the Chavkin lab demonstrated that in dorsal raphe nucleus serotonin neurons, GIRK currents are potentiated by KOR agonist U69,593, but that prior exposure to repeated forced swim stress, GIRK conductance was reduced and channels were phosphorylated in a p38 MAP-K dependent manner (Lemos 2012). Additionally, phosphorylation of the GIRK subunit, Kir3.1, was found in VTA dopamine neurons after repeated, high dose U50,488 treatment (Ehrich 2015). This Kir3.1 phosphorylation suggests that the GIRK1 subunit is expressed in dopamine neurons, however several studies indicate that the predominate and obligate subunit in VTA dopamine neurons for GIRK channel function is GIRK2 or Kir3.2, encoded by the gene KCNJ6 (Karschin 1996, Cruz 2004). Due to the importance of GIRK channels in facilitating KOR regulation of neuronal excitability and the importance of KOR on dopamine neurons in the behavioral aversion to KOR agonists, we hypothesized that disrupting GIRK currents in VTA dopamine neurons would disrupt aversion to the KOR agonist U50,488. Because GIRK2 is the predominantly expressed subunit in VTA dopamine neurons, we predicted that knockdown of this channel would disrupt expression of GIRKs throughout the VTA, and thus KOR regulation of VTA soma and dendrites.

This chapter utilizes a vector-based CRISPR-SaCas9 system developed by Dr. Avery Hunker in the Zweifel lab (Hunker 2020). This method allowed us to target multiple potassium channels in a short time-frame, avoiding the prolonged and expensive process of developing knockout mice. The advantages of this method are speed, cost, and post-development

knockdown reducing compensatory mechanisms. Disadvantages include incomplete knockdown and challenges in confirming the knockout because small indel deletions lead to mutations too small to detect with in situ hybridization techniques and only confirmable with more expensive and technically challenging FACS sort and sequencing. The data presented herein are incomplete and do not support the hypotheses discussed above, however, they do provide a basis for future research, suggest some paths to take (and avoid), and demonstrate implementation of the novel CRISPR-SaCas9 system. The discussion will address what, if any, conclusions can be made from these data and suggest some follow up studies for the Chavkin lab and collaborators.

Methods

Guide RNA design *(modified from Hunker, et al 2020)* sgRNAs were designed first by attaining the full-length sequence of each gene from the UCSC genome browser database (http://genome.ucsc.edu/) (Kent et al., 2002). The exons of each splice isoform of each gene were identified and aligned (Mouse Genome Informatics database). For all genes the first (most 5') exon was present in all known splice isoforms and was selected as the sequence to search for PAMs. The Exon 1 sequence was uploaded to the CRISPOR website (crispor.tefor.net) to determine possible sgRNAs and PAM sequences for SaCas9 enzyme. The final sgRNA was chosen based on specificity, probability of frameshift mutations, and location on exon. Each sgRNA was ordered as short oligos (Integrated DNA Technologies (IDT)) with a 5'CACC- 3' overhang on the forward primer, and a 5'-AAAC 3' overhang on the reverse primer to facilitate integration into the AAV1-FLEX-SaCas9-sgRNA vector. The following are oligos used for the generation of the sgRNAs in this study. (*Rosa*) forward: AAAGGCTAACCTGGTGTGTGG, reverse: GGAGCGGGAGAAATGGATATG; (*KCNJ6*) forward: CCCATCCTTCCTCACGTACCT, reverse: AGGTACGTGAGGAAGGATGGG-C; (*KCNC4*)

forward: GGGATCCGCCAACCAGGCAAG, reverse: CTTGCCTGGTTGGCGGATCCC; (*KCNA2*) forward: GAAGCAATGGAGATGTTTCGG, reverse: CCGAAACATCTCCATTGCTTC.

Cloning AAV-FLEX-SaCas9-sgRNA Constructs The pAAV1-FLEX-SaCas9-sgRNA plasmid (Hunker, 2020) was digested overnight with BsaI-HFv2 and gel purified (Qiaquick Gel Extraction Kit, QIAGEN). The ordered sgRNA oligos were resuspended to a concentration of 100uM. The oligos were phosphorylated at 37C for 30min using the following reaction: 1uL of each 100uM oligo, 1uL T4 ligase buffer (NEB), 0.5uL phosphonucleotide kinase (PNK, NEB) and 6.5uL H2O. To anneal the oligos, the entire reaction was placed at 100C for 5 minutes and allowed to slowly return to room temperature. 50 ng of digested pAAV-FLEX-SaCas9-sgRNA and 1uL T4 ligase (NEB) were added directly to the reaction and incubated at room temperature for 2 hours. 2uL of the reaction was placed in 50 µl of XL-1 Blue competent cells (Agilent) and heat shocked at 42˚C for 45 second. 5 mL of LB was added and the transformed bacteria was allowed to grow at 37˚C in shaking incubator for 75 minutes prior to plating. Colonies were grown in LB + AMP for 14-18 hours and minipreps (QIAGEN) were performed to extract DNA and identify transformed colonies containing the edited plasmid. A restriction digest using BsaI-HFv2 and HindIII-HF was performed to screen for positive colonies. One positive colony was selected and the DNA was extracted using a maxiprep kit (Invitrogen). The insertion of the sgRNA was confirmed via Sanger sequencing (Genewiz).

Surgery Mice were anesthetized with isoflurane and mounted on a model 1900 stereotaxic alignment system (David Kopf Instruments). Adenoassociated viruses used for this project (AAV1-FLEX-SaCas9-sgRNA, AAV1-FLEX-eYFP, and AAV1-FLEX-ChR2 (Zweifel lab)) were injected into the VTA of DAT-IRES-Cre heterozygous mouse (Coordinates: A/P -3.3, M/L

+/- 0.5, D/V -4.4). For voltammetry and behavior experiments, experiments were completed 6 weeks after surgery to allow ample time for both gene editing and depletion of axonally expressed ion channels.

Voltammetry Preparation of NAc slices for fast-scan cyclic voltammetry assays were completed as previously described in this dissertation. To evoke dopamine release from ChR2-expressing terminals, a Prizmatix LED (473 nm) was placed in the bath proximal, but above the slice and carbon fiber microelectrode. Trains of blue-light (25 mW; 20 Hz; 10 ms pulses; varying pulse number) were generated using two PCI multifunction data acquisition cards and software written in LabVIEW version 7.1 (National Instruments). To facilitate comparison between dopamine release volume in Rosa control vs. K+ channel CRISPR mice, we kept the recording electrode the same for each cohort. For the oxo-m experiments, a single 0.2 ms pulse of 473 nm light was used to stimulate dopamine release (Shin 2015).

Animal Behavior For both conditioned-place preference and aversion assays, mice were trained in a balanced, two-chamber plexiglass apparatus with two different floorings (bars and metal mesh) and a removable opening between both chambers. All conditioning and testing sessions lasted 30 min and were recorded on video for analysis in Ethovision version 3.0 (Noldus). Mice were placed in the opening between both chambers, halfway between each and allowed to roam freely for thirty minutes. Their initial preference was then measured and used to determine drug pairing. For cocaine CPP assays, the saline-paired compartment was set as the more-preferred chamber from the pre-test and the cocaine-paired compartment was set as the least-preferred chamber. Mice received cocaine (5 mg/kg, i.p) in the morning and saline (10 mL/kg, i.p.) in the afternoon 4 h after the morning training for two consecutive days. CPP was assessed on day 4 by allowing the mice to roam freely in both compartments and recording the time spent in each.

Preference scores were calculated by subtracting the time spent in the cocaine-paired compartment post test minus the pre-test. For U50,488 CPA assays, the saline-paired compartment was set as the least-preferred chamber from the pre-test and the cocaine-paired compartment was set to the more-preferred chamber. Mice received saline in the morning (10 mL/kg, i.p.) and U50,488 (2.5 mg/kg, i.p.) in the afternoon 4 h after the morning training for two consecutive days. CPA was assessed on day 4 by allowing the mice to roam freely in both compartments and recording the time spent in each. Scores were calculated by subtracting the time spent in the U50,488-paired compartment post test minus the pre-test. Locomotor scores were calculated using Ethovision tracking; distance traveled during 30-minute cocaine pairing was measured during the first AM cocaine session.

Electrophysiology GIRK currents were recorded as previously described (Chapter 4), except dopamine neurons were identified by eYFP expression.

Drugs TEA (10 mM, concentration nonspecific for all potassium channels - Sigma Aldrich), 4-AP (100 μM concentration specific to delayed rectifier family - Sigma Aldrich), and α-Dendrotoxin (30 nM concentration specific for Kv1.1,1.2,1.6- Alomone Labs) were washed onto the slice for 10 min prior to washing on 100 nM U69,593. Oxotremorine (oxo-M), quinpirole, and baclofen were purchased from Tocris. U69,593, U50,488, and cocaine hydrochloride were provided by NIDA Drug Supply.

Results

Non-specific potassium channel blockers TEA and 4-AP attenuate pharmacological inhibition of dopamine release by KOR agonist. To assess the contribution of presynaptic, voltage-gated potassium channels to the inhibition of dopamine release by KOR, we assessed the impact of potassium channel blockers TEA, 4-AP, and α-dendrotoxin on this effect. After

electrically stimulated dopamine release was stabilized to a baseline, aCSF contaning TEA, 4-AP, or α-DTX was washed on the slice and a new baseline was calculated. TEA and 4-AP both increased basal dopamine release (Fig 3A), but α-DTX did not, consistent with its increased specificity (Dolly & Parcej 1996). After a new baseline was established in the presence of the channel blocker, aCSF containing either TEA, 4-AP, or α-DTX *and* U69,593 was washed onto the slice. The time course of stimulated dopamine release, as percent of baseline, compares the inhibition of dopamine release in the presence of U69,593 and vehicle alone or in the presence of U69,593 and a channel blocker. Both 4-AP and TEA attenuate the inhibition of release, whereas α-DTX does not appear to shift the ability of U69,593 to inhibit dopamine release (Fig3 B-G). The percent inhibition was quantified by averaging the final four stimulations after drug treatment (14-20 minutes after wash-on). U69,593 in the presence of 4-AP and TEA was significantly less than the inhibition in vehicle or α-DTX (One-way ANOVA, F statistic 47.4, $p < 0.0001$; Tukey's multiple comparisons: Veh vs. 4-AP $p < 0.0001$, Veh vs. TEA $p < 0.0001$, Veh. vs. α-DTX $p = 0.08$).

AAV-FLEX-SaCas9-sgRNA[K Channel] constructs were expressed in dopamine neurons. We injected DIO SaCas9 virus expressing guide RNAs targeting either voltage-gated potassium channels KCNA2 and KCNC4 or the GIRK channel KCNJ6 (Figure 5.1A) into the VTA of DAT-IRES-Cre mice along with either AAV1-FLEX-ChR2-eYFP or AAV1-FLEX-eYFP and preserved the VTA for immnohistochemistry. The HA-tag (red) overlaps with cells expressing ChR2-eYFP and TH antibody staining dopamine neurons for all three potassium channel viruses and for the control virus targeting the Rosa locus (Figure 5.1B). The HA+ cells confirm viral transduction, but do not confirm knockdown, which must be done functionally with physiology and/or with sequencing (*see Figure 5.4*).

Selective, CRISPR-mediated knockdown of KCNA2, but not Rosa control or KCNJ6 increase optically evoked dopamine release Mice co-injected with the DIO SaCas9 and DIO ChR2 virus were used for ex vivo fast-scan cyclic voltammetry. We measured nA of dopamine in response to a short train of blue light (20 Hz, 5 pulses, 10 ms pulse; 473 nm) applied through an LED fiberoptic directly onto the slice in the bath (Figure 5.2D). The evoked DA release was significantly higher in samples taken from the KCNA2 mice as compared to the Rosa controls, but not for the KCNJ6 mice, or the KCNC4 mice (One-way ANOVA F statistic 7.55; Tukey's multiple comparison: Rosa vs. KCNA2 ($p = 0.003$), KCNJ6 ($p = 0.995$), KCNC4 ($p = 0.315$).

Selective, CRISPR-mediated knockdown of voltage-sensitive potassium channels Kv1.2 (KCNA2) and Kv3.4 (KCNC4) does not alter inhibition of dopamine release by KOR agonist U69,593 or D2R agonist quinpirole We optically stimulated dopamine release from NAc terminals expressing ChR2 in animals coinjected with AAV1-FLEX-SaCas9-sgRNA with guide RNAs targetting either KCNA2, KCNC4, or KCNJ6 (experimental) or the Rosa locus (control). After establishing a stable baseline of dopamine release, we washed on 100 nM U69,593 or 100 nM dopamine to determine if genetic knockdown of the potassium channels changed the efficacy of the compound. Neither the % Inhibition of dopamine release by Quinpirole (E) or U69,593 (F) in experimental groups were significantly different from controls.

Knockdown of KCNA2 gene increases optically evoked dopamine release. Preliminary data (Figure 5.2D) indicated that knocking down expression of the Kv1.2 potassium channel increases optically evoked dopamine release in the NAc core. To test this, I stimulated release with a series of increasing pulses (fixed frequency at 20 Hz and pulse width of 10 ms) and measured the evoked dopamine release in slices taken from mice injected with either ChR2

and the Rosa CRISPR virus or ChR2 and the KCNA2 CRISPR virus. KCNA2 CRISPR significantly increased the evoked dopamine current in response to 5, 10, and 20 pulses of blue light (Figure 5.3A). These data indicate a potential role of the Kv1.2 channel in regulating dopamine outflow at the terminal and indicate it may be important for regulating reward-related behavior and responses to salient, motivational stimuli.

Knockdown of KCNA2 gene does not alter the effect of muscarinic acetylcholine agonist oxotremorine on evoked dopamine release. Prior literature indicated that oxo-M can modulate axonal outflow by blocking voltage-gated potassium channels, thus increasing neurotransmitter release (Shin 2015). To determine if the increase in dopamine release caused by oxo-M is, in part, regulated by a Kv1 current, we applied oxo-M to the slice after establishing a stable baseline of light-evoked release (1 pulse, 20 ns pulse width). The dopamine release increased in response to oxo-M in slices taken from both ROSA and KCNA2 CRISPR treated animals, indicating that knockdown of the Kv1.2 channel does not disrupt muscarinic regulation of dopamine release (Figure 5.3B).

Knockdown of KCNA2 gene does not change novelty locomotor response, cocaine conditioned place preference, or operant learning for food. In an effort to determine if the increased dopamine release detected in the KCNA2 knockout animals modified dopamine-mediated behaviors, we assessed cocaine responding and operant behavior. To assess cocaine behaviors, we used a conditioned place-preference assay. Mice were allowed to freely roam a two-chambered box. We calculated their preference for each compartment, then on two subsequent days, injected them with 5 mg/kg cocaine before placing them for 30 minutes into their less-preferred chamber and saline before placing them in their previously preferred chamber. We calculated their locomotor behavior during the first cocaine pairing (Figure 5.3C).

On a fourth day, we allowed the mice to again freely roam the containers and determined the shift in their preference after two cocaine pairings. Their cocaine preference is calculated as cocaine paired chamber post-pairing minus cocaine paired chamber pre-paring (Post-Pre, in seconds). Both the control and KCNA2 knockdown mice developed a significant preference for the cocaine paired chamber (Figure 5.3D).

GFP+ Cells from animals co-injected with the AAV1-FLEX-KASH-GFP and AAV1-FLEX-SaCas9-SgRNA(KCNJ6) developed multiple insertion and deletion (indel) mutations. Five weeks after we injected the KASH-GFP and CRISPR viruses into the VTA of DAT-IRES-Cre mice, we rapidly decapitated the mice, took VTA punches, and flash froze the tissue in liquid nitrogen. The tissue was processed for nuclear isolation and FACS sorting (Hunker 2020) and the KCNJ6 gene was sequenced using Sanger sequencing of regions isolated with primers developed for exon one of KCNJ6 in the guide RNA targeting region. The most frequent mutations found in the targeted region are listed (Figure 5.4 A) for GFP+ and GFP- cells. The most comment mutations included a two base-pair deletion (15.5%) and a single base-pair insertion (8.1%); the frequencies of mutation were calculated (Figure 5.4C). Although one three-base pair deletion was detected, the majority of the detected indels would lead to a frameshift and, thus, likely loss of function mutation (Figure 5.4 C). Less than one percent of the GFP- cells contained a mutation (Figure 5.4 A,D). To verify knockdown of the protein, functional assays are necessary to confirm that there is a loss of channel function.

Knockdown of KCNJ6 attenuates the $BaCl_2$ sensitive current evoked in response to G_i agonists baclofen. We co-injected AAV1-FLEX-eYFP with AAV1-FLEX-SaCas9-sg(*Rosa*) or AAV1-FLEX-SaCas9-sg(*KCNJ6*) into DAT-IRES-Cre mice and patched from eYFP+ neurons in the VTA. A voltage ramp from -120 mV to -60 mV elicits a large Ba^{2+}-sensitive

current that is attributable to conductance through GIRKs. When the $GABA_B$ agonist baclofen is applied to slices taken from animals injected with the rosa control virus, both the peak inward current and conductance of the Ba^{2+}-sensitive GIRK current increased (paired, two-tailed t-test; $p < 0.05$). Our preliminary data (n=3 cells; 3 mice) indicate that baclofen does not cause a consistent increase in peak current or conductance recorded from eYFP+ dopamine neurons after co-injection with the virus targeting KCNJ6. This is preliminary functional measurement of a knockdown of GIRK current, indicating that the viral strategy of knocking down the GIRK2 subunit may be an effective means to knockdown fucntional GIRK channels in dopamine neurons. To quantitate this shift in current, we subtracted the peak current at baseline from the peak current after baclofen (ΔPeak Current; Figure 5.4 I) and saw that there was a trend toward a difference between the two groups (unpaired t-test, $p = 0.092$). We similarly compared the change in conductance (ΔConductance, Figure 5.4 I), and determined that there was a trend toward a significant effect of treatment at reducing the GIRK conductance elicited by baclofen (unpaired t-test, $p=0.051$).

Knockdown of KCNJ6 may functionally disrupt U69,593-elicited GIRK currents without disrupting conditioned place aversion. The ultimate goal in using this construct is to assess the role of KOR-activated GIRK currents in VTA dopamine neurons and their contribution to behavioral responses to stress. Although the prior data demonstrated genetic and functional validation of knockdown, the effect of GIRK knockdown on KOR-mediated effects has not been tested. The data here are very preliminary and inconclusive, but indicate there may be rationale for continued study. We recorded GIRK currents using the same voltage paradigm as shown for baclofen (Figure 5.4 E,G), but instead recorded the currents before and after treatment with KOR agonist U69,593 (Example traces Figure 5.5 A,C). We detected increases in

the Ba^{2+}-sensitive peak current and conductance in 3 of 4 cells tested from the animals treated with the Rosa control construct (Figure 5.5B). This study is incomplete, and 1 of 2 cells tested from the mice treated with the KCNJ6 construct showed an increase current in response to U69,593 (Figure 5.5D). Further studies are needed to increase the n and determine the proportion of eYFP+ cells in animals co-injected with the KCNJ6 construct that have a loss of GIRK current, thus determining the extent of the knockdown. It is also possible that GIRK3 homomers, or GIRK1/GIRK3 heteromers can form and couple with KORs in dopamine neurons in the absence of GIRK2; leaving the possibility that KOR signaling at GIRKs is more intact in the *KCNJ6* knockdown than baclofen signaling.

Two-thirds of eYFP positive neurons also express HA tag. To ensure that our strategy of co-injecting AAV1-FLEX-eYFP with the AAV1-FLEX-SaCas9-sgRNA constructs is effective, we used immunohistochemistry to estimate the overlap in the viral expression after coinjecting the two viruses at a ratio of 4:1 (SaCas9:eYFP). We used an anti-HA antibody to detect the HA tag on the SaCas9 and detected eYFP with the natural fluorophore, without boosting. We counted cells after merging the eYFP and HA (Red) channel in ImageJ, finding that 66% of eYFP+ neurons also contain HA (Figure 5.5E). HA+ neurons overlapped 85% with eYFP+ (Figure 5.5E). This degree of overlap indicates that although the majority of eYFP positive cells are likely expressing the SaCas9 virus, about one third of eYFP+ cells may not have SaCas9 express and thus gene editing. Therefore, a proportion of the eYFP+ cells we patch are likely to not have a CRISPR-mediated gene editing event in the KCNJ6 locus. To further confirm that the KCNJ6 knockdown is effective, it will be necessary to patch enough neurons to ensure that at least two-thirds of the neurons patched have a loss of GIRK current after AAV1-

FLEX-SaCas9(*Kcnj6)*. It may also be effective to change the dilution so that the eYFP virus does not spread significantly beyond the virus with which it is co-injected.

Preliminary study indicates that knockdown of KCNJ6 does not prevent conditioned place aversion to KOR agonist U50,488. To determine if KOR activation of GIRK currents are necessary for the development of a conditioned place aversion to U50,488, we tested U50,488 CPA in Rosa controls and KCNJ6 knockdown mice. A pre-test, in which animals freely roamed two chambers with different flooring, determined each mouse's preferred context. On two subsequent days, they received saline paired with their least-preferred chamber for thirty minutes in the morning and 2.5 mg/kg paired with their preferred chamber for thirty minutes in the afternoon. On the fourth day, the mice freely roamed the two compartments and their preference was calculated. These scores are reported as Post-Pre in the chamber paired with U50,488. The control mice post-pre scores were significantly different from zero, or no preference (one-sample T and Wilcoxon test, $p = 0.019$). The KCNJ6 knockdown mice did not show a significant aversion to the U50,488-paired chamber, but there was a trend (One-sample T and Wilcoxon test, $p = 0.067$). We also ran two animal controls for each group that received saline in both chambers. These mice slightly decreased their time in the pre-test preferred chamber, but not to the same extent as the U50,488 paired chamber, suggesting the aversion seen in experimental animals was real (data not shown; One-sample T and Wilcoxon test, Rosa $p = 0.072$ and $p = 0.53$; significantly underpowered). These data indicate that KCNJ6 may not be necessary for KOR-mediated aversion, and thus not be the molecular mechanism by which KOR on dopamine neurons facilitates a CPA. Further testing is necessary to validate the extent of viral expression and knockdown, the degree to which loss of GIRK2 eliminates U69,593-mediated currents in dopamine neurons, and the slight shift in preference seen in the saline-saline animals.

Discussion

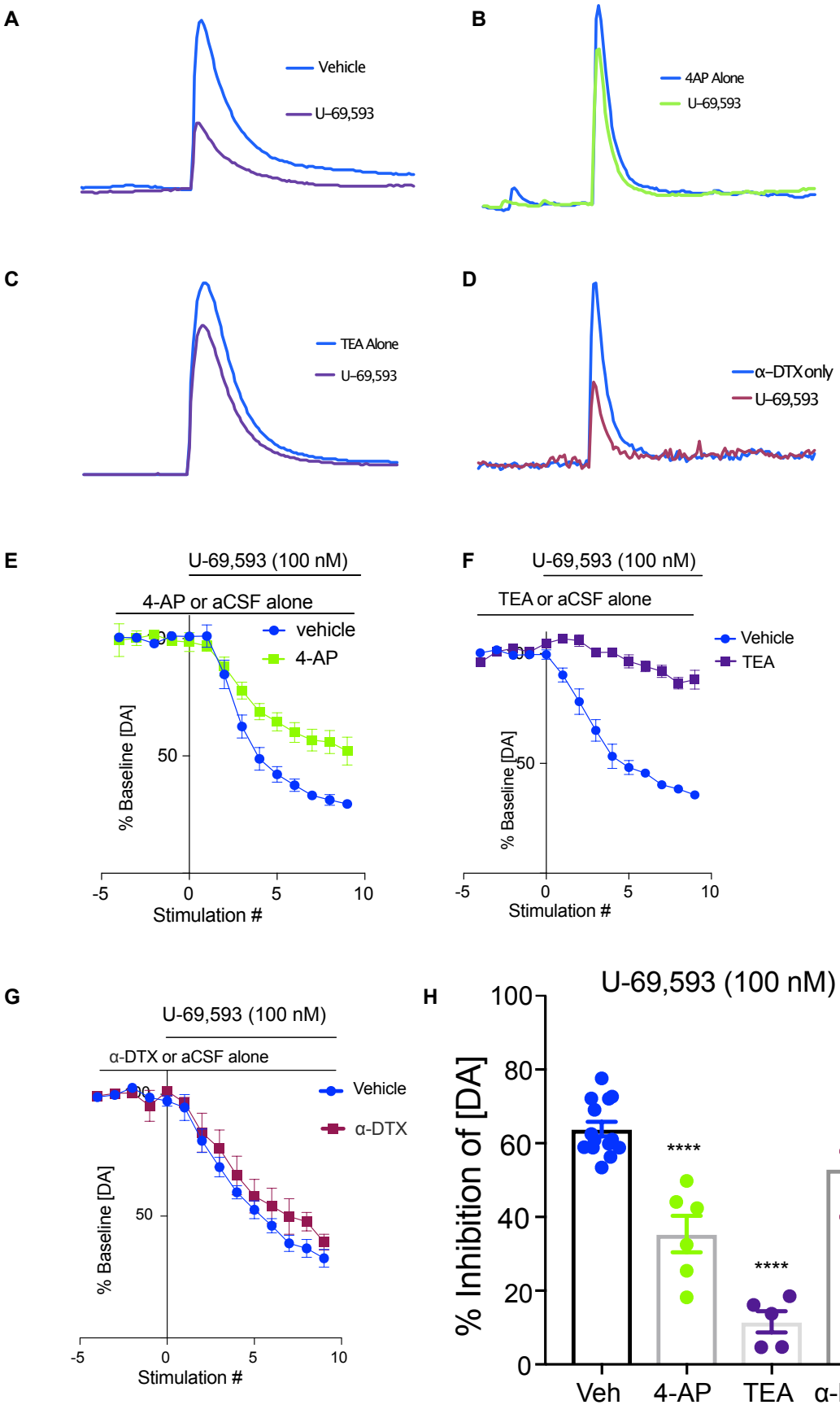
The first question addressed in this study was if presynaptic inhibition of dopamine release by the KOR was facilitated by increased potassium conductances through voltage-sensitive potassium channels. We first tested this hypothesis with non-specific channel blockers and found strong evidence that a potassium channel contributes to this KOR-mediated inhibition (Figure 5.1). Both the K_V1 and K_V4 families are regulated by 4-AP, and mRNA expression data show that the genes KCNA2 and KCNC4 which encode $K_V1.2$ and $K_V3.4$ are two of the most highly expressed of these K^+ channel families in dopamine neurons. For this reason, in addition to some suggestive evidence in the literature (Fulton 2011, Ritter 2011) we chose to target KCNA2 and KCNC4 as putative potassium-channel mediators of KOR terminal inhibition in dopamine neurons. We were unable to demonstrate a shift in the sensitivity to KOR agonist U69,593 or D2 agonist quinpirole after knocking down either channel. Fulton 2011 demonstrated that $K_V1.2$ knockdown caused a slight attenuation in the Quinpirole inhibition (~10-15%) that was not recapitulated in our findings, however we did replicate their finding that knockdown of the KCNA2 gene increases dopamine outflow. These data present several possibilities: (1) in the six-week period between viral injection, gene deletion, and channel protein depletion, significant compensatory mechanisms account for the loss of the K_V channel subunit in question including redundant mechanisms through other K_V channels, DAT regulation, or VGCCs, (2) neither of these channels is important for KOR regulation of presynaptic terminals, (3) heteromeric channels are responsible for the effect and thus a single subunit knockdown strategy is ineffective. Further investigation into the role of VGCCs is necessary to assess the first and second possibility. To test the third possibility, double knockouts or a knock-in of pore-blocking mutated form of the K_V subunits could be used.

The second major finding investigated was the effect of KCNA2 knockdown of dopamine mediated behaviors due to its increase in basal dopamine outflow. Our finding that reduction of $K_V1.2$ currents in dopamine terminals increases outflow was a replication of previous work done in a germline, global knockout (Fulton 2011). These data suggest that the $K_V1.2$ subunit is particularly important for regulating axonal outflow in wild-type dopamine neurons, leading to a prediction that loss of this regulation may change dopamine-mediated behaviors which are regulated at the point of neurotransmitter release. Although the knockdown did not affect cocaine place preference or locomotion, it may be the case that more complex learning tasks like probability discounting would reveal how increased dopamine outflow due to loss of $K_v1.2$ may change behavior. Future studies should use in vivo dopamine imaging and operant behavior studies to characterize the role of $K_v1.2$ in dopamine-mediated behaviors.

The study assessing the knockdown of GIRK2 channels in dopamine neurons had mixed results. The sequencing data demonstrate multiple likely loss-of-function indel mutations caused by the AAV1-FLEX-SaCas9-sgRNA(*Kcnj6*). The data also demonstrate that the KCNJ6 CRISPR virus reduces the Ba^{2+}-sensitive current elicited after application of baclofen. However, our hypothesis that GIRK2 channels were necessary for the expression of U50,488 conditioned place aversion did not hold up to scrutiny in our preliminary data. There are several possibilities: (1) Not enough neurons had a loss of KCNJ6 to cause a shift in behavior, (2) GIRK2 subunit is not necessary for KOR regulation despite the genetic data indicating its the predominant Kir3 channel, and (3) GIRK channels are not necessary for U50,488 conditioned place aversion. Based on the very early data measuring U69,593 elicited GIRK currents in the KCNJ6 knockdowns, we saw one cell with and one cell without a potentiation in Ba^{2+}-sensitive current. This suggests that either the first or second possibility may be the case. Our IHC data support the

possibility that the SaCas9 virus did not infect as many neurons in the VTA as the eYFP virus, and thus may not have infected enough neurons to substantially shift behavior. Additional knockdown studies disrupting GIRK1 could serve to demonstrate a potential other mechanism of action: KOR may act on a small population of GIRK channels in VTA dopamine neurons, with low expression levels but substantial input to KOR-regulated behaviors. Novel in situ hybridization methods with larger signal boosting like RNAscope will allow investigators to follow up on this question by trying to detect GIRK1 in dopamine neurons, as its expression has been previously suggested with phospho-antibody studies (Ehrich 2015).

The data in this chapter present multiple opportunities for follow up study, further exploring the mechanisms by which KORs regulate dopamine neurons.


A
Vehicle
U-69,593
B
4AP Alone
U-69,593
C
TEA Alone
U-69,593
D
α-DTX only
U-69,593
E
U-69,593 (100 nM)
4-AP or aCSF alone
vehicle
4-AP
% Baseline [DA]
50
-5
0
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10
Stimulation #
F
U-69,593 (100 nM)
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U-69,593 (100 nM)
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Figure 5.1: Non-specific potassium channel blockers attenuate the inhibition of dopamine release by KOR agonist U69,593. A. Example trace of dopamine release after electrical stimulation in vehicle and after U69,593 (100 nM) **B,E.** Pretreatment of NAc slices with 4-AP attenuates the ability of KOR-agonist, U69,593 to inhibit dopamine release. **C, F.** Pretreatment of NAc slices with TEA (1 mM) significantly attenuates the ability of KOR-agonist, U69,593, to inhibit dopamine release. **D, G.** Pretreatment of NAc slices with alpha dendrotoxin (100 nM) has no significant effect on the ability of U69,593 to inhibit dopamine release. **H.** Summary data indicating that the %-inhibition by U69,593 in the presence of both 4-AP and TEA is significantly different from vehicle, whereas alpha dendrotoxin is not. Due to the concentration of TEA and 4-AP used, this indicates that a voltage-sensitive potassium channel *not* in the Shaw family contributes to KOR-mediated presynaptic inhibition of dopamine release (Veh vs. 4AP $p < 0.0001$, Veh vs. TEA $p < 0.0001$, Veh vs. DTX $p = 0.08$).

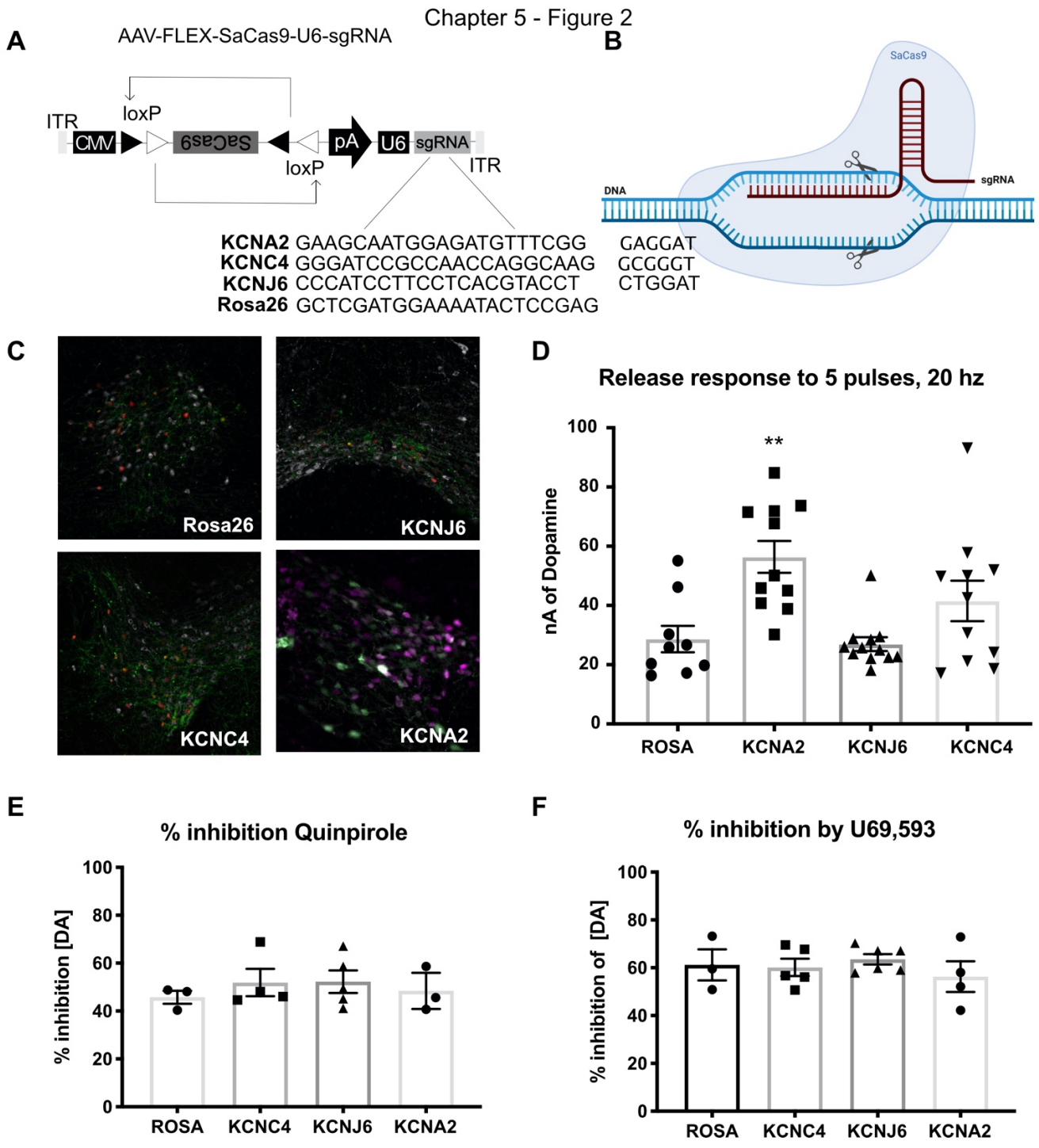

Figure 5.2: Selective knockdown of potassium channels in dopamine neurons using AAV-FLEX-SaCas9-U6-SgRNA. A-B. The AAV virus expresses an invirted SaCas9 enzyme under a CMV promoter, as well as the sgRNA under a U6 promoter. Viruses were cloned using the

AAV-FLEX-SaCas9 backbone and cloning the sgRNA in front of the U6 promoter. The SaCas9 enzyme recognizes PAM sequences, and if a complementary sgRNA strand is expressed, it will recognize the DNA and "clip" the genome upstream of the PAM sequence. The sgRNA sequence and te corresponding PAM within each gene are listed in A. All sgRNA sequences are from Exon 1 of each gene. **C.** Immunohistochemistry of VTA sections taken from the same brains as live NAc imaging to confirm viral expression. ChR2 expression is in green, TH expression in far red, and HA tag recognizing the SaCas9 expression in red (Alexa-555). Substantial overlap between ChR2 and HA indicates that most neurons which expressed ChR2 also expressed the SaCas9 and sgRNA. There were TH+ neurons which did not express either virus, indicating that the ChR2 strategy to only excite neurons which took up virus likely reduced variability in the results. **D.** Stimulating dopamine release with a 5 pulse, 20 hz train of blue light produced release in the NAc Core of mice coinjected with AAV1-DIO-ChR2-eYFP and their respective CRISPR construct. DA responses from mice injected with AAV-FLEX-SaCas9-U6-sgRNA-KCNA2, the CRISPR construct targeting Kv1.2, had significant increases in DA release. There was a trend toward an increase in DA response in the mice injected with AAV-FLEX-SaCas9-U6-sgRNA-KCNC4, the CRISPR construct targeting Kv3.4. Mice injected with construct targeting KCNJ6 (GIRK2) were not significantly different from those with guide RNAs targeting the control ROSA locus. **E, F.** The % inhibition by D2R agonist Quinpirole (100 nM) and KOR agonist U69,593 (100 nM) were not changed by knockdown of the potassium channels.

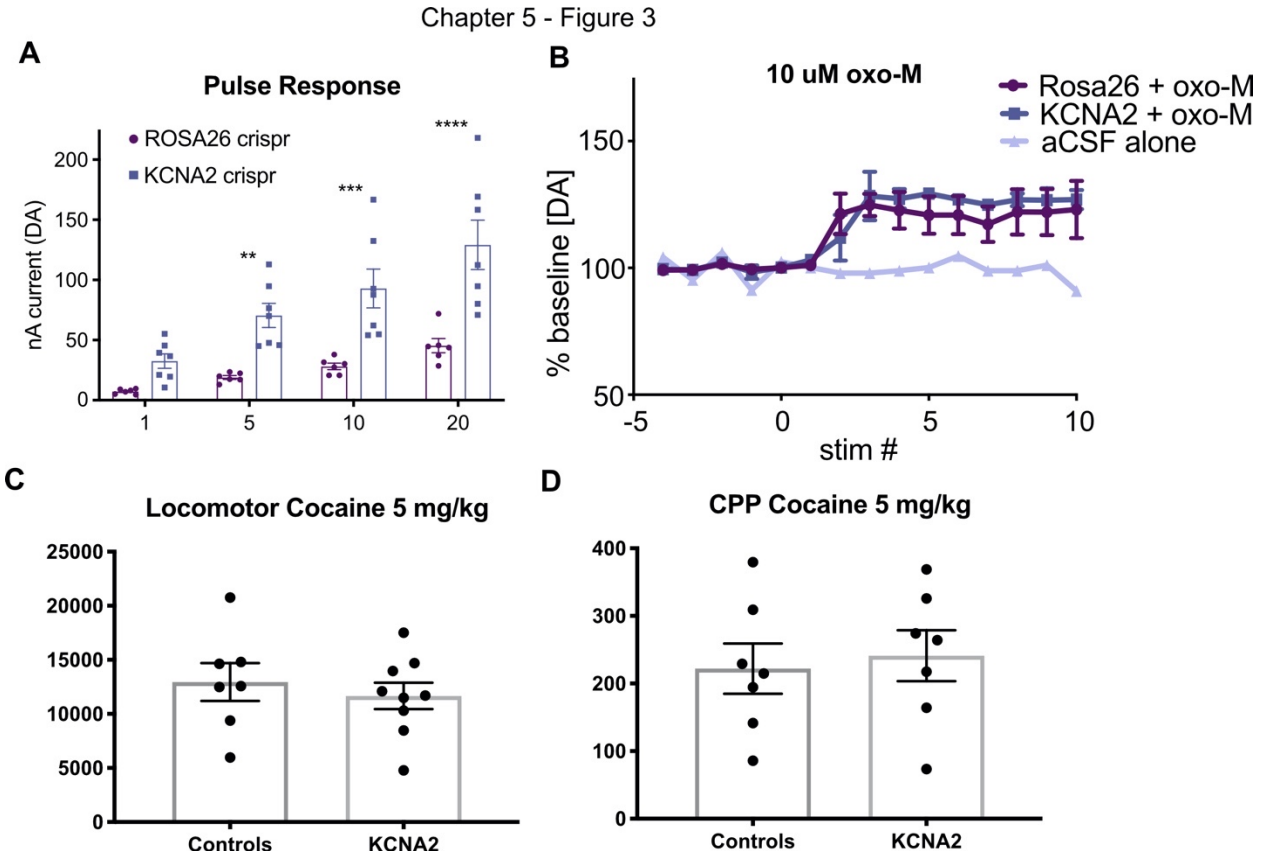

Figure 5.3 Assessment of the KCNA2 knockdown on the regulation of evoked dopamine release and dopamine-mediated behaviors. (A) Dopamine current evoked by increasing number of blue-light pulses at 20 Hz. At 5,10, and 20 pulses, slices taken from KCNA2 knockdown mice exhibited significantly more light-evoked dopamine release than Rosa controls (Two-Way ANOVA; Interaction $p = 0.07$, multiple comparisons Rosa vs. KCNA 2: one pulse $p = 0.118$, five pulses $p = 0.004$, ten pulses $p = 0.0005$, twenty pulses $p < 0.0001$). (B) Time-course of change in DA release after treatment with oxotremorine (Oxo-M) or vehicle showing that oxo-M increased dopamine release above baseline in both Rosa controls and KCNA2 knockdown mice. (C) Distance traveled in 30 minutes after treatment with 5 mg/kg Cocaine; there is no difference between control and KCNA2 group. (D) Preference scores for cocaine-paired chamber after cocaine conditioned place preference assay; Both Rosa control and KCNA2 knockdown mice developed a place preference for cocaine (*Behavior collaboration with Carlie Neiswanger*).

Chapter 5 - Figure 4

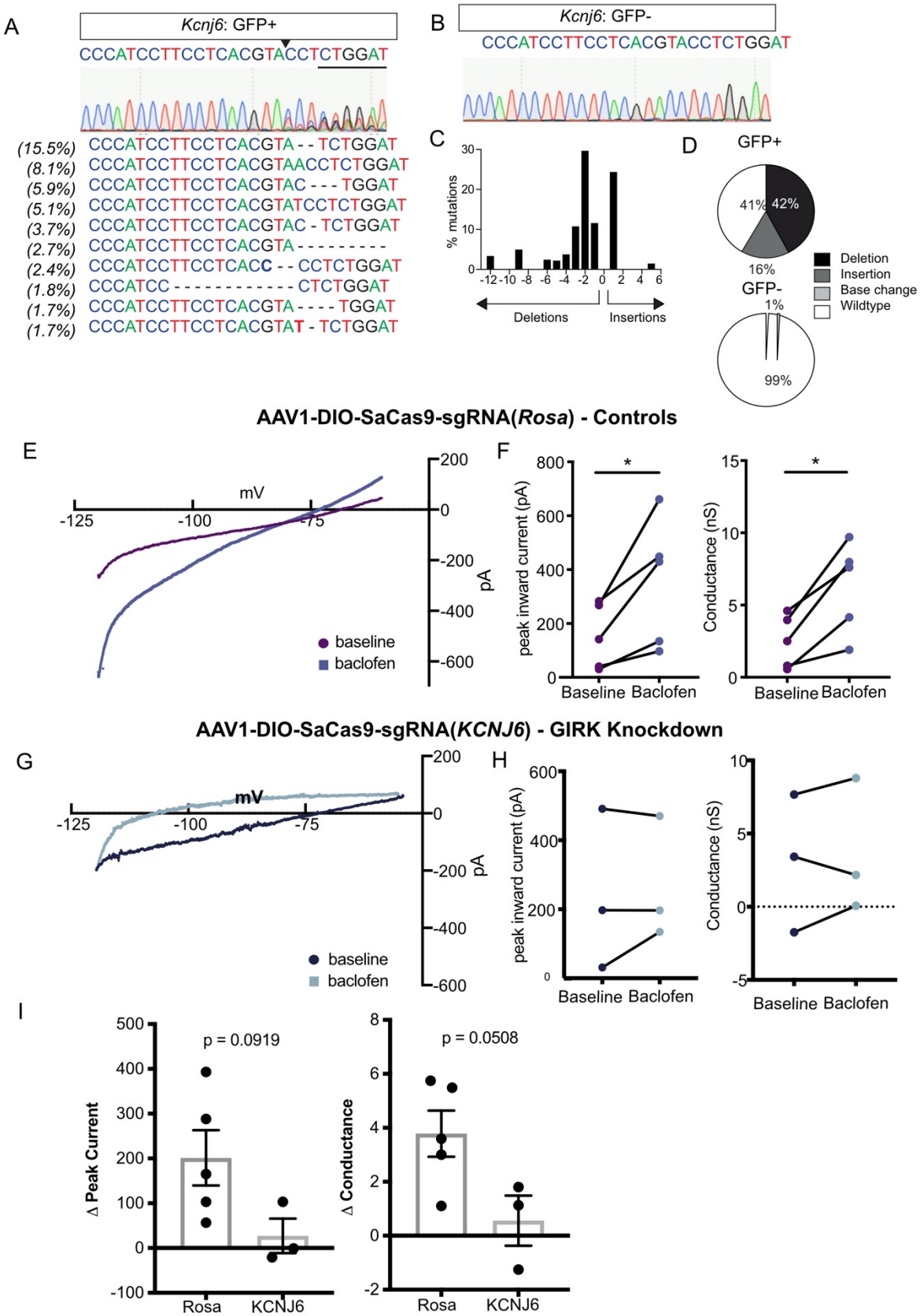

Figure 5.4 Genetic and functional validation of AAV1-FLEX-SaCas9-sgRNA(*Kcnj6*) (A) Sequencing of GFP+ nuclei. Top: sgKcnj6 sequence with PAM underlined and SaCas9 cut site indicated by black arrow. Middle: Sanger sequencing results displaying multiple peaks beginning at the cut site. Bottom: Top ten mutations at cut site with the percent of total reads they on which they occur on the left. Base changes: bolded. Insertions: underlined. Deletions: marked with a "-". (B) Top: Sequencing of GFP- nuclei displaying no evidence of mutations at SaCas9 cut site. (C) Frequency distribution of insertions and deletions for *Kcnj6* from GFP+ nuclei. (D) Percent of wildtype, deletions, insertions, and base changes as percent of total reads for GFP+ and GFP- nuclei. (E) Example trace showing a Ba^{2+}-sensitive GIRK current potentiated by baclofen (50 μM) taken from eYFP-labeled VTA dopamine neuron in slices from Rosa control mice. (F) Both the peak inward current and conductance significantly increased after baclofen treatment in Rosa control mice (paired t-test; current p = 0.031; conductance p = 0.011). (G) Example trace showing a Ba^{2+}-sensitive GIRK current potentiated by baclofen (50 μM) taken from eYFP-labeled VTA dopamine neuron in slices from KCNJ6 knockdown mice. (H) Neither the peak inward current nor the conductance increased significantly after baclofen treatment in KCNJ6 knockdown mice (paired t-test; current p = 0.554; conductance p = 0.608; *a note: low n, underpowered and groups should be added to prior to formal publication*). (I) Change in peak current calculated as peak current (baclofen) minus peak current (baseline) and change in conductance calculated as conductance (baclofen) minus conductance (baseline); there is not a decrease in the $\underline{\Delta}$ Current and $\underline{\Delta}$ Conductance between Rosa controls and KCNJ6 knockdown, but it is not statistically significant (current p = 0.09; conductance p = 0.051).

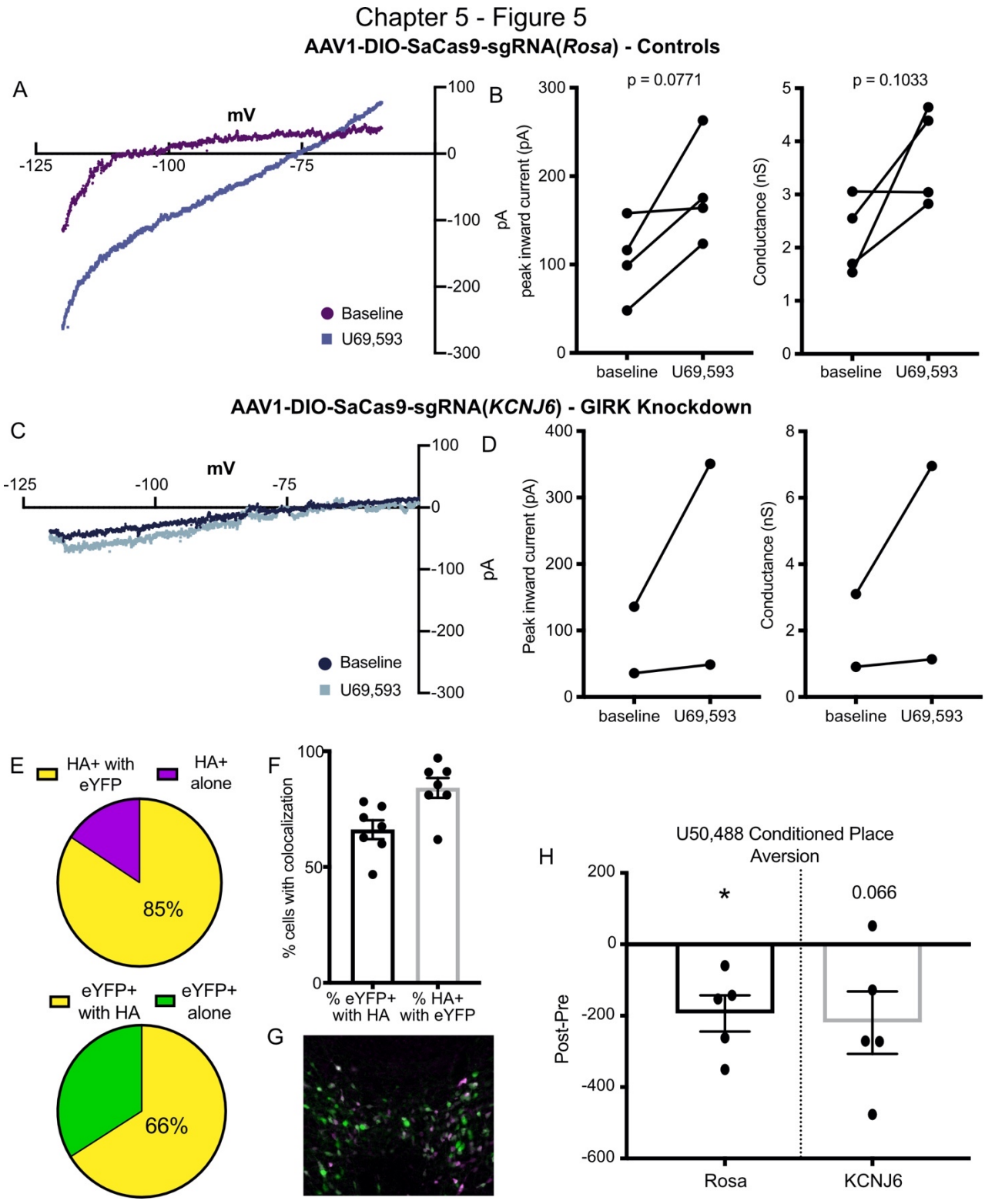

Figure 5.5 Impact of KCNJ6 knockdown on KOR signaling and U50,488 Conditioned Place Aversion (A) Example trace showing a Ba^{2+}-sensitive GIRK current potentiated by U69,593 (1

μM) taken from eYFP-labeled VTA dopamine neuron in slices from Rosa control mice. (B) Quantitation of the peak inward current and conductance before and after U69,593 treatment in Rosa control mice, the differences between the baseline and post-U69,593 values are not statistically significant (two-tailed t-test: current p = 0.077; conductance p = 0.1033). (C) Example trace showing a Ba^{2+}-sensitive GIRK current after treatment with U69,593 (1 μM) taken from eYFP-labeled VTA dopamine neuron in slices from KCNJ6 knockdown mice. (D) Quantitation of the peak inward current and conductance before and after U69,593 treatment in KCNJ6 knockdown mice. (E) Pi charts showing the percent overlap of eYFP within HA+ cells (top) and percent overlap of HA within eYFP+ cells bottom) (F) Quantitation of the percent overlap from seven imaged VTA sections taken from two DAT-IRES-Cre mice co-injected with AAV1-FLEX-SaCas9-sgRNA(*Kcnj6*) and AAV1-FLEX-eYFP. (G) Representative image showing VTA with eYFP in green and HA tag in magenta. Most magenta overlaps with green. (H) U50,488-induced conditioned place aversion scores, calculated as post test minus pre test times in the U50,488-paired side for Rosa controls and KCNJ6 knockdown mice (One-sample T and Wilcoxon test, Rosa p = 0.019; KCNJ6 p = 0.066). (*Behavior in collaboration with Ben Land, PhD and Carlie Neiswanger*).

Closing Remarks & Conclusions

This dissertation demonstrates novel insights into the mechanisms of norBNI and advances in tool and technology use in its depiction of HyPerRed and application of the AAV1-FLEX-SaCas9 CRISPR constructs. It expands upon the mechanisms and constraints of long-acting kappa opioid receptor antagonists, such as norBNI, by characterizing sex differences in the JNK-ROS mechanism, exploring the transferability of this mechanism to other receptor systems, and determining the extent of receptor inactivation in different neuronal compartments of dopamine neurons. The lack of long-acting norBNI effect, in conjunction with the finding the nalfurafine inhibits dopamine release despite not being a dysphoric compound, are counter to the prevailing hypothesis that dynorphin release in the NAc causing dopamine inhibition is responsible stress and drug-withdrawal associated decreases in mood. We also utilized and optimized a new tool in neuroimaging, HyPerRed, to answer questions about localized ROS generation downstream of GPCR activation in dopamine neurons. This study both helped answer an experimental question about norBNI's action in dopamine terminals, but also helped determine the timecourse and dynamics of KOR agonist-induced ROS in living brain tissue. Although inconclusive, the studies using CRISPR SaCas9 viral strategies to excise potassium channel subunits from dopamine neurons outline a workflow for using these tools and present a variety of follow up experiments. The $K_V1.2$ (*Kcna2)* channel knockdown showed robust increases in dopamine release, and may be a helpful model for studying reward valuation. The GIRK2 (*Kcnj6*) channel knockdown was functionally and genetically validated and, perhaps in combination with a GIRK1 knockdown, will be helpful in elucidating the p38 MAP Kinase regulation of VTA dopamine neurons and DRN serotonin neurons. Although plodding, hopefully these small new pieces of knowledge and attempts at new techniques support the next student who follows down this road.

Katie Reichard

Ms. Reichard is a scientist, educator, and advocate, with experience in neuroscience research, political organizing, project management, and teaching. She approaches her work by merging significant experience in neuroscience research and data analysis with consistent social justice and advocacy work, working to make scientific and academic workplaces more equitable.

Education

University of Washington in Seattle, WA (2015 – defense June 2020) PhD in Neuroscience
Colorado College in Colorado Springs, CO (2008-2012) BA, *cum laude,* Biology. Phi Beta Kappa

Research Experience

University of Washington Seattle, WA
Graduate Researcher. Advisor: Charles Chavkin, PhD 2015-June 2020

- Used whole cell voltage clamp electrophysiology and electrochemistry to record changes in dopamine neuron excitability in response to Kappa Opioid Receptor (KOR) signaling.
- Skills: Patch-clamp electrophysiology, fast scan cyclic voltammetry, HEK293 cell culture, immunocytochemistry, survival stereotaxic surgery in mice, plasmid cloning, western blot, qPCR.
- Mentored students: Keionna Newton, Paulo Sotero de Menezes

National Institute of Mental Health - Bethesda, MD
Postbaccalaureate Fellow. Advisor: Barry Kaplan, PhD 2010, 2012-13

- Standardized a biochemical assay to determine protein(s) involved in axonal trafficking of nuclear-encoded mitochondrial RNA. Assessed behavioral impacts of disrupted trafficking in transgenic mice.
- Skills: qPCR, light & fluorescence microscopy, neuron culture maintenance, protein quantification, mouse behavior (open field, elevated plus maze, fear conditioning, forced swim test, Y-maze)

Colorado College Colorado Springs, CO
Undergraduate Researcher. Advisor: Nancy Huang, PhD 2011

- Knockout screen of E3 Ubiquitin Ligases resulting in embryonic lethality C. *elegans* to determine role of protein degradation in anterior-posterior patterning of early embryo.
- Skills: Fluorescence microscopy, C. elegans genetics, plate-making, yeast two hybrid screen, dsRNA synthesis, RNAi (injection & feeding)

Higher Education & Teaching Experience

ENGAGE Board of Directors, UW College of the Environment Seattle, WA
Public science communication course instructor & board member Jan 2018- Present

- Lead instructor for graduate seminar in science communication (2019) and advisory instructor for 2020 seminar.
- Updated curriculum and planned weekly lesson plans while helping students develop public talks and blog posts using skills like analogy, storytelling, and improvisation.
- Led "Future of Engage" committee to help expand course within UW.

Neurobiology Undergraduate Program, UW Biology Department Seattle, WA
Teaching Assistant - Neurobiology 301 2017

- Lead instructor for hands-on neurophysiology lab sessions teaching extracellular cockroach recordings, intracellular leech recordings, and circuits using ohm's law.
- Led students through writing four graduate-level lab reports, teaching science writing, scientific method, figure making, and basic hypothesis testing and descriptive statistics
- Standard TA activities including: office hours, test review sessions, and grading

2U, Inc Landover, MD
Associate Course Producer 2014-2015

- Working with a producer and faculty member: planned, filmed, and built out graduate level courses for degree programs delivered online.
- Project Manager for Counseling@Northwestern, MPH@George Washington, Nursing@Simmons, Nursing@Georgetown, and Accounting@American University course builds
- Coded within and built out course structure on Moodle-based learning management system

Center City PCS Washington DC
Middle School Science Teacher (6th-8th) 2013-2014

- Taught three separate curriculum to three separate grade bands, developing Next Gen Science Standards' aligned lesson plans, including weekly hands-on labs for Earth Sciences, Life Sciences, and Physical Sciences
- Adapted curriculum for students with Individualized Educations Plans, English Language Learners

DC Public Schools - Nalle Summer Academy Washington DC
Teaching Fellow Summer 2013

Colorado College Quantitative Reasoning Center Colorado Springs, CO
Learning Assistant **-** *BY131 Cell & Molecular Biology* 2011

Additional Volunteer & Professional Experience

Pharmacology Diversity Committee Seattle, WA
Committee Member March 2019 - Present

UW Graduate and Professional Student Senate Seattle, WA
Senator, Science & Policy Committee Co-Chair 2017

- Received Research America Grant to organize Candidate Forum in Washington's 5th Congressional District focused on science policy and the opioid crisis.
- Helped organize the annual White Paper Project, allowing scientists to partner with community groups to write data-driven white papers

Scientists Advocating for Representation, Justice & Equity (SARJE) Seattle, WA
Founder, Project Manager 2016-Present

- Coordinated with UW School of Medicine to facilitate climate survey and write a white paper with student and post-doc perspectives and recommendations.
- Organized to bring Robin DiAngelo to UW Pharmacology and Neuroscience departments to present racial justice workshop
- Formed an organization with representation from seven STEM departments to build ally-ship and accountability around diversity within UW scientific community

UW Neuroscience Community Outreach Group Seattle, WA
Outreach Coordinator, Classroom Volunteer 2015-2019

Publications

In revisions: Reichard KL, Newton KA, Rivera ZMG, Sotero de Menezes P, Schattauer SS, Land BB, Chavkin C. "Regulation of kappa opioid receptor inactivation depends on sex and cellular site of action."

Heymann G, Jo YS, **Reichard KL**, McFarland N, Chavkin C, Palmiter RD, Soden ME, Zweifel LS. "Synergy of Distinct Dopamine Projection Populations in Behavioral Reinforcement". *Neuron* 2019 Dec.

Abraham, A. D., Schattauer, S. S., **Reichard, K. L.**, Cohen, J. H., Fontaine, H. M., Song, A. J., Johnson, S., Land, B.B., Chavkin, C. "Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion". *Journal of Neuroscience* 2018 Aug.

Schattauer SS, Land BB, **Reichard KL**, Abraham AD, Burgeno, LM. Kuhar JR, Phillips PEM, Ong SE, Chavkin

C. "Peroxiredoxin 6 mediates Gαi protein-coupled receptor inactivation by cJun kinase". *Nature Communications* 2017 Sept; 8(743).

Kar A, Sun CY, **Reichard K,** Gervasi NM, Pickel J, Nakazawa K, Kaplan BB. "Dysregulation of the axonal trafficking of nuclear-encoded mitochondrial mRNA alters neuronal mitochondrial activity and mouse behavior." Developmental Neurobiology. 2014 Mar;74(3):333-50.

Posters & Presentations

Reichard KL, Sotero de Menezes P, Chavkin C. **"**norBNI Does Not Act as a Long-Acting Antagonist in Nucleus Accumbens Dopamine Terminals**".** Kappa Therapeutics, Seattle WA, April 2019

Reichard KL, Sotero de Menezes P, Abraham AD, Chavkin C. **"**Differential pre- and post-synaptic K+ channel regulation by kappa opioid receptors affect dopamine neuron physiology". Society for Neuroscience, San Diego, November 2018.

Reichard, K. Levinstein MR, Voelker L, Mesa N, Rusch C, Steger JS. "Scientist Advocates: Shifting the culture toward justice, equity, and diverse representation through community organizing, data transparency, and education." Society for Neuroscience, San Diego, November 2018.

Reichard, K. "Building a Better Opioid". UW Science Now Series with Town Hall Seattle. May 2018.

Reichard, K., Schauttauer, SS., Burgeno, LM., Steger, JS., Abraham, AD., Land, BB., Chavkin, C. "NorBNI inactivates Dopamine D2 receptors on VTA nerve terminals by stimulating ROS production through a JNK/PRDX6 mechanism". Poster. Kappa Therapeutics, Philadelphia, PA, April 2017.

Reichard, K., Kar, A., Sun, C., Gioio, A., Gervasi, N. Kaplan, B. "Dysregulated axonal transport of nuclear-encoded mitochondrial mRNA alters axon length and animal behavior." NIH Post-Baccalaureate Poster Day, Bethesda MD May 2013.

Reichard, K., Lin, A., Desai, A., Schwartz, K., Heng, P., Huang, N. "RNAi Screen of Predicted E3 Ubiquitin Ligases that may Mark MEX-3 for Degradation in the C. elegans Embryo". Poster. American Society for Cell Biology Annual Meeting, Denver, CO, December 2011.

Reichard, K., Natera-Naranjo, O., Gioio, A.E., Kar, A., Macgibeny, M., Kaplan, B.B **"**Regulation of axonal ATP Synthase (C1) expression: effects on axonal growth and ROS production." NIH Summer Poster Day, August 2010.

Awards & Grants

- **Bipartisan Civic Engagement Initiative** – Funded by Research America for candidate forum
- **Husky 100 Honoree 2018** - Recognized as one of 100 UW student leaders contributing to campus.
- **AAAS Sponsored Student – CASE Workshop 2018**
- **Honorable Mention - NSF Graduate Research Fellowship Program**
- **NIH Post Baccalaureate Poster Day Award** Awarded to top 15% of presenters
- **Colorado College Laboratory Biology Award** Awarded to two graduating seniors with greatest potential in bench research
- **Colorado College Venture Grant**: "Making Head or Tail of It: The role of MEX-3 in *C. elegans* Anterior/Posterior Development. $1000 PI: Nancy Huang; April 2011

Bibliography

Abraham, A. D., Fontaine, H. M., Song, A. J., Andrews, M. M., Baird, M. A., Kieffer, B. L., Land, B. B., & Chavkin, C. (2018). κ-Opioid Receptor Activation in Dopamine Neurons Disrupts Behavioral Inhibition. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *43*(2), 362–372. doi:10.1038/npp.2017.133

Abraham, A. D., Schattauer, S. S., Reichard, K. L., Cohen, J. H., Fontaine, H. M., Song, A. J., Johnson, S. D., Land, B. B., & Chavkin, C. (2018). Estrogen Regulation of GRK2 Inactivates Kappa Opioid Receptor Signaling Mediating Analgesia, But Not Aversion. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *38*(37), 8031–8043. doi:10.1523/JNEUROSCI.0653-18.2018

Adell, A., Garcia-Marquez, C., Armario, A., & Gelpi, E. (1988). Chronic Stress Increases Serotonin and Noradrenaline in Rat Brain and Sensitizes Their Responses to a Further Acute Stress. *Journal of Neurochemistry*, *50*(6), 1678–1681. doi:10.1111/j.1471-4159.1988.tb02462.x

Al-Hasani, R., McCall, J. G., Foshage, A. M., & Bruchas, M. R. (2013). Locus coeruleus kappa-opioid receptors modulate reinstatement of cocaine place preference through a noradrenergic mechanism. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *38*(12), 2484–2497. doi:10.1038/npp.2013.151

Bambico, F. R., Nguyen, N.-T., & Gobbi, G. (2009). Decline in serotonergic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. *European Neuropsychopharmacology*, *19*(3), 215–228. doi: 10.1016/j.euroneuro.2008.11.005

Barker, D. J., Root, D. H., Zhang, S., & Morales, M. (2016). Multiplexed neurochemical signaling by neurons of the ventral tegmental area. *Journal of chemical neuroanatomy*, *73*, 33–42. doi:10.1016/j.jchemneu.2015.12.016

Bayer, H. M., & Glimcher, P. W. (2005). Midbrain dopamine neurons encode a quantitative reward prediction error signal. *Neuron*, *47*(1), 129–141.doi:10.1016/j.neuron.2005.05.020

Becker JB and Chartoff E (2019) Sex Differences in Neural Mechanisms Mediating Reward and Addiction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **44**(1).

Bedini, A., Di Cesare Mannelli, L., Micheli, L., Baiula, M., Vaca, G., De Marco, R., Gentilucci, L., Ghelardini, C., & Spampinato, S. (2020). Functional Selectivity and Antinociceptive Effects of a Novel KOPr Agonist. *Frontiers in pharmacology*, *11*, 188. doi:10.3389/fphar.2020.00188

Belcheva MM, Clark AL, Haas PD, et al. Mu and kappa opioid receptors activate ERK/MAPK

via different protein kinase C isoforms and secondary messengers in astrocytes. *J Biol Chem*. 2005;280(30):27662-27669. doi:10.1074/jbc.M502593200

Berecki G, Motin L, Adams DJ. Voltage-Gated R-Type Calcium Channel Inhibition via Human μ-, δ-, and κ-opioid Receptors Is Voltage-Independently Mediated by Gβγ Protein Subunits. *Mol Pharmacol*. 2016;89(1):187-196. doi:10.1124/mol.115.101154

Berke, Joshua D. "What does dopamine mean?." *Nature neuroscience* vol. 21,6 (2018): 787-793. doi:10.1038/s41593-018-0152-y

Berkley, K. J. (1997). Sex differences in pain. *Behavioral and Brain Sciences*, *20*(3), 371–380. doi: 10.1017/s0140525x97221485

Blackthorn Therapeutics (2020) A Phase 2a, Randomized, Double-blind, Placebo-controlled Proof of Concept Study to Evaluate the Effects of Oral BTRX-335140 Versus Placebo in Subjects With Major Depressive Disorder.

Breese, G. R., Chu, K., Dayas, C. V., Funk, D., Knapp, D. J., Koob, G. F., Lê, D. A., O'Dell, L. E., Overstreet, D. H., Roberts, A. J., Sinha, R., Valdez, G. R., & Weiss, F. (2005). Stress enhancement of craving during sobriety: a risk for relapse. *Alcoholism, clinical and experimental research*, *29*(2), 185–195. doi:10.1097/01.alc.0000153544.83656.3c

Brischoux, F., Chakraborty, S., Brierley, D. I., & Ungless, M. A. (2009). Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(12), 4894–4899. doi:10.1073/pnas.0811507106

Bromberg-Martin, E. S., Matsumoto, M., & Hikosaka, O. (2010). Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron*, *68*(5), 815–834. doi:10.1016/j.neuron.2010.11.022

Brown, C.A., Smith, T., Lucki, I. (2020). Behavioral effects of the kappa opioid receptor partial agonist nalmefene in tests relevant to depression. *European Journal of Pharmacology*, *872, 172948.* doi: 10.1016/j.ejphar.2020

Bruchas, M. R., & Chavkin, C. (2010). Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology*, *210*(2), 137–147. doi: 10.1007/s00213-010-1806-y

Bruchas, M. R., Macey, T. A., Lowe, J. D., & Chavkin, C. (2006). Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. *The Journal of biological chemistry*, *281*(26), 18081–18089. doi: 10.1074/jbc.M513640200

Bruchas, M. R., Land, B. B., Aita, M., Xu, M., Barot, S. K., Li, S., & Chavkin, C. (2007). Stress-

induced p38 mitogen-activated protein kinase activation mediates kappa-opioid dependent dysphoria. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(43), 11614–11623. doi:10.1523/JNEUROSCI.3769-07.2007

Bruchas, M. R., Land, B. B., & Chavkin, C. (2010). The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain research*, *1314*, 44–55. doi: 10.1016/j.brainres.2009.08.062

Bruchas, M. R., Schindler, A. G., Shankar, H., Messinger, D. I., Miyatake, M., Land, B. B., Lemos, J. C., Hagan, C. E., Neumaier, J. F., Quintana, A., Palmiter, R. D., & Chavkin, C. (2011). Selective p38α MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. *Neuron*, *71*(3), 498–511. doi:10.1016/j.neuron.2011.06.011

Brust, T. F., Morgenweck, J., Kim, S. A., Rose, J. H., Locke, J. L., Schmid, C. L., … Bohn, L. M. (2016). Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. *Science Signaling*, *9*(456), ra117. http://doi.org/10.1126/scisignal.aai8441

Buda JJ, Carroll FI, Kosten TR, Swearingen D and Walters BB (2015) A Double-Blind, Placebo-Controlled Trial to Evaluate the Safety, Tolerability, and Pharmacokinetics of Single, Escalating Oral Doses of JDTic. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **40**(9).

Budygin, E. A., Park, J., Bass, C. E., Grinevich, V. P., Bonin, K. D., & Wightman, R. M. (2012). Aversive stimulus differentially triggers subsecond dopamine release in reward regions. *Neuroscience*, *201*, 331–337. doi:10.1016/j.neuroscience.2011.10.056

Butelman E, Negus S, Ai Y, de Costa B and Woods J (1993) Kappa Opioid Antagonist Effects of Systemically Administered Nor-Binaltorphimine in a Thermal Antinociception Assay in Rhesus Monkeys. *The Journal of pharmacology and experimental therapeutics* **267**(3).

Byers SL, Wiles MV, Dunn SL and Taft RA (2012) Mouse estrous cycle identification tool and images. *PLoS One* **7**(4): e35538.

Carlezon WA Jr, Thome J, Olson VG, et al. Regulation of cocaine reward by CREB. *Science*. 1998;282(5397):2272-2275. doi:10.1126/science.282.5397.2272

Carlezon WAJ and Krystal AD (2016) Kappa-Opioid Antagonists for Psychiatric Disorders: From Bench to Clinical Trials. *Depress Anxiety* **33**(10): 895-906.

Carr, G. V., Bangasser, D. A., Bethea, T., Young, M., Valentino, R. J., & Lucki, I. (2010). Antidepressant-like effects of kappa-opioid receptor antagonists in Wistar Kyoto rats. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *35*(3), 752–763. doi:10.1038/npp.2009.183

Carroll FI and Carlezon WA (2013) Development of κ Opioid Receptor Antagonists. *Journal of medicinal chemistry* **56**(6).

Carroll I, Thomas JB, Dykstra LA, Granger AL, Allen RM, Howard JL, Pollard GT, Aceto MD and Harris LS (2004) Pharmacological properties of JDTic: a novel kappa-opioid receptor antagonist. *Eur J Pharmacol* **501**(1-3): 111-119.

Chang, C. Y., Esber, G. R., Marrero-Garcia, Y., Yau, H. J., Bonci, A., & Schoenbaum, G. (2016). Brief optogenetic inhibition of dopamine neurons mimics endogenous negative reward prediction errors. *Nature neuroscience*, *19*(1), 111–116. doi:10.1038/nn.4191

Chartoff E, Sawyer A, Rachlin A, Potter D, Pliakas A and Carlezon WA (2012) Blockade of kappa opioid receptors attenuates the development of depressive-like behaviors induced by cocaine withdrawal in rats. *Neuropharmacology* **62**(1): 167-176.

Chartoff, E. H., Ebner, S. R., Sparrow, A., Potter, D., Baker, P. M., Ragozzino, M. E., & Roitman, M. F. (2016). Relative Timing Between Kappa Opioid Receptor Activation and Cocaine Determines the Impact on Reward and Dopamine Release. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(4), 989–1002. doi:10.1038/npp.2015.226

Chavkin, C., James, I. F., & Goldstein, A. (1982). Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science*, *215*(4531), 413–415. doi: 10.1126/science.6120570

Chavkin, C., & Koob, G. F. (2016). Dynorphin, Dysphoria, and Dependence: the Stress of Addiction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(1), 373–374. doi: 10.1038/npp.2015.258

Chavkin C, Cohen JH and Land BB (2019) Repeated Administration of Norbinaltorphimine Produces Cumulative Kappa Opioid Receptor Inactivation. *Front Pharmacol* **10**: 88.

Chaudhury, D., Walsh, J. J., Friedman, A. K., Juarez, B., Ku, S. M., Koo, J. W., … Han, M.-H. (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature*, *493*(7433), 532–536. doi:10.1038/nature11713

Che, T., Majumdar, S., Zaidi, S. A., Ondachi, P., McCorvy, J. D., Wang, S., Mosier, P. D., Uprety, R., Vardy, E., Krumm, B. E., Han, G. W., Lee, M. Y., Pardon, E., Steyaert, J., Huang, X. P., Strachan, R. T., Tribo, A. R., Pasternak, G. W., Carroll, F. I., Stevens, R. C., … Roth, B. L. (2018). Structure of the Nanobody-Stabilized Active State of the Kappa Opioid Receptor. *Cell*, *172*(1-2), 55–67.e15. doi:10.1016/j.cell.2017.12.011

Che, T., English, J., Krumm, B. E., Kim, K., Pardon, E., Olsen, R., Wang, S., Zhang, S., Diberto, J. F., Sciaky, N., Carroll, F. I., Steyaert, J., Wacker, D., & Roth, B. L. (2020). Nanobody-enabled monitoring of kappa opioid receptor states. *Nature communications*, *11*(1), 1145. doi:0.1038/s41467-020-14889-7

Chefer, V. I., Bäckman, C. M., Gigante, E. D., & Shippenberg, T. S. (2013). Kappa opioid receptors on dopaminergic neurons are necessary for kappa-mediated place aversion. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *38*(13), 2623–2631. doi:10.1038/npp.2013.171

Chen, Z., Tang, Y., Tao, H., Li, C., Zhang, X., & Liu, Y. (2015). Dynorphin activation of kappa opioid receptor reduces neuronal excitability in the paraventricular nucleus of mouse thalamus. *Neuropharmacology, 97*, 2 59-69. doi:10.1016/j.neuropharm.2015 .05.030

Childers, S. R., & Snyder, S. H. (1978). Guanine nucleotides differentiate agonist and antagonist interactions with opiate receptors. *Life Sci, 23*(7), 759-761.

Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 1992;267:1244-1252.

Chung, A. S., Miller, S. M., Sun, Y., Xu, X., & Zweifel, L. S. (2017). Sexual congruency in the connectome and translatome of VTA dopamine neurons. *Scientific Reports*, *7*(1), 11120. doi:10.1038/s41598-017-11478-5

Clark JJ, Sandberg SG, Wanat MJ, Gan JO, Horne EA, Hart AS, Akers CA, Parker JG, Willuhn I, Martinez V, Evans SB, Stella N and Phillips PEM (2010) Chronic microsensors for longitudinal, subsecond dopamine detection in behaving animals. *Nat Methods* **7**(2): 126-129.

Clayton, C. C., Xu, M., & Chavkin, C. (2009). Tyrosine phosphorylation of Kir3 following kappa-opioid receptor activation of p38 MAPK causes heterologous desensitization. *The Journal of Biological Chemistry*, *284*(46), 31872–31881. http://doi.org/10.1074/jbc.M109.053793

Cohen, A., & George, O. (2013). Animal models of nicotine exposure: relevance to second-hand smoking, electronic cigarette use, and compulsive smoking. *Frontiers in psychiatry*, *4*, 41. doi:10.3389/fpsyt.2013.00041

Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B., & Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature*, *482*(7383), 85–88. doi:10.1038/nature10754

Cole, R. L., Konradi, C., Douglass, J., & Hyman, S. E. (1995). Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron*, *14*(4), 813–823. doi:10.1016/0896-6273(95)90225-2

Coussens, N. P., Sittampalam, G. S., Jonson, S. G., Hall, M. D., Gorby, H. E., Tamiz, A. P.,

McManus, O. B., Felder, C. C., & Rasmussen, K. (2019). The Opioid Crisis and the Future of Addiction and Pain Therapeutics. *The Journal of pharmacology and experimental therapeutics*, *371*(2), 396–408. doi:10.1124/jpet.119.259408

Crowley, N. A., Bloodgood, D. W., Hardaway, J. A., Kendra, A. M., McCall, J. G., Al-Hasani, R., McCall, N. M., Yu, W., Schools, Z. L., Krashes, M. J., Lowell, B. B., Whistler, J. L., Bruchas, M. R., & Kash, T. L. (2016). Dynorphin Controls the Gain of an Amygdalar Anxiety Circuit. *Cell reports*, *14*(12), 2774–2783. doi:10.1016/j.celrep.2016.02.069

Cruz, H. G., Ivanova, T., Lunn, M.-L., Stoffel, M., Slesinger, P. A., & Lüscher, C. (2004). Bi-directional effects of GABAB receptor agonists on the mesolimbic dopamine system. *Nature Neuroscience*, *7*(2), 153–159. doi: 10.1038/nn1181

Cyranowski, J. M., Frank, E., Young, E., & Shear, M. K. (2000). Adolescent Onset of the Gender Difference in Lifetime Rates of Major Depression. *Archives of General Psychiatry*, *57*(1), 21. doi: 10.1001/archpsyc.57.1.21

Daaka Y, Pitcher JA, Richardson M, Stoffel RH, Robishaw JD and Lefkowitz RJ (1997) Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A* **94**(6): 2180-2185.

Danjo, T., Yoshimi, K., Funabiki, K., Yawata, S., & Nakanishi, S. (2014). Aversive behavior induced by optogenetic inactivation of ventral tegmental area dopamine neurons is mediated by dopamine D2 receptors in the nucleus accumbens. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(17), 6455–6460. doi:10.1073/pnas.1404323111

DeVos SL and Miller TM (2013) Direct Intraventricular Delivery of Drugs to the Rodent Central Nervous System. *Journal of visualized experiments : JoVE*(75).

Di Chiara, G., & Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(14), 5274–5278.

Dolly JO, Parcej DN. Molecular properties of voltage-gated K+ channels. *J Bioenerg Biomembr*. 1996;28(3):231-253. doi:10.1007/BF02110698.

Dominguez R, Hu E, Zhou M and Baudry M (2009) 17beta-estradiol-mediated neuroprotection and ERK activation require a pertussis toxin-sensitive mechanism involving GRK2 and beta-arrestin-1. *J Neurosci* **29**(13): 4228-4238.

Dunn, A. D., Reed, B., Erazo, J., Ben-Ezra, A., & Kreek, M. J. (2019). Signaling Properties of Structurally Diverse Kappa Opioid Receptor Ligands: Toward in Vitro Models of in Vivo Responses. *ACS chemical neuroscience*, *10*(8), 3590–3600. doi:10.1021/acschemneuro.9b00195

Ehrich, J. M., Messinger, D. I., Knakal, C. R., Kuhar, J. R., Schattauer, S. S., Bruchas, M. R., . . Chavkin, C. (2015). Kappa Opioid Receptor-Induced Aversion Requires p38 MAPK Activation in VTA Dopamine Neurons. *J Neurosci, 35*(37), 12917-12931. doi:10.1523/jneurosci.2444-15.2015

Endoh T, Tajima A, Suzuki T, Kamei J, Suzuki T, Narita M, Tseng L and Nagase H (2000) Characterization of the Antinociceptive Effects of TRK-820 in the Rat. *European journal of pharmacology* **387**(2).

Ermakova YG, Bilan DS, Matlashov ME, Mishina NM, Markvicheva KN, Subach OM, Subach FV, Bogeski I, Hoth M, Enikolopov G and Belousov VV (2014) Red Fluorescent Genetically Encoded Indicator for Intracellular Hydrogen Peroxide. *Nature communications*.

Escobar, A., Casanova, J. P., Andrés, M. E., & Fuentealba, J. A. (2020). Crosstalk Between Kappa Opioid and Dopamine Systems in Compulsive Behaviors. *Frontiers in pharmacology*, *11*, 57. doi:10.3389/fphar.2020.00057

Faber, E. S., & Sah, P. (2004). Opioids inhibit lateral amygdala pyramidal neurons by enhancing a dendritic potassium current. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *24*(12), 3031–3039. doi:10.1523/JNEUROSCI.4496-03.2004

Falcon, E., Browne, C. A., Leon, R. M., Fleites, V. C., Sweeney, R., Kirby, L. G., & Lucki, I. (2016). Antidepressant-like Effects of Buprenorphine are Mediated by Kappa Opioid Receptors. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(9), 2344–2351. doi:10.1038/npp.2016.38

Fellinger, L., Jo, Y. S., Hunker, A. C., Soden, M. E., & Zweifel, L. S. (2020). A midbrain dynorphin circuit promotes threat generalization. *bioRxiv*, 2020.2004.2021.053892. doi:10.1101/2020.04.21.053892 **[* Preprint]**

Ferrari, L. L., Agostinelli, L. J., Krashes, M. J., Lowell, B. B., Scammell, T. E., & Arrigoni, E. (2016). Dynorphin inhibits basal forebrain cholinergic neurons by pre- and postsynaptic mechanisms. *The Journal of physiology*, *594*(4), 1069–1085. doi:10.1113/JP271657

Fisher AB (2011) Peroxiredoxin 6: A Bifunctional Enzyme With Glutathione Peroxidase and Phospholipase A_2 Activities. *Antioxidants & redox signaling* **15**(3).

Ford, C. P., Mark, G. P., & Williams, J. T. (2006). Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. *J Neurosci, 26*(10), 2788-2797. doi:10.1523/jneurosci.4331-05.2006

Ford, D. E., & Erlinger, T. P. (2004). Depression and C-Reactive Protein in US Adults. *Archives of Internal Medicine*, *164*(9), 1010. doi: 10.1001/archinte.164.9.1010

Franklin K and Paxinos G (2008a) *The Mouse Brain in Stereotaxic Coordinates*. 3 ed.

Fuchs, P. & McKenna, J. (2008). Aversive stimuli. In N. J. Salkind (Ed.), *Encyclopedia of educational psychology* (Vol. 1, pp. 89-89). Thousand Oaks, CA: SAGE Publications, Inc. doi: 10.4135/9781412963848.n24

Fulton, S., Thibault, D., Mendez, J. A., Lahaie, N., Tirotta, E., Borrelli, E., … Trudeau, L.-E. (2011). Contribution of Kv1.2 voltage-gated potassium channel to D2 autoreceptor regulation of axonal dopamine overflow. *The Journal of Biological Chemistry*, *286*(11), 9360–9372. doi:10.1074/jbc.M110.153262

Gähwiler, B. H., & Brown, D. A. (1985). GABAB-receptor-activated K+ current in voltage-clamped CA3 pyramidal cells in hippocampal cultures. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(5), 1558–1562. doi:10.1073/pnas.82.5.1558

Gillett, K., Harshberger, E., & Valdez, G. R. (2013). Protracted withdrawal from ethanol and enhanced responsiveness stress: regulation via the dynorphin/kappa opioid receptor system. *Alcohol (Fayetteville, N.Y.)*, *47*(5), 359–365. doi:10.1016/j.alcohol.2013.05.001

Gilpin, N. W., Roberto, M., Koob, G. F., & Schweitzer, P. (2014). Kappa opioid receptor activation decreases inhibitory transmission and antagonizes alcohol effects in rat central amygdala. *Neuropharmacology*, *77*, 294–302. doi:10.1016/j.neuropharm.2013.10.005

Gold, P. W., & Chrousos, G. P. (2002). Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. *Mol Psychiatry, 7*(3), 254-275. doi:10.1038/sj.mp.4001032

Gold, P. W., Goodwin, F. K., & Chrousos, G. P. (1998). Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (1). *N Engl J Med, 319*(6), 348-353. doi:10.1056/nejm198808113190606

González, D., Riba, J., Bouso, J. C., Gómez-Jarabo, G., & Barbanoj, M. J. (2006). Pattern of use and subjective effects of Salvia divinorum among recreational users. *Drug and Alcohol Dependence*, *85*(2), 157–162. doi: 10.1016/j.drugalcdep.2006.04.001

Grahn, R., Will, M., Hammack, S., Maswood, S., Mcqueen, M., Watkins, L., & Maier, S. (1999). Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. *Brain Research*, *826*(1), 35–43. doi: 10.1016/s0006-8993(99)01208-1

Groblewski, P. A., Zietz, C., Willuhn, I., Phillips, P. E., & Chavkin, C. (2015). Repeated stress exposure causes strain-dependent shifts in the behavioral economics of cocaine in rats. *Addiction biology*, *20*(2), 297–301. doi:10.1111/adb.12123

Gross, J.D., Kaski, S.W., Schmidt, K.T. *et al.* Role of RGS12 in the differential regulation of kappa opioid receptor-dependent signaling and behavior. *Neuropsychopharmacol.* 44, 1728–1741 (2019). doi:10.1038/s41386-019-0423-7

Han, J.-S., & Xie, C.-W. (1982). Dynorphin: Potent analgesic effect in spinal cord of the rat. *Life Sciences*, *31*(16-17), 1781–1784. doi: 10.1016/0024-3205(82)90209-0

Heymann, G., Jo, Y. S., Reichard, K. L., Mcfarland, N., Chavkin, C., Palmiter, R. D., … Zweifel, L. S. (2020). Synergy of Distinct Dopamine Projection Populations in Behavioral Reinforcement. *Neuron*, *105*(5). doi: 10.1016/j.neuron.2019.11.024

Hille, B. (2007). *Ion channels of excitable membranes* (3rd ed.). Sunderland, MA: Sinauer.

Ho, J. H., Stahl, E. L., Schmid, C. L., Scarry, S. M., Aubé, J., & Bohn, L. M. (2018). G protein signaling-biased agonism at the κ-opioid receptor is maintained in striatal neurons. *Science signaling*, *11*(542), eaar4309. doi:10.1126/scisignal.aar4309

Holly, E. N., DeBold, J. F., & Miczek, K. A. (2015). Increased mesocorticolimbic dopamine during acute and repeated social defeat stress: modulation by corticotropin releasing factor receptors in the ventral tegmental area. *Psychopharmacology*, *232*(24), 4469–4479. doi:10.1007/s00213-015-4082-z

Horan P, Taylor J, Yamamura H and Porreca F (1992) Extremely Long-Lasting Antagonistic Actions of Nor-Binaltorphimine (nor-BNI) in the Mouse Tail-Flick Test. *The Journal of pharmacology and experimental therapeutics* **260**(3).

Hunker AC, Soden ME, Krayushkina D, Heymann G, Awatramani R, Zweifel LS. Conditional Single Vector CRISPR/SaCas9 Viruses for Efficient Mutagenesis in the Adult Mouse Nervous System. *Cell Rep*. 2020;30(12):4303-4316.e6. doi:10.1016/j.celrep.2020.02.092

Hurd, Y. L., & Herkenham, M. (1993). Molecular alterations in the neostriatum of human cocaine addicts. *Synapse (New York, N.Y.)*, *13*(4), 357–369. doi:10.1002/syn.890130408

Inui S (2015) Nalfurafine Hydrochloride to Treat Pruritus: A Review. *Clinical, cosmetic and investigational dermatology*.

Isola, R., Zhang, H., Tejwani, G.A. *et al.* Acute nicotine changes dynorphin and prodynorphin mRNA in the striatum. *Psychopharmacology* 201, 507–516 (2009). doi:10.1007/s00213-008-1315-4

Jans, L. A. W., Riedel, W. J., Markus, C. R., & Blokland, A. (2006). Serotonergic vulnerability and depression: assumptions, experimental evidence and implications. *Molecular Psychiatry*, *12*(6), 522–543. doi: 10.1038/sj.mp.4001920

Jarman SK, Haney AM and Valdez GR (2018) Kappa opioid regulation of depressive-like behavior during acute withdrawal and protracted abstinence from ethanol. *PLoS One* **13**(9): e0205016.

Jo, Y. S., Heymann, G., & Zweifel, L. S. (2018). Dopamine Neurons Reflect the Uncertainty in

Fear Generalization. *Neuron*, *100*(4), 916–925.e3. doi:10.1016/j.neuron.2018.09.028

Kalivas, P. W. (1985). Interactions between neuropeptides and dopamine neurons in the ventromedial mesencephalon. *Neuroscience & Biobehavioral Reviews*, *9*(4), 573–587. doi: 10.1016/0149-7634(85)90004-1

Karkhanis, A. N., Rose, J. H., Weiner, J. L., & Jones, S. R. (2016). Early-Life Social Isolation Stress Increases Kappa Opioid Receptor Responsiveness and Downregulates the Dopamine System. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(9), 2263–2274. doi:10.1038/npp.2016.21

Karp, J. F., Butters, M. A., Begley, A. E., Miller, M. D., Lenze, E. J., Blumberger, D. M., Mulsant, B. H., & Reynolds, C. F., 3rd (2014). Safety, tolerability, and clinical effect of low-dose buprenorphine for treatment-resistant depression in midlife and older adults. *The Journal of clinical psychiatry*, *75*(8), e785–e793. doi:10.4088/JCP.13m08725

Karschin, C., Dissmann, E., Stühmer, W., & Karschin, A. (1996). IRK(1-3) and GIRK(1-4) inwardly rectifying K+ channel mRNAs are differentially expressed in the adult rat brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *16*(11), 3559–3570. doi:10.1523/JNEUROSCI.16-11-03559.1996

Kenakin, T., & Miller, L. J. (2010). Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacological reviews*, *62*(2), 265–304. doi:10.1124/pr.108.000992

Kenakin T (2005) New Concepts in Drug Discovery: Collateral Efficacy and Permissive Antagonism. *Nature reviews Drug discovery* **4**(11).

Kessler, R. C. (1997). The effects of stressful life events on depression. *Annu Rev Psychol, 48*, 191-214. doi:10.1146/annurev.psych.48.1.191

Kirby, L. G., Pan, Y. Z., Freeman-Daniels, E., Rani, S., Nunan, J. D., Akanwa, A., & Beck, S. G. (2007). Cellular effects of swim stress in the dorsal raphe nucleus. *Psychoneuroendocrinology*, *32*(6), 712–723. doi:10.1016/j.psyneuen.2007.05.001

Kivell, B. M., Paton, K. F., Kumar, N., Morani, A. S., Culverhouse, A., Shepherd, A., Welsh, S. A., Biggerstaff, A., Crowley, R. S., & Prisinzano, T. E. (2018). Kappa Opioid Receptor Agonist Mesyl Sal B Attenuates Behavioral Sensitization to Cocaine with Fewer Aversive Side-Effects than Salvinorin A in Rodents. *Molecules (Basel, Switzerland)*, *23*(10), 2602. doi:10.3390/molecules23102602

Kivell, B., Uzelac, Z., Sundaramurthy, S., Rajamanickam, J., Ewald, A., Chefer, V., Jaligam, V., Bolan, E., Simonson, B., Annamalai, B., Mannangatti, P., Prisinzano, T. E., Gomes, I., Devi, L. A., Jayanthi, L. D., Sitte, H. H., Ramamoorthy, S., & Shippenberg, T. S. (2014). Salvinorin A regulates dopamine transporter function via a kappa opioid receptor and

ERK1/2-dependent mechanism. *Neuropharmacology*, *86*, 228–240. doi:10.1016/j.neuropharm.2014.07.016

Koob, G. F. (2008). A role for brain stress systems in addiction. *Neuron, 59*(1), 11-34. doi:10.1016/j.neuron.2008.06.012

Koob G. F. (2013). Addiction is a Reward Deficit and Stress Surfeit Disorder. *Frontiers in psychiatry*, *4*, 72. doi:10.3389/fpsyt.2013.00072

Krystal AD, Pizzagalli DA, Smoski M, et al. A randomized proof-of-mechanism trial applying the 'fast-fail' approach to evaluating κ-opioid antagonism as a treatment for anhedonia. *Nat Med*. 2020;26(5):760-768. doi:10.1038/s41591-020-0806-7

Kudryavtseva, N. N., Gerrits, M. A., Avgustinovich, D. F., Tenditnik, M. V., & Van Ree, J. M. (2004). Modulation of anxiety-related behaviors by mu- and kappa-opioid receptor agonists depends on the social status of mice. *Peptides*, *25*(8), 1355–1363. doi:10.1016/j.peptides.2004.05.005

Kudryavtseva, N. N., Gerrits, M. A., Alekseenko, O. V., & Van Ree, J. M. (2005). Chronic cocaine injections attenuate behavioral response of kappa-opioid receptors to U-50,488H agonist. *Bulletin of experimental biology and medicine*, *140*(3), 320–322. doi:10.1007/s10517-005-0479-8

Kuzmin, A. V., Semenova, S., Gerrits, M. A., Zvartau, E. E., & Ree, J. M. V. (1997). κ-Opioid receptor agonist U50,488H modulates cocaine and morphine self-administration in drug-naive rats and mice. *European Journal of Pharmacology*, *321*(3), 265–271. doi: 10.1016/s0014-2999(96)00961-2

Lacey, M. G., Mercuri, N. B., & North, R. A. (1988). On the potassium conductance increase activated by GABAB and dopamine D2 receptors in rat substantia nigra neurones. *The Journal of physiology*, *401*, 437–453. doi:10.1113/jphysiol.1988.sp017171

Land, B. B., Bruchas, M. R., Schattauer, S., Giardino, W. J., Aita, M., Messinger, D., Hnasko, T. S., Palmiter, R. D., & Chavkin, C. (2009). Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(45), 19168–19173. doi:10.1073/pnas.0910705106

Land, B. B., Bruchas, M. R., Lemos, J. C., Xu, M., Melief, E. J., & Chavkin, C. (2008). The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *28*(2), 407–414. doi:10.1523/JNEUROSCI.4458-07.2008

Le Merrer, J., Becker, J. A., Befort, K., & Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiological reviews*, *89*(4), 1379–1412. Doi: 10.1152/physrev.00005.2009

Leander JD (1988) Buprenorphine Is a Potent Kappa-Opioid Receptor Antagonist in Pigeons and Mice. *European journal of pharmacology* **151**(3).

Lemos, J. C., Roth, C. A., & Chavkin, C. (2011). Signaling events initiated by kappa opioid receptor activation: quantification and immunocolocalization using phospho-selective KOR, p38 MAPK, and K(IR) 3.1 antibodies. *Methods in molecular biology (Clifton, N.J.)*, *717*, 197–219. doi:10.1007/978-1-61779-024-9_11

Lemos, J. C., Roth, C. A., Messinger, D. I., Gill, H. K., Phillips, P. E., & Chavkin, C. (2012). Repeated stress dysregulates κ-opioid receptor signaling in the dorsal raphe through a p38α MAPK-dependent mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *32*(36), 12325–12336. doi:10.1523/jneurosci.2053-12.2012

Li, C., Pleil, K. E., Stamatakis, A. M., Busan, S., Vong, L., Lowell, B. B., . . . Kash, T. L. (2012). Presynaptic inhibition of gamma-aminobutyric acid release in the bed nucleus of the stria terminalis by kappa opioid receptor signaling. *Biol Psychiatry, 71*(8), 725-732.

Li, W., Sun, H., Chen, H., Yang, X., Xiao, L., Liu, R., Shao, L., & Qiu, Z. (2016). Major Depressive Disorder and Kappa Opioid Receptor Antagonists. Translational perioperative and pain medicine, 1(2), 4–16.

Lin, S., Boey, D., Lee, N., Schwarzer, C., Sainsbury, A., & Herzog, H. (2006). Distribution of prodynorphin mRNA and its interaction with the NPY system in the mouse brain. *Neuropeptides*, *40*(2), 115–123. doi: 10.1016/j.npep.2005.11.006

Liu, J. J., Sharma, K., Zangrandi, L., Chen, C., Humphrey, S. J., Chiu, Y.-T., … Mann, M. (2018). In vivo brain GPCR signaling elucidated by phosphoproteomics. *Science (New York, N.Y.)*, *360*(6395). Doi:http://doi.org/10.1126/science.aao4927

Llamosas, N., Ugedo, L., & Torrecilla, M. (2017). Inactivation of GIRK channels weakens the pre- and postsynaptic inhibitory activity in dorsal raphe neurons. *Physiological reports*, *5*(3), e13141. doi:10.14814/phy2.13141

Llamosas, N., Bruzos-Cidón, C., Rodríguez, J. J., Ugedo, L., & Torrecilla, M. (2015). Deletion of GIRK2 Subunit of GIRK Channels Alters the 5-HT1A Receptor-Mediated Signaling and Results in a Depression-Resistant Behavior. *The international journal of neuropsychopharmacology*, *18*(11), pyv051. doi:10.1093/ijnp/pyv051

Lovell, K. M., Frankowski, K. J., Stahl, E. L., Slauson, S. R., Yoo, E., Prisinzano, T. E., Aubé, J., & Bohn, L. M. (2015). Structure-activity relationship studies of functionally selective kappa opioid receptor agonists that modulate ERK 1/2 phosphorylation while preserving G protein over βarrestin2 signaling bias. *ACS chemical neuroscience*, *6*(8), 1411–1419. doi:10.1021/acschemneuro.5b00092

Lowe JD, Sanderson HS, Cooke AE, Ostovar M, Tsisanova E, Withey SL, Chavkin C,

Husbands SM, Kelly E, Henderson G and Bailey CP (2015) Role of G Protein-Coupled Receptor Kinases 2 and 3 in mu-Opioid Receptor Desensitization and Internalization. *Mol Pharmacol* **88**(2): 347-356.

Lutz, P. E., & Kieffer, B. L. (2013). Opioid receptors: distinct roles in mood disorders. *Trends in neurosciences*, *36*(3), 195–206. https://doi.org/10.1016/j.tins.2012.11.002

Mahar, I., Bambico, F. R., Mechawar, N., & Nobrega, J. N. (2014). Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects. *Neuroscience & Biobehavioral Reviews*, *38*, 173–192. doi: 10.1016/j.neubiorev.2013.11.009

Margolis, E. B., Hjelmstad, G. O., Bonci, A., & Fields, H. L. (2005). Both kappa and mu opioid agonists inhibit glutamatergic input to ventral tegmental area neurons. *Journal of Neurophysiology*, *93*(6), 3086–3093. http://doi.org/10.1152/jn.00855.2004

Margolis, E. B., Lock, H., Chefer, V. I., Shippenberg, T. S., Hjelmstad, G. O., & Fields, H. L. (2006). Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. *Proc Natl Acad Sci U S A, 103*(8), 2938-2942. doi:10.1073/pnas.0511159103

Margolis, E. B., Wallace, T. L., Van Orden, L. J., & Martin, W. (2020). Differential effects of novel kappa opioid receptor antagonists on dopamine neurons using acute brain slice electrophysiology. *bioRxiv*, 2020. doi:10.1101/2020.04.24.059352 **[preprint]**

Martel, P., Leo, D., Fulton, S., Bérard, M., & Trudeau, L. E. (2011). Role of Kv1 potassium channels in regulating dopamine release and presynaptic D2 receptor function. *PloS one*, *6*(5), e20402. doi:10.1371/journal.pone.0020402

McDermott, C. M., & Schrader, L. A. (2011). Activation of κ opioid receptors increases intrinsic excitability of dentate gyrus granule cells. *The Journal of physiology*, *589*(Pt 14), 3517–3532. doi:10.1113/jphysiol.2011.211623

McLaughlin JP, Marton-Popovici M, Chavkin C. (2003) Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J Neurosci. 2003;23:5674–5683.

McLaughlin, J. P., Li, S., Valdez, J., Chavkin, T. A., & Chavkin, C. (2006a). Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *31*(6), 1241–1248. doi: 10.1038/sj.npp.1300872

McLaughlin, J. P., Land, B. B., Li, S., Pintar, J. E., & Chavkin, C. (2006b). Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. *Neuropsychopharmacology : official publication*

of the American College of Neuropsychopharmacology, *31*(4), 787–794. doi:10.1038/sj.npp.1300860

Melief, E. J., Miyatake, M., Carroll, F. I., Béguin, C., Carlezon, W. A., Jr, Cohen, B. M., Grimwood, S., Mitch, C. H., Rorick-Kehn, L., & Chavkin, C. (2011). Duration of action of a broad range of selective κ-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. *Molecular pharmacology*, *80*(5), 920–929. doi:10.1124/mol.111.074195

Mello, N. K., & Negus, S. S. (1998). Effects of Kappa Opioid Agonists on Cocaine- and Food-Maintained Responding by Rhesus Monkeys. *Journal of Pharmacology and Experimental Therapeutics, 286*(2), 812-824.

Meneses, D., Vega, A. V., Torres-Cruz, F. M., & Barral, J. (2016). KV1 and KV3 Potassium Channels Identified at Presynaptic Terminals of the Corticostriatal Synapses in Rat. *Neural plasticity*, *2016*, 8782518. doi:10.1155/2016/8782518

Miller R. J. (1998). Presynaptic receptors. *Annual review of pharmacology and toxicology*, *38*, 201–227. doi:10.1146/annurev.pharmtox.38.1.201

Minneman, K. P., & Iversen, I. L. (1976). Enkephalin and opiate narcotics increase cyclic GMP accumulation in slices of rat neostriatum. *Nature, 262*(5566), 313-314.

Mitchell, J.M., Liang, M.T. & Fields, H.L. A single injection of the kappa opioid antagonist norbinaltorphimine increases ethanol consumption in rats. *Psychopharmacology* 182, 384–392 (2005). doi:10.1007/s00213-005-0067-7

Mo, B., Feng, N., Renner, K., & Forster, G. (2008). Restraint stress increases serotonin release in the central nucleus of the amygdala via activation of corticotropin-releasing factor receptors. *Brain research bulletin*, *76*(5), 493-498.doi:10.1016/j.brainresbull.2008.02.011

Mores, K. L., Cummins, B. R., Cassell, R. J., & van Rijn, R. M. (2019). A Review of the Therapeutic Potential of Recently Developed G Protein-Biased Kappa Agonists. *Frontiers in pharmacology*, *10*, 407. doi:10.3389/fphar.2019.00407

Morley, J. E., Levine, A. S., & Rowland, N. E. (1983). Stress induced eating. *Life Sciences*, *32*(19), 2169–2182. doi: 10.1016/0024-3205(83)90415-0

Morris, B., Haarmann, I., Kempter, B., Höllt, V., & Herz, A. (1986). Localization of prodynorphin messenger RNA in rat brain by in situ hybridization using a synthetic oligonucleotide probe. *Neuroscience Letters*, *69*(1), 104–108. doi: 10.1016/0304-3940(86)90423-4

Muschamp, J. W., & Carlezon, W. A., Jr (2013). Roles of nucleus accumbens CREB and dynorphin in dysregulation of motivation. *Cold Spring Harbor perspectives in medicine*, *3*(2), a012005. doi:10.1101/cshperspect.a012005

Nagi, K., & Pineyro, G. (2014). Kir3 channel signaling complexes: focus on opioid receptor signaling. *Frontiers in cellular neuroscience*, *8*, 186. doi:10.3389/fncel.2014.00186

Nealey, K. A., Smith, A. W., Davis, S. M., Smith, D. G., & Walker, B. M. (2011). κ-opioid receptors are implicated in the increased potency of intra-accumbens nalmefene in ethanol-dependent rats. *Neuropharmacology*, *61*(1-2), 35–42. doi:10.1016/j.neuropharm.2011.02.012

Negus, S. S., Morrissey, E. M., Rosenberg, M., Cheng, K., & Rice, K. C. (2010). Effects of kappa opioids in an assay of pain-depressed intracranial self-stimulation in rats. *Psychopharmacology*, *210*(2), 149–159. doi:0.1007/s00213-009-1770-6

Nestler, E. J., & Malenka, R. C. (2004). The addicted brain. *Scientific American*, *290*(3), 78–85.

Nestler E. J. (2004). Molecular mechanisms of drug addiction. *Neuropharmacology*, *47 Suppl 1*, 24–32. Doi: 10.1016/j.neuropharm.2004.06.031

Norman, K. J., Seiden, J. A., Klickstein, J. A., Han, X., Hwa, L. S., DeBold, J. F., & Miczek, K. A. (2015). Social stress and escalated drug self-administration in mice I. Alcohol and corticosterone. *Psychopharmacology*, *232*(6), 991–1001. doi:10.1007/s00213-014-3733-9

Paredes, R. G. (2014). Opioids and sexual reward. *Pharmacology Biochemistry and Behavior*, *121*, 124–131. doi: 10.1016/j.pbb.2013.11.004

Paris, J. J., Reilley, K. J., & McLaughlin, J. P. (2011). Kappa Opioid Receptor-Mediated Disruption of Novel Object Recognition: Relevance for Psychostimulant Treatment. *Journal of addiction research & therapy*, *S4*, 007. doi:10.4172/2155-6105.S4-007

Pascoli, V., Terrier, J., Hiver, A., & Luscher, C. (2015). Sufficiency of Mesolimbic Dopamine Neuron Stimulation for the Progression to Addiction. *Neuron, 88*(5), 1054-1066. doi:10.1016/j.neuron.2015.10.017

Perreault, M. L., Graham, D., Scattolon, S., Wang, Y., Szechtman, H., & Foster, J. A. (2007). Cotreatment with the kappa opioid agonist U69593 enhances locomotor sensitization to the D2/D3 dopamine agonist quinpirole and alters dopamine D2 receptor and prodynorphin mRNA expression in rats. *Psychopharmacology*, *194*(4), 485–496. doi: 10.1007/s00213-007-0855-3

Pfeiffer, A., Brantl, V., Herz, A., & Emrich, H. M. (1986). Psychotomimesis mediated by kappa opiate receptors. *Science, 233*(4765), 774-776.

Picetti, R., Saiardi, A., Abdel Samad, T., Bozzi, Y., Baik, J. H., & Borrelli, E. (1997). Dopamine D2 receptors in signal transduction and behavior. *Critical reviews in neurobiology*, *11*(2-3), 121–142. Doi:10.1615/critrevneurobiol.v11.i2-3.20

Pignatelli, M., Umanah, G., Ribeiro, S. P., Chen, R., Karuppagounder, S. S., Yau, H. J., Eacker,

S., Dawson, V. L., Dawson, T. M., & Bonci, A. (2017). Synaptic Plasticity onto Dopamine Neurons Shapes Fear Learning. *Neuron*, *93*(2), 425–440. doi:10.1016/j.neuron.2016.12.030

Polter, A. M., Bishop, R. A., Briand, L. A., Graziane, N. M., Pierce, R. C., & Kauer, J. A. (2014). Poststress block of kappa opioid receptors rescues long-term potentiation of inhibitory synapses and prevents reinstatement of cocaine seeking. *Biological psychiatry*, *76*(10), 785–793. doi:10.1016/j.biopsych.2014.04.019

Polter, A. M., Barcomb, K., Chen, R. W., Dingess, P. M., Graziane, N. M., Brown, T. E., & Kauer, J. A. (2017). Constitutive activation of kappa opioid receptors at ventral tegmental area inhibitory synapses following acute stress. *eLife*, *6*, e23785. doi:10.7554/eLife.23785

Prisinzano, T. E. (2005). Psychopharmacology of the hallucinogenic sage Salvia divinorum. *Life Sciences*, *78*(5), 527–531. doi: 10.1016/j.lfs.2005.09.008

Przewlocki, R., Lason, W., Konecka, A., Gramsch, C., Herz, A., & Reid, L. (1983). The opioid peptide dynorphin, circadian rhythms, and starvation. *Science*, *219*(4580), 71–73. doi: 10.1126/science.6129699

Raffa, R. B., Stagliano, G. W., Ross, G., Powell, J. A., Phillips, A. G., Ding, Z., & Rawls, S. M. (2008). The kappa-opioid receptor antagonist nor-BNI inhibits cocaine and amphetamine, but not cannabinoid (WIN 52212-2), abstinence-induced withdrawal in planarians: an instance of 'pharmacologic congruence'. *Brain research*, *1193*, 51–56. doi:.1016/j.brainres.2007.12.001

Rhinehart, E. M., Nentwig, T. B., Wilson, D. E., Leonard, K. T., Chaney, B. N., & Grisel, J. E. (2018). Sex and β-Endorphin Influence the Effects of Ethanol on Limbic *Gabra2* Expression in a Mouse Binge Drinking Model. *Frontiers in genetics*, *9*, 567. doi:10.3389/fgene.2018.00567

Rifkin, R. A., Huyghe, D., Li, X., Parakala, M., Aisenberg, E., Moss, S. J., & Slesinger, P. A. (2018). GIRK currents in VTA dopamine neurons control the sensitivity of mice to cocaine-induced locomotor sensitization. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(40), E9479–E9488. doi:10.1073/pnas.1807788115

Rice ME (1999) Use of Ascorbate in the Preparation and Maintenance of Brain Slices. *Methods (San Diego, Calif)* **18**(2).

Richard-Lalonde, M., Nagi, K., Audet, N., Sleno, R., Amraei, M., Hogue, M., Balboni, G., Schiller, P. W., Bouvier, M., Hébert, T. E., & Pineyro, G. (2013). Conformational dynamics of Kir3.1/Kir3.2 channel activation via δ-opioid receptors. *Molecular pharmacology*, *83*(2), 416–428. doi:10.1124/mol.112.081950

Ritter, D. M., Ho, C., O'Leary, M. E., & Covarrubias, M. (2012). Modulation of Kv3.4 channel

N-type inactivation by protein kinase C shapes the action potential in dorsal root ganglion neurons. *The Journal of physiology*, *590*(1), 145–161. doi:10.1113/jphysiol.2011.218560.

Riven, I., Iwanir, S., & Reuveny, E. (2006). GIRK Channel Activation Involves a Local Rearrangement of a Preformed G Protein Channel Complex. *Neuron, 51*(5), 561-573. doi:10.1016/j.neuron.2006.08.017

Rorick-Kehn LM, Witcher JW, Lowe SL, Gonzales CR, Weller MA, Bell RL, Hart JC, Need AB, McKinzie JH, Statnick MA, Suico JG, McKinzie DL, Tauscher-Wisniewski S, Mitch CH, Stoltz RR and Wong CJ (2014) Determining Pharmacological Selectivity of the Kappa Opioid Receptor Antagonist LY2456302 Using Pupillometry as a Translational Biomarker in Rat and Human. *The international journal of neuropsychopharmacology* **18**(2).

Rose, J. H., Karkhanis, A. N., Chen, R., Gioia, D., Lopez, M. F., Becker, H. C., McCool, B. A., & Jones, S. R. (2016). Supersensitive Kappa Opioid Receptors Promotes Ethanol Withdrawal-Related Behaviors and Reduce Dopamine Signaling in the Nucleus Accumbens. *The international journal of neuropsychopharmacology*, *19*(5), pyv127. doi:10.1093/ijnp/pyv127

Rüedi-Bettschen, D., Rowlett, J. K., Spealman, R. D., & Platt, D. M. (2010). Attenuation of cocaine-induced reinstatement of drug seeking in squirrel monkeys: kappa opioid and serotonergic mechanisms. *Psychopharmacology*, *210*(2), 169–177. doi:10.1007/s00213-009-1705-2

Saunders, B. T., Richard, J. M., Margolis, E. B., & Janak, P. H. (2018). Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nature neuroscience*, *21*(8), 1072–1083. doi:10.1038/s41593-018-0191-4

Saunders, B. T., Richard, J. M., Margolis, E. B., & Janak, P. H. (2018). Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nature neuroscience*, *21*(8), 1072–1083. https://doi.org/10.1038/s41593-018-0191-4

Schattauer, S. S., Bedini, A., Summers, F., Reilly-Treat, A., Andrews, M. M., Land, B. B., & Chavkin, C. (2019). Reactive oxygen species (ROS) generation is stimulated by κ opioid receptor activation through phosphorylated c-Jun N-terminal kinase and inhibited by p38 mitogen-activated protein kinase (MAPK) activation. *Journal of Biological Chemistry*, *294*(45), 16884–16896. doi: 10.1074/jbc.ra119.009592

Schattauer SS, Kuhar J, Song A and Chavkin C (2017a) Nalfurafine Is a G-protein Biased Agonist Having Significantly Greater Bias at the Human Than Rodent Form of the Kappa Opioid Receptor. *Cellular signalling* **32**.

Schattauer SS, Land BB, Reichard KL, Abraham AD, Burgeno LM, Kuhar JR, Phillips PEM,

Ong SE and Chavkin C (2017b) Peroxiredoxin 6 mediates Galphai protein-coupled receptor inactivation by cJun kinase. *Nat Commun* **8**(1): 743.

Schenk, S., Partridge, B., & Shippenberg, T. S. (1999). U69593, a kappa-opioid agonist, decreases cocaine self-administration and decreases cocaine-produced drug-seeking. *Psychopharmacology*, *144*(4), 339–346. doi:10.1007/s002130051016

Schenk, S., Partridge, B., & Shippenberg, T. S. (2000). Reinstatement of extinguished drug-taking behavior in rats: effect of the kappa-opioid receptor agonist, U69593. *Psychopharmacology*, *151*(1), 85–90. doi:10.1007/s002130000476

Schindler, A. G., Messinger, D. I., Smith, J. S., Shankar, H., Gustin, R. M., Schattauer, S. S., . . . Chavkin, C. (2012). Stress produces aversion and potentiates cocaine reward by releasing endogenous dynorphins in the ventral striatum to locally stimulate serotonin reuptake. *J Neurosci, 32*(49), 17582-17596. doi:10.1523/jneurosci.3220-12.2012

Schlosburg, J. E., Whitfield, T. W., Jr, Park, P. E., Crawford, E. F., George, O., Vendruscolo, L. F., & Koob, G. F. (2013). Long-term antagonism of κ opioid receptors prevents escalation of and increased motivation for heroin intake. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *33*(49), 19384–19392. doi:10.1523/JNEUROSCI.1979-13.2013

Schmitz, S. K., King, C., Kortleven, C., Huson, V., Kroon, T., Kevenaar, J. T., Schut, D., Saarloos, I., Hoetjes, J. P., de Wit, H., Stiedl, O., Spijker, S., Li, K. W., Mansvelder, H. D., Smit, A. B., Cornelisse, L. N., Verhage, M., & Toonen, R. F. (2016). Presynaptic inhibition upon CB1 or mGlu2/3 receptor activation requires ERK/MAPK phosphorylation of Munc18-1. *The EMBO journal*, *35*(11), 1236–1250. doi:10.15252/embj.201592244

Schoenecker J, Takemori A and Portoghese P (1987) Opioid Agonist and Antagonist Activities of Monofunctional Nitrogen Mustard Analogues of Beta-Chlornaltrexamine. *Journal of medicinal chemistry* **30**(5).

Schultz, W., Dayan, P., & Montague, P. R. (1997). A neural substrate of prediction and reward. *Science (New York, N.Y.)*, *275*(5306), 1593–1599. doi:10.1126/science.275.5306.1593

Schultz W. (1998). Predictive reward signal of dopamine neurons. *Journal of neurophysiology*, *80*(1), 1–27. doi:10.1152/jn.1998.80.1.1

Schwarzer C. (2009). 30 years of dynorphins--new insights on their functions in neuropsychiatric diseases. *Pharmacology & therapeutics*, *123*(3), 353–370. doi:10.1016/j.pharmthera.2009.05.006

Sinha, R. (2007). The role of stress in addiction relapse. *Current Psychiatry Reports*, *9*(5), 388–395. doi: 10.1007/s11920-007-0050-6

Shippenberg, T. S., Zapata, A., & Chefer, V. I. (2007). Dynorphin and the pathophysiology of drug addiction. *Pharmacology & therapeutics*, *116*(2), 306–321. doi:10.1016/j.pharmthera.2007.06.011

Shirayama Y, Ishida H, Iwata M, Hazama GI, Kawahara R and Duman RS (2004) Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. *J Neurochem* **90**(5): 1258-1268.

Simmons, M. L., & Chavkin, C. (1996). k-Opioid receptor activation of a dendrotoxin-sensitive potassium channel mediates presynaptic inhibition of mossy fiber neurotransmitter release. *Mol Pharmacol, 50*(1), 80-85.

Smith C, Medzihradsky F, Hollingsworth P, DeCosta B, Rice K and Woods J (1990) Nor-binaltorphimine Is a Reversible, Noncompetitive Opioid Antagonist in the Mouse Vas Deferens With High Affinity for Kappa Receptors in Monkey Brain Membranes. *Progress in clinical and biological research* **328**.

Smith, J. S., Schindler, A. G., Martinelli, E., Gustin, R. M., Bruchas, M. R., & Chavkin, C. (2012). Stress-induced activation of the dynorphin/κ-opioid receptor system in the amygdala potentiates nicotine conditioned place preference. *The Journal of neuroscience*, *32*(4), 1488–1495. doi: 10.1523/JNEUROSCI.2980-11.2012

Smythies J (2000) Redox Aspects of Signaling by Catecholamines and Their Metabolites. *Antioxidants & redox signaling* **2**(3).

Spanagel, R., Herz, A., & Shippenberg, T. S. (1992). Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(6), 2046–2050. doi:10.1073/pnas.89.6.2046

Sperling, R. E., Gomes, S. M., Sypek, E. I., Carey, A. N., & McLaughlin, J. P. (2010). Endogenous kappa-opioid mediation of stress-induced potentiation of ethanol-conditioned place preference and self-administration. *Psychopharmacology (Berl), 210*(2), 199-209. doi:10.1007/s00213-010-1844-5

Stelly, C. E., Haug, G. C., Fonzi, K. M., Garcia, M. A., Tritley, S. C., Magnon, A. P., Ramos, M., & Wanat, M. J. (2019). Pattern of dopamine signaling during aversive events predicts active avoidance learning. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(27), 13641–13650. doi:10.1073/pnas.1904249116

Stokes AH, Hastings TG and Vrana KE (1999) Cytotoxic and Genotoxic Potential of Dopamine. *Journal of neuroscience research* **55**(6).

Stuber, G. D., Roitman, M. F., Phillips, P. E., Carelli, R. M., & Wightman, R. M. (2005). Rapid

dopamine signaling in the nucleus accumbens during contingent and noncontingent cocaine administration. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *30*(5), 853–863. doi:10.1038/sj.npp.1300619

Svingos, A. L., Chavkin, C., Colago, E. E., & Pickel, V. M. (2001). Major coexpression of kappa-opioid receptors and the dopamine transporter in nucleus accumbens axonal profiles. *Synapse (New York, N.Y.)*, *42*(3), 185–192. doi:10.1002/syn.10005

Taussig, R., Iniguez-Lluhi, J. A., & Gilman, A. G. (1993). Inhibition of adenylyl cyclase by Gi alpha. *Science, 261*(5118), 218-221.

Tejeda, H. A., Counotte, D. S., Oh, E., Ramamoorthy, S., Schultz-Kuszak, K. N., Bäckman, C. M., Chefer, V., O'Donnell, P., & Shippenberg, T. S. (2013). Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *38*(9), 1770–1779. doi:10.1038/npp.2013.76

Thal DM, Yeow RY, Schoenau C, Huber J and Tesmer JJG (2011) Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. *Mol Pharmacol* **80**(2):

Ting JT, Daigle TL, Chen Q and Feng G (2014) Acute Brain Slice Methods for Adult and Aging Animals: Application of Targeted Patch Clamp Analysis and Optogenetics. *Methods in molecular biology (Clifton, NJ)* **1183**.

Trifilieff, P., & Martinez, D. (2013). Kappa-opioid receptor signaling in the striatum as a potential modulator of dopamine transmission in cocaine dependence. *Frontiers in psychiatry*, *4*, 44. doi:10.3389/fpsyt.2013.00044

Trudeau, L. E., Hnasko, T. S., Wallén-Mackenzie, A., Morales, M., Rayport, S., & Sulzer, D. (2014). The multilingual nature of dopamine neurons. *Progress in brain research*, *211*, 141–164. doi:10.1016/B978-0-444-63425-2.00006-4

Tye, K. M., Mirzabekov, J. J., Warden, M. R., Ferenczi, E. A., Tsai, H. C., Finkelstein, J., . . . Deisseroth, K. (2013). Dopamine neurons modulate neural encoding and expression of depression-related behaviour. *Nature, 493*(7433), 537-541. doi:10.1038/nature11740

Tyree, S. M., & de Lecea, L. (2017). Lateral Hypothalamic Control of the Ventral Tegmental Area: Reward Evaluation and the Driving of Motivated Behavior. *Frontiers in systems neuroscience*, *11*, 50. https://doi.org/10.3389/fnsys.2017.00050

Uhari-Väänänen, J., Raasmaja, A., Bäckström, P., Oinio, V., Carroll, F. I., Airavaara, M., … Piepponen, P. (2018). The κ-opioid receptor antagonist JDTic decreases ethanol intake in alcohol-preferring AA rats. *Psychopharmacology*, *235*(5), 1581–1591. doi: 10.1007/s00213-018-4868-x

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., . . .

Mailman, R. B. (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther, 320*(1), 1-13. doi:10.1124/jpet.106.104463

Valentino, R. J., Bangasser, D., & Van Bockstaele, E. J. (2013). Sex-biased stress signaling: the corticotropin-releasing factor receptor as a model. *Molecular pharmacology*, *83*(4), 737–745. doi:10.1124/mol.112.083550

Van't Veer, A., Smith, K. L., Cohen, B. M., Carlezon, W. A., Jr, & Bechtholt, A. J. (2016). Kappa-opioid receptors differentially regulate low and high levels of ethanol intake in female mice. *Brain and behavior*, *6*(9), e00523. doi:10.1002/brb3.523

Van't Veer, A., & Carlezon, W. A. J. (2013). Role of kappa-opioid receptors in stress and anxiety-related behavior. *Psychopharmacology*, *229*(3), 435–452. doi: 10.1007/s00213-013-3195-5

Votinov, M., Pripfl, J., Windischberger, C., Moser, E., Sailer, U., & Lamm, C. (2015). A functional polymorphism in the prodynorphin gene affects cognitive flexibility and brain activation during reversal learning. *Frontiers in behavioral neuroscience*, *9*, 172. doi:10.3389/fnbeh.2015.00172

Walker, B. M., & Koob, G. F. (2008). Pharmacological evidence for a motivational role of kappa-opioid systems in ethanol dependence. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *33*(3), 643–652. doi:10.1038/sj.npp.1301438

Wee, S., Orio, L., Ghirmai, S., Cashman, J. R., & Koob, G. F. (2009). Inhibition of kappa opioid receptors attenuated increased cocaine intake in rats with extended access to cocaine. *Psychopharmacology*, *205*(4), 565–575. doi:10.1007/s00213-009-1563-y

Wee, S., & Koob, G. F. (2010). The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology*, *210*(2), 121–135. doi:10.1007/s00213-010-1825-8

Whitfield, T. W., Jr, Schlosburg, J. E., Wee, S., Gould, A., George, O., Grant, Y., Zamora-Martinez, E. R., Edwards, S., Crawford, E., Vendruscolo, L. F., & Koob, G. F. (2015). κ Opioid receptors in the nucleus accumbens shell mediate escalation of methamphetamine intake. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *35*(10), 4296–4305. doi:10.1523/JNEUROSCI.1978-13.2015

Wickman, K. D., Iñiguez-Lluhi, J. A., Davenport, P. A., Taussig, R., Krapivinsky, G. B., Linder, M. E., … Clapham, D. E. (1994). Recombinant G-protein βγ-subunits activate the muscarinic-gated atrial potassium channel. *Nature*, *368*(6468), 255–257. doi: 10.1038/368255a0

Williams JT, North RA and Tokimasa T (1988) Inward rectification of resting and

opiate-activated potassium currents in rat locus coeruleus neurons. *J Neurosci* **8**(11): 4299-4306.

Wimpey, T. L., & Chavkin, C. (1991). Opioids activate both an inward rectifier and a novel voltage-gated potassium conductance in the hippocampal formation. *Neuron*, *6*(2), 281–289. doi: 10.1016/0896-6273(91)90363-5

Wright, E. C., Parks, T. V., Alexander, J. O., Supra, R., & Trainor, B. C. (2018). Activation of kappa opioid receptors in the dorsal raphe have sex dependent effects on social behavior in California mice. *Behavioural brain research*, *351*, 83–92. doi:10.1016/j.bbr.2018.05.011

Yeomans, M. R., & Gray, R. W. (2002). Opioid peptides and the control of human ingestive behaviour. *Neuroscience & Biobehavioral Reviews*, *26*(6), 713–728. Doi: 10.1016/s0149-7634(02)00041-6

Young, A. M., Ahier, R. G., Upton, R. L., Joseph, M. H., & Gray, J. A. (1998). Increased extracellular dopamine in the nucleus accumbens of the rat during associative learning of neutral stimuli. *Neuroscience*, *83*(4), 1175–1183. doi:10.1016/s0306-4522(97)00483-1

Zweifel, L. S., Fadok, J. P., Argilli, E., Garelick, M. G., Jones, G. L., Dickerson, T. M., Allen, J. M., Mizumori, S. J., Bonci, A., & Palmiter, R. D. (2011). Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nature neuroscience*, *14*(5), 620–626. https://doi.org/10.1038/nn.2808