DETERMINATION OF THE ROLE OF VENTRAL TEGMENTAL AREA SGK1 CATALYTIC ACTIVITY AND PHOSPHORYLATION IN DRUG BEHAVIOR

By

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ABSTRACT

DETERMINATION OF THE ROLE OF VENTRAL TEGMENTAL AREA SGK1 CATALYTIC ACTIVITY AND PHOSPHORYLATION IN DRUG BEHAVIOR

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Substance use disorder is a chronic, relapsing disease that affects 20.3 million people in the United States. Despite its prevalence, treatments remain inadequate in part due to our limited understanding of the neuroadaptations induced by drug use. Drugs of abuse are known to regulate the activity of the mesolimbic dopamine (DA) system, a key circuit for drug action and reward. Specifically, drug-induced changes in ventral tegmental area (VTA) cellular activity and gene regulation have been linked to behavioral outputs associated with addiction. Previous work determined that serum- and glucocorticoid-inducible kinase 1 (SGK1) mRNA expression, catalytic activity, and phosphorylation were increased by chronic administration of cocaine or morphine; however, it was unknown if these changes contributed to drug reward behaviors. In this thesis, I utilized transgenic and viral-mediated SGK1 manipulations to determine the impact of altered SGK1 expression and function on cocaine and morphine related-behaviors, primarily assessed by cocaine conditioned place preference (CPP) and voluntary morphine intake using a two-bottle choice task. I first established that that while SGK1 is transcriptionally upregulated and biochemically modified by chronic-drug administration, SGK1 deletion in either the VTA or in DA neurons was not capable of altering drug reward behaviors. Though SGK1 gene deletion did not alter reward, I next showed that viral-mediated overexpression of SGK1 mutants in the VTA of adult mice produced behaviorally relevant effects on cocaine and morphine reward. Specifically, intra-VTA infusion of a catalytically

inactive SGK1 mutant (K127Q) significantly decreased cocaine CPP and morphine preference, suggesting that decreased VTA SGK1 activity is sufficient to impair drug reward. To more fully understand the role of VTA SGK1 in behaviors relevant to addiction, I manipulated SGK1 expression in a cell type-specific manner to determine whether SGK1 activity in VTA DA or GABA neurons drove the observed behavioral effects. Utilizing novel Cre-dependent viral constructs, I found that reduced SGK1 activity in VTA DA neurons significantly decreases cocaine CPP, while this same manipulation in VTA GABA neurons had no effect. Interestingly, this manipulation did not alter morphine preference. Future studies seek to determine a potential mechanism for these behavioral effects using *ex vivo* slice electrophysiology, and parallel studies currently explore the potential effects of a similarly regulated SGK1 phosphorylation site (Ser78) in drug-related behaviors. Altogether, these studies will allow for the identification of the specific cells and circuits that are critical for SGK1-mediated effects on drug reward and intake, a necessary step in assessing the feasibility of SGK1 inhibition as a novel therapeutic avenue for addiction.

For my family.

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KEY TO ABBREVIATIONS

AAV	adeno-associated virus
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMY	amygdala
ANOVA	analysis of variance
BMK1	big mitogen-activated protein kinase 1
CPP	conditioned place preference
Cre	Cre recombinase
CREB	cAMP response element-binding protein
D_1R	dopamine receptor type 1
D_2R	dopamine receptor type 2
DA	dopamine
DAT	dopamine transporter
EGR1	early growth response protein 1
ENaC	epithelial sodium channel
EPSC	excitatory postsynaptic current
ERK	extracellular signal-regulated kinase
ETV5	e-twenty- six version 5
FR1	fixed ratio of 1
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GC	guanylate cyclase
GDI	guanosine nucleotide dissociation inhibitor

GFP	green fluorescent protein
GIRK	G protein-gated inwardly rectifying potassium channel
GluA1	glutamate (AMPA) receptor subunit 1
GluA2	glutamate (AMPA) receptor subunit 2
GRASP1	glutamate receptor interacting protein (GRIP)-associated protein 1
GRIP	glutamate receptor interacting protein
HSV	herpes simplex virus
i.p.	intraperitoneal
i.t.	intrathecal
IACUC	University Institutional Animal Care and Use Committee
ICS	intracranial self-stimulation
IF	interfascicular nucleus of the ventral tegmental area
I_h	hyperpolarization-activated current
IPSC	inhibitory postsynaptic current
IRS2	insulin receptor substrate 2
KCNAB2	voltage-gated potassium channel subunit β-2
KO	knockout
LDT	laterodorsal tegmentum nucleus
LHb	lateral habenula
LTD	long-term depression
LTD_{GABA}	GABAergic long-term depression
LTP	long-term potentiation
LTP_{GABA}	GABAergic long-term potentiation

mEPSC	miniature excitatory postsynaptic current (EPSC)
mIPSC	miniature inhibitory postsynaptic current (IPSC)
MOR	μ-opioid receptor
mPFC	medial prefrontal cortex
MSN	medium spiny neuron
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
NAc	nucleus accumbens
NAc lShell	nucleus accumbens lateral shell
NAc mShell	nucleus accumbens medial shell
NDRG	n-myc downstream regulated gene
NEDD4-2	neural precursor cell-expressed developmentally downregulated gene 4-2
NET	norepinephrine transporter
NFDM	nonfat dairy milk
NMDAR	*N*-methyl-D-aspartic acid receptor
NO	nitric oxide
PB	parabrachial nucleus of the ventral tegmental area
PBS	phosphate buffered saline
PBS-T	phosphate-buffered saline (PBS) with 0.1% Tween-20
PDK1	3-phosphoinositide-dependent kinase 1
PFC	prefrontal cortex
PI3K	phosphoinositide 3 kinase
PKC	protein kinase C

PN	paranigral nucleus of the ventral tegmental area
pNDRG	phospho-n-myc downstream regulated gene
PPTg	pedunculopontine tegmental nucleus
PR	progressive ratio
PSD-95	postsynaptic density-95
pSer78	phospho-SGK1 at Ser78
RL	rostral linear nucleus of the ventral tegmental area
RM	repeating measures
RMTg	rostromedial mesopontine tegmental nucleus
SERT	serotonin transporter
SGK1	serum- and glucocorticoid-inducible kinase 1
SK	small-conductance calcium-activated potassium channel
SRF	serum response factor
TBC	two-bottle choice
TH	tyrosine hydroxylase
VGAT	vesicular γ-aminobutyric acid (GABA) transporter
VGLUT2	vesicular glutamate transporter 2
VP	ventral pallidum
VTA	ventral tegmental area
WT	wild-type

Chapter 1. Introduction

Note: Some portions of this chapter were published in Doyle and Mazei-Robison, 2020.

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Substance Use Disorder

Drug addiction is a prevalent issue in the United States, totaling more than $740 billion annually in costs related to lost employment, crime, and medical care (NIDA, 2020a). While the initiation of drug use is often volitional, the transition to addiction is debilitating due to its chronic, relapsing nature (Volkow and Morales, 2015). The term “addiction” itself does not refer to a diagnosis, but rather, substance use disorder is the clinical diagnosis, as determined by *The Diagnostic and Statistical Manual of Mental Disorders: DSM-5*. This diagnostic manual defines a constellation of behavioral, physiological, and cognitive symptoms split into 11 criteria (Table 1). Broadly, these criteria encompass decreased control of drug consumption, social consequences due to substance use, drug use in risky situations, and pharmacological effects, together depicting continued substance use despite their significant negative consequences (APA, 2013). A patient must meet at least 2 criteria to be diagnosed with substance use disorder, and the diagnosis is further defined by classifying it based on the specific substance being misused (APA, 2013).

Prominent examples of subcategories for substance use disorder include alcohol

Table 1. List of criteria for the diagnosis of substance use disorder.

Category	Criteria Details
Decreased control of drug consumption	Consumption of the substance in greater quantities or over greater periods of time than initially intended
	Continued drug use despite a desire to decrease or regulate use, which may include multiple unsuccessful attempts to reduce or discontinue use
	A significant amount of time is devoted to acquiring, using, and recovering the effects of the substance
	The individual displays a powerful urge or desire for the substance, known as craving
Social consequences	Failure to perform responsibilities related to roles at work, school, or home due to drug use
	Substance use is continued even though social or interpersonal conflicts arise from or are worsened by the effects of the drug
	Activities related to social engagements, work, or hobbies may be decreased or stopped due to drug use
Drug use in risky situations	Drug use occurs in physically hazardous situations
	Substance use is continued despite physical or psychological issues that arise from or are worsened by the drug
Pharmacological effects	The individual exhibits tolerance, defined as a reduction in drug effect when the usual dose is consumed or a significant increase in dose required to achieve the desired effect
	The individual exhibits withdrawal, defined as a decrease in blood or tissue concentration of the substance following prolonged and heavy use

use disorder, tobacco use disorder, and disorders related to an illicit drug. In 2018, alcohol use disorder affected 5.1% of U.S. adults, and 18.5% of the population aged 26 years or older reported tobacco use in the last month, 62.3% of whom reported daily use, suggesting that a large portion of this subpopulation may qualify as having tobacco use disorder (SAMHSA, 2019). In the same year, substance use disorder related to an illicit drug (cannabis use disorder, stimulant use disorder, opioid use disorder, etc.) affected 2.2% of adults ≥26 year of age (SAMHSA, 2019). Opioid use disorder and stimulant use disorder are among the highest prevalence for substance use disorders related to illicit drugs in the U.S. (SAMHSA, 2019), and including both legal and illegal drugs of abuse, opioids and cocaine are ranked highest for risk of harm, which includes physical harm, societal harm, and risk of dependence (Morgan et al., 2013; Nutt et al., 2007). For these reasons, understanding the causes and molecular underpinnings of these disorders is of great interest to the medical community.

Opioid Use Disorder

Opioid drugs have been used medically for centuries for their potent analgesic properties (Fields, 2011). This class of drugs includes naturally occurring compounds derived from the opium poppy (*Papaver somniferum*) such as codeine and morphine as well as many synthetic derivatives such as heroin, oxycodone, and fentanyl. Though opioids remain among the most effective medications for acute pain relief, there are serious side effects that can occur with long-term opiate use, such as tolerance, physical dependence, and addiction (Ballantyne and LaForge, 2007). In the United States misuse of prescription drugs, and specifically pain-relieving opioids, has increased greatly since 1992 (Compton and Volkow, 2006; Han et al., 2017; Manchikanti et al., 2010). Despite a

recent strengthening of regulations that has decreased the number of opioid prescriptions, opioid-related deaths continue to rise, indicating that these measures alone are inadequate to combat the crisis (Manchikanti et al., 2018; Volkow and Koroshetz, 2019). Opioid use disorder is diagnosed when prolonged, compulsive opioid use occurs without a medical purpose or as misuse in the context of a medical condition (APA, 2013). In 2018, 1.6 million adults aged 26 years or older were categorized as having opioid use disorder, about 0.7% of the U.S. population (SAMHSA, 2019). Although the ethics of chronic pain treatment and the potential for over or under use of opioid drugs can be debated (Dowell et al., 2016; Fields, 2011), there is no question that chronic opioid use causes neuroadaptations that lead to undesirable effects.

Stimulant Use Disorder

Similar to opioids, cocaine is a naturally occurring compound, extracted from leaves of the shrub *Erthroxylum coca*, and the use of cocaine can be traced back for hundreds of years (Drake and Scott, 2018; Goldstein et al., 2009). Medically, cocaine was used as the first local anesthetic (Calatayud and González, 2003), and due to its ability to induce vasoconstriction, it is still utilized today during medical procedures (Drake and Scott, 2018). Though cocaine has key uses in a medical context, it is strictly regulated due to its known abuse liability as a psychostimulant. Repeated cocaine use causes serious side effects including addiction and tolerance, when used at high doses. Like other substance use disorders, stimulant use disorder, specified for cocaine, is diagnosed when cocaine is used compulsively for extended periods of time despite facing significant negative consequences. In the U.S., 3.5 million adults ≥26 years of age used cocaine in

2018, and 0.4% of the population qualified for diagnosis of stimulant use disorder due to cocaine use (SAMHSA, 2019). Additionally, the number of cocaine-only overdose deaths have remained of significant concern, though relatively constant over the last 20 years (NIDA, 2020b).

However, with the emergence of the opioid epidemic, cocaine-related overdose deaths associated with opioid use have risen significantly since 2014 (NIDA, 2020b), highlighting the prevalence of multiple drug use (polysubstance use) within individuals with substance use disorders. In fact, most drug users do not exclusively use a single substance but rather polysubstance use is the norm (Leri et al., 2003; Liu et al., 2018b). These individuals are particularly vulnerable, as polysubstance use is associated with greater risk of harm (Butelman and Kreek, 2017; Leeman et al., 2016). The prevalence and increased risk of polysubstance use suggests that the most efficacious therapies for the treatment of substance use disorders should span drug classes.

Treatments and limitations

The pervasiveness and substantial economic burden of drug use clearly emphasize the scale of substance use disorder in the United States. To combat this issue, treatments are available for both opioid use disorder and stimulant use disorder. For opioid use disorder, treatments frequently involve a combination of pharmacological and behavioral interventions. Pharmacotherapies, including methadone and buprenorphine, are commonly used as replacement therapy, where daily treatment decreases opioid seeking and taking to promote abstinence (Strang et al., 2020). While not as broadly implemented, behavioral therapies, including cognitive-behavioral therapy and

contingency management programs, are being more frequently adopted in combination with pharmacological interventions (Strang et al., 2020). Though the additional benefits from behavioral therapies is debated (Amato et al., 2011; Fiellin et al., 2006), pharmacotherapies have been proven as effective treatments for opioid use, although there are significant limitations and barriers to their use (Mattick et al., 2014; NIDA, 2018). Methadone and buprenorphine act at as agonists at opioid receptors, so while they are designated as Essential Medications by the World Health Organization (WHO, 2019), these pharmacotherapies have abuse liability (Dole and Nyswander, 1965; Jasinski et al., 1978; Mello and Mendelson, 1985). Moreover, the common forms of these medications require daily dosing, often overseen in person, and lasting for as long as is necessary for each individual, up to years. This is on top the fact that availability of treatment centers and doctors with abuse expertise are limited, adding access as an additional barrier to treatment. Development of novel therapeutics with reduced abuse liability may be a key factor in treating opioid use disorder.

Treatments for stimulant use disorder remain significantly more limited. While a number of pharmacological interventions are currently under consideration for treating cocaine use, efficacy has generally been poor and none are currently approved by the U.S. Food and Drug Administration (Kampman, 2019; Negus and Henningfield, 2015; NIDA, 2018; Perez-Mana et al., 2011). Instead, behavioral therapies, including cognitive-behavioral therapy and contingency management programs, remain the only line of treatment (Kampman, 2019; NIDA, 2018). While these forms of behavioral therapies promote abstinence and treatment retention (Higgins et al., 1994; Higgins et al., 2000; Rawson et al., 2006), factors, such as environmental stress and drug craving, contribute

to high rates of relapse (Paliwal et al., 2008; Sinha, 2011). Pharmacotherapies in combination with behavioral therapies would likely increase success of stimulant use disorder treatment programs.

Thus, there is significant room for improvement in the treatment of substance use disorders, especially for stimulant use disorder and treatments that take polysubstance use into consideration (Butelman and Kreek, 2017). Treatments for addiction remain inadequate, in part due to our limited understanding of the drug-induced neuroadaptations underlying addiction. This represents a significant knowledge gap and barrier for the development of novel therapeutics. To inform better therapeutics, animal models represent a critical tool for studying the effect of chronic drug use on the central nervous system. Utilizing these models, we can better delve into key brain regions and circuits underlying addiction. The mesocorticolimbic dopamine (DA) system is one such circuit due to its critical role in drug reward and drug-related behaviors.

Mesocorticolimbic reward circuitry

Ventral Tegmental Area anatomy and connectivity

Both opioids and cocaine exert their rewarding effects through the mesocorticolimbic DA system. This circuit can be simplified as the ventral tegmental area (VTA) and its primary targets. The VTA has been widely studied in drug addiction due to its central role in reward-related behaviors. This heterogeneous region is composed of 60-65% DA, 30-35% γ-aminobutyric acid (GABA), and 2-3% glutamate neurons (Nair-Roberts et al., 2008; Swanson, 1982). While neuronal cell types do not strictly localize to one subnucleus of the VTA, they are organized in gradients, particularly across anterior-

posterior and medial-lateral axes. Specifically, the ratio of GABA to DA neurons is higher in posterior sections of the VTA compared to anterior sections (Nair-Roberts et al., 2008) and glutamatergic neurons are concentrated in medial subnuclei (Yamaguchi et al., 2007) (Figure 1). However, recent studies have shown that the VTA is even more heterogeneous than previously thought. VTA DA neurons have been historically defined using two main criteria: 1) expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and 2) presence of a large hyperpolarization-activated (I_h) current. However, VTA DA neurons are now known to be capable of expressing and releasing multiple neurotransmitters (recently reviewed (Barker et al., 2016; Morales and Margolis, 2017)). These combinatorial DA neurons have been classified as dual DA-glutamate when co-expressing TH and vesicular glutamate transporter 2 (VGLUT2) or dual DA-GABA through co-expression of TH and vesicular GABA transporter (VGAT) or glutamic acid decarboxylase (GAD) (Barker et al., 2016). Additionally, I_h current fails to fully distinguish between VTA DA and non-dopamine neurons, as there are subsets of DA neurons that express little to no I_h current (Chieng et al., 2011; Margolis et al., 2006). Thus, our understanding of drug-adaptations of VTA DA neurons may be incomplete, as many of the foundational studies preceded the identification of these subpopulations. The functional relevance of subpopulations of VTA DA neurons is currently an active area of research.

VTA DA neurons fire in characteristic patterns of either slow, single spike tonic firing (2-4 Hz) or fast, multispike phasic firing (>15 Hz, also termed burst firing) (Grace and Bunney, 1984a, b; Grace and Onn, 1989; Juarez and Han, 2016). Within target regions, tonic firing results in low levels of DA release while phasic firing leads to transient

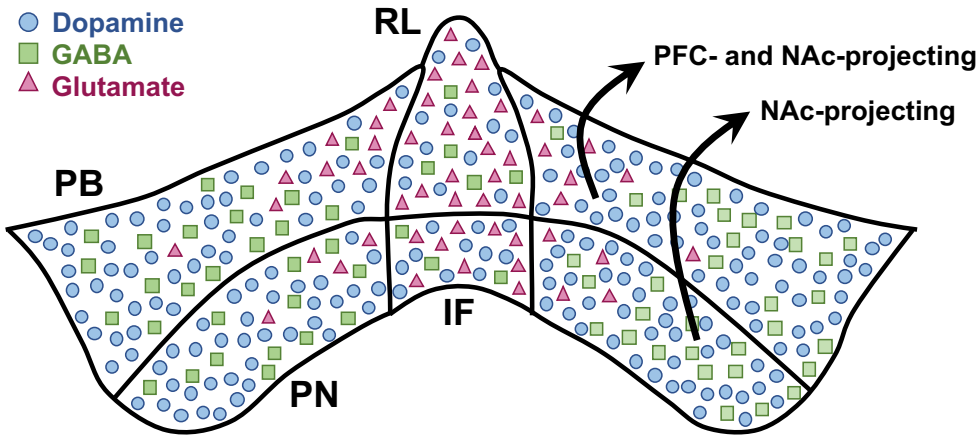

Figure 1. VTA anatomy and neuronal cell types.
The VTA is subdivided into several nuclei. These are the parabrachial nucleus (PB), paranigral nucleus (PN), rostral linear nucleus (RL), and interfascicular nucleus (IF). Overall, the VTA is composed of 60-65% DA, 30-35% GABA, and 2-3% glutamate neurons. Neuronal cell types are intermixed within the VTA, but they are organized across axes. In the medial-lateral axis, glutamatergic neurons are concentrated in medial nuclei, while DA and GABA neurons are more lateral. In the anterior-posterior axis, anterior sections of the VTA are lower in concentration of GABA neurons compared to posterior sections.

spikes in elevated DA (Grace, 1995; Tsai et al., 2009). Individual dopaminergic projections from the VTA largely innervate only one region (Swanson, 1982), with major targets including the nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala (Morales and Margolis, 2017; Sesack and Grace, 2010; Swanson, 1982), and hippocampus (McNamara et al., 2014; Morales and Margolis, 2017) (Figure 2). These outputs are roughly organized topographically in the medial-lateral axis. Since the NAc receives dense VTA DA projections, much work has focused on characterizing these neurons. For example, DA cell bodies located in more lateral portions of the VTA project to the NAc lateral shell (lShell), while more medial neurons project to the NAc core and NAc medial shell (mShell) (Breton et al., 2019; Lammel et al., 2008; Lammel et al., 2014). There is a clear separation between DA projections terminating in NAc subregions, where

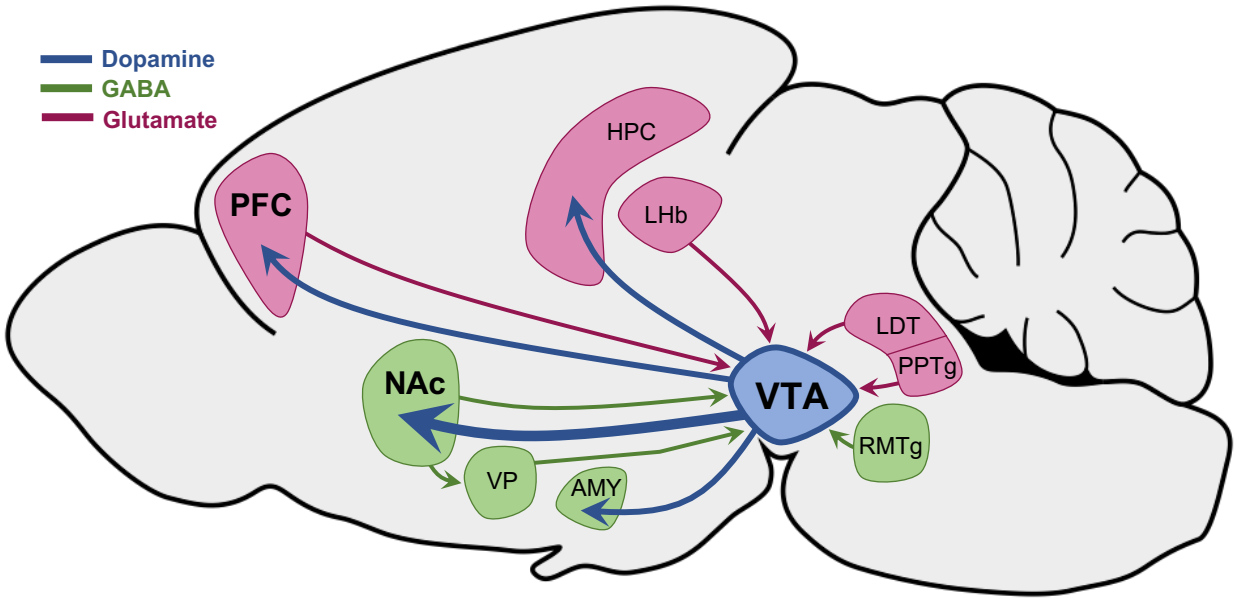

Figure 2. VTA afferent and efferent projections.
The ventral tegmental area (VTA) sends dopaminergic projections (blue) to the prefrontal cortex (PFC), amygdala (AMY), hippocampus (HPC), and a particularly dense number to the nucleus accumbes (NAc). In addition to these efferent projections, the VTA receives glutamatergic input (red) from the PFC, lateral habenula (LHb), pedunculopontine tegmental nucleus (PPTg), and laterodorsal tegmentum nucleus (LDT). GABAergic input (green) is received from the NAc, ventral pallidum (VP), and rostromedial mesopontine tegmental nucleus (RMTg).

lateral DA arborizations in the lShell minimally overlap with medial DA arborizations concentrated in the mShell (Beier et al., 2015). Moreover, these subpopulations are functionally distinct. For example, VTA DA projections targeting the ventral portion of the mShell are excited by unexpected aversive stimuli, in contrast to the inhibition seen in all other projection targets (de Jong et al., 2019). Due to VTA neuronal heterogeneity, it will be especially important to take factors such as cell type and projection into account when interpreting VTA DA adaptations in response to drugs of abuse.

The VTA also receives input from a variety of brain regions (Figure 2). Prominent afferents to VTA DA neurons include excitatory input originating from the medial PFC (mPFC), lateral habenula, bed nucleus of the stria terminalis, pedunculopontine

tegmentum (PPTg), and laterodorsal tegmentum nucleus (LDT) (Morales and Margolis, 2017; Sesack and Grace, 2010) as well as inhibitory input arising from the NAc, rostromedial mesopontine tegmental nucleus (RMTg), lateral hypothalamus, and ventral pallidum (VP) (Morales and Margolis, 2017; Sesack and Grace, 2010). There is also complexity at this level of regulation, as illustrated by examination of inhibitory inputs from the NAc to VTA DA neurons (Beier et al., 2015; Watabe-Uchida et al., 2012). NAc lShell neurons project indirectly to disinhibit lateral VTA DA neurons via synapses onto VTA GABAergic interneurons resulting in reinforcement, as mice form a real-time place preference to lShell terminal stimulation and will self-stimulate these terminals (Yang et al., 2018). In contrast, mShell neurons predominately project directly to medial VTA DA neurons, inhibiting their activity (Yang et al., 2018). These studies demonstrate that inputs are capable of targeting discrete DA subpopulations and influencing a variety of behaviors, from aversion to reward (Lammel et al., 2014), demonstrating the complexity of this circuit and highlighting the need for further research.

Reward in the mesocorticolimbic dopamine system

Dopaminergic projections from the VTA that innervate the NAc have been a focus of reward research as they are critical for signaling reward prediction error, stimuli salience, associative learning, and motivation (Keiflin and Janak, 2015; Schultz et al., 1993). DA signaling in the NAc is achieved through binding to dopamine receptors on NAc GABAergic medium spiny neurons (MSNs), making up 95% of all neuronal cell types in the NAc (Russo and Nestler, 2013). MSNs can be categorized into largely nonoverlapping dopamine receptor type 1 (D_1R) and dopamine receptor type 2 (D_2R)

expressing populations (Gangarossa et al., 2013). Both receptor subtypes are metabotropic; however, D_1Rs are $G_{s/olf}$ coupled while the D_2Rs are $G_{i/o}$ coupled (Surmeier et al., 2007). Canonically, D_1R-expessing MSNs feed directly back onto the VTA, designated as the “direct” pathway and ultimately increase thalamocortical drive, while the D_2R-MSNs signal first through the ventral pallidum, termed “indirect” and ultimately decrease thalamocortical drive (Russo and Nestler, 2013). However, recent studies have shown NAc outputs to the ventral pallidum that are comprised of both D_1R- and D_2R-expressing MSNs (Kupchik et al., 2015; Smith et al., 2013), suggesting that a clear delineation of D_1R- vs. D_2R-expressing circuits is not universal but rather these may be complex, overlapping circuits within the mesocorticolimbic DA system.

Within the dopaminergic VTA to NAc circuit, baseline tonic firing of VTA DA neurons causes DA release and binding to high-affinity D_2Rs while stimuli-induced phasic firing causes high levels of DA release and allows for binding to low-affinity D_1Rs (Garris et al., 1994; Richfield et al., 1989). Moreover, activation of this projection is inherently rewarding, as rodents will self-stimulate the VTA-NAc pathway, both electrically and optogenetically (Olds and Milner, 1954; Witten et al., 2011). Conversely, lesioning or chemogenic inhibition of VTA DA neurons disrupts reward self-administration (Corre et al., 2018; Phillips and Fibiger, 1978; Roberts et al., 1977; Roberts and Koob, 1982). These patterns of firing enable the coding of stimuli based on VTA DA neuron activity.

This signaling is critical in natural reward behaviors. Food rewards increase both VTA DA neuron firing and extracellular DA in the NAc (Bassareo and Di Chiara, 1997; Schultz et al., 1993), and phasic activation of VTA DA neurons reinforces food-seeking behavior (Adamantidis et al., 2011). While this circuit exists to enhance motivation for

actions necessary to survival, such as seeking food, water, and sex, it can be appropriated by drugs of abuse. Virtually all classes of drugs of abuse similarly increase VTA DA output in the NAc (Di Chiara and Imperato, 1988). More specifically, DA action at its receptors in the NAc is critical for drug reward behaviors. Systemic or intra-NAc infusion of either D_1R or D_2R antagonists increase cocaine self-administration (Caine and Koob, 1994; Ettenberg et al., 1982; Koob et al., 1987; Maldonado et al., 1993). This effect is understood as a reduction in the reinforcing properties of cocaine, as low doses of cocaine parallel this increase in responding (Pickens and Thompson, 1968), supporting DA release and action at its receptors in the NAc as a mediator of behavior.

Though drugs act through distinct mechanisms, there are overlapping effects of chronic use on neuroadaptations in VTA DA neurons. Broadly, these drug-induced adaptations include: synaptic plasticity – persistent changes in glutamatergic and GABAergic synaptic transmission (Bellone et al., 2020; Francis et al., 2019; Langlois and Nugent, 2017; Lüscher and Malenka, 2011); structural plasticity – long-lasting changes in neuronal morphology (Russo et al., 2010); and cellular plasticity – homeostatic changes in intracellular signaling cascades (Nestler, 2004; Williams et al., 2001). Because changes in VTA gene transcription and cellular activity have been linked to altered reward-related behaviors, an understanding of these maladaptive changes is key to the development of novel therapies for treating substance use disorder.

Opioid effects on VTA DA neurons

Acute opioid mechanism of action

Like other classes of drugs of abuse, acute systemic or intra-VTA infusion of

opioids such as morphine causes increased DA release into the NAc (Di Chiara and Imperato, 1988; Leone et al., 1991). This effect is primarily achieved through opioid binding to $G_{i/o}$-coupled μ-opioid receptors (MOR) located on VTA GABA neurons. Opioid binding hyperpolarizes presynaptic VTA GABA neurons and decreases their spontaneous firing rate, consequently disinhibiting DA neuron firing (Gysling and Wang, 1983; Johnson and North, 1992; Matthews and German, 1984; Ostrowski et al., 1982) (Figure 3). Moreover, opioid-induced increases in DA spontaneous firing rate can be blocked by systemic or local infusion of the MOR antagonist naloxone, consistent with a prominent inhibitory role of opioid action at MORs on GABA neurons (Gysling and Wang, 1983; Matthews and German, 1984). These MOR-mediated effects significantly contribute to opioid reward behaviors, as intra-VTA infusion of MOR agonists alone is capable of forming a conditioned place preference (CPP) (Bals-Kubik et al., 1993) and small interfering RNA knockdown of MORs in the VTA and substantia nigra prevents heroin CPP (Zhang et al., 2009). However, while the canonical model describes opioid action exclusively on VTA GABA neurons, postsynaptic MOR expression has been described on a subset of VTA DA neurons as well, where opioid peptides can elicit both excitation and inhibition of these neurons (Margolis et al., 2014). Though the contribution of the MOR-expressing DA subpopulation to behavior is not well understood, the possibility that these neurons could respond differentially to rewarding vs. aversive stimuli (Brischoux et al., 2009; Lammel et al., 2012; Margolis et al., 2014) is an area worthy of future study.

Opioid effects on VTA DA synaptic plasticity

While research has focused on the effects of acute morphine on VTA DA

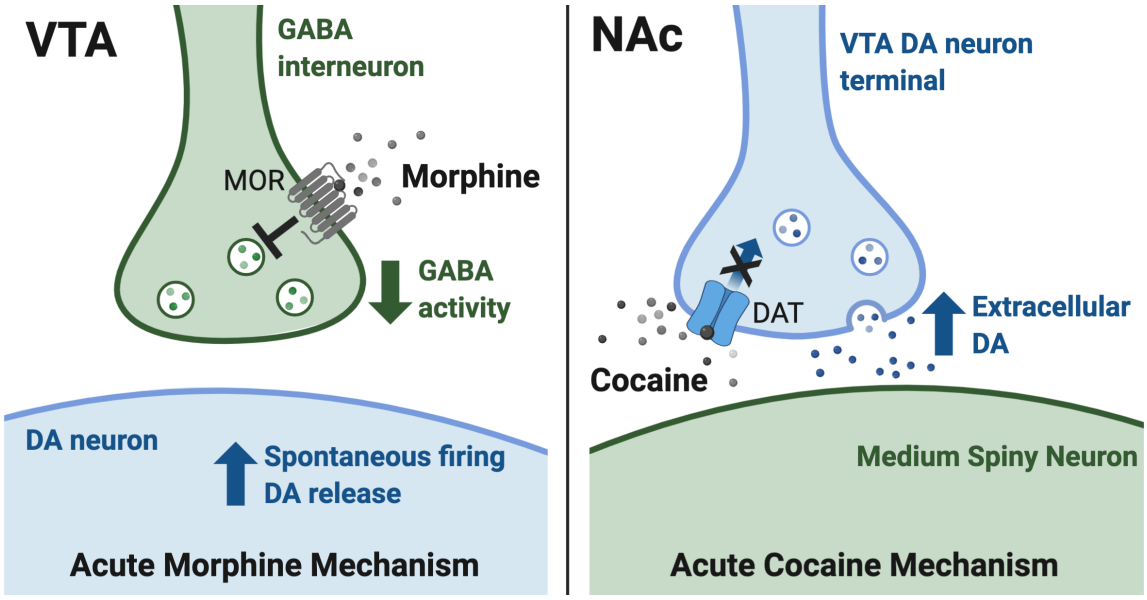

Figure 3. Acute drug mechanisms of action.
Acute morphine (left). Acute morphine promotes the release of dopamine (DA) through disinhibition of VTA DA neurons. Morphine binds to $G_{i/o}$-coupled μ-opioid receptors (MOR) located on GABA interneurons, causing hyperpolarization and decreased firing. Reduced GABAergic input onto VTA DA neurons then results in increased spontaneous firing and dopamine output in target regions.
Acute cocaine (right). Acute cocaine blocks the dopamine transporter (DAT) located on VTA DA terminals in the NAc. Cocaine blocks the normal reuptake of DA and this action allows DA to accumulate in the synapse, increasing extracellular levels. Both drug mechanisms result in increased extracellular DA concentrations in the NAc.

physiology, relatively little is known about the long-term consequences of opioid exposure. Since opioids are generally taken chronically, understanding whether similar mechanisms are induced by acute and chronic opioid exposure is critical to our understanding of opioid abuse. Similar to acute treatment, chronic morphine administration increases VTA DA neuronal firing, as morphine-dependent rats show a significantly increased *in vivo* firing rate and burst event frequency compared to drug-naïve rats (Georges et al., 2006). Supporting this finding, similar effects are found in mice, both *in vivo* and in *ex vivo* slices (Koo et al., 2012; Mazei-Robison et al., 2011). This increased activity is in part mediated through potassium channel regulation, as the peak

and sustained components of potassium currents are significantly decreased in VTA DA neurons of morphine pelleted mice (Koo et al., 2012). Chronic morphine also decreases VTA mRNA expression of potassium channel subunits (Mazei-Robison et al., 2011). Specifically, voltage-gated potassium channel subunit β-2 (KCNAB2) and G protein-gated inwardly rectifying potassium (GIRK) channel 3 show decreased permissive epigenetic markers and binding of RNA polymerase II at the gene promoters, consistent with decreased transcription (Mazei-Robison et al., 2011). These findings support enhanced excitability of VTA DA neurons following chronic morphine exposure; however, further research is needed to uncover the opioid-induced adaptations to ion channel expression and function that contribute to changes in cellular activity.

Altered VTA DA-glutamatergic plasticity has also been demonstrated following chronic morphine administration. Glutamatergic transmission in the VTA is critical for opioid responses, as pharmacological inhibition prevents expression of heroin self-administration (Xi and Stein, 2002) and morphine CPP (Harris et al., 2004), and glutamatergic signaling is required for morphine-driven increases in VTA DA spontaneous firing rate and burst firing (Jalabert et al., 2011). Thus, much work has sought to define the regulation of the glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and *N*-methyl-D-aspartic acid receptor (NMDAR)) that mediate excitatory postsynaptic currents (EPSCs) in the VTA following drug exposure. Specifically, a single dose of morphine increases AMPA/NMDA ratio on VTA DA neurons, consistent with an increase in long-term potentiation (LTP) (Authement et al., 2016; Baimel and Borgland, 2015; Saal et al., 2003), an effect similar to that observed with other drugs of abuse (Saal et al., 2003; Ungless et al., 2001). This is

achieved in part through increased synaptic localization of the AMPARs, measured through labeling of their constituent subunits (e.g. GluA1, GluA2, etc.). Chronic escalating morphine injections increase GluA1 protein in the VTA (Fitzgerald et al., 1996) and VTA GluA1 overexpression potentiates morphine CPP (Carlezon et al., 1997), establishing this induction as behaviorally relevant. Ultrastructural studies using the same morphine administration paradigm have found increased GluA1 synaptic labeling in VTA DA neurons (Lane et al., 2008), consistent with increased LTP (Figure 4).

Acute morphine treatment also drives insertion of calcium-permeable GluA2-lacking AMPARs in exchange for calcium-impermeable GluA2-containing AMPARs (Authement et al., 2016; Baimel and Borgland, 2015; Brown et al., 2010). These results are linked to DA neuronal activity, as direct optogenetic activation of VTA DA neurons induces AMPAR redistribution and exchange of subunits similar to that following drug exposure (Brown et al., 2010), though further work is needed determine if these changes also occur in the context of chronic morphine administration. Together, these opioid-induced adaptations in receptor expression mediate enhanced glutamatergic transmission onto VTA DA neurons, which is thought to contribute to the development of addiction (Wolf, 2016).

In addition to altering VTA DA neuron glutamatergic plasticity, drugs of abuse, including ethanol, nicotine, and cocaine, alter inhibitory synaptic plasticity in the VTA (Liu et al., 2005; Melis et al., 2002; Niehaus et al., 2010; Nugent et al., 2007). Unsurprisingly, acute opioids also regulate VTA DA neuron GABAergic responses (recently reviewed (Langlois and Nugent, 2017)). VTA DA GABAergic LTP (LTP_{GABA}) can be evoked by high-frequency electrical stimulation and results in facilitation of inhibitory postsynaptic

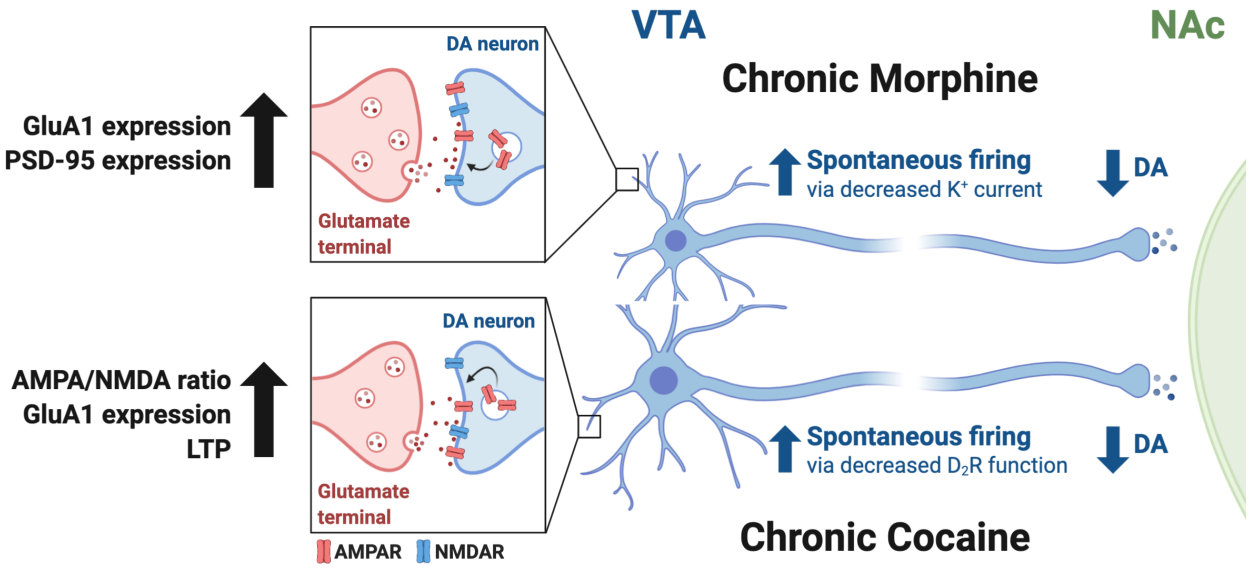

Figure 4. Chronic drug-induced VTA DA-glutamatergic and cellular plasticity. **Chronic morphine (top).** Following chronic administration of morphine, glutamatergic input onto VTA DA neurons is potentiated. Specifically, VTA DA neurons show enhanced synaptic labeling of GluA1 subunits and increased expression of PSD-95. Alterations in cellular plasticity also occur with long-term use. Unique to opioids, the soma size of VTA DA neurons is significantly reduced with long-term use. Additionally, spontaneous firing is increased, partially due to decreased potassium channel regulation. Though firing is increased, electrically evoked DA is decreased in the NAc.
Chronic cocaine (bottom). Repeated cocaine administration causes an increase in VTA DA AMPA/NMDA ratio, increased GluA1 expression, and the facilitation of LTP. These adaptations are associated with potentiated glutamatergic plasticity, similar to chronic morphine administration. In the context of cellular plasticity, VTA DA neurons do not show altered soma size in response to chronic cocaine treatment. They do increase spontaneous firing, but this is primary due to a decrease in the function of D_2-autoreceptor function. Finally, like morphine, chronic cocaine also decreases DA output in the NAc.

currents (IPSCs) onto VTA DA neurons (Nugent et al., 2007). This form of inhibitory plasticity is driven by NMDAR-dependent release of nitric oxide (NO) (Nugent et al., 2007). NO acts as a retrograde messenger to activate guanylate cyclase (GC) in presynaptic GABAergic terminals, resulting in increased GABA release and initiating LTP_{GABA} (Nugent et al., 2007). Acute *in vivo* morphine exposure blocks the induction of LTP_{GABA} through disrupted NO−GC−protein kinase G (PKG) signaling (Niehaus et al.,

2010; Nugent et al., 2009; Nugent et al., 2007). Amplitude and frequency of miniature IPSCs (mIPSCs) are also decreased following acute morphine exposure (Authement et al., 2016), consistent with evidence that LTP_{GABA} is maintained presynaptically through a steady increase in GABA release (Nugent et al., 2007) and further supporting decreased inhibitory control of VTA DA neurons following opioid exposure. These data support disrupted inhibitory regulation of VTA DA neurons, contributing to increased VTA DA neuronal activity in response to acute morphine treatment (Figure 5).

Long-term depression (LTD) of GABAergic synapses onto VTA DA neurons (LTD_{GABA}) has also been characterized (Dacher and Nugent, 2011). LTD_{GABA} is NMDA-independent and is induced postsynaptically through D_2R activation (Dacher et al., 2013; Dacher and Nugent, 2011). Downstream of the D_2R, inositol triphosphate receptor activation causes a local increase in intracellular calcium, resulting in internalization of $GABA_A$ receptors through protein kinase A signaling (Dacher et al., 2013). Acute morphine treatment blocks the induction of LTD_{GABA}, thereby disrupting normal inhibitory control through a second mechanism, though the exact mechanism of action is not well understood (Dacher and Nugent, 2011). Taken together, these studies indicate that opioids such as morphine both disrupt inhibitory input and strengthen excitatory input onto VTA DA neurons, altering the normal excitatory to inhibitory balance to increase VTA DA neuronal activity.

Opioid effects on VTA DA structural plasticity

While opioid-induced changes in VTA gene regulation and signaling related to DA-glutamatergic plasticity and their link to behavior are being actively investigated,

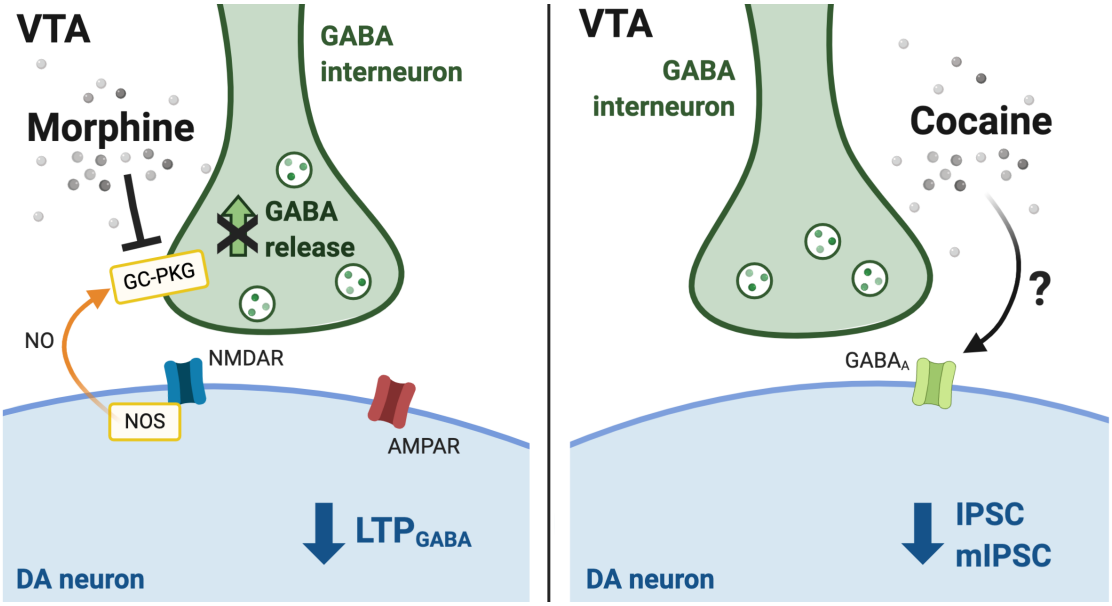

Figure 5. Drug-induced VTA DA-GABAergic plasticity.
Morphine (Left). Acute morphine treatment prevents long-term potentiation of GABAergic (LTP_{GABA}) synapses on VTA DA neurons. LTP_{GABA} derives from NMDAR-dependent release of nitric oxide (NO) via activation of nitric oxide synthase (NOS). NO acts as a retrograde messenger to activate guanylate cyclase (GC) in presynaptic GABAergic terminals, resulting in increased GABA release and initiating LTP_{GABA}. Morphine prevents LTP_{GABA} and disrupts normal inhibitory signaling in the VTA by blocking NO−GC−protein kinase G (PKG) signaling.
Cocaine (Right). Chronic cocaine injections reduce the amplitude of evoked IPSCs and mIPSCs of VTA DA neurons, contributing to VTA DA glutamatergic long-term potentiation (LTP). This adaptation occurs through $GABA_A$ signaling, as pharmacological inhibition of $GABA_A$ channels prevents cocaine-induced LTP. However, the details of this mechanism remain poorly understood.

molecular changes also contribute to structural plasticity in the VTA. One of the best characterized adaptations following chronic opioid administration is decreased VTA DA neuron soma size, with no changes seen in neighboring substantia nigra DA neurons or non-dopamine VTA neurons (TH-negative, putative GABA neurons) (Sklair-Tavron et al., 1996). This effect has been seen with passive morphine administration across rodent species (Chu et al., 2007; Mazei-Robison et al., 2011; Russo et al., 2007; Sklair-Tavron et al., 1996; Spiga et al., 2003) as well as with heroin self-administration in rats (Russo et

al., 2007). This morphological change is transient, as soma size is decreased at 1 and 14 days of withdrawal but returns to baseline by 30 days (Russo et al., 2007). Critically, post-mortem samples from human heroin users also show a significant decrease in VTA DA soma size, suggesting this adaptation is translationally relevant (Mazei-Robison et al., 2011). The decrease in soma size is dependent on MOR activation, as effects could be blocked with concomitant systemic administration of the MOR antagonist naltrexone (Sklair-Tavron et al., 1996). Moreover, neurotrophic factor signaling cascades appear critical for size changes, as decreased insulin receptor substrate 2 (IRS2)-Akt-mammalian target of rapamycin complex-2 (mTORC2) signaling promotes decreased soma size while increased activity within the pathway prevents morphine-induced changes (Mazei-Robison et al., 2011; Russo et al., 2007).

Changes in VTA DA soma size have also been linked with neuronal activity (Coque et al., 2011). Like acute treatment, chronic morphine increases spontaneous and burst firing rates of VTA DA neurons, effects that are correlated with decreased soma size (Koo et al., 2012; Mazei-Robison et al., 2011). However, electrically evoked DA release into the NAc is decreased by chronic morphine, exhibiting decreased output (Mazei-Robison et al., 2011). These seemingly contradictory results are consistent with recent work by Liu et al. demonstrating that VTA deletion of mammalian target of rapamycin (mTOR) similarly decreased electrically evoked DA release in the NAc shell (Liu et al., 2018a). In addition to these adaptations in electrophysiological properties and output, decreased soma size has been correlated with changes in reward behavior (Mazei-Robison et al., 2011; Russo et al., 2007). Specifically, both morphine CPP and soma size are decreased 1 and 14 days following morphine pellet exposure, but both measures are similar to sham

surgical controls following 30 days of withdrawal (Russo et al., 2007). Similarly, these behavioral effects can be mimicked with biochemical manipulations of the IRS2-Akt-mTORC2 pathway that also influence VTA DA soma size. VTA overexpression of dominant-negative mutants of IRS2 or AKT are capable of decreasing morphine CPP and VTA DA soma size (Russo et al., 2007), supporting a link between soma size, neuronal activity, and reward behavior.

Structural plasticity also includes changes in dendritic spine density and dendritic morphology; however, these measures have been technically challenging to assess due to the complexity of the VTA. DA neurons in the VTA form a dense network of cell bodies and processes, making isolation of single neurons technically difficult to attain with transgenic-driven viral approaches that label the majority of VTA DA neurons. In contrast, Golgi staining achieves sparse labeling but requires a counterstain to identify the neurotransmitter profile of the selected cell. Stemming from these challenges, VTA spine adaptations in response to chronic opioid use remain wholly uncharacterized. However, the length of VTA DA neuron processes is significantly decreased following chronic morphine treatment (Sklair-Tavron et al., 1996), suggesting this may be a fruitful avenue to pursue if the technical challenges can be overcome. VTA biochemical studies also hint at potential opioid-induced changes in spine structure. Postsynaptic density-95 (PSD-95) is thought to play a critical role in synaptic assembly and maturation, as overexpression causes increased GluA1 synaptic clustering and spine density in primary hippocampal cultured neurons (El-Husseini et al., 2000). PSD-95 gene and protein expression is increased in the VTA of rats following morphine CPP (Wang et al., 2014), consistent with increased GluA1 synaptic labeling during morphine exposure (Lane et al., 2008). Given

the connection between spine morphology and synaptic function, investigation of VTA DA spine density and dendritic morphology should be a promising future research direction.

Cocaine effects on VTA DA neurons

Acute cocaine mechanism of action

Like morphine, acute systemic treatment with cocaine increases DA concentrations in the NAc (Di Chiara and Imperato, 1988). This effect is achieved primarily by blocking the dopamine transporter (DAT) located on VTA DA terminals to prevent the normal reupdate of DA, resulting in increased extracellular concentrations (Kuhar et al., 1991; Ritz et al., 1987) (Figure 3). DAT knockout mice display a delay in acquisition of cocaine self-administration and reduced motivation to earn rewards (Rocha et al., 1998; Thomsen et al., 2009a), and transgenic mice expressing a cocaine-insensitive DAT mutant do not form a CPP for cocaine and fail to self-administer cocaine (Chen et al., 2006; Thomsen et al., 2009b). Further, DAT inhibition is correlated with self-administration of drugs pharmacologically similar to cocaine (Ritz et al., 1987). These data implicate the dopaminergic system as the driver of cocaine reward.

Despite the focus on DA, it is important to note that cocaine inhibits other monoamine transports, including the norepinephrine transporter (NET) and serotonin transporter (SERT) (Drake and Scott, 2018; Ritz et al., 1990), as well as voltage-gated sodium channels (Drake and Scott, 2018). In fact, DAT knockout mice are still capable of self-administering cocaine and forming a cocaine CPP (Medvedev et al., 2005; Rocha et al., 1998; Sora et al., 1998), and research utilizing NET and SERT knockout mice supports partial contribution of these additional monoamines to cocaine reward (Rocha,

2003). The presence of cocaine reward behaviors in DAT knockout mice may be explained by significant adaptions in DA homeostasis resulting from gene deletion (Giros et al., 1996; Jones et al., 1991). Critically, similar adaptations are not observed in mice expressing a cocaine-insensitive DAT mutant, which do not display cocaine CPP or self-administration (Chen et al., 2006; Thomsen et al., 2009b). Taken together, while NET and SERT inhibition play a role in cocaine reward, evidence suggests that DAT inhibition is the primary mechanism underlying the reinforcing properties of cocaine.

In addition to DA reuptake inhibition, acute cocaine alters VTA DA neuronal firing rate to modulate DA release within the NAc. In response to acute cocaine, tonic VTA DA activity itself is capable of increasing extracellular DA levels in the NAc due to DAT inhibition; however, phasic firing is responsible for the rapid rise, as NAc DA levels are attenuated by VTA NMDAR inhibition (Sombers et al., 2009), consistent with increased extracellular VTA glutamate following acute administration (Kalivas and Duffy, 1995). While phasic firing greatly increases DA concentrations in the NAc, systemic cocaine then results in a transient decrease in VTA DA spontaneous firing rate and activity (Calipari et al., 2017; Einhorn et al., 1988; Lacey et al., 1990). This effect on cell firing occurs through DA binding to D_2-autoreceptors located on VTA DA neurons (Ford, 2014; Lacey et al., 1990). While VTA DA neuron activity is acutely decreased with drug onboard, spontaneous firing is increased at baseline 24 hours after an acute injection (Creed et al., 2016). Here, cocaine drives the insertion of calcium-impermeable NMDARs, reducing the function of small-conductance calcium-activated potassium (SK) channels (Creed et al., 2016), critical regulators of VTA DA firing pattern (Grace and Bunney, 1983).

Cocaine effects on VTA DA synaptic plasticity

Chronic cocaine treatment increases *in vivo* VTA DA firing. This enhanced baseline firing rate is increased at 1 and 3 days after cocaine self-administration (Marinelli et al., 2003) and can be correlated with drug intake. Specifically, rats that responded at high levels during cocaine self-administration show greater *in vivo* VTA DA firing rate and burst activity compared to low responders (Marinelli and White, 2000), indicating a correlation between VTA DA activity and behavioral responding. Repeated cocaine exposure decreases D_2-autoreceptor function (Hearing et al., 2012; Henry et al., 1989), contributing to increased VTA DA activity following chronic cocaine administration. However, both changes in cell firing and D_2R function return to baseline after 10 days of withdrawal (Hearing et al., 2012; Marinelli et al., 2003). At the same time, basal levels of extracellular DA in the NAc is decreased hours after unlimited access to cocaine self-administration (Weiss et al., 1992), and electrically evoked DA is decreased at 1 and 7 days of withdrawal from binge self-administration compared to saline controls (Mateo et al., 2005). Similar effects can also be seen following investigator administered cocaine (Parsons et al., 1991); however, decreased DA was not seen until 10 days of withdrawal, highlighting the difference between self-administered and investigator administered paradigms. These studies in rodent models are translationally relevant, as human cocaine users show a reduction in dopamine release compared to controls (Ashok et al., 2017). In addition to reduced dopamine output, cocaine's ability to inhibit the DAT is reduced following binge or long-access cocaine self-administration paradigms (Mateo et al., 2005; Siciliano et al., 2018). Overall this work demonstrates that chronic cocaine administration decreases DA release in the NAc as well as causes cocaine to be less efficacious at

blocking DA reuptake, associated with tolerance and increased intake in animal models.

VTA DA synaptic plasticity is also significantly regulated following chronic cocaine treatment. VTA DA-glutamatergic plasticity has been a key area of interest, as VTA glutamatergic transmission is critical for cocaine-related behaviors. Intra-VTA pharmacological inhibition of glutamate receptor function prevents locomotor sensitization to cocaine (Kalivas and Alesdatter, 1993; Li et al., 1999) and the formation of cocaine CPP (Harris and Aston-Jones, 2003). Like morphine, both acute and chronic cocaine increase VTA DA AMPA/NMDA ratio (Borgland et al., 2004; Chen et al., 2008; Saal et al., 2003; Ungless et al., 2001). It is important to again note that duration of effects can differ based on method of drug administration. Rats that self-administer cocaine show increased AMPA/NMDA ratio for at least 3 months after the last session while chronic intraperitoneal (i.p.) injections produce an effect that lasts 5 but not 10 days (Borgland et al., 2004; Chen et al., 2008), and rats that received passive yoked infusions showed no difference in AMPA/NMDA ratio from naïve controls (Chen et al., 2008). The potentiation of AMPA/NMDA ratio seen following chronic cocaine administration is achieved through increased AMPAR current (Borgland et al., 2004). This is consistent with work demonstrating that VTA GluA1 expression is increased 1 day after chronic treatment (Choi et al., 2011; Fitzgerald et al., 1996) and GluA1 knockout mice fail to show the expected cocaine-induced increases in AMPAR/NMDAR ratio (Dong et al., 2004). Moreover, VTA viral-mediated overexpression of GluA1 enhances motivation to earn cocaine rewards while a phosphodeficient GluA1 mutant (S845A) decreases self-administration (Choi et al., 2011), further supporting the behavioral relevance of VTA DA AMPAR function in cocaine reward. As AMPA/NMDA ratio is only a correlate of enhanced

LTP, others have gone on to test altered LTP directly. Consistent with increased AMPA/NMDA ratio, VTA DA neurons show a facilitation of LTP following chronic cocaine injections (Liu et al., 2005).

In addition to overall expression, synaptic localization of AMPAR subunits also contributes to altered VTA DA-glutamatergic plasticity. Specifically, VTA DA synaptic labeling of GluA1 subunits is increased 3 days after chronic cocaine exposure (Lane et al., 2011) (Figure 4). The synaptic localization of calcium permeable AMPARs is also a significant marker of plasticity, though much of the current research has focused on neuroadaptations following acute exposure. Acute cocaine drives the insertion of calcium-permeable GluA2-lacking AMPARs in exchange for calcium-impermeable GluA2-containing AMPARs (Bellone and Luscher, 2006), resulting in a redistribution of GluA2 subunits away from the postsynaptic density (Brown et al., 2010). Together, VTA glutamatergic plasticity plays a critical role in drug reward behavior and has been linked to cocaine addiction (Wolf, 2016).

On top of altered AMPAR subunit trafficking, reduced GABAergic input onto VTA DA neurons also contributes to enhanced LTP following cocaine exposure. Specifically, repeated cocaine exposure reduces evoked IPSC and mIPSC amplitudes (Liu et al., 2005). This reduced inhibitory input can be mimicked by pharmacologically blocking $GABA_A$ channels with bicuculline, as $GABA_A$ inhibition produces a potentiation of glutamatergic transmission in saline treated animals (Liu et al., 2005). Conversely, application of the $GABA_A$ agonist diazepam blocks LTP in cocaine treated mice (Liu et al., 2005), further supporting the necessity and sufficiently of modified GABAergic input on to VTA DA neurons for enhanced glutamatergic transmission (Figure 5). Overall,

chronic cocaine decreases inhibitory input onto VTA DA neurons while increasing excitatory input. These effects are similar to those following opioid exposure, disrupting the normal excitatory to inhibitory balance to increase neuronal activity.

Cocaine effects on VTA DA structural plasticity

VTA structural plasticity following cocaine exposure remains largely understudied. VTA DA soma size is not altered by chronic cocaine administration (Mazei-Robison et al., 2014). Rather, adaptations to VTA spines are an active area of investigation, though studies are limited due to challenges presented by VTA structure. Acute cocaine exposure increases spines on Type I, but not Type II, VTA neurons and is consistent with increased AMPA/NMDA ratio in only Type I neurons (Sarti et al., 2007). However, cell type was originally based on morphology (Phillipson, 1979), and while Type 1 and Type 2 cells were largely TH-positive, whether differences in projection or neurotransmitter profile define cocaine-induced spine changes remains unclear. As detailed earlier, the density and complexity of VTA DA neuronal structure makes spine studies technically challenging. Going forward, our knowledge of drug-induced neuroadaptations within the VTA will likely be most improved by taking into account the complexity of VTA neurons, such as differences between populations defined by their neurotransmitter expression and projection target.

Heterogeneity of VTA DA neurons in response to drugs of abuse

There is considerable heterogeneity even within VTA DA neuronal populations, contributing to the diversity of effects seen in response to drugs of abuse (Juarez and

Han, 2016). Defining projection-specific effects of drugs of abuse is challenging, as broadly speaking, VTA neuronal cell types are widely mixed (Morales and Margolis, 2017). Even within VTA DA neurons, DA projection populations fail to exclusively localize to a single subregion (Beier et al., 2015; Lammel et al., 2014) and biochemical markers for VTA DA populations are still under investigation (Poulin et al., 2018; Poulin et al., 2020; Simmons et al., 2019). However, differences in baseline biochemical and electrophysiological properties have been characterized in VTA DA neurons projecting to the NAc vs. PFC (Lammel et al., 2008; Lammel et al., 2011). For example, PFC-projecting DA neurons lack functional somatodendritic D_2Rs, once thought of as a hallmark characteristic of VTA DA neurons (Lammel et al., 2008), implying functional distinctions based on projection target. There are also differences in excitatory synaptic plasticity, where PFC- and NAc mShell-projecting neurons have increased AMPA/NMDA ratio compared to NAc lShell-projecting neurons (Lammel et al., 2011).

These properties translate to divergent effects following acute drug administration. Acute cocaine treatment causes an increase in AMPA/NMDA ratio in NAc-projecting but not PFC-projecting neurons (Lammel et al., 2011). These findings are consistent with ultrastructural studies showing an increase in both the density of plasmalemmal GluA1 and the percentage of GluA1 labeled synapses of VTA DA neurons in the paranigral (PN) but not parabrachial (PB) subregions following acute cocaine (Lane et al., 2010). While projection target is not strictly defined, the PN predominately contains cell bodies of NAc-projecting DA neurons while the PB contains both NAc- and PFC-projecting DA neurons, suggesting that differences are likely based on their projection target. This work has been expanded to chronic cocaine, where acute withdrawal (72 hours) from repeated cocaine

administration increased the percentage of synapses containing GluA1 subunits in both PB and PN subregions; however, the PB showed an increase in plasmalemmal labeling while the PN has a decrease (Lane et al., 2011). Projection-specific differences in cocaine-induced glutamatergic plasticity may also clarify earlier work on VTA DA spine regulation. As mentioned previously, VTA neurons classified as Type I, but not Type II, show both increased AMPA/NMDA ratio and spine density following acute cocaine administration (Sarti et al., 2007), though further work is needed to confirm differences in projection target.

While projection differences in glutamatergic plasticity remain understudied in the context of opioid treatment, there is evidence for circuit-level regulation following acute treatment. This is illustrated in findings that a single session of heroin self-administration predominantly activates VTA DA neurons projecting to the NAc mShell over those projecting to the NAc lShell (Corre et al., 2018). Thus, there is ample evidence for both basal differences in VTA subpopulations based on projection target as well as in responses to cocaine and opioid drugs.

Instead, a significant amount of effort has now focused on VTA DA subpopulation structural plasticity in the context of chronic opioid use. Building on early results showing decreased dendritic length by chronic morphine treatment (Sklair-Tavron et al., 1996), Lane et al. observed changes in VTA DA, but not non-dopaminergic, dendrite diameter, with diameter decreased in the PN but increased in the PB subregions of the VTA (Lane et al., 2008). Again, as the PN is largely NAc-projecting DA neurons while the PB contains both NAc- and PFC-projecting DA neurons, these data support functional differences of DA subpopulations based on their projection target. Given this evolution in the field toward

projection-specific information, recent work has investigated changes in VTA DA soma size, focusing on the NAc and PFC as target regions (Figure 6). A decrease in the surface area of VTA DA neurons projecting to the NAc following chronic morphine administration replicated previous studies (Mazei-Robison et al., 2011; Russo et al., 2007; Sklair-Tavron et al., 1996); however, this change was only seen in neurons projecting to the NAc mShell, as neurons projecting to the NAc core did not differ from sham treated mice (Simmons et al., 2019). Surprisingly, chronic morphine increased the soma size of VTA DA neurons projecting to the PFC (Simmons et al., 2019). While unexpected, these contrasting changes in soma size parallel increased dendrite diameter in PB but decreased dendrite diameter in PN regions mentioned earlier (Lane et al., 2008). As decreases in surface area have been linked to increased firing (Coque et al., 2011; Mazei-Robison et al., 2011), these opposing changes likely correlate with neuron activity and output, highlighting and further supporting circuit-specific changes within populations of VTA DA neurons.

Sex differences in drug reward behaviors

Future work should also consider sex as a biological variable. Though there are no intrinsic differences in VTA DA neuron electrophysiological properties (Chung et al., 2017), sex differences in drug reward and drug-related behaviors exist (Becker, 2016; Becker and Koob, 2016). For opioids, female rodents self-administer more oxycodone, morphine, and heroin compared to males (Cicero et al., 2003; Phillips et al., 2019; Roth et al., 2004). Similar effects are observed for cocaine reward, as female rodents will form CPP at lower doses of cocaine than males (Russo et al., 2003). Further, during withdrawal periods, female rodents show a higher persistence of incubation of craving following

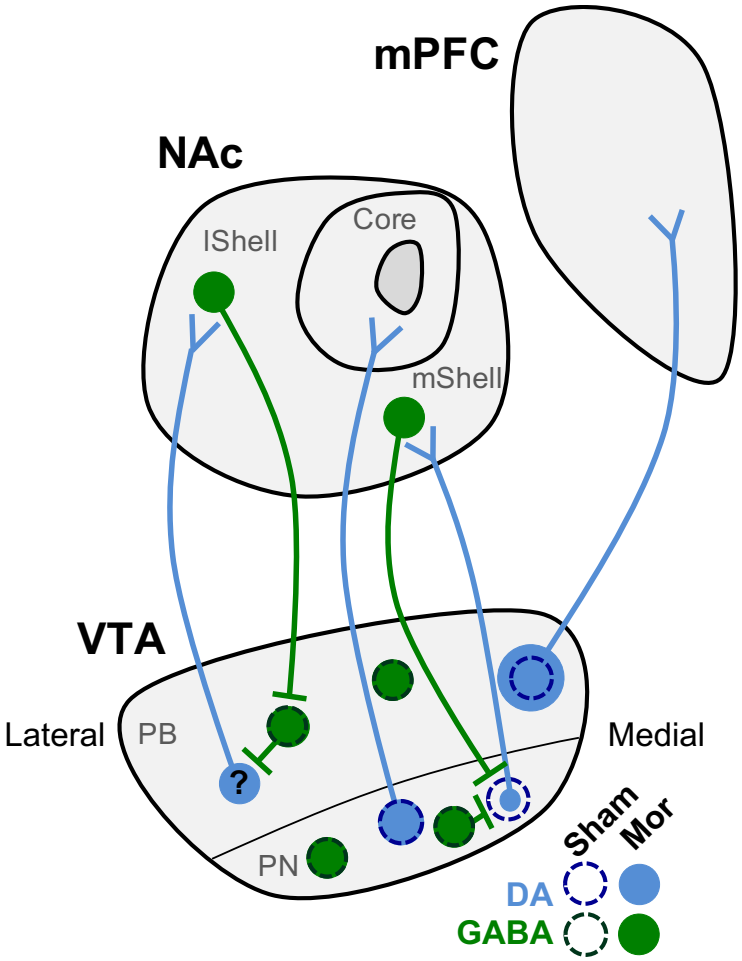

Figure 6. Morphine alters VTA DA soma size in a projection-specific manner. Chronic opioid exposure alters VTA DA, but not GABA, neuron soma size. However, the direction of change is projection specific. DA neurons projecting to the NAc medial shell (mShell) show the decrease in soma size while those projecting to the core are not different from sham controls. It remains unknown whether neurons projecting to the NAc lateral shell (lShell) show a decrease in soma size as well. In contrast, VTA DA neurons projecting to the mPFC show a surprising increase in soma size.

cocaine self-administration (Kerstetter et al., 2008; Nicolas et al., 2019) and enhanced mesolimbic responses to drug-associated contexts in the absence of drug (Calipari et al., 2017). While there have been pioneering studies illustrating sex differences in drug reward behaviors, much of the seminal work characterizing drug-induced changes in synaptic and molecular plasticity was conducted in male rodents. Together, these data

support robust sex differences in addiction-related behavior and highlight the necessity of determining whether sex-specific neuroadaptations exist in the mesolimbic reward circuit.

Drug-induced changes in VTA cellular plasticity

While opioids and cocaine exert their effects on the mesocorticolimbic dopamine system through distinct mechanisms of action, there are similarities in their effects following chronic administration. These are particularly evident in paralleled enhancement of VTA DA-glutamatergic plasticity. Both drugs of abuse increase AMPA/NMDA ratio, with a significant contribution through increased GluA1 trafficking to the synapse. Additionally, increased spontaneous firing and decreased DA output in the NAc are similarly altered by both chronic cocaine and morphine administration. Therefore, identification of novel upstream regulators of drug-induced VTA DA plasticity may provide greater therapeutic potential, particularly in the context of polysubstance use. Cocaine and morphine produce robust changes in the VTA transcriptome (Heller et al., 2015; Lepack et al., 2020; Walker et al., 2018), contributing to drug-induced changes in cellular plasticity. As changes in VTA gene transcription and expression have been linked to changes in drug-related behaviors in mice (Lüscher and Malenka, 2011; Wolf, 2016), transcriptional profiles shared between different drug classes may reveal key druggable targets.

RNA-sequencing provides an unbiased method for identifying genome-wide changes in drug-induced gene transcription. Using RNA-sequencing to identify novel gene targets, our group previously identified serum- and glucocorticoid-inducible kinase 1 (SGK1) as one of only five genes similarly upregulated in the VTA of mice treated with

chronic cocaine or morphine (Heller et al., 2015). *sgk1* has been previously shown to be transcriptionally induced by chronic morphine in the VTA in a microarray study (McClung et al., 2005), further supporting the validity of our genome-wide finding. In addition to regulation at the level of mRNA, SGK1 catalytic activity and phosphorylation at Ser78 are significantly increased by chronic, but not acute, drug treatment (Heller et al., 2015). Given the largely distinct changes in gene expression produced by chronic morphine and cocaine in the VTA, SGK1 represents an intriguing gene of interest due to its shared regulation, highlighting it as a target worthy of further investigation. However, despite evidence for SGK1 transcriptional and protein-level regulation, the functional relevance of SGK1 activity, particularly in the central nervous system, remains poorly understood.

Serum- and glucocorticoid-inducible kinase 1 (SGK1) structure and function in the periphery

SGK1 was originally discovered as an immediate early gene transcriptionally induced by both serum and glucocorticoids (Webster et al., 1993). This early work was seminal in sequencing SGK1, allowing for its identification as a serine/threonine kinase and member of the AGC family, in which it shares homology with other members (Pearce et al., 2010). Specifically, SGK1's sequence includes a highly conserved ATP-binding motif, Gly-X-Gly-X-X-Gly, followed by a downstream Lys 17 residue (Webster et al., 1993) (Figure 7). Like other AGC family members, such as AKT and protein kinase C (PKC), maximal kinase activity is achieved through phosphorylation of the activation segment and hydrophobic motif (Kobayashi and Cohen, 1999; Kobayashi et al., 1999; Park et al., 1999). Within SGK1, these phosphorylation events occur at Thr256 and Ser422, by 3-

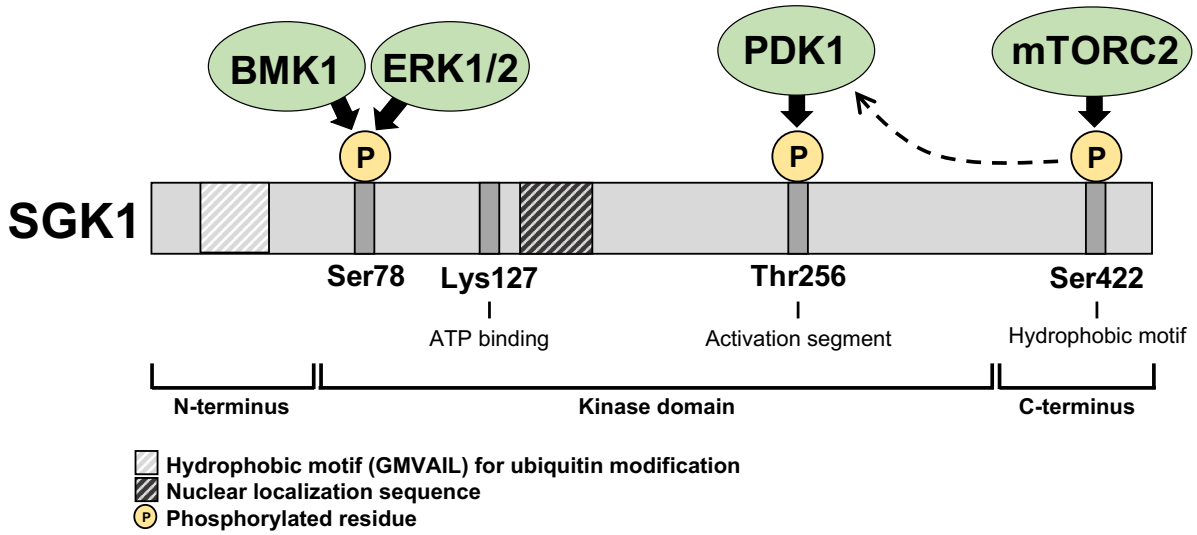

Figure 7. SGK1 structure and relationship to function.
In the context of protein trafficking, SGK1 contains a hydrophobic motif (GMVAIL, AAs 19-24) critical for ubiquitination of the kinase, which has been linked to efficient localization to the plasma membrane. A nuclear localization sequence (AAs 131-141) has also been identified for trafficking to the nucleus. For kinase activity, SGK1 contains 2 critical phosphorylation sites at Thr256 and Ser422. Phosphorylation of both is required for maximal kinase activity. Phosphorylation at Ser422 occurs first via mTORC2, then recruiting PDK1 to phosphorylate SGK1 at Thr256. A kinase dead mutant can be generated by mutating Lys127, a critical residue for ATP binding. Finally, a more novel phosphorylation site exists at Ser78; however, the functional relevance of this site remains poorly understood.

phosphoinositide-dependent kinase 1 (PDK1) and mTORC2 respectively (Garcia-Martinez and Alessi, 2008; Park et al., 1999). Specifically, phosphorylation at Ser422 leads to the recruitment of PDK1 for phosphorylating of Thr265 (Biondi et al., 2001), resulting in maximal kinase activity. In addition to phosphorylation sites related to kinase activity, a more novel phosphorylation site at Ser78 (pSer78) has also been identified and is regulated by extracellular signal-regulated kinase (ERK) 1/2 and big mitogen-activated protein kinase 1 (BMK1, also known as ERK5) (Hayashi et al., 2001; Lee et al., 2006); however, the functional implication of pSer78 remains unclear.

Most of what is known about SGK1 comes from studying its expression and

regulation in the periphery. This kinase is highly conserved, with expression reported in many mammalian species, sharks, birds, and *Caenorhabditis elegans*, with orthologs also expressed in yeast (Lang et al., 2006; Rensel and Schlinger, 2020). In the periphery, SGK1 has been implicated in a variety of cellular processes, including cell survival, volume regulation, and stress (Lang et al., 2006). SGK1 is one of three isoforms of SGK (Kobayashi et al., 1999). SGK1 is presumably expressed in all tissues, as all tissues tested to date express *sgk1*; however, transcript levels vary by tissue (Lang et al., 2006). SGK2 and SGK3 transcripts are similarly expressed in virtually all tissues, though s*gk2*'s expression is primarily in epithelial tissues and s*gk3*'s expression is greatest in the embryo and in adult heart and spleen tissue (Lang et al., 2006). Additionally, SGK1 has 2 identified splice variants, SGK1.1 and SGK1.2, which differ from SGK1 exclusively in their first exons (Arteaga et al., 2008; Simon et al., 2007). mRNA levels of SGK1.2 are very low in analyzed tissues, so this splice variant remains largely unexplored (Arteaga et al., 2008). SGK1.1, on the other hand, has been characterized as a brain-specific variant (Arteaga et al., 2008). As recent work demonstrates that *sgk1* and *sgk1.1* can be differently regulated by stress (Cooper et al., 2017), transcript variant should be taken into consideration for studies in the central nervous system. SGK1 transcript expression remains best characterized of all isoforms and splice variants and, therefore, SGK1 has been the focus of numerous studies examining regulation and function.

SGK1 expression is tightly and rapidly controlled at both the mRNA and protein levels. Transcript expression and degradation occurs within 30 minutes (Waldegger et al., 1997), and protein level degradation is similarly rapid, with a half-life of about 30 minutes (Brickley et al., 2002). This protein level degradation is controlled through an N-

terminal hydrophobic motif, Gly-Met-Val-Ala-Ile-Leu (GMVAIL), a site for ubiquitin modification, and degradation is independent of activation status (Bogusz et al., 2006). This mechanism is unique from other AGC family kinases and is theorized to limit protein accumulation following *sgk1* induction (Bogusz et al., 2006).

At the protein level, subcellular localization of SGK1 remains poorly characterized. There is evidence for SGK1 localization to many subcellular compartments, including the plasma membrane (Brickley et al., 2002), nucleus (Buse et al., 1999), mitochondria (Cordas et al., 2007; Park et al., 1999), and endoplasmic reticulum (Arteaga et al., 2006; Bogusz et al., 2006). In addition to its role in SGK1 degradation, the N-terminus plays an important role in the localization of SGK1, with the plasma membrane as a prominent example. *In vitro*, polyubiquitinated SGK1 exists almost exclusively at the plasma membrane compared to the cytoplasm (Brickley et al., 2002). Deletion of the N-terminus (∆60SGK1) significantly reduces the amount of membrane-associated SGK1 (Brickley et al., 2002), consistent with N-terminal expression of the GMVAIL ubiquitin modification motif (Bogusz et al., 2006) and suggesting that the N-terminal modifications may play a critical role in efficient subcellular localization. Kinase activity does not determine this membrane vs. cytoplasmic localization (Brickley et al., 2002). In addition to the GMVAIL ubiquitin modification motif, SGK1 contains a nuclear localization sequence, and regulation of cytoplasmic to nuclear localization is dependent on phosphoinositide 3 (PI3)-kinase pathway phosphorylation (Buse et al., 1999; Maiyar et al., 2003; Park et al., 1999). Additional work is required to better characterize SGK1 localization in response to stimuli, as this information is critical to understanding SGK1's role in cellular processes.

Functionally, SGK1 is capable of regulating a broad range of ion channels,

transporters, and transcription factors (Reviewed in (Lang et al., 2006)). In the periphery, SGK1 has been extensively characterized for its ability to modulate sodium regulation in the renal system via modifications to epithelial sodium channel (ENaC) trafficking and activity (Debonneville et al., 2001; Snyder et al., 2002; Wang et al., 2001). Results are inconclusive on whether SGK1 directly phosphorylates the ENaC (Diakov and Korbmacher, 2004), but the mechanism best defined in through phosphorylation of neural precursor cell-expressed developmentally downregulated gene 4-2 (NEDD4-2), a member of the family of ubiquitin ligases (E3s) (Figure 8). NEDD4-2 is capable of regulating ENaC abundance at the membrane through ubiquitination, thus signaling the channel for membrane removal and later degradation (Lang et al., 2006). SGK1 phosphorylation of NEDD4-2 decreases this ubiquitination, allowing greater abundance of ENaC at the cell membrane (Lang et al., 2006). While regulation of the ENaC has been the primary focus of research, SGK1 has been shown to regulate a variety of other proteins (Reviewed in (Lang et al., 2006)), implicating SGK1 in the modulation of a variety of cellular functions.

Further, though SGK1 has been shown to regulate a wide range of proteins and cellular functions, SGK1 expression itself is not required for survival, as whole body knockout (KO) mice are viable (Fejes-Toth et al., 2008). Rather, SGK1 KO mice only show a phenotype when significantly physiologically challenged. Again using sodium regulation as a known prominent example of SGK1 function, when placed on a low salt diet, SGK1 KO mice display impaired salt excretion (Wulff et al., 2002) and deficits in ENaC processing (Fejes-Toth et al., 2008). As SGK1 shares overlapping substrates with other AGC family kinases, compensation by another family member may explain the

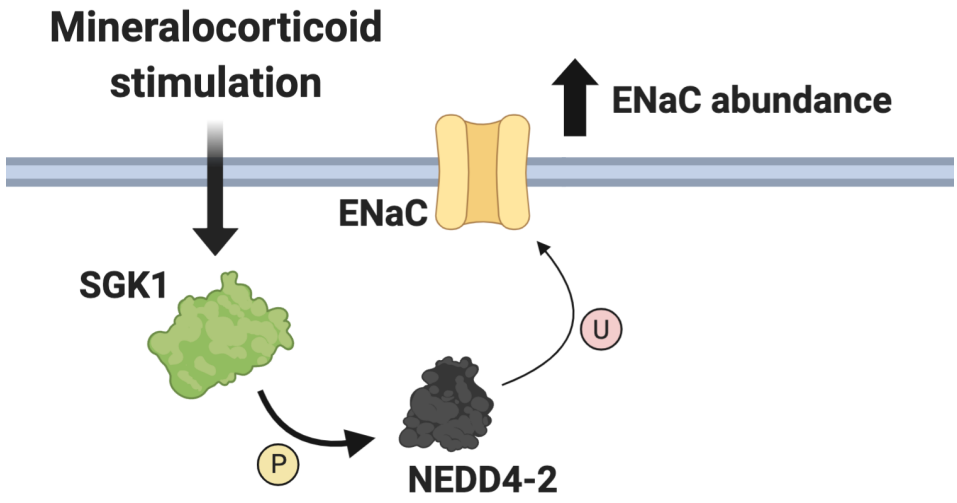

Figure 8. Renal system SGK1 signaling in ENaC regulation.
In the renal system, SGK1 has been well characterized for its role in sodium regulation through ENaC signaling. In response to mineralocorticoid signaling, SGK1 phosphorylates NEDD4-2, a ubiquitin ligase. This phosphorylation event decreases NEDD4-2's activity, reducing its ubiquitination of ENaC. As this would normally tag the channel for removal and degradation, SGK1 therefore indirectly increases ENaC abundance at the plasma membrane.

viability and phenotype of SGK1 KO mice (Brunet et al., 2001; Burchfield et al., 2004; Di Cristofano, 2017; Kobayashi and Cohen, 1999; Pearce et al., 2010). Altogether, more information is needed to better understand the impact of SGK1 expression on physiological function.

SGK1 function in the central nervous system

SGK1 is expressed throughout the brain, with transcript or protein expression shown in the central extended amygdala (Befort et al., 2008), hypothalamus (Deng et al., 2018; Sato et al., 2008), hippocampus (Husain et al., 2015; Lee et al., 2003; Tsai et al., 2002), VTA (Cooper et al., 2017; Heller et al., 2015; McClung et al., 2005), locus coeruleus (McClung et al., 2005), PFC (Licznerski et al., 2015; Piechota et al., 2010;

Yuen et al., 2011), and striatum (Avey et al., 2018; Piechota et al., 2010; Slezak et al., 2013). Further, *sgk1* transcription can be regulated by a variety of different stimuli (Lang et al., 2010). These stimuli include seizures (Husain et al., 2015), stress (Cooper et al., 2017; Koya et al., 2005; Licznerski et al., 2015; Yuen et al., 2011), learning (Lee et al., 2007; Lee et al., 2006; Tsai et al., 2002), and drugs of abuse (Gao et al., 2015; Heller et al., 2015; McClung et al., 2005; Nichols and Sanders-Bush, 2002; Piechota et al., 2010). While altered *sgk1* expression has been identified in response to a wide range of stimuli, much of this information was gathered from large-scale screening studies. In fact, most of these studies did not expand upon mRNA findings with protein-level changes. Therefore, within the central nervous system, little is known about the regulation of SGK1 catalytic activity or phosphorylation and its potential impact on behavior.

The hippocampus has been a region of particular interest in the study of SGK1 function, as the transcription of hippocampal SGK1 can be regulated by learning tasks. In the Morris water maze, rats categorized as fast learners showed increased *sgk1* expression compared to average learners, while slow learner showed a reduction in gene expression (Tsai et al., 2002). However, due to SGK1's status as an immediate early gene, robust increases in *sgk1* mRNA are expected. Rather, protein level regulation may be a better proxy for stimuli-induced SGK1 function, with changes in SGK1 catalytic activity being of particular interest. Following training in the Morris water maze, phosphorylation of SGK1 at both Thr256 and Ser422 was significantly increased in the hippocampus (Lee et al., 2006). As stated earlier, phosphorylation at both of these sites is required for maximal kinase activity. Moreover, viral-mediated overexpression of wild-type SGK1 in the dorsal hippocampus enhanced learning in the Morris water maze (Tsai

et al., 2002), while overexpression of SGK1 mutants with decreased catalytic activity (T256A or S422A) impaired learning (Lee et al., 2006; Lee et al., 2003; Tsai et al., 2002; Tyan et al., 2008). Reduced hippocampal SGK1 catalytic activity though S422A overexpression similarly caused deficits in other context-dependent learning tasks, specifically novel object recognition task and contextual fear-conditioning task (Lee et al., 2003). These studies illustrate two key points: 1) increased hippocampal SGK1 catalytic activity promotes learning and memory, supporting a functional role for SGK1 activity in the brain and 2) SGK1 activity can be manipulated in the brain using viral vectors.

Another regulator of *sgk1* expression is stress. In the PFC, SGK1 was transcriptionally downregulated in human patients diagnosed with posttraumatic stress disorder (Licznerski et al., 2015). Moreover, SGK1 total protein levels were significantly reduced in rats with a high number of escape failures in a model of learned helplessness compared to naïve controls, consistent with the human post-traumatic stress disorder findings (Licznerski et al., 2015). To further assess the role of PFC SGK1 catalytic activity, viral-mediated overexpression studies found that decreasing SGK1 catalytic activity (S422A) increased escape failures while overexpression of wild-type SGK1 decreased escape failures (Licznerski et al., 2015). Together, these data suggest that deficits in PFC SGK1 catalytic activity contribute to the expression of PTSD-like behaviors.

In a different animal model of stress, acute stress resulting from a forced swim, Yuen et al. observed increased SGK1 protein in the PFC (Yuen et al., 2011). Critically, this induction of SGK1 expression was also behaviorally relevant, as protein interference through either intravenous or intra-PFC injections of TAT-SGK1 peptides was capable of blocking the expected stress-enhanced performance on a T-maze task (Yuen et al.,

2011). Here, an inferred induction in PFC SGK1 catalytic activity drove enhanced learning, consistent with SGK1 function promoting hippocampal-dependent learning. While different forms of stress have been associated with both increases and decreases in SGK1 catalytic activity, restoring SGK1 function to baseline is capable of reversing the observed behavioral changes, implicating SGK1 as an important mediator of stress-induced behavior.

Along with the canonical sites (Thr256 and Ser422) critical for catalytic activity, SGK1 contains additional phosphorylation sites. These includes a more novel Ser78 site in the N-terminal domain, although the implication of phosphorylation at this site in terms of protein function remains unclear. However, there is evidence that phosphorylation of SGK1 at Ser78 is physiologically relevant and contributes to behavior. Phosphorylation of SGK1 at Ser78 was increased in the hippocampus following contextual learning tasks (Morris water maze and contextual fear-conditioning training) (Lee et al., 2007; Lee et al., 2006). Moreover, hippocampal viral mediated-overexpression of a phosphodeficient Ser78 mutant (S78A) impaired retention of fear memories following contextual fear conditioning and overexpression of a phosphomimetic mutant (S78D) facilitated learning of contextual fear conditioning (Lee et al., 2007). Moreover, pSer78 effects may be distinct from catalytic activity, as S78A expression did not affect Morris water maze learning, in contrast to catalytic site phosphodeficient mutants (Lee et al., 2006; Lee et al., 2003; Tsai et al., 2002; Tyan et al., 2008). These data suggest that SGK1 catalytic activity and pSer78 may be separately regulated and have distinct behavioral effects.

Though the behavioral effects of manipulating SGK1 catalytic activity and pSer78 in the hippocampus and PFC are promising, the mechanisms thought which altered SGK1

expression or activity influence behavior remain unknown. The exception for this is PFC SGK1 regulation of enhanced working memory. In this example, the forced swim task induced a corticosterone-dependent induction of SGK1 protein, resulting in increased AMPA insertion and function (Yuen et al., 2011) (Figure 9). Like in the periphery, SGK1 did not directly phosphorylate AMPAR but instead altered trafficking via phosphorylation of intermediate proteins. Specifically, this result was likely achieved though SGK1 phosphorylation of guanosine nucleotide dissociation inhibitor (GDI) (Liu et al., 2010), known to regulate Rab4 cycling between the cytoplasm and membrane. This channel trafficking was linked to function, as SGK1 regulated miniature EPSCs (mEPSCs) of cultured PFC neurons. SGK1 siRNA expression prevented a corticosterone-induced increase in mEPSC amplitude, while constitutively active SGK1 (S422D) increased mEPSC amplitude (Yuen et al., 2011). Further, TAT-SGK1 peptides, capable of blocking enhanced working memory when infused into rat PFC, also prevented the increase in synaptic GluA1 labeling (Yuen et al., 2011). This regulation of glutamatergic plasticity is consistent with data demonstrating that electrical induction of hippocampal LTP increased SGK1 phosphorylation at Ser422, and viral-mediated overexpression of an SGK1 mutant with reduced catalytic activity (S422A) impaired the late phase of LTP (Ma et al., 2006). Further, PSD-95 was upregulated in the hippocampus of mice overexpressing a constitutively active SGK1 mutant (S422D) (Ma et al., 2006), and decreased SGK1 catalytic activity (K127Q or S422A) was capable of altering spine density in the hippocampus and PFC (Licznerski et al., 2015; Steffke et al., 2020). These finding support the role of SGK1 in regulating AMPAR trafficking, LTP, and gene transcription following stimuli-induced activation and phosphorylation.

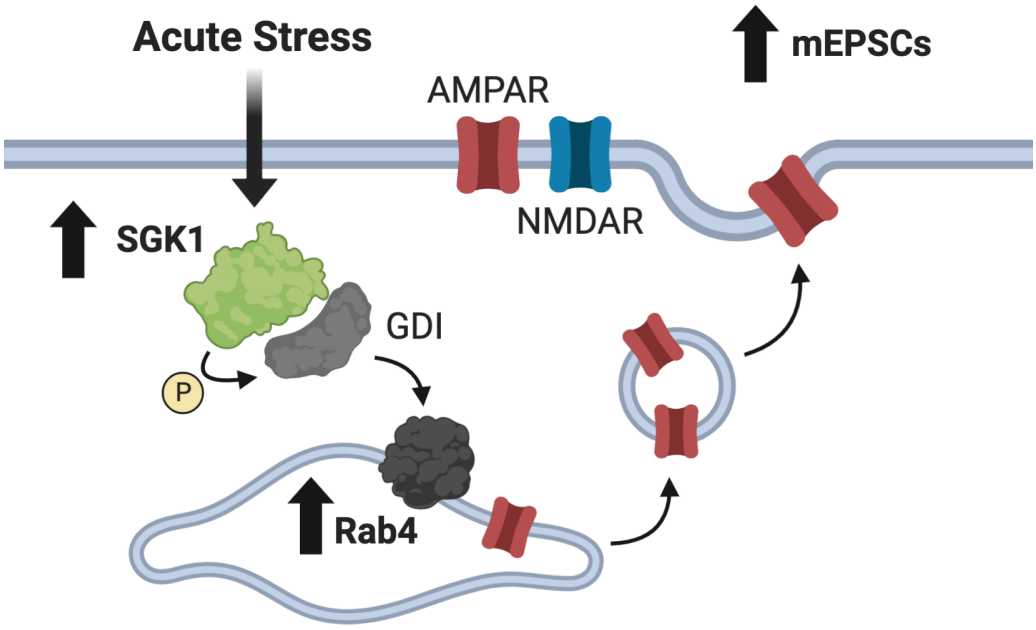

Figure 9. Acute stress increases PFC AMPAR insertion through SGK1 signaling. In the PFC, acute stress increases SGK1 levels through a corticosterone-dependent signaling pathway. This likely results in SGK1 phosphorylation of GDI, a known regulator of Rab4 function, Rab4 activity is increased by stress via an SGK1-dependent mechanism. Since Rab4 is critical for vesicle recycling from early endosomes to the plasma membrane, this increased activity results in increased GluA1 synaptic labeling and increased mEPSCs, indicative of potentiated glutamatergic transmission.

In addition to regulation of glutamatergic plasticity, SGK1 is capable of influencing gene transcription following stimuli-induced activation and phosphorylation. As stated above, performance in the Morris water maze task can be regulated by SGK1 catalytic activity (Lee et al., 2006; Lee et al., 2003; Tsai et al., 2002; Tyan et al., 2008). During this learning task, hippocampal SGK1 had been shown to indirectly regulate mRNA expression of the transcription factor early growth response protein 1 (EGR1, also known as Zif268) through phosphorylation of serum response factor (SRF) or cAMP response element-binding protein (CREB) (Tyan et al., 2008). These phosphorylation events were

linked to SGK1 catalytic activity, as phosphorylation of SRF and CREB were enhanced by constitutively active SGK1 expression (S422D) but attenuated by reduced SGK1 activity (S422A) in a cell culture model (Tyan et al., 2008). Similarly, SGK1 pSer78 has been linked to the regulation of transcriptional machinery (Lee et al., 2007), though a signaling cascade has not been defined. Because SRF and CREB are themselves transcription factors, these data implicate SGK1 as a mediator of the transcription of a wide variety of genes via stimuli-induced phosphorylation of transcriptional machinery. Together the above studies support a role for central nervous system SGK1 protein function in behavior; however, they also highlight the lack of knowledge surrounding SGK1 protein regulation and its consequences. This knowledge gap is partially evident in drug addiction research.

SGK1 in the context of drugs of abuse

While SGK1 expression and function in the context of stress and learning provide strong evidence for SGK1's ability to alter animal behavior, this protein remains largely unstudied in the effects of drugs of abuse. Transcriptionally, virtually all classes of drug of abuse are capable of inducing SGK1 expression across a variety of brain regions within the mesocorticolimbic dopamine system. These include cocaine (Gao et al., 2015; Heller et al., 2015), amphetamine (dela Pena et al., 2013), methamphetamine (dela Pena et al., 2013; Piechota et al., 2010), methylphenidate (dela Pena et al., 2013), morphine (Avey et al., 2018; Heller et al., 2015; McClung et al., 2005; Slezak et al., 2013), heroin (Piechota et al., 2010), ethanol (Piechota et al., 2010), and lysergic acid diethylamide (Nichols and Sanders-Bush, 2002). However, nearly all studies have stopped at the level of

transcriptional regulation, leaving the functional role of SGK1 catalytic activity and phosphorylation at Ser78 unexplored following drug administration.

As stated earlier, our lab previously identified *sgk1* as one of only five gene to be transcriptionally upregulated in the VTA of adult mice following chronic cocaine or morphine administration (Heller et al., 2015), consistent with earlier work showing VTA *sgk1* induction following chronic morphine administration (McClung et al., 2005). SGK1 has a brain-specific isoform, SGK1.1 (Arteaga et al., 2008). However, SGK1 and SGK1.1 have been shown to undergo divergent levels of regulation in the VTA following stress (Cooper et al., 2017). Consistent with these data, chronic morphine and cocaine administration increase VTA *sgk1* levels but *sgk1.1* remains unchanged (unpublished, Michelle Mazei-Robison). For this reason, research has focused on SGK1 regulation and function following drug administration.

Since literature from the stress and learning and memory fields have provided strong evidence for protein-level changes, regulation of SGK1 catalytic activity and phosphorylation at Ser78 following chronic cocaine or morphine administration was next assessed. Chronic administration of either drug significantly increased VTA SGK1 catalytic activity, as measured by phosphorylated n-myc downstream regulated gene (NDRG) (pNDRG), an exclusive substrate of SGK1 (Murray et al., 2004; Murray et al., 2005), and pSer78 1 hour after the last injection (Heller et al., 2015). These biochemical changes were not observed 24 hours after the last injection nor were there changes in total SGK1 protein at either time point (Heller et al., 2015), consistent with SGK1's tight regulation at both mRNA and protein levels. These identified changes in catalytic activity and phosphorylation were not seen if only a single injection of drug was administered,

and if a morphine pellet or binge cocaine paradigm were used as methods of drug delivery, levels of catalytic activity and phosphorylation were robustly increased (Heller et al., 2015). These data indicate that chronic, but not acute, cocaine or morphine administration is capable of inducing VTA SGK1 catalytic activity and phosphorylation.

Given the identification of increased VTA SGK1 catalytic activity, early work sought to test if altered catalytic activity was capable of modulating drug-related behaviors. Using viral-mediated overexpression, a constitutively active SGK1 mutant (S422D) was bilaterally infused into the VTA of adult male mice, increasing the overall catalytic activity of SGK1 in this brain region. In cocaine and morphine locomotor sensitization paradigms, mice overexpressing constitutively active SGK1 (S422D) showed altered locomotor activity in response to repeated injections of drug compared to GFP-expressing controls (Heller et al., 2015), indicating that the activity of VTA SGK1 is capable of regulating drug-related behaviors. However, VTA overexpression of the S422D mutant did not alter drug reward, as measured by cocaine and morphine CPP (Heller et al., 2015). These data suggest that increased catalytic activity may not be capable of further driving drug reward behavior; however, this experiment is limited in that it only assessed one side of the question. It remains unknown if decreased catalytic activity is capable of disrupting drug reward. This early work was critical in identifying SGK1 as a key protein regulated following chronic cocaine and morphine administration, specifically that chronic drug administration increases VTA SGK1 catalytic activity and phosphorylation at Ser78. However, no other studies have sought to characterize the role of SGK1 in drug reward or other drug-related behaviors. Therefore, it is unknown if SGK1 catalytic activity and phosphorylation promote drug-related behaviors.

Hypothesis and specific aims

Substance use disorder is a prevalent issue in the United States (NIDA, 2020a; SAMHSA, 2019), yet treatments remain inadequate. Moreover, polysubstance use is common within this population and puts these individuals at a higher risk of harm (Butelman and Kreek, 2017; Leeman et al., 2016; Leri et al., 2003; Liu et al., 2018b). To combat this problem, genes similarly regulated across multiple drug classes may represent more efficacious targets for pharmacotherapies used in treating substance use disorders. We previously demonstrated that chronic cocaine or morphine administration both result in increased VTA SGK1 mRNA, catalytic activity, and phosphorylation at Ser78 (Heller et al., 2015). While these biochemical changes have been identified, the impact of this regulation on drug-related behaviors remains poorly understood. Given the induction of *sgk1* expression and protein-level changes in response to drug treatment, *I hypothesized that decreased VTA and DA sgk1 expression would be capable of altering cocaine and morphine reward-related behaviors*. In Specific Aim 1, I tested this hypothesis using VTA- and DA-specific SGK1 knockout mouse models to investigate the role of VTA SGK1 gene expression in mediating cocaine CPP and morphine preference in a two-bottle choice task.

SGK1 catalytic activity, specifically, has been linked to altered stress- and learning-related behaviors (Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008; Yuen et al., 2011). As VTA SGK1 catalytic activity is significantly increased following chronic treatment with cocaine or morphine (Heller et al., 2015), I next focused on altered catalytic activity as a potential mediator of drug reward. As stated earlier, our lab previously showed that VTA overexpression of a constitutively active

SGK1 mutant (S422D) did not change cocaine or morphine CPP; however, the effect of decreased catalytic activity remains unknown. Therefore, *I hypothesized that decreased VTA SGK1 activity would be sufficient to drive changes in drug reward behaviors*. I evaluated this hypothesis in Specific Aim 2 through viral-mediated overexpression of an SGK1 mutant lacking catalytic activity (K127Q). Behavioral studies were used to examine the impact of the resulting decreased catalytic activity in the VTA as well as in VTA DA vs. GABA neurons, the major neuronal populations (Nair-Roberts et al., 2008; Swanson, 1982).

Together these Specific Aims test my central hypothesis, that *increased VTA SGK1 phosphorylation and catalytic activity caused by chronic cocaine and morphine treatment drives drug-related behaviors in a cell type-specific manner*. This is a critical gap in knowledge, as SGK1 is posed as a promising therapeutic target due to its shared regulation across 2 classes of drugs of abuse. Altogether, this work will increase our understanding of the role of VTA SGK1 activity in drug-related behaviors, a necessary step in assessing the feasibility of SGK1 inhibition as a novel therapeutic avenue for the treatment of substance use disorders.

Chapter 2. Behavioral effects of SGK1 knockout in VTA and dopamine neurons

Introduction

The abuse of illicit drugs is a substantial economic burden in the US (Cartwright, 2008), yet treatments for addiction remain inadequate, in part due to our limited understanding of the drug-induced neuroadaptations underlying addiction. The mesocorticolimbic dopamine (DA) system plays a critical role in drug reward and addiction. Chronic drug use induces neuroadaptations within the ventral tegmental area (VTA) (Juarez and Han, 2016), with changes in gene expression and cell activity linked to addiction-like behaviors in mice (Lammel et al., 2014; Volkow and Morales, 2015). Using RNA-sequencing, we previously identified serum- and glucocorticoid-inducible kinase 1 (SGK1) as one of the few genes transcriptionally upregulated in the VTA by both chronic psychostimulants (cocaine) and opiates (morphine) (Heller et al., 2015), positioning SGK1 as a potential drug-induced mediator of reward behaviors.

SGK1 is an immediate early gene originally discovered through its transcriptional regulation by serum and glucocorticoids (Webster et al., 1993). This serine/threonine kinase is part of the AGC family of kinases, which also includes AKT and protein kinase C (PKC) (Webster et al., 1993). Similar to these other AGC family members, maximal kinase activity is achieved through phosphorylation of the activation segment and hydrophobic motif: in SGK1, this occurs at Thr256 and Ser422, by 3-phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) respectively (Garcia-Martinez and Alessi, 2008; Park et al., 1999). A unique phosphorylation site at Ser78 has also been identified and is regulated by extracellular signal-regulated kinase (ERK) signaling and epidermal growth factor (EGF) stimulation

(Hayashi et al., 2001; Lee et al., 2006). In the periphery, SGK1 has been well characterized for its ability to modulate the function of a broad range of ion channels and transcription factors (Lang et al., 2006). Of note, whole body SGK1 knockout is not developmentally lethal, but mice show deficits in the regulation of salt excretion (Wulff et al., 2002) and impaired epithelial sodium channel processing (Fejes-Toth et al., 2008) when placed on a low salt diet. While SGK1 is widely expressed in the brain (Befort et al., 2008; Deng et al., 2018; Heller et al., 2015; Licznerski et al., 2015; McClung et al., 2005; Piechota et al., 2010; Sato et al., 2008; Tsai et al., 2002), its protein-level regulation and functional role in the central nervous system remain poorly understood.

In the brain, *sgk1* gene expression is induced by virtually all classes of drugs of abuse in a variety of regions critical to drug reward behaviors (Gao et al., 2015; Heller et al., 2015; McClung et al., 2005; Nichols and Sanders-Bush, 2002; Piechota et al., 2010). However, much of this information was collected as part of large-scale screening studies and most did not include protein-level or behavioral assays. Therefore, the significance of gene regulation remains largely unexplored, though SGK1 is capable of modulating animal behavior. Specifically, in the prefrontal cortex (PFC) and hippocampus viral-mediated overexpression of SGK1 mutants with reduced catalytic activity (T256A or S422A) or phospho-deficiency (S78A) were capable of impairing stress- and learning-induced behaviors (Lee et al., 2007; Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008; Yuen et al., 2011). Expanding on known drug-induced mRNA regulation, we previously identified increases in VTA SGK1 catalytic activity and phosphorylation at Ser78 following chronic, but not acute, cocaine or morphine administration (Heller et al., 2015). Further, VTA overexpression of

constitutively active SGK1 (S422D) alters locomotor sensitization to cocaine and morphine (Heller et al., 2015), indicating a behaviorally relevant role for SGK1 signaling in drug-related behaviors.

Given the induction of VTA SGK1 regulation by drugs of abuse as well as the capacity for SGK1 catalytic activity and phosphorylation to regulate animal behavior, we sought to determine the effect of VTA SGK1 deletion on cocaine and morphine reward. Moreover, the VTA contains a diverse neuronal population, composed of 60-65% DA, 30-35% gamma-aminobutyric acid (GABA), and 2-3% glutamate neurons (Morales and Margolis, 2017; Nair-Roberts et al., 2008; Swanson, 1982). Therefore, it is important to take neuronal cell type into account. Because VTA DA neuronal activity and output are critical modulators of drug reward (Volkow and Morales, 2015), we also investigated a DA neuron-specific SGK1 knockout. To investigate the effects of region- and cell type-specific SGK1 deletion on drug reward, novel VTA- and DA neuron-SGK1 knockout mouse models were utilized to assess altered cocaine reward via cocaine conditioned place preference and morphine preference via morphine two-bottle choice task.

Materials and methods

Mice

Initial floxed SGK1 (FlxSGK1) breeder mice (Fejes-Toth et al., 2008) were obtained from A. Náray-Fejes-Tóth (Dartmouth University) and bred homozygously ($SGK1^{Flx/Flx}$) for VTA SGK1 knockout experiments. To generate dopamine SGK1 knockout, FlxSGK1 mice were crossed with heterozygous dopamine transporter (DAT)-Cre-recombinase (Cre) mice (Jackson Laboratories, 006660). All mice were fully backcrossed to the c57Bl/6

background, and genotyping was performed at 3−4 weeks using the following primers for FlxSGK1 and DAT-Cre mouse lines:

SGK1 Down: 5'-AAAGCTTATCTCAAACCCAAACCAA-3'

SGK1 WT-Up: 5'-CTCATTCCAGACCGCTGACAAG-3'

SGK1 Mutant-Up: 5'-CTCAGTCTCTTTTGGGCTCTTT-3'

DAT Common: 5'- TGGCTGTTGGTGTAAAGTGG-3'

DAT WT Reverse: 5'- GGACAGGGACATGGTTGACT-3'

DAT Mutant Reverse: 5'- CCAAAAGACGGCAATATGGT-3'

Experiments utilized male and female mice (8−15 weeks). Mice were housed at 22−25°C on a standard 12 hr light-dark cycle with food and water ad libitum, and all behavior was measured during the light phase. All experiments were approved by Michigan State University Institutional Animal Care and Use Committee (IACUC) and were carried out in accordance with the guidelines set in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Drugs

Cocaine was obtained from Sigma. For all conditioned place preference experiments, cocaine was dissolved in 0.9% sterile saline. Male mice were conditioned with 12.5 mg/kg cocaine and females with 10 mg/kg cocaine. Because female rodents have been shown to form a conditioned preference at lower doses of cocaine compared to males (Russo et al., 2003), a lower dose of drug was chosen for the females in order to achieve equivalent preference scores.

Morphine sulfate was generously provided by the NIDA Drug Supply Program. For all morphine two-bottle choice experiments, morphine was used at 0.05 mg/mL and dissolved in vehicle solution (0.2% sucrose in water).

Viral-mediated gene transfer

Stereotaxic surgeries were completed following established procedures(Heller et al., 2015; Kaska et al., 2017). Briefly, mice were anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine) and received bilateral intra-VTA infusions (0.5 µl) of rAAV2/CMV-Cre-GFP or rAAV2/TR-eGFP (University of North Carolina GTC Vector Core) at established coordinates (-3.2 mm A/P, +1.0 mm M/L, −4.6 mm D/V, 7° angle). Mice were allowed to recover for at least 21 days before the start of behavioral experiments to allow for Cre-mediated gene deletion and the degradation of remaining SGK1 in infected cells. Following behavioral studies, viral targeting was confirmed by standard histological methods.

Viral targeting

Mice were sacrificed and brains postfixed in 10% formalin for 3 days before cryo-preservation in 30% sucrose-phosphate buffered saline (PBS). Brains were then sliced into 30 µm sections and GFP labeling was used to confirm viral targeting. Mice with GFP expression outside of the VTA or with unilateral hits were excluded from analysis.

Quantitative Real-Time PCR (qPCR)

At least 3 weeks following stereotaxic surgeries for VTA SGK1 knockdown, mice were

sacrificed and VTA and NAc were microdissected and stored at -80°C until processing. RNA was isolated and purified using RNAeasy micro-columns (Qiagen), and cDNA was created using a high capacity reverse transcription kit (Applied Biosystems). Changes in *sgk1* gene expression were determined via RT-PCR using Power SYBR green (CFX connect, BioRad) and published primers for SGK1 and GAPDH (Cooper et al., 2017):

SGK1 F: 5'-ATCGTGTTAGCTCCAAAGC-3'

SGK1 R: 5'-GTCTGTGATCAGGCATAGC-3'

GAPDH F: 5'-AGGTCGGTGTGAACGGATTTG-3'

GAPDH R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'

Tyrosine hydroxylase (TH) and Cre were used to confirm tissue punch and viral targeting of the VTA:

TH F: 5'-CAGAGCAGGATACCAAGCAGG-3'

TH R: 5'-CTCGAATACCACAGCCTCCAA-3'

Cre F: 5'-GAACGAAAACGCTGGTTAGC-3'

Cre R: 5'-CCCGGCAAAACAGGTAGTTA-3'

All samples were run in triplicate and normalized to GAPDH before analysis using the ΔΔCt method.

Two-bottle voluntary choice (TBC) tasks

To assess voluntary drinking, mice were singly housed with access to two 50 mL conical tubes fitted with sipper tops following established procedures (Cooper et al., 2017; Kaska et al., 2017). Briefly, every morning fluid consumption was measured and bottle placement was switched to account for individual side bias. Mice that displayed a >30%

side/bottle preference during morphine preference were excluded from analysis. Mice were allowed to habituate to both bottles containing only water for 4 days, then water bottles were replaced for sucrose or morphine preference assessment for an additional 4 days. For sucrose preference, water bottles were replaced with bottles containing either 1% sucrose or water. For morphine preference, water bottles were replaced with bottles containing a 0.2% sucrose solution with either 0.05 mg/mL morphine sulfate or 0.01 mg/mL quinine (Sigma, 22640, bitter taste control) (Belknap et al., 1993; Forgie et al., 1988). Drinking was measured to determine solution preference over time. Results are reported as percent preference (solution consumed/total fluid consumed x 100), average preference (4 day average of percent preference), and fluid intake (volume normalized to mouse body weight, 4 day average).

Cocaine conditioned place preference (CPP)

Cocaine CPP was performed as previously described (Heller et al., 2015). Briefly, mice were placed in the center of a 3-chambered CPP box (San Diego Instruments) and allowed to freely explore for 20 min to pretest for chamber bias. Mice with >20% bias were excluded from analysis. During two conditioning days, mice were restricted to one chamber for 30 min and received a control (saline) i.p. injection in the morning and a cocaine injection (Sigma, 12.5 mg/kg for males or 10 mg/kg for females) in the opposite chamber in the afternoon. On test day, mice were placed in the center chamber and allowed to freely explore for 20 min. The CPP score was calculated as the time (sec) spent in the drug-paired minus saline-paired chamber. Locomotor activity was determined by total beam breaks during each session, and averages of conditioning sessions were

used for analysis.

Open field task

Mice were placed in a 38cm x 38cm arena and distance traveled was measured for 1 hr using video-tracking software (TopScan Suite, CleverSys). Anxiety measures were calculated as the percent of time spent in the center and periphery of the area during the first 10 min of the session.

Body measurements

Body weights were taken once a week from 7−12 weeks of age, and body length was measured from the tip of the nose to the base of the tail at 12 weeks.

Statistics

Full statistical analyses and results are listed in Table 2. All statistical analyses were performed using GraphPad Prism, and all values are represented as mean±SEM. An unpaired t-test (two-tailed) was used to compare the means of 2 groups and a one-way analysis of variance (ANOVA) was used to compare the means of 3 groups, followed by a Tukey or Dunnett post-hoc test when appropriate. A two-way ANOVA was used when comparing 2 independent variables and a two-way ANOVA with repeated measures (RM) when 2 independent variables with repeating measures were compared, followed by a Tukey and Sidak or Dunnett post-hoc test when appropriate, respectively. Males and females were combined for biochemical validation but always analyzed separately for behavior. Significance was defined as *$p<0.05$ and **$p<0.01$.

Results

Validation of VTA-specific SGK1 knockdown

To create a conditional model of VTA-specific SGK1 deletion, male and female mice with homozygous floxed SGK1 gene (FlxSGK1) were bilaterally injected in VTA with AAV2-Cre-GFP (AAV-Cre) to knockdown SGK1 expression within this specific brain region or with AAV2-eGFP as a vector control (Figure 10A). Following surgery, VTA *sgk1* mRNA expression was significantly decreased in mice that received the Cre-expressing virus compared to GFP controls (Figure 10B, all statistical analyses and results are detailed in Table 2), confirming decreased gene expression. Thus, intra-VTA AAV-Cre infusion resulted in SGK1 gene knockdown, providing a useful model for determining the behavioral consequences of reduced SGK1 expression and function within the VTA.

VTA SGK1 knockdown does not alter natural or drug reward behavior

Because previous studies have demonstrated an effect of manipulated VTA gene expression on natural reward and ingestive behaviors (Bolaños et al., 2003; de Jong et al., 2015; Hommel et al., 2006), we first sought to characterize potential changes in sucrose preference induced by VTA SGK1 deletion. Following viral surgery to knock down VTA SGK1 expression, male and female mice were placed in a sucrose two-bottle choice (TBC) task to assess the impact of viral manipulation on sucrose preference. During acclimation to two water bottles, there was no significant difference between groups in total water intake, indicating that fluid intake was not altered by homozygous VTA SGK1 deletion (Figure 11A). Natural reward was unchanged, as no difference was seen in preference for sucrose over the course of 4 days (average percent sucrose preference in

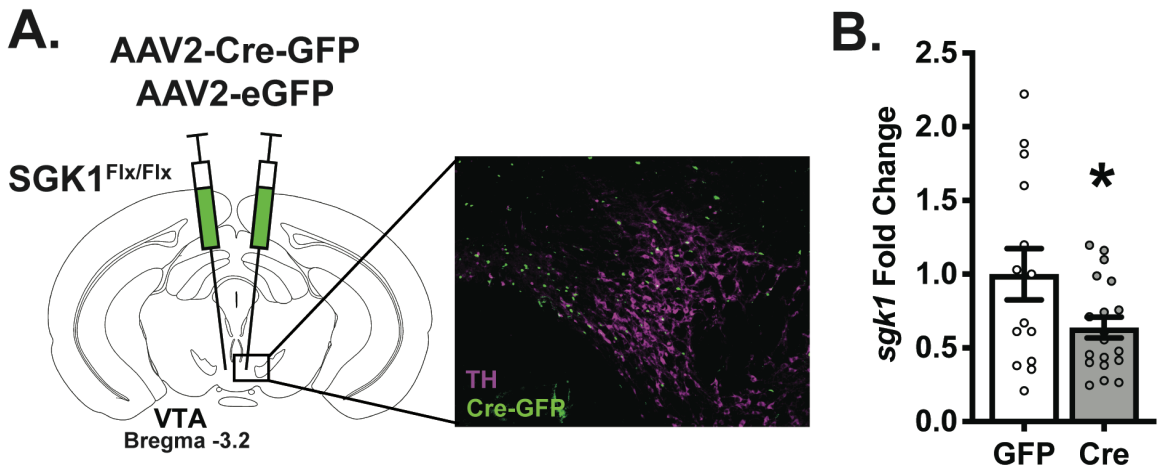

Figure 10. Establishment of VTA SGK1 knockdown mouse model.
A. Representative image of AAV2-Cre-GFP viral infection in VTA of FlxSGK1 mice.
B. VTA SGK1 knockdown (Cre) decreased VTA SGK1 mRNA expression compared to controls (GFP) (n=14-19, unpaired t-test, p=0.0424).

males GFP:75.58±3.33 Cre:79.79±2.02 and females GFP:78.77±3.06 Cre:78.31±2.49) or average fluid intake during the task (Figure 11B). There was a significant interaction of virus x time in female mice (Figure 11B-C); however, a Sidak post-hoc test did not determine any significant differences between groups. While interesting, this effect may have been driven by a low number of mice in the control group, and, therefore, an additional cohort of mice is needed to confirm this observation. These data are consistent with the fact that whole body SGK1 knockout mice do not display a phenotype until physiologically challenged (Fejes-Toth et al., 2008; Wulff et al., 2002). Since acute drug treatment is not sufficient to induce significant changes in VTA SGK1 biochemistry (Heller et al., 2015), natural rewards may not adequately engage SGK1 signaling pathways to drive deficits in reward behavior in our gene knockdown model.

Given the significant effects of chronic drug treatment on VTA SGK1 biochemistry and VTA constitutively active-SGK1 overexpression on drug reward (Heller et al., 2015),

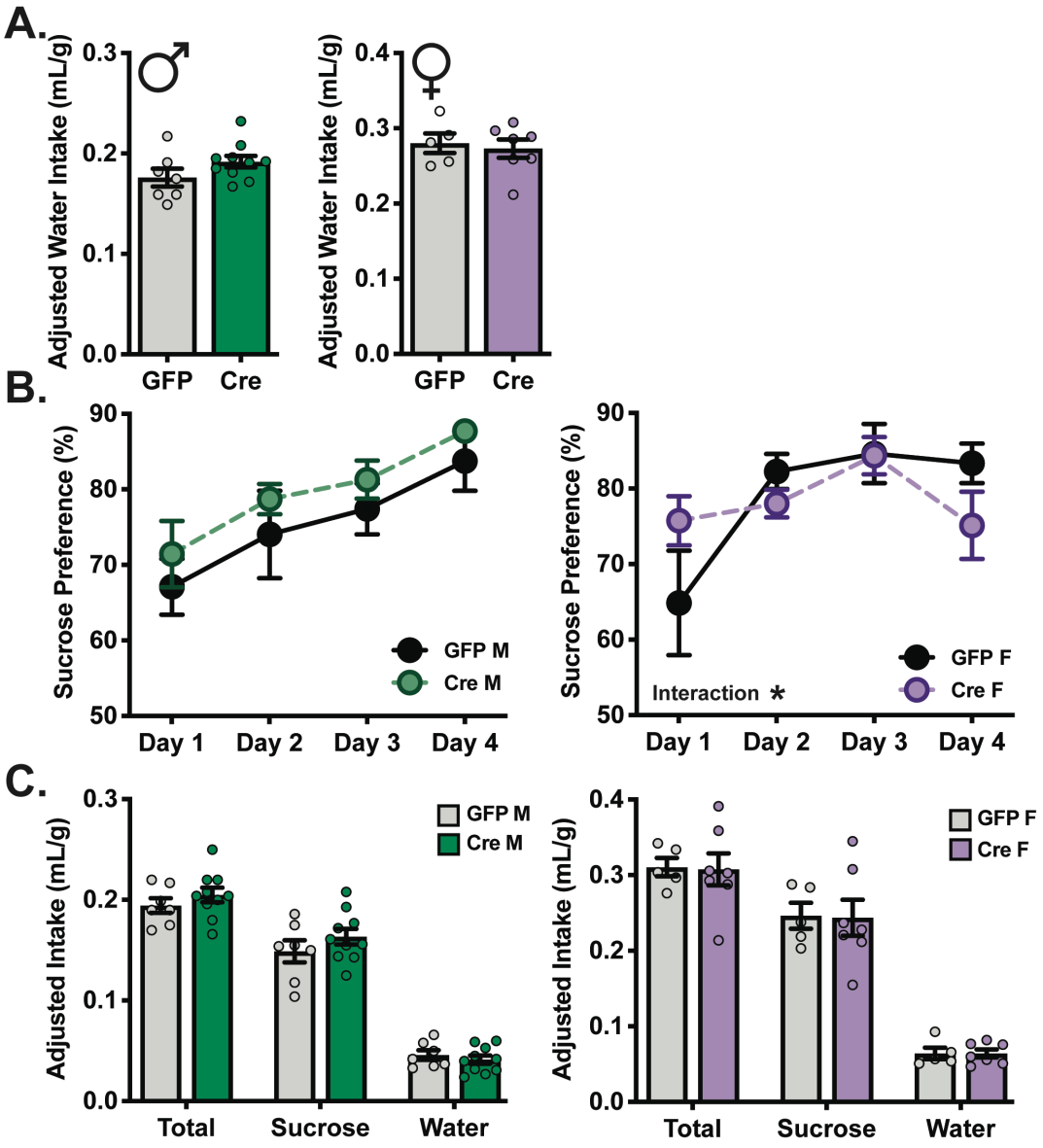

Figure 11. Fluid intake and natural reward are not altered by VTA SGK1 knockdown.

A. Water intake was not altered by VTA knockdown of SGK1 (Cre) compared to controls (male n=7-10, unpaired t-test; female n=5-7, unpaired-test).

B. Natural reward measured via sucrose preference (1%) was not changed by VTA SGK1 expression (male n=7-10, two-way ANOVA with repeated measures; female n=5-7, two-way ANOVA with repeated measures, time x virus interaction p=0.0126 but no significant effects in a Sidak post-hoc test).

C. Intake of fluids is not altered by VTA SGK1 knockdown during sucrose preference test (male n=7-10 unpaired t-test; female n=5-7, unpaired t-test).

we next determined whether VTA SGK1 deletion caused a deficit in morphine preference in the TBC task and cocaine reward in conditioned place preference (CPP). Similar to sucrose reward, morphine preference was not significantly different in either male or female homozygous VTA SGK1 knockdown mice compared to GFP controls (Figure 12A) (average percent morphine preference in males GFP:72.04±2.10 Cre:74.64±1.78 and females GFP:72.89±2.50 Cre:66.83±1.83) nor in heterozygous knockdown males (Figure 13). Fluid intake during the task was not changed by VTA SGK1 knockdown in male or female mice (Figure 12B).

SGK1 activity and phosphorylation were also increased by chronic cocaine treatment (Heller et al., 2015), so we next examined the effects of altered VTA SGK1 expression on cocaine reward using CPP. Female mice were treated with a moderately lower dose of cocaine (10 mg/kg) compared to males (12.5 mg/kg) due to evidence for higher drug sensitivity for female rodents in CPP (Calipari et al., 2017; Russo et al., 2003). Following conditioning, GFP controls formed a preference for the drug-paired chamber, as expected; however, neither male nor female homozygous VTA SGK1 knockdown mice showed a significant difference in time spent in the drug-paired chamber compared to controls, indicative of intact drug reward (Figure 12C). Cocaine increased locomotor activity as expected, but SGK1 knockdown did not alter average total beam breaks between groups during either the saline or cocaine conditioning sessions (Figure 12D). In all, VTA SGK1 knockdown did not appreciably alter reward behavior; however, while our viral expression was region-specific, it was not cell type-specific and knockdown in both VTA DA and GABA neurons could mask potential effects.

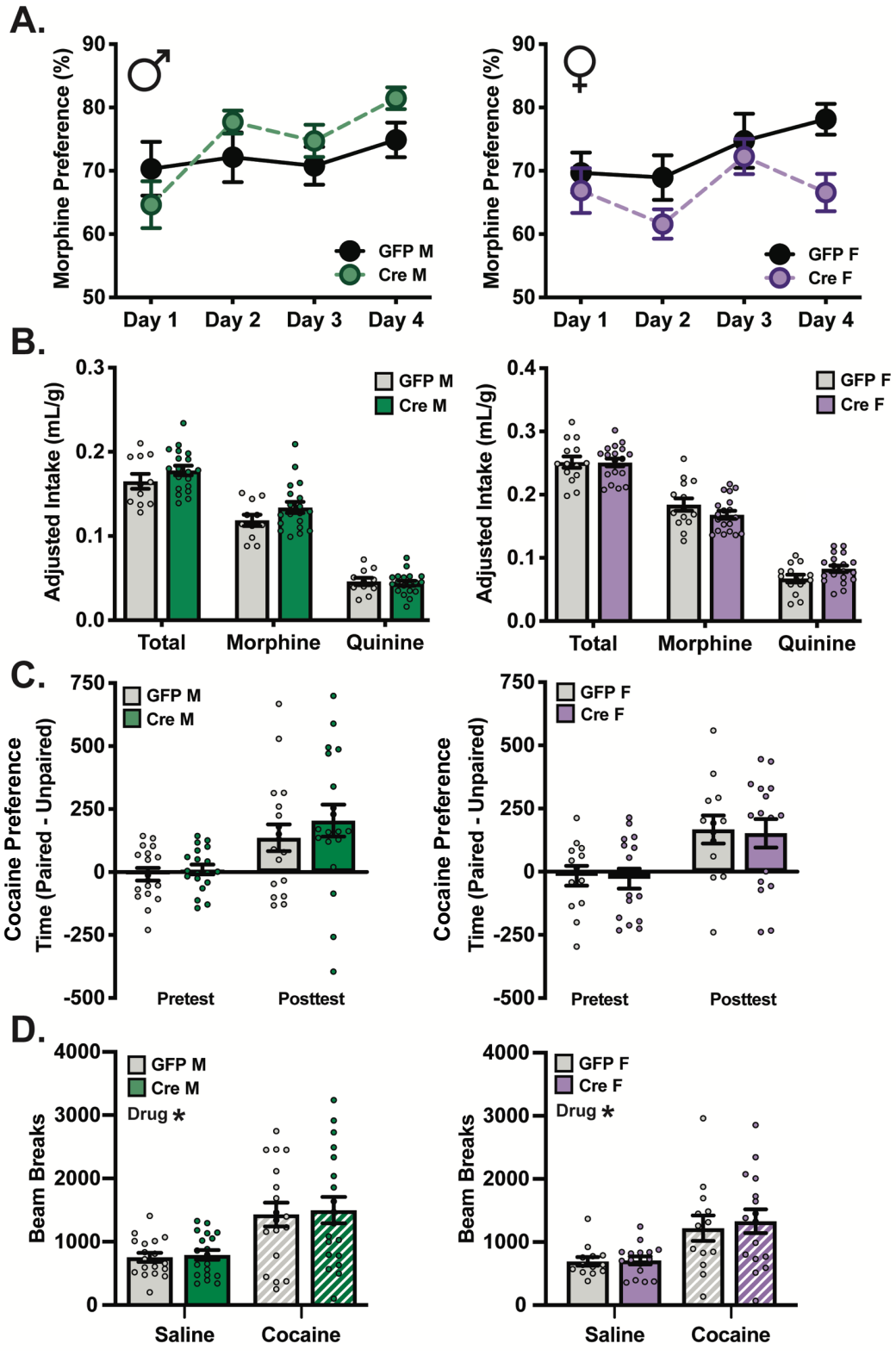

Figure 12. VTA SGK1 knockdown does not alter or morphine or cocaine reward behaviors.

Figure 12. (cont'd).
A. Morphine preference knockdown (Cre) (0.05 mg/mL morphine vs. 0.01 mg/mL quinine) was not significantly altered by VTA SGK1 compared to GFP controls (male n=11-19, two-way ANOVA with repeated measures; female n=14-19, two-way ANOVA with repeated measures).
B. Fluid intake between groups was not different during morphine TBC (male n=11-19, unpaired t-test; female n=14-19, quinine: p= 0.0650).
C. Cocaine CPP in male (12.5 mg/kg) or female (10 mg/kg) mice was not altered by VTA SGK1 knockdown (Cre) compared to controls (GFP) (male n=18-19, unpaired t-test; female n=13-16, unpaired t-test).
D. Two day average locomotor activity measured during saline and cocaine conditioning sessions was not different between VTA SGK1 knockdown (Cre) and control (GFP) groups (male n=18-19, two-way ANOVA followed by Tukey post-hoc, main effect of drug: $p<0.0001$; female n=13-16, two-way ANOVA followed by Tukey post-hoc, main effect of drug: $p=0.0002$).

DA neuron-specific SGK1 deletion alters body weight and locomotor activity

Given the neuronal heterogeneity in the VTA (Nair-Roberts et al., 2008; Swanson, 1982) and the critical role for VTA DA activity and output in drug behavior (Volkow and Morales, 2015), we next evaluated the effects of SGK1 deletion in DA neurons. To exclusively delete expression from DA neurons, FlxSGK1 mice were crossed with dopamine transporter (DAT)-Cre mice to create a DA neuron-specific SGK1 deletion. To first determine if baseline physiology and behavior are altered by developmental DA SGK1 knockout (DA SGK1 KO), we tracked body weight of heterozygous and homozygous DA SGK1 KO mice compared to littermates across early adulthood. Both sexes showed significant effects of time x genotype interaction, while females had an additional significant main effect of genotype (Figure 14A). Overall, DA KO of SGK1 significantly decreased body weight of both male and female mice compared to their control littermates (Figure 14A). Since homozygous DA SGK1 KO males showed a non-significant decrease in body length at 12 weeks of age (body length in centimeters Con:9.69±0.11 KO:9.48±0.07, n=7-10), the effect of gene knockout on weight may be

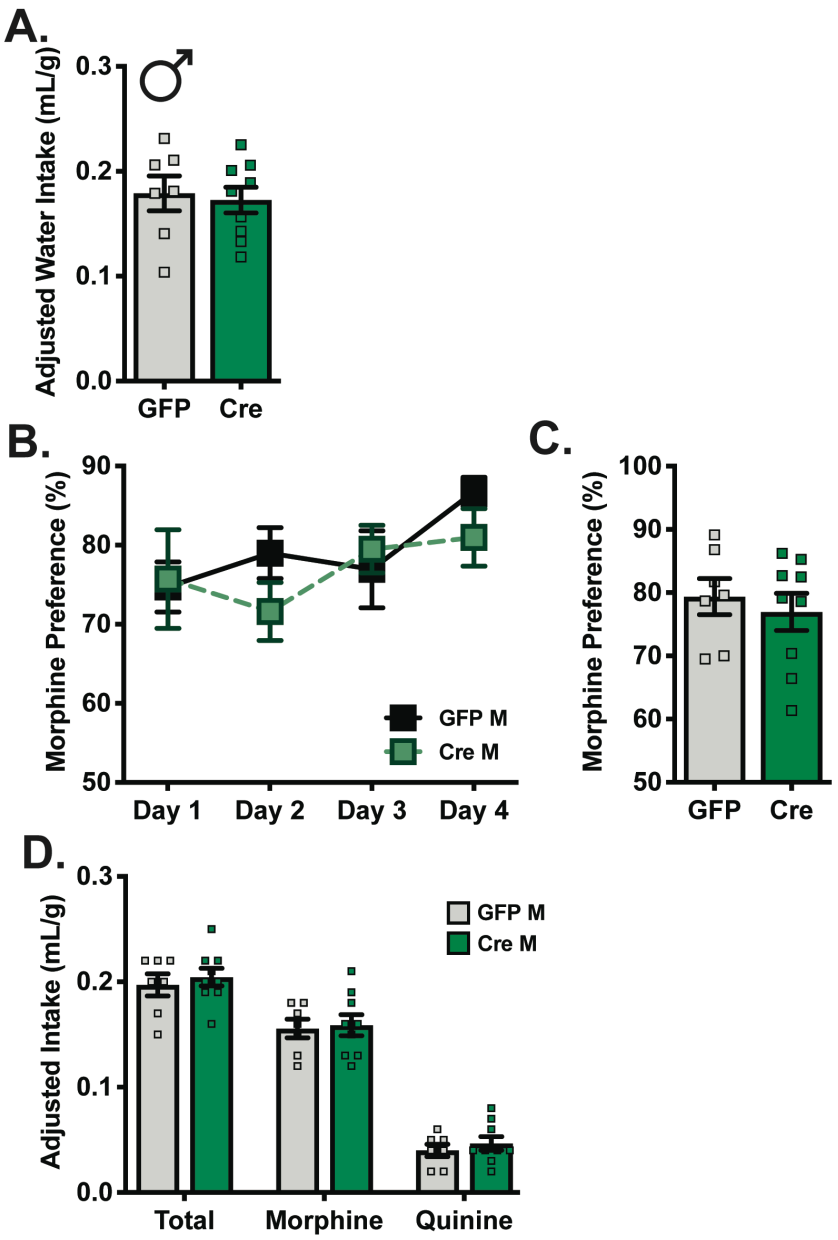

Figure 13. Heterozygous VTA SGK1 knockdown does not alter morphine preference in males.

A. No change in water intake was observed in males with heterozygous VTA SGK1 knockdown (Cre) compared to GFP controls (n=7-9, unpaired t-test).

B. Daily morphine preference in a TBC task was not changed by heterozygous knockdown of SGK1 in the VTA of males (n=7-9, two-way ANOVA with repeated measures).

C. Average morphine preference was similarly unaffected in VTA SGK1 knockdown mice compared to GFP controls (n=7-9, unpaired t-test).

D. Heterozygous SGK1 knockdown did not alter fluid intake during the morphine TBC task compared to controls (GFP) (n=7-9, unpaired t-test).

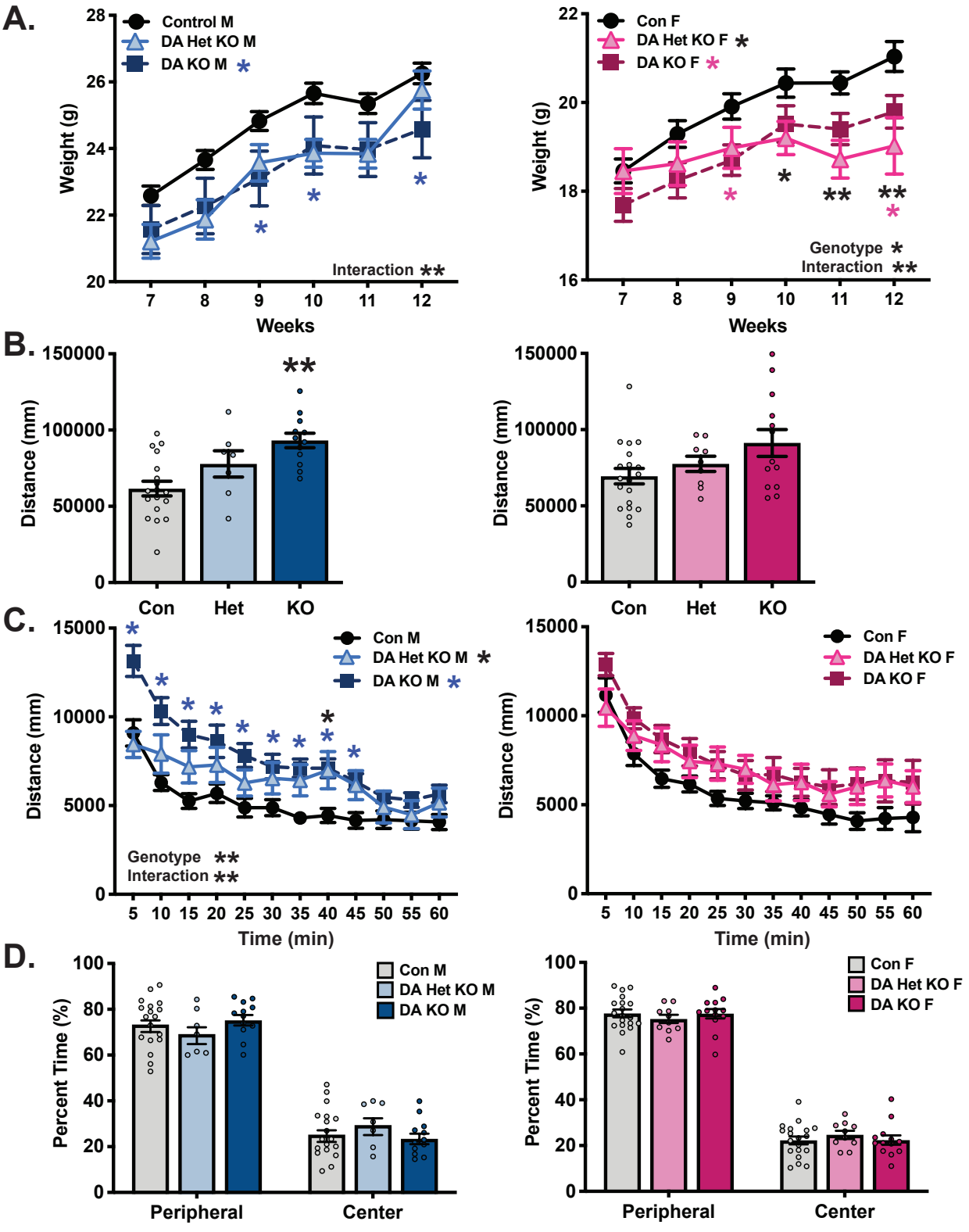

Figure 14. Body weight and locomotor activity are altered by DA SGK1 KO in both male and female mice.

Figure 14. (cont'd).
A. There was a significant decrease in body weight of DA SGK1 KO mice compared to littermate controls over early adulthood in both males and females (7-12 weeks of age) (male con=23, het=7, KO=12, two-way ANOVA with repeated measures followed by Sidak post-hoc, males time x genotype interaction: $p=0.0011$ and main effect of genotype: $p=0.0566$; female con=19, het=9, KO=13, two-way ANOVA with repeated measures followed by Sidak post-hoc, time x genotype interaction: $p<0.0001$ and main effect of genotype: $p=0.0328$).
B. SGK1 knockout in DA neurons increased locomotor activity in an open field task in males but not females (male con=18, het=7, KO=12, one-way ANOVA followed by Dunnett post-hoc, main effect: $p=0.0006$, Con vs KO: $p=0.0003$; female con=19, het=9, KO=13, one-way ANOVA, main effect: $p=0.0579$).
C. Locomotor activity was similarly increased over time in a habituation curve for DA SGK1 KO males but not females (male con=18, het=7, KO=12, two-way ANOVA with repeated measured followed by Dunnett post-hoc, main effect of genotype: $p=0.0006$, time x genotype interaction: $p<0.0001$; female con=19, het=9, KO=13, two-way ANOVA with repeated measures).
D. During the open field task, there was no effect of DA KO on center time in male or female mice (male con=18, het=7, KO=12, one-way ANOVA; female con=19, het=9, KO=13, one-way ANOVA).

caused by a reduction in body length or a shift in metabolism, though additional cohorts would be required to confirm this observation. Locomotion is regulated in part by activity of DA neurons (Langston et al., 1984; Ungerstedt, 1971), so we next sought to determine if locomotor activity was altered in an open field task. Male mice had a significant main effect of genotype, where homozygous DA SGK1 KO mice displayed a robust increase in total distance traveled during this task (Figure 14B). In contrast, females did not display a significant increase in total locomotor activity (Figure 14B). These effects were also seen in the habituation curve, where only male DA SGK1 KO locomotor activity was consistently higher than control littermates for the duration of the task (Figure 14C). Neither sex showed a difference in center time during the open filed task, which may suggest that anxiety-like behaviors are unaltered by DA SGK1 KO (Figure 14D). Overall, gene knockout of SGK1 in DA neurons caused a significant decrease in body weight in

male and female mice and increased locomotor activity in males. We next sought to determine if reward-related behaviors were altered in this cell type-specific model.

Reward behaviors, both natural and drug, are not changed in DA SGK1 KO mice

As DA is a critical mediator of reward behavior (Volkow and Morales, 2015), we first examined altered natural reward in homozygous DA SGK1 KO mice. To test this behavior, we again used the sucrose TBC task and observed no difference in fluid intake during water bottle acclimation (Figure 15A). Parallel to the VTA-specific knockdown model, DA SGK1 KO mice showed no difference in sucrose preference over 4 days (average percent sucrose preference in males Con:72.67±1.88 KO:76.64±3.70 and females Con:73.46±2.10 KO:78.07±1.59) or average fluid intake during the task (Figure 15B-C).

To observe morphine and cocaine reward behaviors, we utilized morphine TBC and cocaine CPP, as in previous experiments. First, in the morphine TBC task, there was no effect of homozygous DA SGK1 KO on morphine preference (average percent morphine preference in males Con:66.31±2.68 KO:63.63±2.97 and females Con:72.01±2.44 KO:71.68±2.57) or average fluid intake (Figure 16A-B), consistent with VTA-specific SGK1 knockdown (Figure 11A-B). In the CPP task, controls formed a preference for the cocaine-paired chamber as expected, and both male and female homozygous DA SGK1 KO mice showed no difference from littermate controls in time spent in the cocaine-paired chamber, indicative of intact drug reward processing (Figure 16C). Locomotor activity during the conditioning sessions showed an overall main effect of drug treatment for both male and female mice, but no significant effects were observed

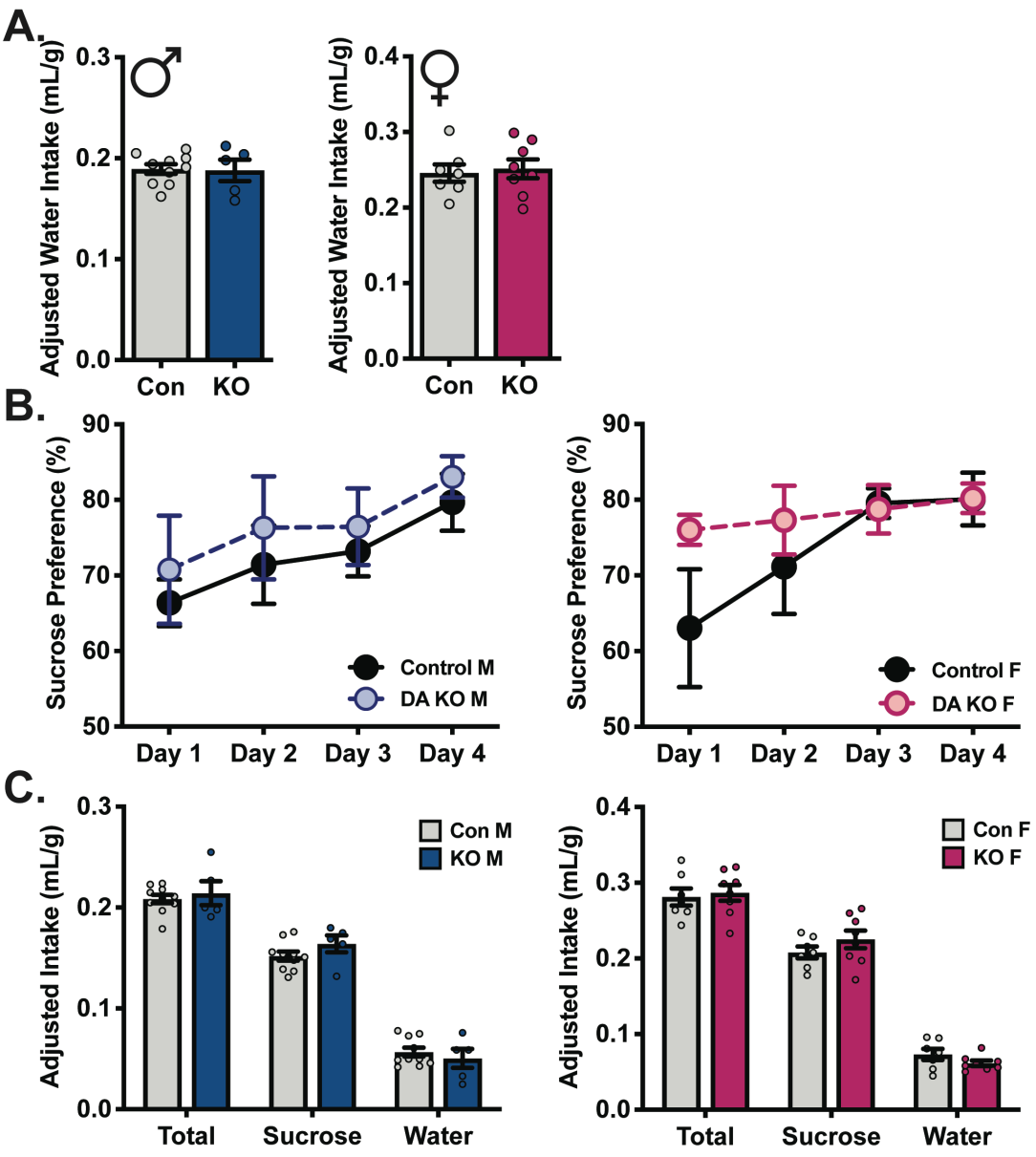

Figure 15. Fluid intake and preference for natural reward is not altered by DA SGK1 KO.

A. Water intake was not altered by DA SGK1 KO compared to controls (males n=9-13, unpaired t-test; females n=9-12, unpaired t-test).

B. No differences in sucrose preference (1%) were seen in DA SGK1 KO mice compared to littermate controls (male n=5-10, two-way ANOVA with repeated measures; female n=7-8, two-way ANOVA with repeated measures).

C. Average intake volumes were not altered during sucrose TBC task across genotype or sex (male n=5-10, unpaired t-test; female n=7-8, unpaired t-test).

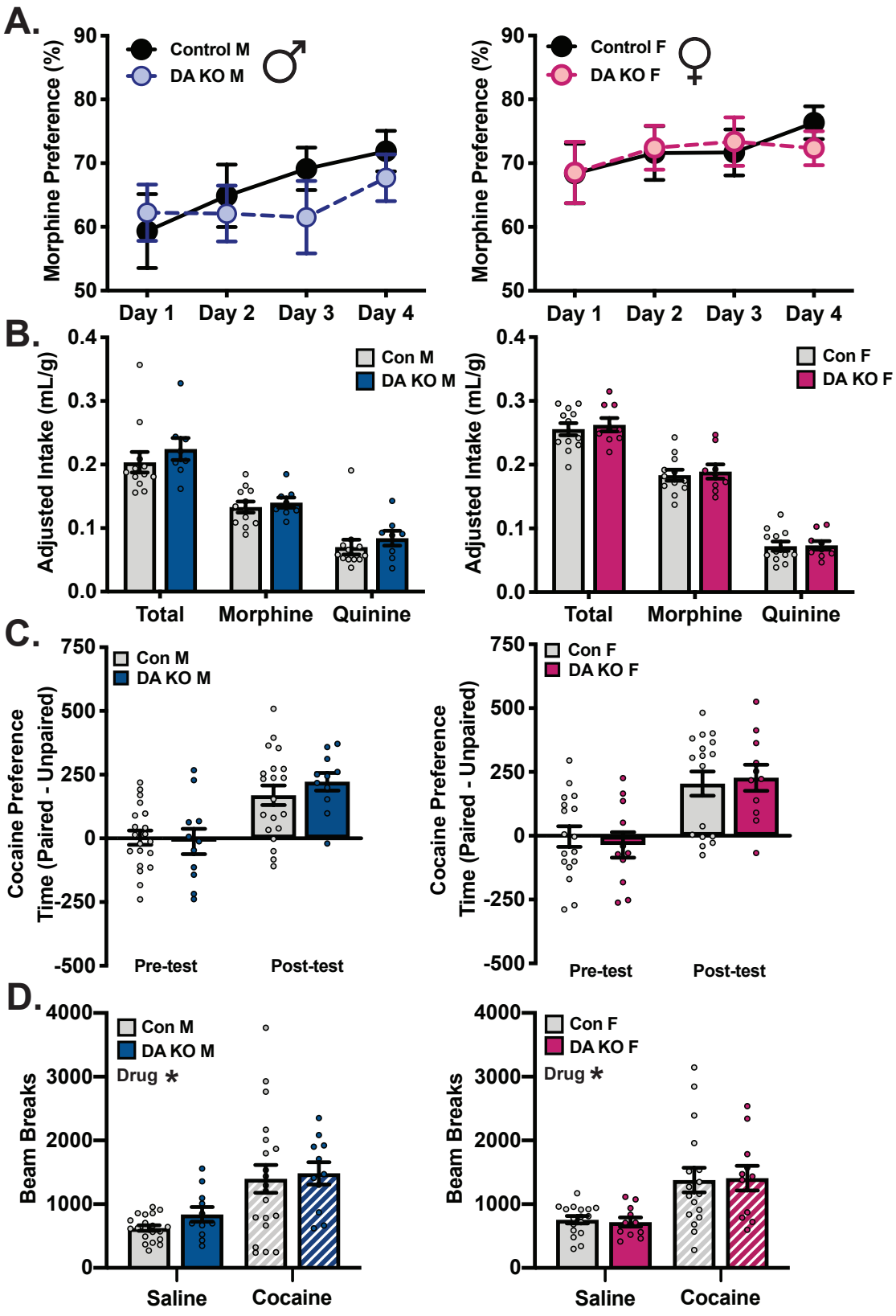

Figure 16. DA SGK1 KO does not alter morphine or cocaine reward.

Figure 16. (cont'd).
A. Morphine preference (0.05 mg/mL morphine vs. 0.01 mg/mL quinine) was not altered by DA SGK1 KO compared to controls (male n=8-12, two-way ANOVA with repeated measure; female n=9-12, two-way ANOVA with repeated measures).
B. Intake of fluids was not changed by genotype during morphine TBC task (male n=8-12, unpaired t-test; female n=9-12, unpaired t-test).
C. Cocaine reward measured by conditioned place preference (CPP) was not altered in male (12.5 mg/kg) or female (10 mg/kg) DA SGK1 KO mice (males n=11-20, unpaired t-test; females n=11-17, unpaired t-test).
D. There was not a main effect of genotype on locomotor activity during CPP conditioning sessions (males n=11-20, two-way ANOVA followed by Tukey post-hoc, males main effect of drug: $p=0.0001$; females n=11-17, two-way ANOVA followed by Tukey post-hoc, main effect of drug: $p<0.0001$).

between genotypes (Figure 16D). These data indicate that while DA SGK1 KO modestly altered baseline measurements of body weight and locomotor activity in an open field task (Figure 14), behaviors related to natural and drug reward, as assessed by TBC and CPP, were not be impaired by SGK1 knockout.

Discussion

The main objective of these studies was to characterize the effect of VTA- and DA neuron-specific SGK1 gene deletion on drug-related behaviors. Since VTA SGK1 mRNA, catalytic activity, and phosphorylation are upregulated by chronic cocaine and morphine treatment (Heller et al., 2015), we predicted that drug reward-related behaviors would be particular vulnerable to the effects of SGK1 deletion; however, neither VTA knockdown nor DA neuron-specific deletion was sufficient to disrupt morphine preference or cocaine reward. These data may suggest that SGK1 knockout does not directly affect drug reward behaviors. Other studies have identified stimuli-induced changes in VTA gene regulation or function without subsequent effects on behavior. For example, mRNA expression of the transcription factor E-twenty- six version 5 (ETV5) was found to be decreased in the

VTA following food restriction (Boender et al., 2012), but viral-mediated overexpression of ETV5 did not alter operant self-administration of sucrose pellets (Boender et al., 2014). Similarly, decreased VTA dopamine receptor 2 (D_2R) expression and sensitivity has been observed following psychostimulant self-administration (Marinelli and White, 2000; Sun et al., 2015); however, VTA knockdown of D_2R did not alter the number of sucrose or cocaine rewards earned during operant self-administration, though D_2R knockdown did increase motivation to earn rewards when rats were tested under a progressive ratio (de Jong et al., 2015). Together these studies demonstrate a disconnect between stimuli-induced gene regulation and an effect of experimenter manipulation.

However, as SGK1 shares overlapping substrates with other AGC family kinases, opportunistic compensation may explain a lack of observable effect in our models (Brunet et al., 2001; Burchfield et al., 2004; Di Cristofano, 2017; Kobayashi and Cohen, 1999; Pearce et al., 2010). Of note, opportunistic compensation of AKT and SGK1 has been characterized in breast cancer cells (Sommer et al., 2013). It is possible that a similar method for compensation occurred in our novel neuronal models, masking potential effects. Alternatively, compensation may have occurred at a circuit level. SGK1 is capable of regulating a variety of ion channels and transporters in the periphery (Lang et al., 2006), but the role of SGK1 in the brain is poorly understood. The electrophysiological effects of SGK1 KO are unknown in the VTA or in DA neurons and remain an area of interest. Further research is needed to determine if molecular or circuit level compensation is protective of SGK1 KO effects.

While SGK1 expression itself was not required for these drug-reward behaviors, recent work has highlighted the functional requirement for SGK1 catalytic activity and

phosphorylation, where experimental manipulation of SGK1 catalytic activity and phosphorylation in the PFC (Licznerski et al., 2015; Yuen et al., 2011) and hippocampus (Lee et al., 2007; Lee et al., 2006; Lee et al., 2003; Tsai et al., 2002; Tyan et al., 2008) have been linked to altered stress- and learning-induced behavior. Specifically, in the PFC, viral-mediated overexpression of a catalytically inactive SGK1 mutant (S422A) increased escape failures during a learned helplessness task (Licznerski et al., 2015). Similarly, SGK1 protein interference in the PFC via infusion of TAT-SGK1 peptides prevented stress-enhanced performance in a T-maze (Yuen et al., 2011). Both cases illustrate SGK1's ability to orchestrate behavior in rodent models of stress. In the hippocampus, catalytically inactive SGK1 (S422A) overexpression impaired escape latency in the Morris water maze (Tsai et al., 2002; Tyan et al., 2008) while phospho-deficient (S78A) overexpression decreased retention of contextual fear conditioning (Lee et al., 2007), suggesting that hippocampal SGK1 is functionally required for these learning tasks. These viral-mediated overexpression and protein interference methods for assessing the role of SGK1 protein function may be fundamentally different from KO models. The presence of SGK1 protein in these models, both endogenous and overexpressed, may not have engaged opportunistic compensation pathways, such that deficiencies in SGK1 function related to catalytic activity and phosphorylation were capable of impacting behavior where gene knockouts were not.

Despite a lack of drug reward effects, developmental DA SGK1 KO caused a significant decrease in body weight in male and female mice and increased open field locomotor activity in males. While changes in locomotor activity were not seen with our conditional VTA-specific knockdown, there were key differences between the two models,

namely timing and region-specificity of knockdown. First, our models differed in adult vs. developmental SGK1 gene deletion. The decreased body weight and increased activity found in the male DA SGK1 KO mice is consistent with literature demonstrating that developmental modification of the DA system causes changes in weight and locomotor activity. Characterization of homozygous dopamine transporter (DAT) KO mice showed decreased body weight and robustly increased locomotor activity in an open field assay (Giros et al., 1996). Additionally, this developmental model displayed significant adaptations in DA homeostasis as a result of gene deletion (Giros et al., 1996). Altered homeostasis is a caveat of developmental models that is important to keep in mind for the interpretation of these data.

Secondly, though the VTA represents a well characterized hub of DA neurons in the brain, DA neurons are expressed in other regions, including the substantia nigra, hypothalamus, periaqueductal gray, and olfactory bulbs (Bjorklund and Dunnett, 2007; Dahlstroem and Fuxe, 1964). The DA SGK1 KO mice used in these studies did not have gene deletion confined to the VTA, so SGK1 KO in another dopaminergic region may have contributed to the observed effects on locomotor behavior and weight. For example, DA signaling from the substantia nigra has been characterized as a critical mediator of locomotor activity (Langston et al., 1984; Ungerstedt, 1971). It is possible that SGK1 KO in this dopaminergic population resulted in the observed increase in open field locomotor activity.

Further, the modest effects found in both our models were consistent with the phenotype of whole body SGK1 KO mice, which showed only mild physiological effects at baseline (Fejes-Toth et al., 2008; Wulff et al., 2002). Instead, physiological challenges

are required to show deficits, examples of which have been best characterized in the renal system. Whole body SGK1 KO mice were viable and largely comparable to WT mice at baseline; however, when mice are placed on a salt-restricted diet, KO mice showed impaired salt retention in response to the change in diet (Wulff et al., 2002) and was linked to disrupted epithelial sodium channel (ENaC) processing (Fejes-Toth et al., 2008). Given mild KO effects compounded with the fact that acute cocaine or morphine treatment does not induce changes in VTA SGK1 catalytic activity or phosphorylation (Heller et al., 2015), more chronic paradigms may be needed to assess reward behavior following long-term drug administration, such as locomotor sensitization or self-administration.

In summary, our data suggest that SGK1 expression in the VTA or in DA neurons is not required for early stages of drug reward or intake. Strategies that utilize mutant-SGK1 overexpression or interfere with the function of endogenous protein expression may be necessary to uncover the role of SGK1 in the context of drug-related behaviors. Altogether, this work provides novel information about SGK1 expression and builds on a limited knowledgebase for the function of SGK1 in the central nervous system, both necessary to understanding the impact of SGK1 regulation on drug-reward behaviors.

Table 2. Statistics table for Chapter 2 results.

	Description	Sex	Group size	Statistical test	Main effect	t- or f-statistic	p-value	Post-hoc test	Post-hoc comparison	p-value
Figure 10. Establishment of VTA SGK1 knockdown mouse model.										
Fig 10B	VTA KD *sgk1* mRNA	Both	GFP: 14 Cre: 19	Unpaired t-test		t=2.117, df=31	p= 0.0424			
Figure 11. Fluid intake and natural reward are not altered by VTA SGK1 knockdown.										
Fig 11A	VTA KD Average water intake	Male	GFP: 7 Cre: 10	Unpaired t-test		t=1.571, df=15	p=0.1370			
		Female	GFP: 5 Cre: 7	Unpaired t-test		t=0.3936, df=10	p=0.7021			
Fig 11B	VTA KD Daily sucrose preference	Male	GFP: 7 Cre: 10	Two-way ANOVA with RM	Time x Virus Time Virus	F (3, 45) = 0.009888 F (2.098, 31.48) = 13.64 F (1, 15) = 1.321	p=0.9986 p<0.0001 p=0.2685			
		Female	GFP: 5 Cre: 7	Two-way ANOVA with RM	Time x Virus Time Virus	F (3, 30) = 4.277 F (2.294, 22.94) = 8.968 F (1, 10) = 0.01378	p=0.0126 p=0.0009 p=0.9089	Sidak	GFP vs. Cre Day 1 GFP vs. Cre Day 2 GFP vs. Cre Day 3 GFP vs. Cre Day 4	p=0.6031 p=0.5654 p=>0.9999 p=0.4674
In text	VTA KD Average sucrose preference	Male	GFP: 7 Cre: 10	Unpaired t-test		t=1.149, df=15	p=0.2685			
		Female	GFP: 5 Cre: 7	Unpaired t-test		t=0.1161, df=10	p=0.9099			
Fig 11C	VTA KD Average total fluid intake	Male	GFP: 7 Cre: 10	Unpaired t-test		t=0.9788, df=15	p=0.3432			
		Female	GFP: 5 Cre: 7	Unpaired t-test		t=0.1052, df=10	p=0.9183			
	Average sucrose intake	Male	GFP: 7 Cre: 10	Unpaired t-test		t=1.109, df=15	p=0.2848			
		Female	GFP: 5 Cre: 7	Unpaired t-test		t=0.08385, df=10	p=0.9348			
	Average water intake	Male	GFP: 7 Cre: 10	Unpaired t-test		t=0.7222, df=15	p=0.4813			

Table 2. (cont'd).

		Female	GFP: 5 Cre: 7	Unpaired t-test		t=0.006383, df=10	p=0.9950
Figure 12. VTA SGK1 knockdown does not alter or morphine or cocaine reward behaviors.							
Fig 12A	VTA KD Daily morphine preference	Male	GFP: 11 Cre: 19	Two-way ANOVA with RM	Time x Virus Time Virus	F (3, 84) = 2.257 F (3, 84) = 5.748 F (1, 28) = 0.8411	p=0.0877 p=0.0013 p=0.3669
		Female	GFP: 14 Cre: 19	Two-way ANOVA with RM	Time x Virus Time Virus	F (3, 93) = 1.247 F (3, 93) = 3.887 F (1, 31) = 4.027	p=0.2972 p=0.0115 p=0.0536
In text	VTA KD Average morphine preference	Male	GFP: 11 Cre: 19	Unpaired t-test		t=0.9169, df=28	p=0.3670
		Female	GFP: 14 Cre: 19	Unpaired t-test		t=2.006, df=31	p=0.0536
Fig 12B	VTA KD Average total fluid intake	Male	GFP: 11 Cre: 19	Unpaired t-test		t=1.281, df=28	p=0.2107
		Female	GFP: 14 Cre: 19	Unpaired t-test		t=0.05917, df=31	p=0.9532
	Average morphine intake	Male	GFP: 11 Cre: 19	Unpaired t-test		t=1.502, df=28	p=0.1443
		Female	GFP: 14 Cre: 19	Unpaired t-test		t=1.425, df=31	p=0.1641
	Average quinine intake	Male	GFP: 11 Cre: 19	Unpaired t-test		t=0.4314, df=28	p=0.6694
		Female	GFP: 14 Cre: 19	Unpaired t-test		t=1.913, df=31	p=0.0650
Fig 12C	VTA KD CPP posttest scores	Male	GFP: 18 Cre: 19	Unpaired t-test		t=0.8243, df=35	p=0.4154
		Female	GFP: 13 Cre: 16	Unpaired t-test		t=0.1844, df=27	p=0.8551

Table 2. (cont'd).

Fig 12D	VTA KD CPP conditioning locomotor activity	Male	GFP: 18 Cre: 19	Two-way ANOVA	Drug x Virus Drug Virus	F (1, 70) = 0.01100 F (1, 70) = 21.17 F (1, 70) = 0.1245	p=0.9168 p<0.0001 p=0.7253	Tukey	Saline:GFP vs. Saline:Cre Saline:GFP vs. Cocaine:GFP Saline:GFP vs. Cocaine:Cre Saline:Cre vs. Cocaine:GFP Saline:Cre vs. Cocaine:Cre Cocaine:GFP vs. Cocaine:Cre	p=0.9981 p=0.013 p=0.0044 p=0.0189 p=0.0065 p=0.9882
		Female	GFP: 13 Cre: 16	Two-way ANOVA	Drug x Virus Drug Virus	F (1, 54) = 0.1042 F (1, 54) = 15.47 F (1, 54) = 0.1825	p=0.7481 p=0.0002 p=0.6709	Tukey	Saline :GFP vs. Saline :Cre Saline :GFP vs. Cocaine:GFP Saline :GFP vs. Cocaine:Cre Saline :Cre vs. Cocaine:GFP Saline :Cre vs. Cocaine:Cre Cocaine:GFP vs. Cocaine:Cre	p=0.9999 p=0.0834 p=0.0165 p=0.0747 p=0.0127 p=0.9513
Figure 13. Heterozygous VTA knockdown does not alter morphine preference in males.										
Fig 13A	Heterozygous VTA KD Average water intake	Male	GFP: 7 Cre: 9	Unpaired t-test		t=0.3181, df=14	p=0.7551			
Fig 13B	Heterozygous VTA KD Daily morphine preference	Male	GFP: 7 Cre: 9	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (3, 42) = 1.142 F (3, 42) = 3.131 F (1, 14) = 0.3306 F (14, 42) = 3.318	p=0.3434 p=0.0355 p=0.5745 p=0.0013			
Fig 13C	Heterozygous VTA KD Average morphine preference	Male	GFP: 7 Cre: 9	Unpaired t-test		t=0.5744, df=14	p=0.5748			
Fig 13D	Heterozygous VTA KD Average total fluid intake	Male	GFP: 7 Cre: 9	Unpaired t-test		t=0.5543, df=14	p=0.5881			
	Average morphine intake	Male	GFP: 7 Cre: 9	Unpaired t-test		t=0.2283, df=14	p=0.8227			
	Average quinine intake	Male	GFP: 7 Cre: 9	Unpaired t-test		t=0.7462, df=14	p=0.4679			
Figure 14. Body weight and locomotor activity, but not anxiety, are altered by DA SGK1 KO in both male and female mice.										
Fig 14A	DA SGK1 KO Weight	Males	Con: 23 Het: 7 KO: 12	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (10, 195) = 3.091 F (5, 195) = 185.4 F (2, 39) = 3.094 F (39, 195) = 60.78	p=0.0011 p<0.0001 p=0.0566 p<0.0001	Sidak	7 Control M vs. Het KO M 7 Control M vs. KO M 8 Control M vs. Het KO M 8 Control M vs. KO M 9 Control M vs. Het KO M	p=0.1863 p=0.2526 p=0.0628 p=0.0857 p=0.2404

Table 2. (cont'd).

								9 Control M vs. KO M	p=0.0243
								10 Control M vs. Het KO M	p=0.0602
								10 Control M vs. KO M	p=0.0451
								11 Control M vs. Het KO M	p=0.1339
								11 Control M vs. KO M	p=0.0858
								12 Control M vs. Het KO M	p=0.7887
								12 Control M vs. KO M	p=0.0299
		Females	Con: 19	Two-way ANOVA with RM	Time x Genotype	F (10, 190) = 5.275	p<0.0001	Sidak	7 Control F vs. Het KO F	p=0.9999
			Het: 9		Time	F (5, 190) = 48.48	p<0.0001		7 Control F vs. KO F	p=0.218
			KO: 13		Genotype	F (2, 38) = 3.743	p=0.0328		8 Control F vs. Het KO F	p=0.395
					Subject	F (38, 190) = 27.68	p<0.0001		8 Control F vs. KO F	p=0.0639
									9 Control F vs. Het KO F	p=0.169
									9 Control F vs. KO F	p=0.0263
									10 Control F vs. Het KO F	p=0.0478
									10 Control F vs. KO F	p=0.1138
									11 Control F vs. Het KO F	p=0.0036
									11 Control F vs. KO F	p=0.0643
									12 Control F vs. Het KO F	p=0.0005
									12 Control F vs. KO F	p=0.0217
In text	DA SGK1 KO Body length at 12 weeks of age	Male	Con: 7 KO: 10	Unpaired t-test		t=1.629, df=15	p=0.1241			
Fig 14B	DA SGK1 KO Locomotor activity	Male	Con: 18 Het: 7 KO: 12	One-way ANOVA		F (2, 34) = 9.339	p=0.0006	Dunnett	Con M vs. Het KO M	p=0.1354
									Con M vs. KO M	p=0.0003
		Female	Con: 19 Het: 9 KO: 13	One-way ANOVA		F (2, 38) = 3.074	p=0.0579			
Fig 14C	DA SGK1 KO Habituation curve	Male	Con: 18 Het: 7 KO: 12	Two-way ANOVA with RM	Time x Genotype	F (22, 374) = 2.831	p<0.0001	Dunnett	5 Con M vs. Het KO M	p=0.7396
					Time	F (11, 374) = 37.28	p<0.0001		5 Con M vs. KO M	p<0.0001
					Genotype	F (2, 34) = 9.373	p=0.0006		10 Con M vs. Het KO M	p=0.1735
					Subject	F (34, 374) = 15.17	p<0.0001		10 Con M vs. KO M	p<0.0001
									15 Con M vs. Het KO M	p=0.0851
									15 Con M vs. KO M	p<0.0001
									20 Con M vs. Het KO M	p=0.1808
									20 Con M vs. KO M	p=0.0006
									25 Con M vs. Het KO M	p=0.264
									25 Con M vs. KO M	p=0.0006

Table 2. (cont'd).

							30 Con M vs. Het KO M p=0.1567 30 Con M vs. KO M p=0.0083 35 Con M vs. Het KO M p=0.0535 35 Con M vs. KO M p=0.0011 40 Con M vs. Het KO M p=0.0164 40 Con M vs. KO M p=0.0021 45 Con M vs. Het KO M p=0.0754 45 Con M vs. KO M p=0.0138 50 Con M vs. Het KO M p=0.697 50 Con M vs. KO M p=0.2143 55 Con M vs. Het KO M p=0.9183 55 Con M vs. KO M p=0.2632 60 Con M vs. Het KO M p=0.4356 60 Con M vs. KO M p=0.0941
		Female	Con: 19 Het: 9 KO: 13	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (33, 462) = 0.9163 F (5.135, 215.7) = 31.08 F (3, 42) = 1.978 F (42, 462) = 19.23	p=0.6041 p<0.0001 p=0.1319 p<0.0001
Fig 14D	DA SGK1 KO Anxiety in open field	Male	Center Con: 18 Het: 7 KO: 12	One-way ANOVA		F (2, 34) = 0.8585	p=0.4328
		Male	Peripheral Con: 18 Het: 7 KO: 12	One-way ANOVA		F (2, 34) = 0.8592	p=0.4325
		Female	Center Con: 19 Het: 9 KO: 13	One-way ANOVA		F (2, 39) = 0.4309	p=0.6530
		Female	Peripheral Con: 19 Het: 9 KO: 13	One-way ANOVA		F (2, 39) = 0.4303	p=0.6533

Figure 15. Fluid intake and preference for natural reward is not altered by VTA DA KO.

Table 2. (cont'd).

Fig 15A	DA SGK1 KO Average water intake	Male	Con: 10 KO: 5	Unpaired t-test		t=0.1311, df=13	p=0.8977
		Female	Con: 7 KO: 8	Unpaired t-test		t=0.3296, df=13	p=0.7470
Fig 15B	DA SGK1 KO Daily sucrose preference	Male	Con: 10 KO: 5	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (3, 39) = 0.01410 F (3, 39) = 2.459 F (1, 13) = 1.157 F (13, 39) = 1.217	p=0.9977 p=0.0772 p=0.3017 p=0.3046
		Female	Con: 7 KO: 8	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (3, 39) = 1.045 F (3, 39) = 2.450 F (1, 13) = 3.156 F (13, 39) = 0.6906	p=0.3837 p=0.0780 p=0.0990 p=0.7601
In text	VTA KO Average sucrose preference	Male	Con: 10 KO: 5	Unpaired t-test		t=1.075, df=13	p=0.3018
		Female	Con: 7 KO: 8	Unpaired t-test		t=1.776, df=13	p=0.0991
Fig 15C	DA SGK1 KO Average total fluid intake	Male	Con: 10 KO: 5	Unpaired t-test		t=0.5621, df=13	p=0.5836
		Female	Con: 7 KO: 8	Unpaired t-test		t=0.3539, df=13	p=0.7291
	Average sucrose intake	Male	Con: 10 KO: 5	Unpaired t-test		t=1.383, df=13	p=0.1901
		Female	Con: 7 KO: 8	Unpaired t-test		t=1.183, df=13	p=0.2580
	Average water intake	Male	Con: 10 KO: 5	Unpaired t-test		t=0.7019, df=13	p=0.4951
		Female	Con: 7 KO: 8	Unpaired t-test		t=1.507, df=13	p=0.1557

Figure 16. DA SGK1 KO does not alter morphine or cocaine reward.

Table 2. (cont'd).

Fig 16A	DA SGK1 KO Daily morphine preference	Male	Con: 12 KO: 8	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (3, 54) = 0.5270 F (3, 54) = 1.592 F (1, 18) = 0.5089 F (18, 54) = 1.848	p=0.6656 p=0.2020 p=0.4847 p=0.0427			
		Female	Con: 12 KO: 9	Two-way ANOVA with RM	Time x Genotype Time Genotype Subject	F (3, 57) = 0.2738 F (3, 57) = 1.038 F (1, 19) = 0.008160 F (19, 57) = 2.210	p=0.8441 p=0.3829 p=0.9290 p=0.0111			
In text	VTA KO Average morphine preference	Male	Con: 12 KO: 8	Unpaired t-test		t=0.7137, df=18	p=0.4846			
		Female	Con: 12 KO: 9	Unpaired t-test		t=0.09002, df=19	p=0.9292			
Fig 16B	DA SGK1 KO Average total fluid intake	Male	Con: 12 KO: 8	Unpaired t-test		t=0.8533, df=18	p=0.4047			
		Female	Con: 12 KO: 9	Unpaired t-test		t=0.4895, df=19	p=0.6301			
	Average morphine intake	Male	Con: 12 KO: 8	Unpaired t-test		t=0.5629, df=18	p=0.5804			
		Female	Con: 12 KO: 9	Unpaired t-test		t=0.4213, df=19	p=0.6783			
	Average quinine intake	Male	Con: 12 KO: 8	Unpaired t-test		t=0.8259, df=18	p=0.4197			
		Female	Con: 12 KO: 9	Unpaired t-test		t=0.1430, df=19	p=0.8878			
Fig 16C	DA SGK1 KO CPP posttest scores	Male	Con: 20 KO: 11	Unpaired t-test		t=0.9219, df=29	p=0.3642			
		Female	Con: 17 KO: 11	Unpaired t-test		t=0.3210, df=26	p=0.7508			
Fig 16D	DA SGK1 KO CPP conditioning locomotor activity	Male	Con: 20 KO: 11	Two-way ANOVA	Drug x Genotype Drug	F (1, 58) = 0.1445 F (1, 58) = 17.42	p=0.7052 p=0.0001	Tukey	Saline:Con M vs. Saline:KO M Saline:Con M vs. Cocaine:Con M	p=0.8072 p=0.0018

Table 2. (cont'd).

				Genotype	F (1, 58) = 0.7855	p=0.3791		Saline:Con M vs. Cocaine:KO M	p=0.0038
								Saline:KO M vs. Cocaine:Con M	p=0.1041
								Saline:KO M vs. Cocaine:KO M	p=0.0961
								Cocaine:Con M vs. Cocaine:KO M	p=0.9841
	Female	Con: 17	Two-way ANOVA	Drug x Genotype	F (1, 52) = 0.05082	p=0.8225		Saline:Con F vs. Saline:KO F	p=0.9983
		KO: 11		Drug	F (1, 52) = 18.96	p<0.0001	Tukey	Saline:Con F vs. Cocaine:Con F	p=0.0094
				Genotype	F (1, 52) = 0.0001199	p=0.9913		Saline:Con F vs. Cocaine:KO F	p=0.0173
								Saline:KO F vs. Cocaine:Con F	p=0.0166
								Saline:KO F vs. Cocaine:KO F	p=0.0245
								Cocaine:Con F vs. Cocaine:KO F	p=0.9987

Chapter 3. Ventral tegmental area SGK1 activity regulates cocaine and morphine reward in a cell type-specific manner

Introduction

Serum- and glucocorticoid-inducible kinase 1 (SGK1) was initially identified as an immediate early gene transcriptionally induced by serum and glucocorticoids (Webster et al., 1993). This early work was critical in identifying SGK1 as a serine/threonine kinase and member of the AGC family of kinases, with other prominent members including AKT and protein kinase C (PKC). AGC kinases achieve maximal activity though phosphorylation of the activation segment and hydrophobic motif (Kobayashi and Cohen, 1999; Kobayashi et al., 1999; Park et al., 1999; Pearce et al., 2010). For SGK1, these phosphorylation events occur at Ser422 and Thr256, by 3-phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) respectively (Garcia-Martinez and Alessi, 2008; Park et al., 1999). Additionally, family members share a highly conserved ATP-binding motif (Pearce et al., 2010). In SGK1's sequence, Lys127 is the critical residue for ATP binding, as mutations at this site have been shown to produce a catalytically inactive protein (Park et al., 1999; Snyder et al., 2002; Strutz-Seebohm et al., 2005). SGK1 catalytic activity has been shown to regulate a variety of ion channels, transporters, and transcription factors in the periphery (Lang et al., 2006); however, far less is known about the functional role of SGK1 in the central nervous system.

Expression of SGK1 mRNA and protein is found throughout the brain (Lang et al., 2010), and transcription can be induced by a variety of stimuli, including learning, stress, seizures, and drugs of abuse (Cooper et al., 2017; Gao et al., 2015; Heller et al., 2015;

Husain et al., 2015; Licznerski et al., 2015; McClung et al., 2005; Nichols and Sanders-Bush, 2002; Piechota et al., 2010; Tsai et al., 2002; Yuen et al., 2011). However, nearly all information characterizing SGK1 mRNA regulation was collected as part of large-scale screening investigations, with few studies expanded upon transcript findings with protein-level assays. Given SGK1's classification as an immediate early gene, transcription is likely induced in response to a large number of stimuli and may not directly parallel modifications in protein function. Rather, protein-level regulation, such as altered catalytic activity, may be better measurements for assessing stimuli-induced changes in SGK1 function. In the central nervous system, SGK1 catalytic activity and its potential impact on animal behavior remains poorly understood.

Recent work has demonstrated that SGK1 catalytic activity is capable of modulating learning- and stress-related behaviors. In the hippocampus, training in the Morris water maze task increased SGK1 phosphorylation at both Thr256 and Ser422 (Lee et al., 2006). As phosphorylation at these residues is required for maximal kinase activity, these data suggest that kinase activity was likely increased by training. Moreover, hippocampal SGK1 kinase activity is behaviorally relevant, as mice overexpressing SGK1 mutants with deficits in catalytic activity (T256A or S422A) took longer to find a hidden platform in the Morris water maze task (Lee et al., 2006; Lee et al., 2003; Tsai et al., 2002; Tyan et al., 2008), indicating an impairment in context-dependent learning. Additionally, stress is capable of regulating SGK1 protein in the prefrontal cortex (PFC). In a model of learned helplessness, total protein levels were decreased in rats showing greater escape failures (Licznerski et al., 2015), showing an inverse relationship between SGK1 levels and depressive-like behaviors. Consistent with this finding, PFC overexpression of a

SGK1 mutant with decreased activity (S422A) was found to increase the number of escape failures in a learned helplessness task (Licznerski et al., 2015), implicating decreased PFC SGK1 activity as a contributor to mood disorders. In contrast, acute stress induced by a forced swim increased PFC SGK1 total protein levels (Yuen et al., 2011). Blocking SGK1 function via protein interference (TAT-SGK1) prevented increased performance in a T-maze task, indicative of increased SGK1 activity promoting stress-induced enhancement of working memory (Yuen et al., 2011). While these studies highlight opposing SGK1 regulation by different forms of stress, together, they implicate SGK1 catalytic activity in the central nervous system as an important mediator of learning and stress behaviors.

Though SGK1 function in response to learning and stress has been examined, relatively little is known about SGK1 catalytic activity in the context of drug of abuse. Using RNA-sequencing, we previously identified SGK1 as one of only five genes transcriptionally upregulated in the ventral tegmental area (VTA) by both chronic cocaine and morphine treatment (Heller et al., 2015). Expanding on transcript regulation, VTA SGK1 catalytic activity was significantly upregulated following chronic, but not acute, administration of cocaine or morphine (Heller et al., 2015); however, the functional relevance of VTA SGK1 catalytic activity remains unknown. VTA overexpression of a constitutively active SGK1 mutant (S422D) resulted in disrupted locomotor sensitization in response to cocaine or morphine injections but not changes in drug conditioned place preference (CPP) (Heller et al., 2015). While this study suggests a potential role for SGK1 in drug-related behaviors, it only assessed increased catalytic activity, leaving questions surrounding decreased activity unanswered.

Given that chronic cocaine and morphine increased VTA SGK1 catalytic activity (Heller et al., 2015), in this study we sought to determine whether decreased activity attenuated drug-associated behaviors, primary measured using cocaine CPP and morphine two-bottle choice tasks. Moreover, given that the VTA is a heterogenous brain region, composed of 60-65% dopamine and 30-35% GABA neurons (Nair-Roberts et al., 2008; Swanson, 1982), we assessed cell type-specific decreases in VTA SGK1 activity to determine the neuronal population critical for driving behavioral effects.

Materials and methods

Animals

Herpes simplex virus (HSV) overexpression experiments used adult (8−9 weeks) male c57BL/6J mice purchased from Jackson Laboratory. Mice were allowed to habituate to the animal facility >7 days prior to the start of experiments. Cell type-specific experiments utilized adult (8−15 weeks) male and female heterozygous dopamine transporter (DAT)-Cre recombinase (Cre) mice (Jackson Laboratories, 006660) or vesicular GABA transporter (VGAT)-Cre mice (Jackson Laboratories, 028862), bred inhouse. Transgenic lines were backcrossed to the c57Bl/6J background, and genotype was confirmed at 3−4 weeks using the following primers:

DAT Common Forward: 5’-TGG CTG TTG GTG TAA AGT GG-3’

DAT WT Reverse: 5’-GGA CAG GGA CAT GGT TGA CT-3’

DAT Mutant Reverse: 5’-CCA AAA GAC GGC AAT ATG GT-3’

VGAT Forward: 5’-CTT CGT CAT CGG CGG CAT CTG-3’

VGAT Reverse: 5’-CCA AAA GAC GGC AAT ATG GT-3’

The SGK1 rescue experiment utilized dopamine neuron SGK1 knockout mice, as described in Chapter 2. Briefly, homozygous floxed SGK1 (FlxSGK1) mice (Fejes-Toth et al., 2008), initially obtained from A. Náray-Fejes-Tóth (Dartmouth University), were crossed with DAT-Cre mice to generate a dopamine neuron-specific knockout of SGK1. Mouse genotyping was performed at 3−4 weeks of age using the primers for the DAT-Cre mouse line described above and the following primers for the FlxSGK1 mouse line:

SGK1 Forward: 5'-AAAGCTTATCTCAAACCCAAACCAA-3'

SGK1 WT Reverse: 5'-CTCATTCCAGACCGCTGACAAG-3'

SGK1 Mutant Reverse: 5'-CTCAGTCTCTTTTGGGCTCTTT-3'

All mice were housed at 22−25°C on a 12 hr light-dark cycle with food and water ad libitum and behavioral testing took place during the light phase, unless otherwise noted. For cocaine conditioned place preference (CPP) and locomotor sensitization tasks, mice were allowed to habituate to the behavioral room for 30 min under red light conditions before the start of behavioral measurements. All experiments were approved by Michigan State University Institutional Animal Care and Use Committee (IACUC) and were carried out in accordance with the guidelines set in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Viral constructs

The HSV vectors encoding green fluorescent protein (GFP) or HA-tagged SGK1 (wild-type SGK1, catalytically inactivity K127Q mutant, and constitutively active S422D mutant) have been previously used and validated (Heller et al., 2015; Steffke et al., 2020). To

generate the adeno associated virus (AAV) vectors, restriction sites for AscI and NcoI were first added to the 5' and 3' ends of the HA-SGK1-K127Q transgene (CMV-HA-SGK1-K127Q), respectively via PCR using the following primers purchased from IDT:

Forward: NcoI-HA-SGK1: 5'-GGTGGTCCATGGCTTACCCATACGATGTTC-3'

Reverse: AscI-HA-SGK1: 5'-GCGGCGCGCCAGATCAGAGGAAGGAGTCCATA-3'

HA-SGK1-K127Q was then cloned into the pAAV-Ef1α-DIO-EYFP (Addgene # 27056) vector using AscI (3') and NcoI (5') restriction endonucleases (New England Biosystems), and correct insertion was verified by dideoxysequencing. This cloning resulted in EYFP being replaced by HA-SGK1-K127Q.

HSVs (HSV-CMV-GFP, HSV-HA-SGK1-WT-GFP, HSV-HA-SGK1-K127Q-GFP) were packaged by Dr. Rachael Neve at the Gene Technology Core (Massachusetts General Hospital). AAVs were packaged by the University of North Carolina GTC Vector Core (AAV2-EF1α-DIO-HA-SGK1-K127Q, AAV2-EF1α-DIO-EYFP-WPRE-pA, rAAV2/TR-eGFP). SGK1 expression from all constructs was verified *in vivo* via IHC analysis for GFP and HA-tagged SGK1 following stereotaxic injection into the VTA.

Viral-mediated gene transfer

Stereotaxic surgeries were completed using established procedures (Heller et al., 2015; Kaska et al., 2017). Adult mice (8−10 weeks) were anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine. All mice received bilateral intra-VTA infusions at established coordinates (-3.2 mm A/P, +1.0 mm M/L, −4.6 mm D/V, 7° angle).

For HSV surgeries, 0.5 µl of SGK1 construct or control HSV-CMV-GFP was infused, and 12−24 hours of recovery were provided before the start of behavioral experiments.

For AAV surgeries, 0.4 µl of rAAV2-EF1α-DIO-HA-SGK1-K127Q (AAV-DIO-K127Q) construct or control virus was infused. Control mice were Cre-negative mice that received rAAV2/TR-eGFP or Cre-positive mice that received AAV2-EF1α-DIO-EYFP-WPRE-pA (AAV-DIO-EYFP), and since no effect of Cre expression observed, mice were grouped together. At least 21 days were allowed for recovery and viral expression before the start of behavioral experiments. Following the completion of all behavioral studies, viral targeting was confirmed by standard histological methods.

In the SGK1 rescue experiment utilizing AAVs, animals in the control group were homozygous FlxSGK1 and Cre-negative with AAV-DIO-K127Q infusions. The DA SGK1 knockout (KO) mice were homozygous FlxSGK1 and Cre-positive with AAV-DIO-EYFP expression, and the rescue group consisted of homozygous FlxSGK1 and Cre-positive mice with AAV-DIO-K127Q expression.

Validation of viral expression with immunohistochemistry (IHC)

Following stereotaxic surgery and allowing time for viral expression (>2 days for HSVs and >21 days for AAVs), VTA expression of SGK1 was confirmed using IHC. Briefly, mice with VTA overexpression of SGK1 mutants were transcardially perfused with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were postfixed in the paraformaldehyde solution overnight before cryopreservation in 30% sucrose in PBS. Brains were then sliced into 30 µm sections for use in IHC, and unused sections were stored in 0.01% sodium azide-PBS for long-term storage. For IHC, brain

slices were blocked in a 3% normal donkey serum (NDS) and 0.3% triton-X PBS solution for 60 min prior to the start of an overnight primary antibody incubation. This incubation contained primary antibodies in a 3% NDS and 0.3% tween-20 PBS solution. The following day, slices were rinsed in PBS before a 2 hr incubation in the secondary antibody solution, containing fluorescent secondary antibodies in PBS. Slices were again rinsed in PBS before dehydration using increasing concentrations of ethanol and tissue clearing using Citrisolv. Finally, slices were mounted and coverslipped before imaging. Additionally, control wells lacking primary antibody (no primary controls) were run to confirm specificity of fluorescent signal.

Because specificity issues with total SGK1 antibodies had been previously identified in western blot analyses (unpublished), virally overexpressed SGK1 protein was identified using the HA-tag. For Cre-inducible SGK1 expression, HA-tag co-expression with tyrosine hydroxylase (TH), a marker for DA neurons, was used to confirm cell type-specific SGK1 expression in DAT-Cre and VGAT-Cre mice. Primary antibodies: Tyrosine hydroxylase (1:5000, Millipore, T1299) and HA-tag (C29F4) (1:1000, Cell Signaling, 3724S). Secondary antibodies: Cy3 donkey anti-rabbit (1:500, Jackson ImmunoResearch, 711-165-152) and Cy5 goat anti-mouse (1:500, Jackson ImmunoResearch, 115-175-146).

Viral targeting

Following behavioral testing, mice were sacrificed and their brains postfixed for 3 days in 10% formalin before switching to 30% sucrose-PBS for cryo-preservation. Brains were then sliced into 30 µm sections for targeting analysis. Mice were excluded from analysis

if GFP expression was unilateral or outside of the VTA.

Drugs

Cocaine was purchased from Sigma and dissolved in 0.9% sterile saline for all experiments. For HSV CPP experiments, male mice were conditioned with 7.5 mg/kg cocaine. In AAV CPP experiments, 12.5 mg/kg cocaine was used to condition male mice and 10 mg/kg cocaine was used for female mice. This lower drug dose was selected for females male in order to produce comparable preference scores between the sexes, as female rodents have been shown to form a conditioned preference at lower doses of cocaine (Russo et al., 2003). For self-administration experiments, 0.5 mg/kg/infusion of cocaine was used for studies examining acquisition and the progressive ratio assay. Cocaine doses of 1.0, 0.5, 0.25, 0.063, 0.016, and 0.001 mg/kg/infusion were used during dose-response testing.

The NIDA Drug Supply Program generously provide morphine sulfate. For all morphine two-bottle choice experiments, morphine dissolved in vehicle solution (0.2% sucrose in water). Studies utilizing HSVs used 0.03 mg/mL morphine while AAV studies used 0.1 mg/mL.

Cocaine conditioned place preference (CPP)

Cocaine CPP was performed using established procedures (Heller et al., 2015). In a 3-chambered CPP box (San Diego Instruments), mice were placed in the center chamber and allowed to freely explore for 20 min to test for initial chamber bias. If >20% of time

was spent in the left vs. right chamber, mice were excluded from analysis due to side bias. During the two conditioning days, mice were restricted to the left or right chamber for 30 min and received a control (saline) i.p. injection in the morning and in the afternoon received a cocaine (Sigma) i.p. injection in the opposite chamber. In the posttest, mice were placed in the center chamber and allowed to freely explore for 20 min. The CPP score was calculated as the time (seconds) spent in the drug-paired minus saline-paired chamber. Total beam breaks during each conditioning session were used to calculate locomotor activity, and two-day averages were used to analyze locomotor activity during the conditioning sessions.

For HSV experiments, mice underwent the CPP pretest the day prior to stereotaxic surgery. Following one day of post-surgery recovery, conditioning session began and mice (male) were drug conditioned with 7.5 mg/kg cocaine. In AAV experiments, all behavioral measures took place at least 21 days after stereotaxic surgery. Males were conditioned with 12.5 mg/kg cocaine and females with 10 mg/kg cocaine in order to achieve equivalent preference scores between the sexes. CPP was also run at a lower dose of drug, with male mice conditioned at 7.5 mg/kg cocaine and female mice at 5 mg/kg cocaine.

Cocaine locomotor sensitization

Locomotor sensitization in response to repeated cocaine injections was performed as previously described (Heller et al., 2015). Locomotor activity was measured in a 3-chambered CPP box (San Diego Instruments) and calculated as total beam breaks. One day prior to the start of sensitization, mice were habituated to the boxes for 60 min. Next,

in daily testing sessions mice were first i.p. injected with saline and locomotor behavior was recorded for 30 min. Immediately following the completion of the saline session, mice were injected with cocaine (7.5 mg/kg for males and 5 mg/kg for females) and locomotor behavior was recorded for an additional 30 min. Locomotor sensitization was run for 7 days and data are displayed as total bream breaks on days 1 and 7.

Cocaine self-administration

Cocaine self-administration protocols were adapted from previous methods (Ozburn et al., 2012; Walker et al., 2018). Mice underwent self-administration experiments in operant boxes (MedAssociates), and all animals were individually housed for the duration of all experiments. Each box contained two nose pokes. Selecting the active nose poke resulted in the delivery of a reward and a concurrent light cue (nose poke illumination and house light turned off for the duration of the timeout period, 5 seconds for sucrose reward or 20 seconds for cocaine reward). Action on the inactive nose poke had no consequences.

Mice were first trained to respond for sucrose pellets (food training). Briefly, mice were food restricted to 90% of their free feeding weight and sucrose self-administration was conducted on a fixed ratio of 1 (FR1) schedule in 1 hr sessions for 7 days. The maximum number of rewards that could be earned in a session was set to 50, at which point the session ended automatically. Mice were returned to ad libitum food once acquisition criteria were met. Acquisition was defined as $\geq$15 rewards earned and $\geq$75% accuracy for the active lever, and mice that did not acquire were removed from the experiment.

Following food training, mice were anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine)

and implanted with a chronic indwelling jugular catheter. Following surgery, mice were allowed to recovery for 3 days, and 50 µl of ampicillin (50 mg/mL in 0.9% sterile saline) was administered during the recovery period. Catheter patency was maintained with twice daily infusions of 50 µl of heparinized saline (30 U of heparin in 0.9% sterile saline). Catheter patency was assessed at the start and end of each experiment using 20 µl Brevital (5 mg/mL in 0.9% sterile saline), and catheters were considered functional if the animal lost muscle tone in <5 seconds from the time of infusion.

Cocaine self-administration was conducted (19.6 µl infusion, 0.5 mg/kg/infusion) on a FR1 schedule in 2 hr session for 7−10 days. Mice were limited to 50 rewards per session. Acquisition was again determined as ≥15 rewards earned and ≥75% accuracy for the active lever, and mice that did not acquire were excluded from further experimental assessment. Following acquisition of cocaine self-administration, a cohort of mice underwent self-administration (19.6 µl infusions, 0.5 mg/kg/infusion) on a progressive ratio (PR) schedule in a 2 hr session (Richardson and Roberts, 1996). Additional mice were put through a dose-response analysis. For this, mice self-administered a descending set of doses (19.6 µl infusion, 1.0, 0.5, 0.25, 0.063, 0.016, and 0.001 mg/kg/infusion) on an FR1 schedule in 2 hr sessions (method adapted from (Arena et al., 2019; Ozburn et al., 2012)). Each dose was run for two consecutive days, and the average of responses at each dose were used for analysis. The number of rewards that could be earned in a session were not limited during PR or dose-response testing.

Two-bottle voluntary choice (TBC) tasks

To assess voluntary intake, mice were singly housed with access to two 50 mL conical

tubes fitted with sipper tops following established procedures (Cooper et al., 2017; Kaska et al., 2017). Briefly, bottle volume was measured at the same time each morning and bottle placement was swapped to account for potential side bias. Mice were excluded from analysis if they exhibited >30% preference for one bottle/side during morphine preference testing. For HSV experiments, mice were placed in cages immediately after stereotaxic surgeries and allowed to habituate overnight to both bottles containing only water before the start of preference measurements the next morning. AAV experiments differed in that mice were allowed to recovery from surgery for at least 21 days before starting initial water habituation, lasting 2 days. At the end of bottle habitation for both sets of viral surgeries, water bottles were substituted for preference bottles and assessment occurred for 4 days.

In all studies, fluid intake (mL) was measured to determine preference over time. Results are reported as percent preference (select fluid consumed/total fluid consumed x 100, calculated for each day), average preference (4-day average of daily percent preference), and average fluid intake (4-day averages of individual fluid intakes and total intakes).

Morphine preference

For morphine preference, water bottles were exchanged for bottles containing a vehicle solution of 0.2% sucrose with either morphine sulfate or quinine (Sigma, 22640), a bitter taste control. HSV experiments used 0.03 mg/mL morphine vs. 0.006 mg/mL quinine while AAV experiments used 0.1 mg/mL morphine vs. 0.02 mg/mL quinine (Belknap et al., 1993; Forgie et al., 1988). Male and female mice were run at the same preference doses.

Sucrose preference

In the sucrose preference task, the water bottles were replaced with bottles containing either 1% sucrose or water. All experiments and both sexes used the same percentage of sucrose for preference tasks.

Quinine aversion

To measure quinine aversion, bottles containing 0.1mM quinine or water were used to replace water bottles at the end of the habitation period.

Statistics

The list of full statistical analyses and results can be found in Table 3. GraphPad Prism was used for all statistical analyses, and all values are represented as mean±SEM. The means of two groups were compared using an unpaired t-test (two-tailed), and the means of three groups were compared using a one-way analysis of variance (ANOVA), followed by a Tukey or Dunnett post-hoc test when appropriate. A two-way ANOVA was used when comparing two independent variables, and a two-way ANOVA with repeating measures (RM) when two independent variables with repeated measures were compared, followed by a Tukey and Sidak or Dunnett post-hoc test when appropriate, respectively. The comparison of three independent variables with repeated measures used a three-way ANOVA with repeated measures followed by a Sidak post-hoc test. Males and females were always analyzed separately for behavior. Significance was defined as *$p<0.05$ and **$p<0.01$.

Results

Decreased VTA SGK1 activity reduces conditioned cocaine and morphine reward

Chronic, but not acute, cocaine or morphine administration had been previously shown to increase VTA SGK1 catalytic activity (Heller et al., 2015); however, the functional relevance of SGK1 activity remains unknown. Therefore, we generated an SGK1 mutant with an amino acid substitution at Lys127 to create a catalytically inactive mutant (Park et al., 1999; Snyder et al., 2002; Steffke et al., 2020; Strutz-Seebohm et al., 2005), resulting in decreased SGK1 catalytic activity in the VTA. Adult male mice were bilaterally injected with a herpes simplex virus (HSV) expressing the catalytically inactive SGK1 mutant (K127Q), wild-type SGK1 (WT), or control GFP into the VTA and conditioned cocaine reward was assessed by conditioned place preference (CPP). Following conditioning (7.5 mg/kg cocaine), time spent in the drug paired chamber was significantly decreased in mice with K127Q overexpression compared to GFP controls (Figure 17A), indicative of reduced cocaine reward. Overexpression of WT SGK1 in the VTA had no effect of cocaine reward compared to GFP controls (Figure 17A), suggesting that increased VTA SGK1 protein expression alone did not impact reward. Viral expression did not influence locomotor activity during either the posttest or during conditioning sessions (Figure 17B-C).

Since VTA SGK1 activity was similarly poised to modulate morphine related-behaviors, morphine preference (0.03 mg/mL) in a two-bottle choice task (TBC) was used to assess changes in reward (Experiments completed by Barbara Fallon). Male mice with VTA overexpression of the catalytically inactive SGK1 mutant showed a significant

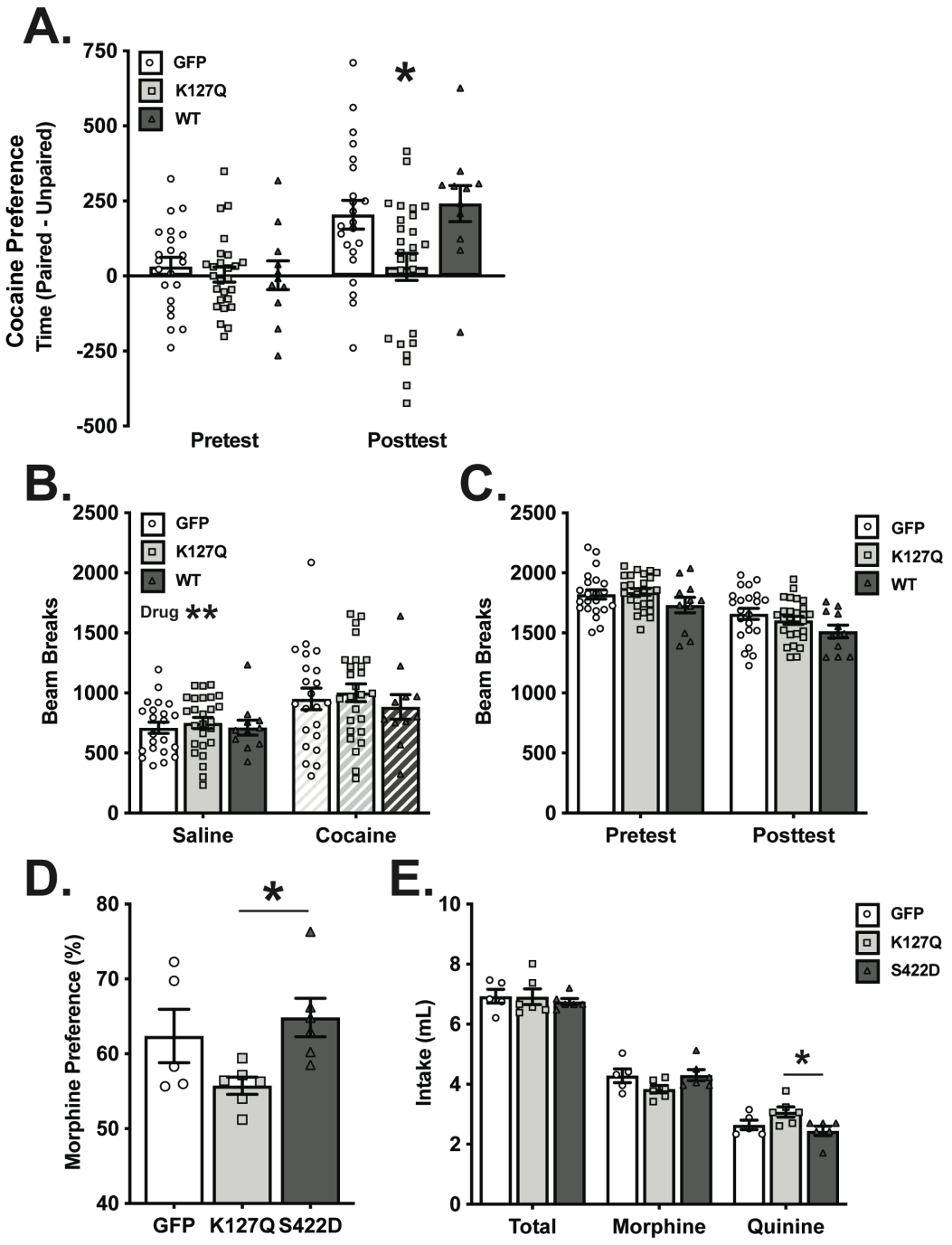

Figure 17. Decreased VTA SGK1 activity is capable of modulating cocaine and morphine reward.

Figure 17. (cont'd).
A. Overexpression of a catalytically inactive SGK1 mutant (K127Q) decreased cocaine CPP compared to GFP controls while WT SGK1 had no effect (GFP=22, K127Q=26, WT=11, one-way ANOVA followed by a Dunnett post-hoc test: p=0.0177).
B. While there was a main effect of drug, locomotor activity during the CPP conditioning sessions was not changed by either overexpression of the K127Q mutant or WT SGK1 (GFP=22, K127Q=26, WT=11, two-way ANOVA followed by a Tukey post-hoc test: p=0.0006).
C. Viral manipulation similarly had no effect on locomotor activity during CPP pretest or posttest sessions (GFP=22, K127Q=26, WT=11, one-way ANOVA).
D. Morphine preference was significantly decreased in mice with decreased VTA SGK1 activity (K127Q) compared to mice with constitutively active SGK1 (S422D) (GFP=5, K127Q=6, S422D=6, one-way ANOVA followed by a Tukey post-hoc test: p=0.0465).
E. Total fluid intake and morphine intake were not altered by viral manipulation, but there was a significant increase in quinine intake of K127Q-expressing mice compared to the S422D group (GFP=5, K127Q=6, S422D=6, one-way ANOVA followed by a Tukey post-hoc-test: p=0.0357).

decrease in preference for the morphine bottle compared to mice expressing a constitutively active SGK1 mutant (S422D) (Figure 17D). There was a non-significant decrease in the K127Q group compared to GFP controls (Figure 17D), and another cohort is needed to confirm this observation. Mice with constitutively active SGK1 were not different from GFP controls, consistent with previous work demonstrating no effect of increased VTA SGK1 activity on morphine CPP (Heller et al., 2015). These data suggest that while decreased activity may be capable of impairing morphine reward, increased activity does not further drive these drug behaviors. Total fluid intake was not different between groups (Figure 17E). Together, these findings indicate that decreased VTA SGK1 catalytical activity is sufficient to impair cocaine reward and suggest that SGK1 is capable of modulating morphine reward.

Reduced SGK1 catalytic activity in VTA DA, but not GABA, neurons alters conditioned cocaine reward and psychomotor activity.

Given that SGK1 catalytic activity is capable of regulating drug reward, we next sought to determine if these effects were cell type-specific. The VTA is a heterogenous brain region made up of 60-65% DA and 30-35% GABA neurons (Nair-Roberts et al., 2008; Swanson, 1982). To alter SGK1 catalytic activity in a cell type-specific manner, we generated novel Cre recombinase (Cre)-dependent K127Q constructs. By overexpressing these constructs in the VTA of adult male and female dopamine transporter (DAT)-Cre or vesicular GABA transporter (VGAT)-Cre mice, SGK1 catalytic activity was decreased specifically in VTA DA or GABA neurons, respectively. Cocaine CPP (12.5 mg/kg for males and 10 mg/kg for females) was again used to determine potential changes in conditioned cocaine reward induced by decreased VTA DA or GABA SGK1 catalytic activity. Following conditioning, control mice formed a preference for the cocaine-paired chamber; however, when catalytic activity was decreased in VTA DA neurons, both male and female mice spent significantly less time in the drug-paired chamber compared to controls, indicating a deficit in cocaine reward (Figure 18A). In contrast, both male and female mice with decreased SGK1 catalytic activity in VTA GABA neurons showed no difference from controls (Figure 18A). Together, these data indicate that it was a reduced catalytic activity in VTA DA, but not GABA, neurons that drove earlier findings (Figure 17A).

In addition to altered cocaine reward, mice with decreased VTA DA SGK1 catalytic activity showed altered psychomotor activity in response injections of cocaine. DA-specific expression elicited enhanced psychomotor effects in both male and female mice

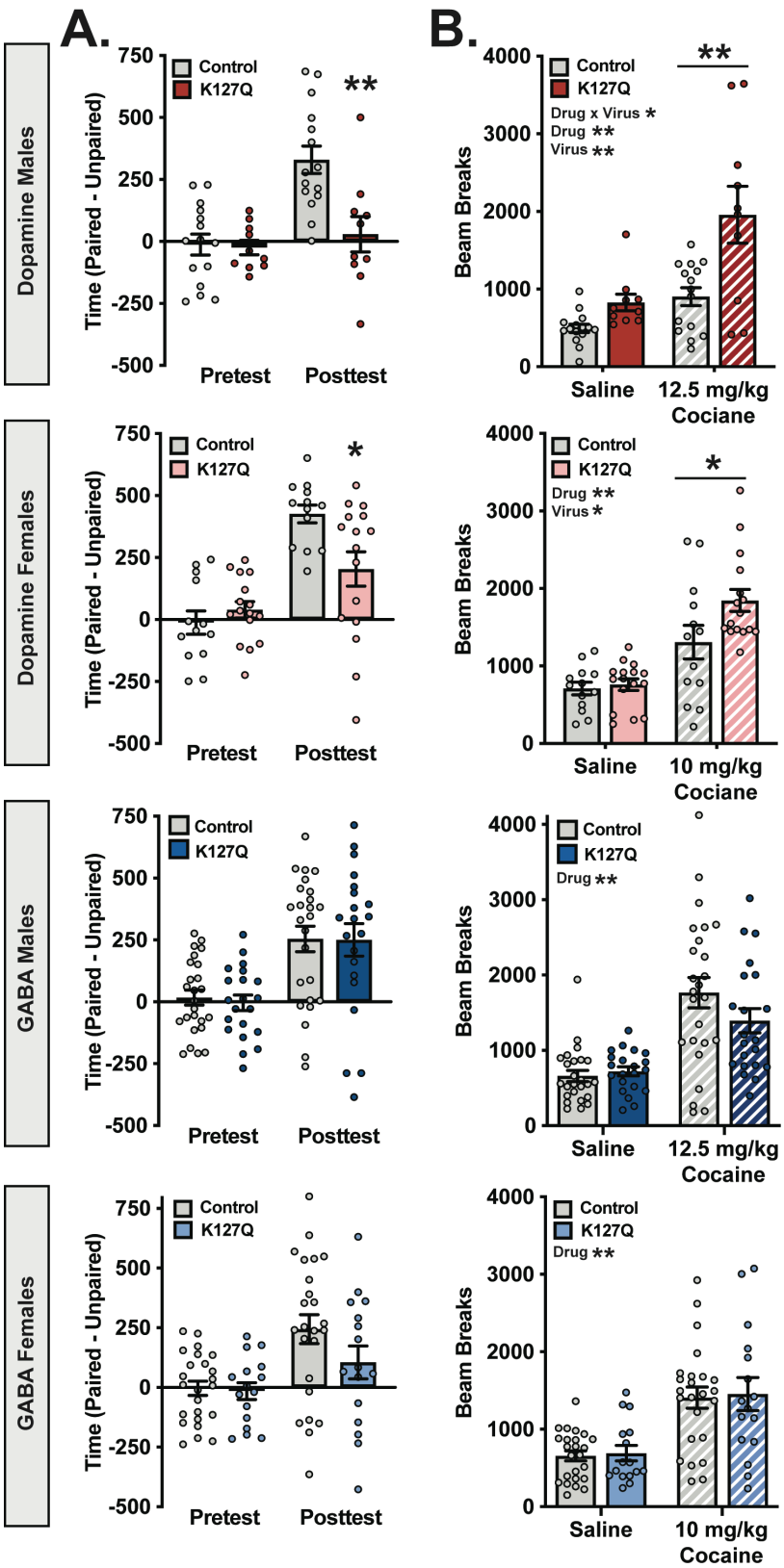

Figure 18. A reduction in SGK1 catalytic activity in VTA DA, but not GABA, neurons drives impaired cocaine reward.

Figure 18. (cont'd).
A. DA expression. Overexpression of a catalytically inactive SGK1 mutant (K127Q) in VTA DA neurons significantly reduced cocaine conditioned reward in male and female mice (male n=10-15, unpaired t-test: p=0.0026; females n=13-16, unpaired t-test: p=0.0134).
GABA expression. Decreased catalytic activity of SGK1 in VTA GABA neurons did not alter cocaine conditioned place preference (male n=21-25, unpaired t-test; females n=16-24, unpaired t-test).
B. DA expression. During CPP conditioning, male and female mice with decreased activity in VTA DA neurons (K127Q) displayed a significant increase in psychomotor activity (male n=10-15, two-way ANOVA followed by a Tukey post-hoc test, significant main effects of drug: $p<0.0001$ and virus: $p=0.0002$ and an interaction of drug x virus:0.0423, post-hoc: $p=0.0005$; females n=13-16, two-way ANOVA followed by a Tukey post-hoc test, significant main effects of drug: $p<0.0001$ and virus: 0.0365, post-hoc: $p=0.0372$).
GABA expression. Psychomotor activity was not changed by VTA GABA neuron expression of the K127Q mutant in male or female mice (male n=21-25, two-way ANOVA followed by a Tukey post-hoc test, main effect of drug: $p<0.0001$; females n=16-24, two-way ANOVA followed by a Tukey post-hoc test, main effect of drug: $p<0.0001$).

(Figure 18B). Specifically, in addition to the expected main effect of drug, both sexes showed main effects of virus; however, only males showed an interaction of virus x drug. GABA-specific K127Q expression did not alter drug-induced locomotor activity during the conditioning sessions (Figure 18B). A modest but significant increase in locomotor activity was observed in male DA K127Q-expressing mice during the pretest, though no difference was found during the posttest (Figure 19A). Changes in pretest or posttest locomotor activity were not observed in female DA or either sex of GABA manipulated mice (Figure 19A-B).

To further investigate enhanced locomotor activity driven by decreased SGK1 catalytic activity, a cohort of mice underwent cocaine locomotor sensitization (7.5 mg/kg cocaine for males and 5 mg/kg for females) to assess changes in drug-induced locomotor activity driven by cell type-specific decreased in VTA SGK1 activity. Male DA-specific

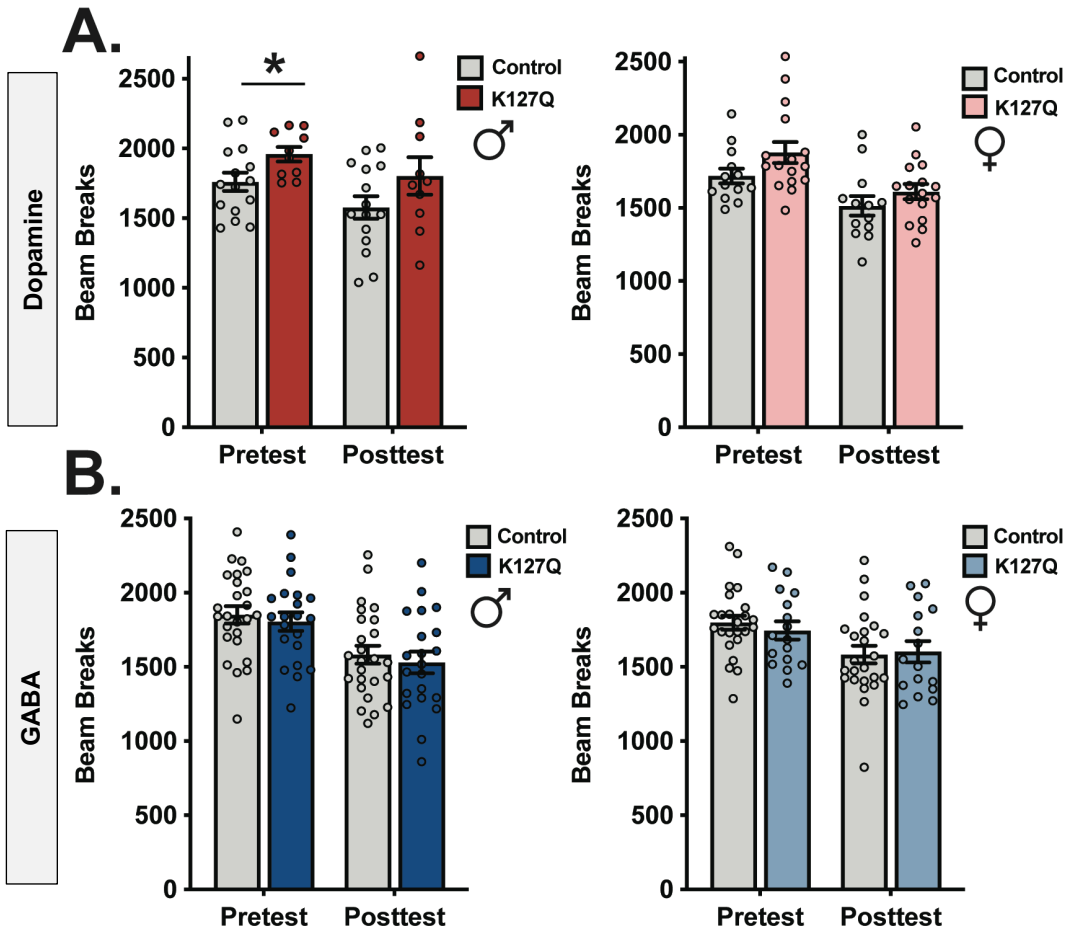

Figure 19. Locomotor activity during conditioned place preference pretest is altered by DA neuron-K127Q expression in male mice.
A. Male mice with decreased SGK1 catalytic activity in VTA DA neurons displayed a significant increase in locomotor activity during the CPP pretest session but not posttest session, and no effect was observed in female mice (males n=10-15, unpaired t-test, pretest:
p=0.0388; females n=13-16, unpaired t-test).
B. Neither male nor female mice with K127Q expression in VTA GABA neurons showed altered locomotor activity during CPP pretest or posttest sessions (males n=21-28, unpaired t-test; females n=16-24, unpaired t-test).

K127Q expression robustly increased cocaine-induced locomotor activity but did not show an effect of time, likely due to the low number of mice (Figure 20A). An additional cohort is needed to determine if there is a main effect of time on the observed psychomotor effect. Females with decreased catalytic activity in VTA DA neurons showed

a main effect of drug and an interaction of day x drug; however, there was not a significant effect of virus on psychomotor activity (Figure 20A). Conversely, mice with decreased activity in VTA GABA neurons again showed an expected main effect of cocaine treatment but viral expression did not have a significant effect on locomotor activity (Figure 20B). These data replicated the psychomotor effect originally observed during CPP conditioning of male DA manipulated mice (Figure 18B) and indicate that this viral manipulation is capable of modulating acute drug-related behaviors. However, female DA K127Q-expressing mice did not replicate a main effect of virus on locomotor activity. As sensitization was performed at a lower cocaine dose than CPP conditioning, we next sought to determine if CPP conditioned at a lower dose was similarly altered by VTA DA SGK1 manipulation.

Following CPP conditioning at a low dose of cocaine (7.5 mg/kg for males and 5 mg/kg for females), differences in cocaine reward were not observed when mice with DA neuron-specific K127Q expression were conditioned at a low dose of cocaine (Figure 21A), suggesting that these effects on reward may be dose-dependent. Interestingly, while no effect of viral expression on CPP was observed at this dose, male mice still exhibited a robust increase in psychomotor activity during conditioning sessions (Figure 21B). No altered psychomotor effect was observed in females (Figure 21B), consistent with the sensitization study (Figure 20A).

A deficit in morphine preference is not driven by decreased SGK1 catalytic activity in VTA DA neurons

As VTA SGK1 catalytic activity was capable of modulating morphine preference,

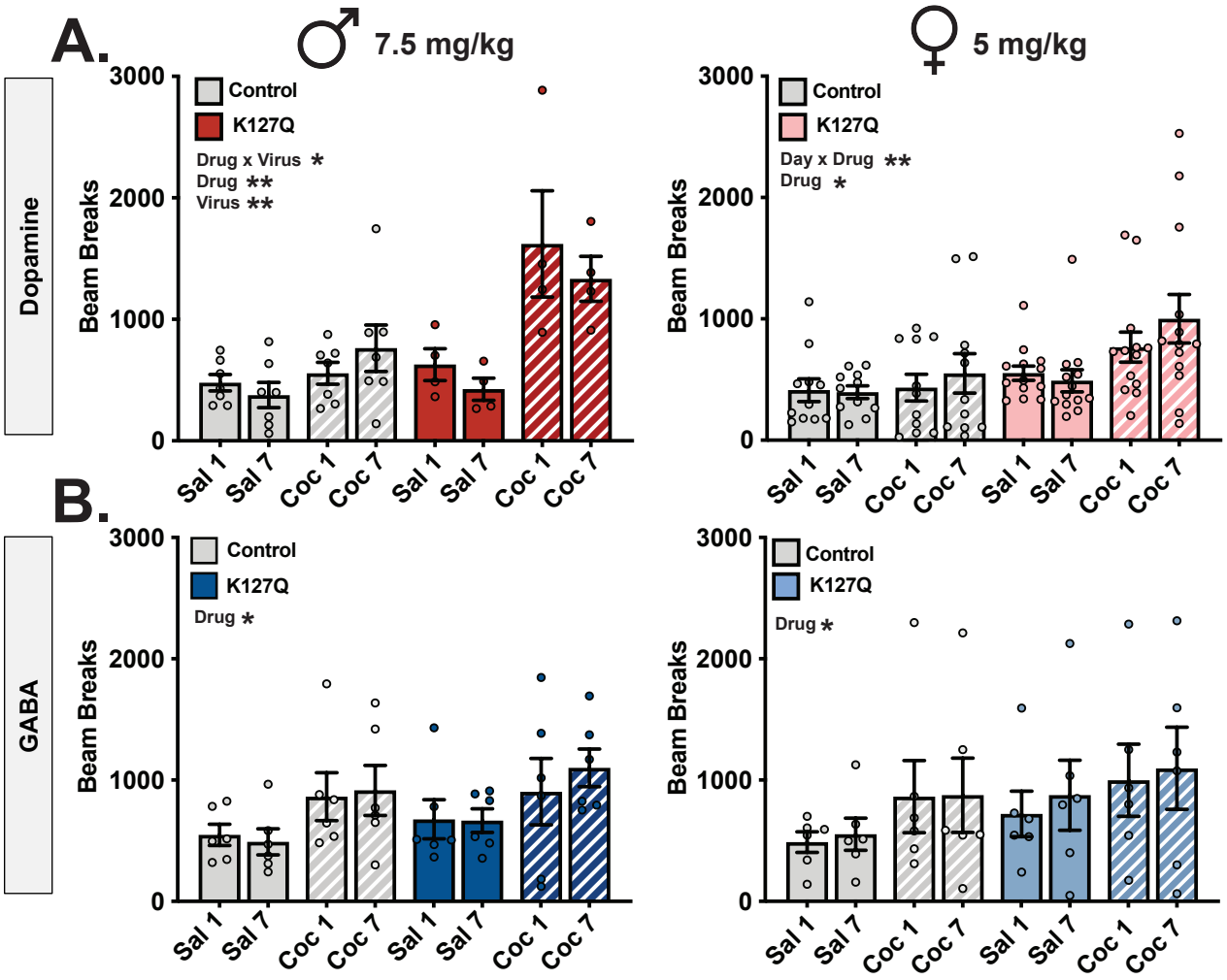

Figure 20. Cocaine-induced psychomotor activity is altered by decreased VTA DA SGK1 catalytic activity.

A. Decreased VTA DA SGK1 catalytic activity altered psychomotor activity in male, but not female, mice (males n=4-7, three-way ANOVA with repeated measured, main effects of drug: p=0.0002, virus: p=0.022, and an interaction of drug x virus: p=0.0047; females n=11-13, three-way ANOVA with repeated measured, main effect of drug: p=0.0168 and an interaction of day x drug: p=0.0037)

B. A main effect of drug was observed in male and female mice with decreased VTA GABA SGK1 catalytic activity, but no effect of virus was detected (males n=6, three-way ANOVA with repeated measured, main effect of drug: p=0.0175; females n=6, three-way ANOVA with repeated measured, main effect of drug: p=0.0246).

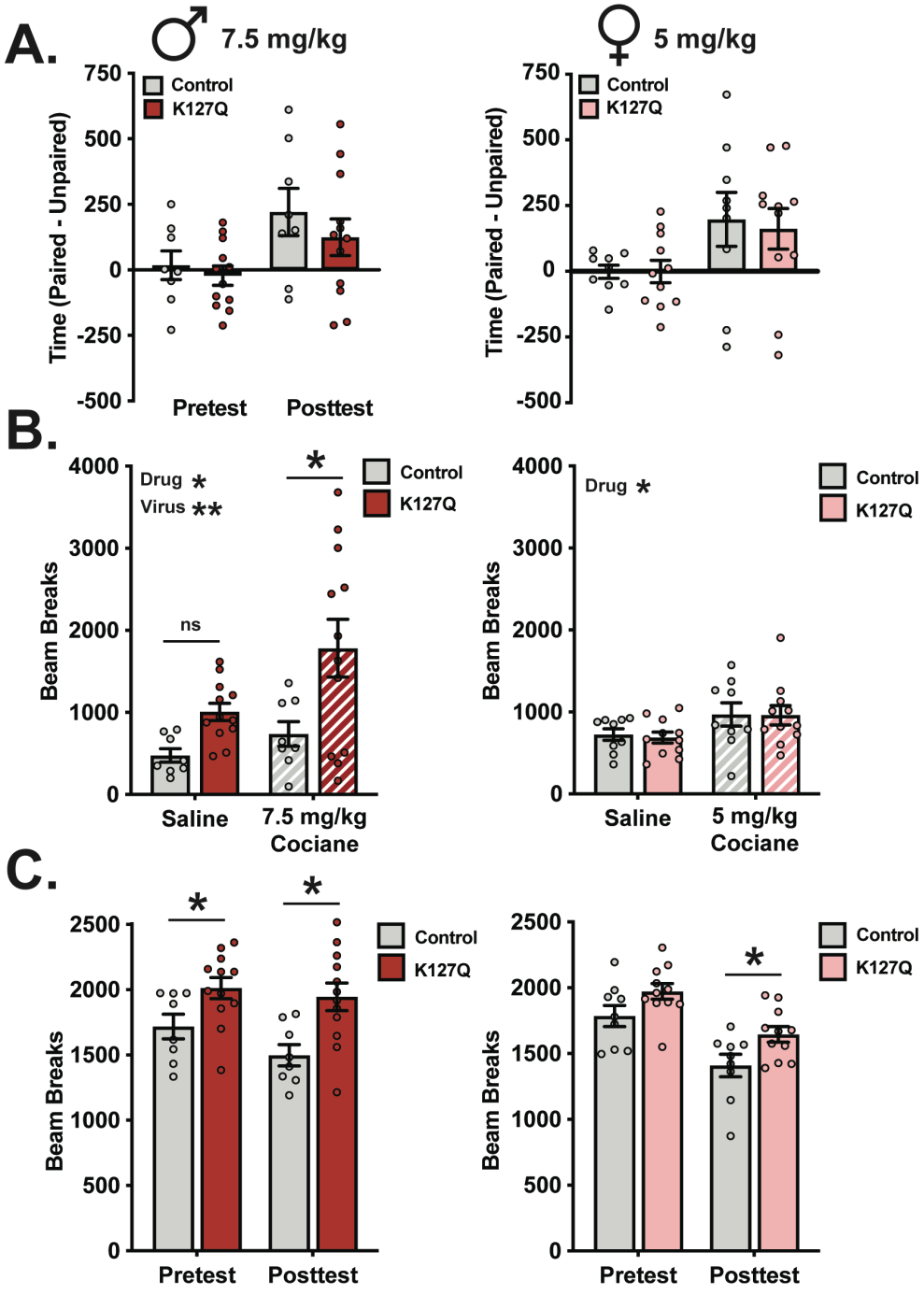

Figure 21. Reduced catalytic activity in VTA DA neurons does not impair conditioned cocaine reward at a low dose.

Figure 21. (cont'd).
A. No impairment in cocaine reward was observed when mice with decreased VTA DA SGK1 catalytic activity were conditioned at a low dose of cocaine (7.5 mg/kg for males, 5 mg/kg for females) (males n=8-12, unpaired t-test; females n=9-11, unpaired t-test).
B. Male mice with VTA DA neuron expression of the K127Q mutant showed enhanced psychomotor activity during conditioned place preference conditioning sessions, with no effect observed in females (males n=8-12, two-way ANOVA followed by a Tukey post-hoc test, main effects of drug: p=0.0349 and virus: p=0.002, post-hoc: p=0.018; females n=9-11, two-way ANOVA, main effect of drug: p=0.0173).
C. Locomotor differences between groups were detected during the pretest and posttest sessions (males n=8-12, unpaired t-test, pretest: p=0.0294, posttest p=0.0068; females n=9-11, unpaired t-test, posttest: p=0.0303).

we next sought to determine if decreased activity specifically within VTA DA neurons was responsible for the observed effect, as with cocaine CPP. Male mice with DA neuron-specific K127Q expression showed no difference from controls in morphine preference (0.1 mg/mL morphine) (Figure 22A), failing to duplicate non-cell type-specific VTA findings (Figure 17D). This result was likely not caused by a change in aversive behavior, as mice with decreased VTA DA SGK1 activity showed quinine aversion (0.1mM) comparable to controls (Figure 22C). Fluid consumption in either task was not changed by this manipulation (Figure 22B,D). Thus, while VTA DA SGK1 catalytic activity was a significant contributor to cocaine CPP, manipulation of this neuronal population via K127Q overexpression does not result in reduced morphine preference.

Decreased SGK1 activity in the VTA neurons does not alter sucrose preference

Expanding on results demonstrating impaired drug reward driven by decreased VTA SGK1 catalytic activity, we next sought to determine if a natural reward was similarly affected. To characterize potential changes in natural reward driven by altered VTA SGK1 catalytic activity, sucrose preference in a two-bottle choice task was assessed following

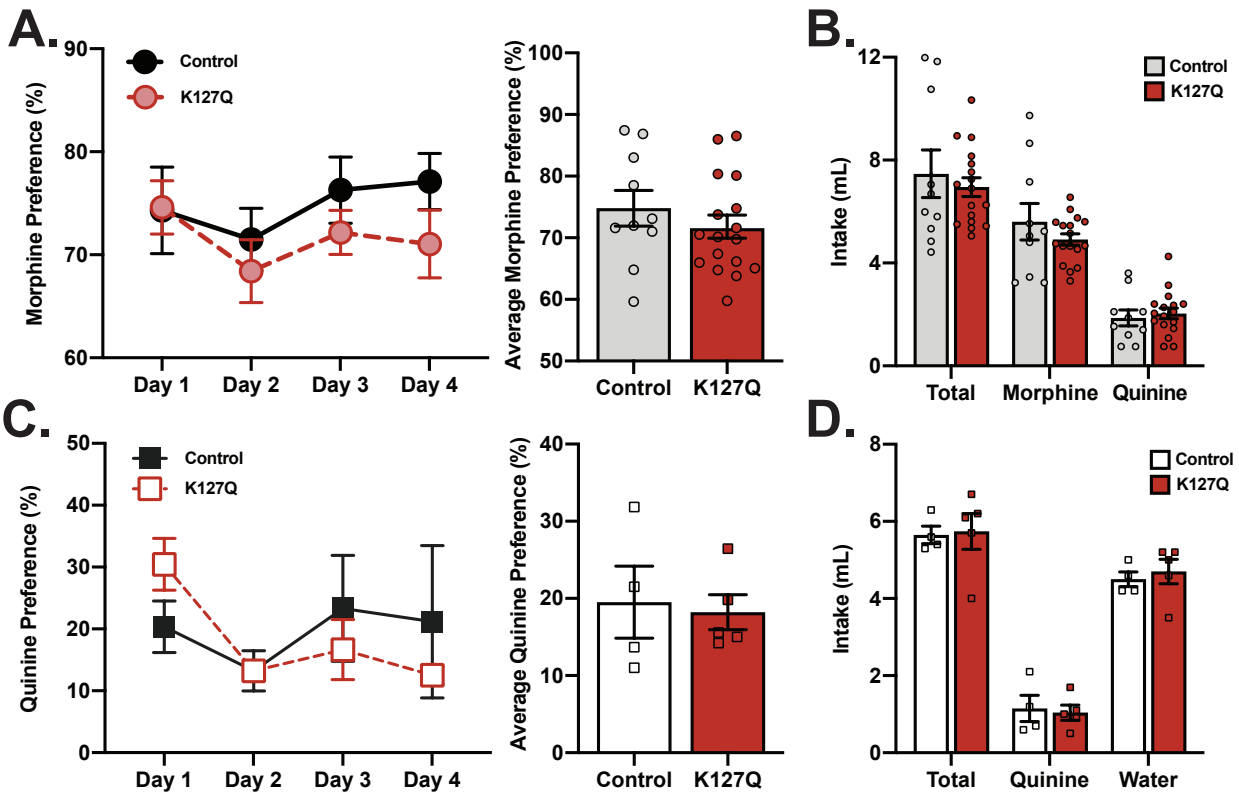

Figure 22. Morphine preference is not changed by decreased VTA DA SGK1 catalytic activity.

A. Decreased SGK1 catalytic activity in VTA DA neurons was not sufficient to impair morphine reward in male mice as measured by preference in a two-bottle choice task (n=10-17, daily and average preference using two-way ANOVA with repeated measures or unpaired t-test, respectively).

B. Fluid intake during the morphine two-bottle choice task was not modulated by DA neuron-specific K127Q expression (n=10-17, unpaired t-test).

C. Daily and average aversion to quinine was unaltered by VTA DA SGK1 manipulation (n=4-5, daily and average preference using two-way ANOVA with repeated measures or unpaired t-test, respectively).

D. Overexpression of the K127Q mutant in VTA DA neurons did not change fluid intake during the quinine two-bottle choice task (n=4-5, unpaired t-test).

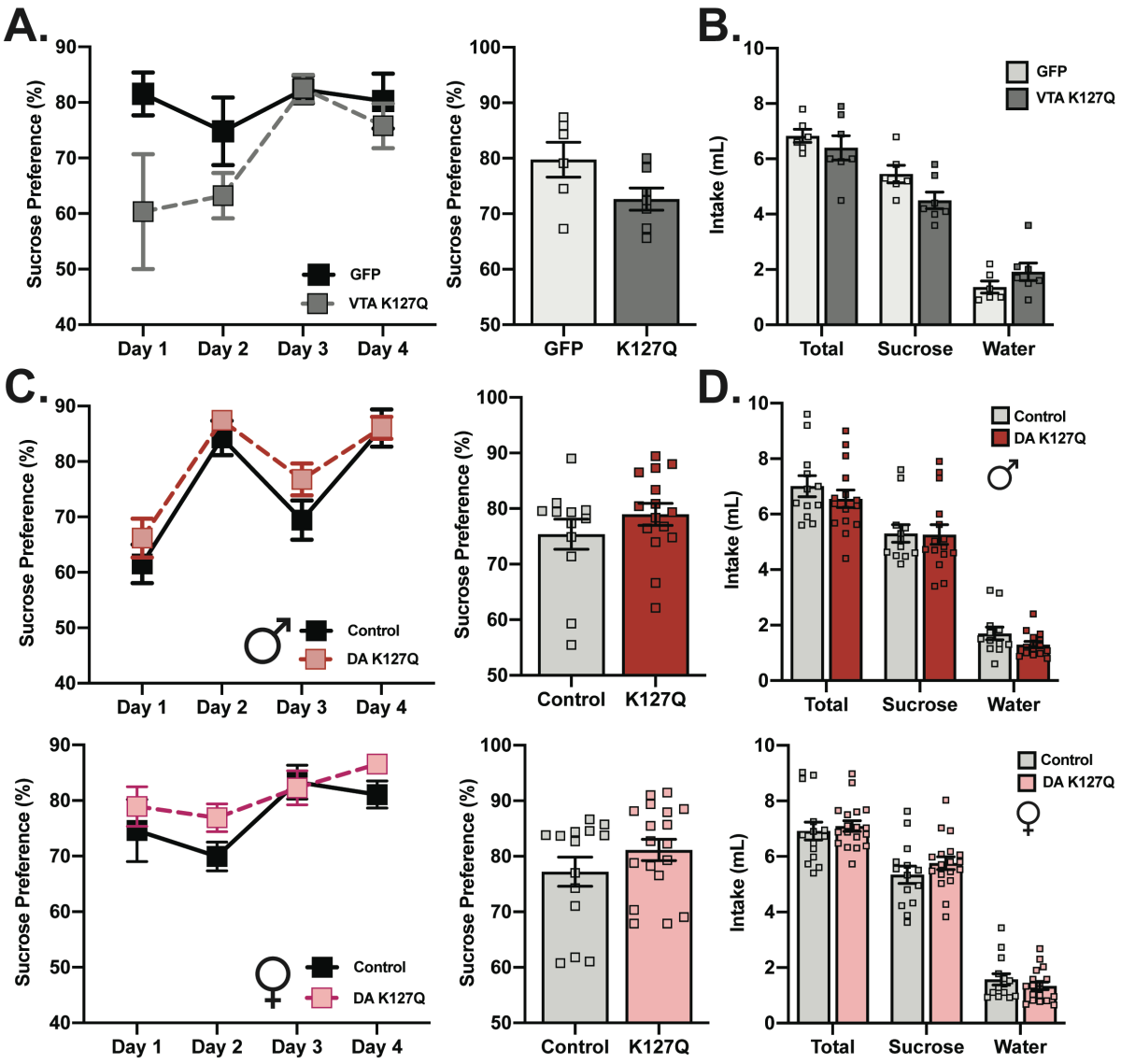

Figure 23. Decreased VTA SGK1 catalytic activity does not regulate sucrose preference.

A. VTA overexpression of catalytically inactive SGK1 (VTA-K127Q) did not alter daily or average sucrose preference in male mice compared to controls (GFP) (n=6-7, two-way ANOVA with repeated measures).

B. VTA overexpression of the catalytically inactive SGK1 mutant did not change fluid intake in male mice during the sucrose two-bottle choice task (n=6-7, unpaired t-test).

C. Daily or average sucrose preference of male or female mice was not changed by decreased SGK1 activity specifically in VTA DA neurons (DA-K127Q) (males: n=12-15, two-way ANOVA with repeated measures; females n= 14-18, two-way ANOVA with repeated measures).

D. Fluid intake during the sucrose two-bottle choice task was not modulated by decreased SGK1 catalytic activity in VTA DA neurons (males: n=12-15, unpaired t-test; females n= 14-18, unpaired t-test).

viral manipulations. A non-cell type-specific decrease in VTA SGK1 catalytic activity via overexpression HSV-K127Q did not significantly alter sucrose preference (Figure 23A). Similarly, both male and female mice with VTA DA-specific decreased activity did not show differences in sucrose preference from controls (Figure 23C). Neither viral manipulation altered total fluid consumption compared (Figure 23B,D). These data suggest that decreased VTA SGK1 catalytic activity may not impair preference for natural rewards.

Cocaine self-administration is not altered by decreased VTA DA SGK1 catalytic activity

To better define changes in cocaine reward driven by reduced SGK1 activity in VTA DA neurons, cocaine self-administration was used to assess additional cocaine reward behaviors, including acquisition, motivation, and responding as a result of drug dose. Male mice with DA neuron-specific expression of the K127Q mutant were first trained to respond for food, where sucrose pellets were earned on a fixed ratio of 1 (FR1) schedule, and mice that did not meet criteria for acquisition of food training were excluded from analysis and further experimentation. Both control mice and those expressing the K127Q mutant were able to acquire the operant behavior, with no observed differences between groups in the percentage of mice to meet acquisition criteria (Figure 24A). Additionally, decreased VTA DA SGK1 catalytic activity did not significantly alter the number of days to meet acquisition or the number of rewards earned during sucrose self-administration sessions (Figure 24B-C), consistent with sucrose preference findings and indicating that the processing of natural reward is intact in DA SGK1 mutant-expressing

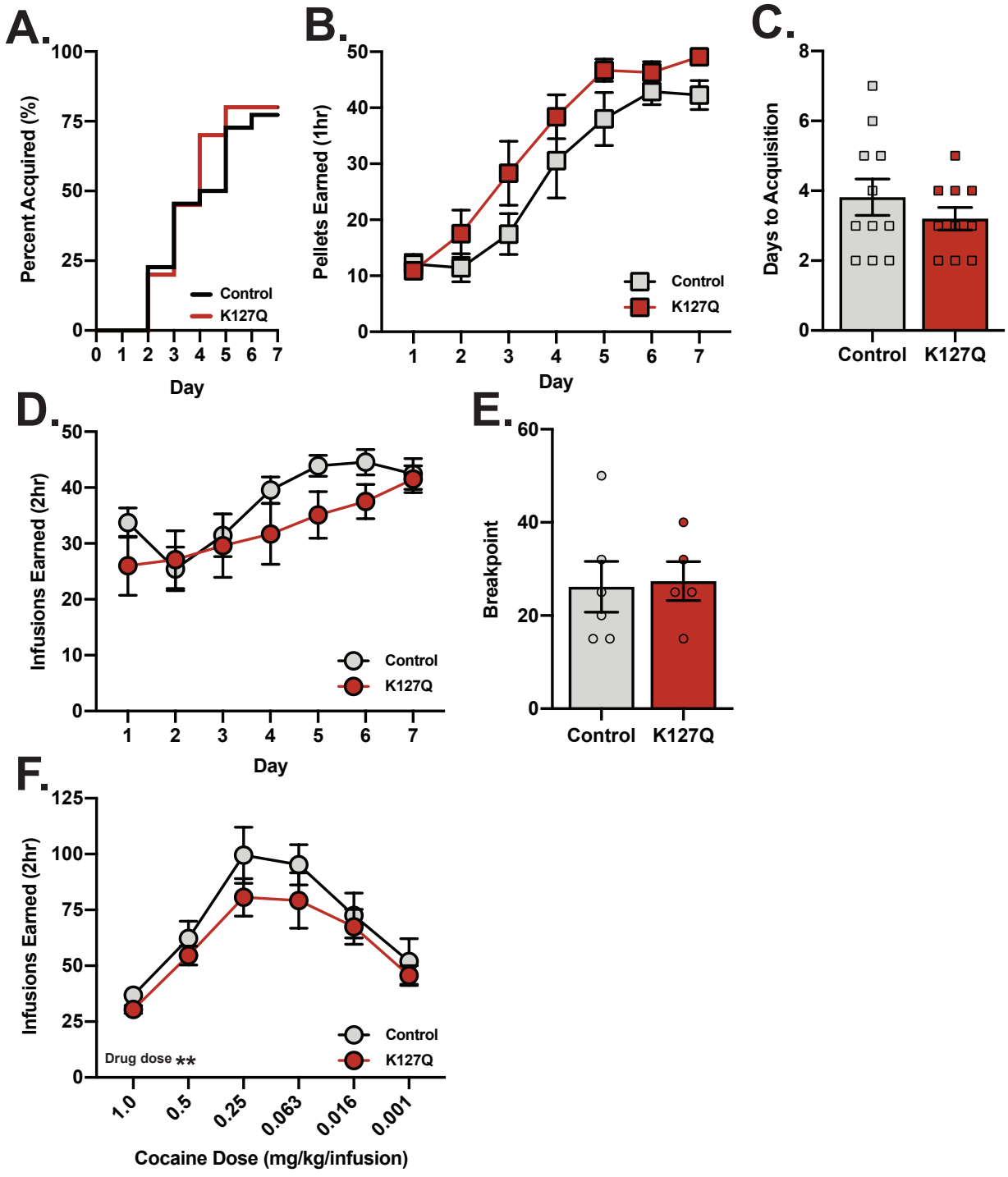

Figure 24. Cocaine intake and motivation to earn a reward are not mediated by VTA DA SGK1 catalytic activity.

Figure 24. (cont'd).
A. When comparing all mice run through the initial food training phase, there was no difference between groups on the number of mice to acquire the operant behavior (n=20-22, Log-rank (Mantel-Cox) test).
B. Of mice that successfully acquired cocaine self-administration, decreased VTA DA SGK1 catalytic activity (K127Q) did not alter the number of rewards earned during food training (n=10-11, two-way ANOVA with repeated measures).
C. Acquisition of sucrose pellet self-administration was not altered by decreased VTA DA SGK1 catalytic activity compared to controls (n=10-11, unpaired t-test).
D. Decreased VTA DA SGK1 catalytic activity did not change cocaine intake compared to controls (n=10-11, two-way ANOVA with repeated measures).
E. Breakpoint in a progressive ratio test was not different between mice with decreased VTA DA SGK1 catalytic activity and controls (n=5-6, unpaired t-test).
D. Responding for cocaine during a dose-response assay showed a main effect of drug dose, but decreased VTA DA SGK1 activity did not change responding compared to controls (n=7-8, two-way ANOVA with repeated measures, main effect of drug dose: p=p<0.0001).

mice.

To assess potential changes to cocaine behavior, mice then underwent cocaine self-administration (0.5 mg/kg/infusion) on an FR1 schedule. Both control and VTA DA SGK1 mutant mice acquired cocaine self-administration, and the number of cocaine infusions earned was not different between groups (Figure 24D), indicating that decreased VTA DA SGK1 activity did not modulate cocaine intake. Next, to determine if motivation to earn a cocaine reward was altered by decreased VTA DA SGK1 catalytic activity, mice were put through progressive ratio (PR) testing (0.5 mg/kg/infusion). The PR breakpoint was not different between control and K127Q-exprssing mice (Figure 24E). These findings suggest that decreased catalytic activity in VTA DA neurons did not alter motivation to obtain cocaine infusions. Finally, given deficits in cocaine CPP at a moderate but not low dose of cocaine, a dose-response assay was next run to determine if effects on self-administration were potentially obscured by the dose administered during acquisition and PR testing. There was a significant main effect of dose on the number of

cocaine infusions earned, replicating previous effects found in the literature (Arena et al., 2019; Ozburn et al., 2012); however, no differences were observed between DA SGK1 mutant mice and controls (Figure 24F). Overall, while cocaine reward was altered in a CPP paradigm, decreased VTA DA catalytic activity did not affect cocaine self-administration behaviors.

K127Q rescue of DA SGK1 gene knockout does not alter conditioned cocaine reward

While viral-mediated overexpression of a catalytically inactive SGK1 mutant (K127Q) has been a critical tool for understanding the role of SGK1 activity in drug-reward behaviors, this method overexpresses the mutant on top of endogenous SGK1 protein expressed by the neuron. Therefore, it remains unknown if the expression of endogenous SGK1 protein contributes to the observed effects of K127Q expression on cocaine CPP and morphine preference. Previous work has demonstrated that DA neuron-specific SGK1 knockout (KO) (DA SGK1 KO) does not affect cocaine CPP (Chapter 2). In order to assess the potential impact of endogenous SGK1 signaling in our viral models, VTA DA SGK1 expression in DA SGK1 KO mice was rescued with K127Q mutant expression. Following conditioning (12.5 mg/kg cocaine in males and 10 mg/kg cocaine in females), control mice formed a preference for the drug-paired chamber as expected (Figure 25A). Mice with DA neuron-specific SGK1 deletion (DA SGK1 KO group) similarly formed a preference for the drug paired chamber that was no different from controls (Figure 25A), replicating previous findings (Chapter 2). When the K127Q mutant was expressed in VTA DA neurons lacking endogenous expression SGK1 (rescue group), mice were no different

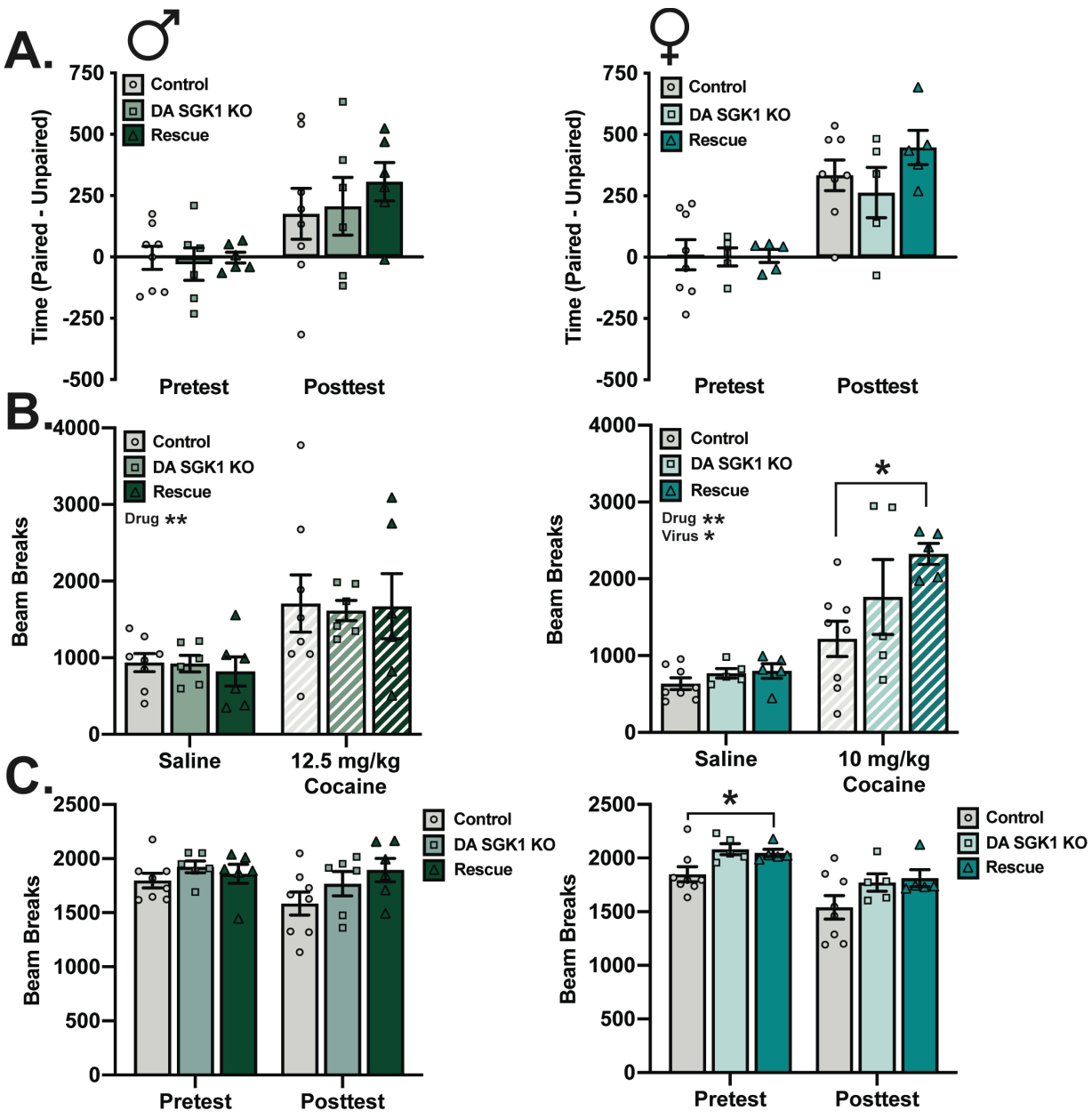

Figure 25. K127Q expression in VTA DA neurons lacking endogenous SGK1 expression does not alter cocaine CPP.

Figure 25. (cont'd).
A. Cocaine CPP was not changed when the SGK1 mutant (K127Q) was overexpressed in VTA DA neurons lacking endogenous SGK1 expression (males con:8, KO:6, rescue:6, one-way ANOVA; females con:8, KO:5, rescue:5, one-way ANOVA).
B. During the CPP conditioning sessions, female, but not male, mice with K127Q expression showed a main effect of virus (males con:8, KO:6, rescue:6, two-way ANOVA, main effect of drug: $p=0.0012$; females con:8, KO:5, rescue:5, two-way ANOVA followed by a Tukey post-hoc test, main effects of drug: $p<0.0011$ and virus: $p=0.0213$, post-hoc: $p=0.0131$).
C. Females in the rescue group showed a significant increase in locomotor activity during the CPP pretest session but not the posttest session (males con:8, KO:6, rescue:6, one-way ANOVA; females con:8, KO:5, rescue:5, one-way ANOVA followed by a Tukey post-hoc test, $p=0.0411$).

from controls (Figure 25A), diverging from impaired CPP effects seen with DA-K127Q expression alone (Figure 18A). Locomotor activity during pre and posttests was not affected by genetic or viral manipulations in male mice, but female rescue mice showed a significant increase in locomotor activity during the CPP pretest (Figure 25C). These data suggest that the effect of viral-mediated overexpression of the K127Q mutant on cocaine CPP is mediated in part by expression of endogenous SGK1 protein.

Additionally, VTA DA neuron-K127Q expression was previously shown to robustly increase cocaine-induced psychomotor activity (Figure 18B). During the CPP condition sessions, all groups exhibited the expected drug-induced increase in locomotor activity. Specifically, in male mice, DA SGK1 KO mice were comparable to controls, replicating earlier findings; however, K127Q expression in mice with VTA DA neurons lacking endogenous SGK1 failed to show enhanced cocaine-induced psychomotor activity compared to the other groups (Figure 25B). Female mice similarly replicated earlier findings of comparable locomotor activity in control and DA SGK1 KO mice, but, surprisingly, an increase in psychomotor activity was seen in rescue females compared to controls (Figure 25B). As only a single cohort was tested, an additional set of mice are

needed to confirm these observations. Together, these data, at least in males, suggest that endogenous SGK1 expression is critical for K127Q effects on cocaine behavior.

Discussion

SGK1 is widely expressed in the brain and its transcription can be induced by virtually all classes of drug of abuse (Gao et al., 2015; Heller et al., 2015; Lang et al., 2010; McClung et al., 2005; Nichols and Sanders-Bush, 2002; Piechota et al., 2010); however, few studies have built on transcriptional findings to characterize protein-level regulation and function. Previous work from our lab identified an upregulation in VTA SGK1 mRNA and catalytic activity following chronic cocaine or morphine administration. Though SGK1 catalytic activity has been identified as a modulator of stress-and learning-related behaviors (Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008; Yuen et al., 2011), its effect on cocaine and morphine reward remained unknown. Thus, this work presents the first evidence for the relevance of SGK1 catalytic activity in modulating drug reward. We found that decreased VTA SGK1 catalytic activity impaired cocaine CPP and modulated morphine preference, while wild-type SGK1 overexpression alone had no effect on CPP. This is consistent with data showing VTA overexpression of a constitutively active SGK1 mutant (S422D) does not further increase cocaine or morphine CPP compared to GFP controls (Heller et al., 2015). Together, these data suggest that while decreasing SGK1 catalytic activity is sufficient to disrupt conditioned cocaine reward and morphine preference, increasing expression or activity are not sufficient to further increase drug reward.

It is known that chronic cocaine increases VTA SGK1 catalytic activity; however,

the neuronal cell type/s of interest remained unidentified. Due to antibody limitations, immunohistochemistry could not be used to identify the population in which SGK1 regulation occurs. Instead, we took a viral-mediated approach, overexpressing a Cre-dependent catalytically inactive SGK1 mutant (K127Q) in either VTA DA or GABA neurons. Decreased activity in VTA DA, but not GABA, neurons was found to impair cocaine CPP, replicating whole VTA findings and identifying VTA DA neurons as the likely neuronal population of interest. Interestingly, both male and female mice with DA-specific expression showed a robust increase in psychomotor activity during CPP conditioning. This effect on cocaine-induced locomotor activity was replicated in a locomotor sensitization assay.

Though decreased SGK1 catalytic activity in VTA DA neurons was capable of disrupting cocaine reward, this same manipulation failed to reduce morphine preference, as was observed in non-cell type-specific VTA manipulations. A DA-independent pathway for opioid reward may contribute to these findings. The VTA contains both DA-dependent and DA-independent reward systems (Ting-A-Kee and van der Kooy, 2012). Evidence of this originated from findings that intracranial self-stimulation (ICS) does not evoke DA release in the NAc of trained rats, suggesting that the reinforcing mechanism underlying ICS does not signal through DA release (Garris et al., 1999). Similarly, reduced NAc DA release or signaling did not ablate opioid reward (Ettenberg et al., 1982; Hnasko et al., 2005; Pettit et al., 1984). However, this is likely dependent on drug state, as systemic or NAc DA receptor blockage is capable of preventing morphine CPP in opiate-dependent but not opiate-naïve rats (Laviolette et al., 2004; Nader and van der Kooy, 1997). Opiate-naïve signaling instead occurs through VTA GABA projections to the pedunculopontine

tegmental nucleus (PPTg) (Bechara and van der Kooy, 1992; Nader and van der Kooy, 1997; Olmstead et al., 1998; Ting-A-Kee and van der Kooy, 2012). These data indicate that while opioid reward relies heavily on DA signaling in opioid-dependent animals, opioid-naïve animals have a contribution of DA-independent pathways. Therefore, to better characterize the role of SGK1 activity in morphine reward behaviors, future studies should implement VTA GABA-specific overexpression of the K127Q mutant as well as conduct VTA DA manipulations in opioid-dependent mice.

We next sought to determine if natural reward was altered by decreased VTA SGK1 activity, using sucrose preference as an example. Neither VTA- nor DA-specific decreases in activity altered sucrose preference in a two-bottle choice task. These data are consistent with no effect of decreased VTA DA SGK1 activity on the numbers of sucrose rewards earned during the food training phase of operant self-administration. These finds are further supported by biochemical data showing that VTA SGK1 activity is not increased following an acute administration of cocaine (Heller et al., 2015). Together these data suggest that natural rewards and acute drug administration may not be sufficient to drive SGK1 signaling pathways.

While male mice with DA neuron-specific decreases in SGK1 activity showed deficits in cocaine CPP, effects were not significant in cocaine self-administration paradigms. Decreased VTA DA SGK1 activity did not alter cocaine intake or motivation to earn rewards. Additionally, while K127Q-expressing mice exhibited decreased drug reward at a moderate but not a low CPP conditioning dose, no differences between groups were seen in dose-dependent responding during cocaine self-administration. Several factors could contribute to these differences in results. First, mice were taught to

respond for sucrose pellets prior to cocaine self-administration, and it is possible that any modest effects on drug acquisition or intake were obscured by this prior training. Second, CPP and self-administration paradigms differ greatly in both route of administration and cocaine dose. On top of self-administered vs. investigator administered methods of cocaine delivery, self-administrating commonly leads to greater drug intake for a greater number of sessions compared to CPP. These factors may contribute to divergent VTA SGK1 regulation. Evidence of this idea is supported with mRNA studies. We previously identified a significant increase in VTA SGK1 transcription following 7 days of cocaine or morphine i.p. injections with 24 hours of withdrawal (Heller et al., 2015). However, RNA-sequencing of VTA tissue from mice that self-administered cocaine or saline showed a significant reduction in SGK1 mRNA following 24 hours of withdrawal (Walker et al., 2018). The findings in this study directly conflict with our pervious results, suggesting that SGK1 may be differently regulated in these two administration paradigms. Therefore, because the self-administration protocol used by Walker et al. is nearly identical to that used in the current study, SGK1 may be downregulated at the time we measured PR and ran the dose-response analysis. If this is true, viral-mediated decreases in catalytic activity would understandably have little effect when activity was already low. However, as SGK1 mRNA does not always track directly with protein regulation (Heller et al., 2015), protein-level analysis is required to confirm this theory.

Though viral-mediated overexpression of SGK1 mutants has been a critical method for studying SGK1 function, endogenous protein is still expressed in these models. As DA SGK1 KO mice with DA neuron-specific K127Q mutant expression do not replicate deficits in cocaine CPP and psychomotor activity, endogenous protein

expression likely contributes to the observed effects. Therefore, pharmacological inhibition represents a future direction for the manipulation of endogenous protein. SGK1 inhibitors have been developed (Ackermann et al., 2011; D'Antona et al., 2015; Halland et al., 2015; Sherk et al., 2008), but there are significant limitations to their use. Particularly for commercially available GSK650394, the most widely used compound, these issues are chiefly in its specificity for SGK1 over other SGK isoforms and poor membrane permeability (Di Cristofano, 2017; Sherk et al., 2008). Once these limitations can be overcome, future work should utilize pharmacological inhibitors in the study of VTA SGK1 function.

In summary, this work demonstrates for the first time that VTA SGK1 is capable of modulating drug reward, as decreasing catalytic activity is sufficient to disrupt cocaine CPP and morphine preference. Further, it appears that SGK1 activity within VTA DA neurons is critical for effects on cocaine CPP and psychomotor activity, though this manipulation did not affect cocaine self-administration. Further, DA neuron-specific overexpression of the K127Q mutant did not drive a decrease in morphine preference, indicating activity in this neuronal population is not critical to morphine reward. These data demonstrate the complexity of SGK1 activity in the VTA. Altogether, this work expands on the limited knowledge of SGK1 function in the central nervous system, critical to understanding the effect of SGK1 catalytic activity on cocaine and morphine reward.

Table 3. Statistics table for Chapter 3 results.

	Description	Sex	Group Size	Statistical Test	Main Effect	t- or f-statistic	p-value	Post-hoc test	Post-hoc Comparison	p-value
Figure 17 - Decreased VTA SGK1 activity is capable of modulating cocaine and morphine reward.										
Fig 17A	VTA K127Q and WT CPP posttest scores	Male	GFP: 22 K127Q: 26 WT: 11	One-way ANOVA		F (2, 56) = 5.187	p=0.0086	Dunnett	GFP vs. K127Q GFP vs. WT	p=0.0177 p=0.8714
Fig 17B	VTA K127Q and WT CPP conditioning locomotor activity	Male	GFP: 22 K127Q: 26 WT: 11	Two-way ANOVA	Interaction Drug Virus	F (2, 112) = 0.1250 F (1, 112) = 12.48 F (2, 112) = 0.5336	p=0.8826 p=0.0006 p=0.5880	Tukey	Saline:GFP vs. Saline:K127Q Saline:GFP vs. Saline:WT Saline:GFP vs. Cocaine:GFP Saline:GFP vs. Cocaine:K127Q Saline:GFP vs. Cocaine:WT Saline:K127Q vs. Saline:WT Saline:K127Q vs. Cocaine:GFP Saline:K127Q vs. Cocaine:K127Q Saline:K127Q vs. Cocaine:WT Saline:WT vs. Cocaine:GFP Saline:WT vs. Cocaine:K127Q Saline:WT vs. Cocaine:WT Cocaine:GFP vs. Cocaine:K127Q Cocaine:GFP vs. Cocaine:WT Cocaine:K127Q vs. Cocaine:WT	p=0.9979 p>0.9999 p=0.1289 p=0.024 p=0.6777 p=0.9994 p=0.2562 p=0.0576 p=0.8517 p=0.3275 p=0.1234 p=0.8009 p=0.9941 p=0.9926 p=0.9076
Fig 17C	VTA K127Q and WT Pretest locomotor activity	Male	GFP: 22 K127Q: 26 WT: 11	One-way ANOVA		F (2, 56) = 1.559	p=0.2193			
	VTA K127Q and WT Posttest locomotor activity	Male	GFP: 22 K127Q: 26 WT: 11	One-way ANOVA		F (2, 56) = 2.172	p=0.1234			
Fig 17D	VTA K127Q and S422D Average morphine preference	Male	GFP: 5 K127Q: 6 S422D: 6	One-way ANOVA		F (2, 14) = 3.745	p=0.0498	Tukey	GFP vs. S422D GFP vs. K127Q S422D vs. K127Q	p=0.7753 p=0.191 p=0.0465
Fig 17E	VTA K127Q and S422D Average total fluid intake	Male	GFP: 5 K127Q: 6 S422D: 6	One-way ANOVA		F (2, 14) = 0.2355	p=0.7932			
	VTA K127Q and S422D Average morphine intake	Male	GFP: 5 K127Q: 6 S422D: 6	One-way ANOVA		F (2, 14) = 2.210	p=0.1465			
	VTA K127Q and S422D Average morphine intake	Male	GFP: 5 K127Q: 6	One-way ANOVA		F (2, 14) = 4.065	p=0.0405	Tukey	GFP vs. S422D GFP vs. K127Q	p=0.6785 p=0.2002

Table 3. (cont'd).

			S422D: 6						S422D vs. K127Q	p=0.0357
Figure 18 - A reduction in SGK1 catalytic activity in VTA DA, but not GABA, neurons drives impaired cocaine reward.										
Fig 18A	DA K127Q CPP posttest scores	Male	Con: 15 K127Q: 10	Unpaired t-test		t=3.378, df=23	p=0.0026			
	DA K127Q CPP posttest scores	Female	Con: 13 K127Q: 16	Unpaired t-test		t=2.646, df=27	p=0.0134			
	GABA K127Q CPP posttest scores	Male	Con: 25 K127Q: 21	Unpaired t-test		t=0.05421, df=44	p=0.9570			
	GABA K127Q CPP posttest scores	Female	Con: 24 K127Q: 16	Unpaired t-test		t=1.485, df=38	p=0.1459			
Fig 18B	DA K127Q CPP conditioning locomotor activity	Male	Con: 15 K127Q: 10	Two-way ANOVA	Interaction Drug Virus	F (1, 46) = 4.362 F (1, 46) = 19.72 F (1, 46) = 16.08	p=0.0423 p<0.0001 p=0.0002	Tukey	Saline:Control vs. Saline:K127Q Saline:Control vs. Cociane:Control Saline:Control vs. Cociane:K127Q Saline:K127Q vs. Cociane:Control Saline:K127Q vs. Cociane:K127Q Cociane:Control vs. Cociane:K127Q	p=0.5312 p=0.2593 p<0.0001 p=0.99 p=0.0006 p=0.0005
	DA K127Q CPP conditioning locomotor activity	Female	Con: 13 K127Q: 16	Two-way ANOVA	Interaction Drug Virus	F (1, 54) = 3.151 F (1, 54) = 37.62 F (1, 54) = 4.600	p=0.0815 p<0.0001 p=0.0365	Tukey	Saline:Control vs. Saline:K127Q Saline:Control vs. Cociane:Control Saline:Control vs. Cociane:K127Q Saline:K127Q vs. Cociane:Control Saline:K127Q vs. Cociane:K127Q Cociane:Control vs. Cociane:K127Q	p=0.9937 p=0.0246 p<0.0001 p=0.0329 p<0.0001 p=0.0372
	GABA K127Q CPP conditioning locomotor activity	Male	Con: 25 K127Q: 21	Two-way ANOVA	Interaction Drug Virus	F (1, 88) = 2.385 F (1, 88) = 39.85 F (1, 88) = 1.237	p=0.1261 p<0.0001 p=0.2691	Tukey	Saline:Control vs. Saline:K127Q Saline:Control vs. Cocaine:Control Saline:Control vs. Cocaine:K127Q Saline:K127Q vs. Cocaine:Control Saline:K127Q vs. Cocaine:K127Q Cocaine:Control vs. Cocaine:K127Q	p=0.99 p<0.0001 p=0.0023 p<0.0001 p=0.0092 p=0.2448
	GABA K127Q CPP conditioning locomotor activity	Female	Con: 24 K127Q: 16	Two-way ANOVA	Interaction Drug Virus	F (1, 76) = 0.001761 F (1, 76) = 32.39 F (1, 76) = 0.09226	p=0.9666 p<0.0001 p=0.7622	Tukey	Saline:Control vs. Saline:K127Q Saline:Control vs. Cocaine:Control Saline:Control vs. Cocaine:K127Q Saline:K127Q vs. Cocaine:Control Saline:K127Q vs. Cocaine:K127Q Cocaine:Control vs. Cocaine:K127Q	p=0.9977 p=0.0002 p=0.0004 p=0.0016 p=0.0022 p=0.9948

Table 3. (cont'd).

Figure 19 - Locomotor activity during CPP pretest is altered by DA neuron-K127Q expression in male mice.									
Fig 19A	DA K127Q CPP pretest locomotor activity	Male	Con: 15 K127Q: 10	Unpaired t-test		t=2.191, df=23	p=0.0388		
	DA K127Q CPP posttest locomotor activity	Male	Con: 15 K127Q: 10	Unpaired t-test		t=1.540, df=23	p=0.1372		
	DA K127Q CPP pretest locomotor activity	Female	Con: 13 K127Q: 16	Unpaired t-test		t=1.734, df=27	p=0.0944		
	DA K127Q CPP posttest locomotor activity	Female	Con: 13 K127Q: 16	Unpaired t-test		t=1.187, df=27	p=0.2454		
Fig 19B	GABA K127Q CPP pretest locomotor activity	Male	Con: 28 K127Q: 21	Unpaired t-test		t=0.5445, df=44	p=0.5889		
	GABA K127Q CPP posttest locomotor activity	Male	Con: 28 K127Q: 21	Unpaired t-test		t=0.5634, df=44	p=0.5760		
	GABA K127Q CPP pretest locomotor activity	Female	Con: 24 K127Q: 16	Unpaired t-test		t=0.6935, df=38	p=0.4922		
	GABA K127Q CPP posttest locomotor activity	Female	Con: 24 K127Q: 16	Unpaired t-test		t=0.2094, df=38	p=0.8353		
Figure 20 - Cocaine-induced psychomotor activity is altered by decreased VTA DA SGK1 catalytic activity.									
Fig 20A	DA K127Q Cocaine locomotor sensitization	Males	Con: 7 K127Q: 4	Three-way ANOVA with RM	Day Drug Virus Day x Drug Day x Virus Drug x Virus Day x Drug x Virus	F (1, 9) = 0.5639 F (1, 9) = 37.82 F (1, 9) = 7.638 F (1, 9) = 0.4191 F (1, 9) = 1.350 F (1, 9) = 13.95 F (1, 9) = 1.292	p=0.4719 p=0.0002 p=0.0220 p=0.5336 p=0.2752 p=0.0047 p=0.2850	Sidak	Day 1:Con Sal vs. Day 7:Con Sal Day 1:K127Q Sal vs. Day 7:K127Q Sal Day 1:Con Coc vs. Day 7:Con Coc Day 1:K127Q Coc vs. Day 7:K127Q Coc Day 1:Con Sal vs. Day 1:K127Q Sal Day 1:Con Coc vs. Day 1:K127Q Coc Day 7:Con Sal vs. Day 7:K127Q Sal Day 7:Con Coc vs. Day 7:K127Q Coc Day 1:Con M Sal vs. Day 1:Con Coc Day 1:K127Q Sal vs. Day 1:K127Q Coc Day 7:Con Sal vs. Day 7:Con Coc Day 7:K127Q Sal vs. Day 7:K127Q Coc	p>0.9999 p=0.9986 p=0.9811 p=0.9726 p>0.9999 p=0.0014 p>0.9999 p=0.2733 p>0.9999 p=0.0017 p=0.2467 p=0.0041
	DA K127Q Cocaine locomotor sensitization	Females	Con: 11 K127Q: 13	Three-way ANOVA with RM	Day Drug Virus Day x Drug Day x Virus	F (1, 22) = 1.485 F (1, 22) = 6.702 F (1, 22) = 3.452 F (1, 22) = 10.51 F (1, 22) = 0.1091	p=0.2359 p=0.0168 p=0.0766 p=0.0037 p=0.7443	Sidak	Day 1:Con Sal vs. Day 7:Con Sal Day 1:K127Q Sal vs. Day 7:K127Q Sal Day 1:Con Coc vs. Day 7:Con Coc Day 1:K127Q Coc vs. Day 7:K127Q Coc Day 1:Con Sal vs. Day 1:K127Q Sal	p>0.9999 p=0.9996 p=0.9559 p=0.1188 p=0.9988

Table 3. (cont'd).

					Drug x Virus	F (1, 22) = 2.491	p=0.1287		Day 1:Con Coc vs. Day 1:K127Q Coc	p=0.5265
					Day x Drug x Virus	F (1, 22) = 1.505	p=0.2329		Day 7:Con Sal vs. Day 7:K127Q Sal	p>0.9999
									Day 7:Con Coc vs. Day 7:K127Q Coc	p=0.1297
									Day 1:Con M Sal vs. Day 1:Con Coc	p>0.9999
									Day 1:K127Q Sal vs. Day 1:K127Q Coc	p=0.7056
									Day 7:Con Sal vs. Day 7:Con Coc	p=0.975
									Day 7:K127Q Sal vs. Day 7:K127Q Coc	p=0.0024
Fig 20B	GABA K127Q	Males	Con: 6	Three-way ANOVA with RM	Day	F (1, 10) = 0.2888	p=0.6027		Day 1:Con Sal vs. Day 7:Con Sal	p>0.9999
	Cocaine locomotor sensitization		K127Q: 6		Drug	F (1, 10) = 8.077	p=0.0175	Sidak	Day 1:K127Q Sal vs. Day 7:K127Q Sal	p>0.9999
					Virus	F (1, 10) = 0.5833	p=0.4627		Day 1:Con Coc vs. Day 7:Con Coc	p>0.9999
					Day x Drug	F (1, 10) = 1.013	p=0.3378		Day 1:K127Q Coc vs. Day 7:K127Q Coc	p=0.9635
					Day x Virus	F (1, 10) = 0.3238	p=0.5819		Day 1:Con Sal vs. Day 1:K127Q Sal	p>0.9999
					Drug x Virus	F (1, 10) = 0.02334	p=0.8816		Day 1:Con Coc vs. Day 1:K127Q Coc	p>0.9999
					Day x Drug x Virus	F (1, 10) = 0.09752	p=0.7612		Day 7:Con Sal vs. Day 7:K127Q Sal	p=0.9996
									Day 7:Con Coc vs. Day 7:K127Q Coc	p=0.9992
									Day 1:Con M Sal vs. Day 1:Con Coc	p=0.8453
									Day 1:K127Q Sal vs. Day 1:K127Q Coc	p=0.9819
									Day 7:Con Sal vs. Day 7:Con Coc	p=0.4851
									Day 7:K127Q Sal vs. Day 7:K127Q Coc	p=0.447
	GABA K127Q	Females	Con: 6	Three-way ANOVA with RM	Day	F (1, 10) = 0.9907	p=0.3430		Day 1:Con Sal vs. Day 7:Con Sal	p>0.9999
	Cocaine locomotor sensitization		K127Q: 6		Drug	F (1, 10) = 6.990	p=0.0246	Sidak	Day 1:K127Q Sal vs. Day 7:K127Q Sal	p=0.9993
					Virus	F (1, 10) = 0.5197	p=0.4875		Day 1:Con Coc vs. Day 7:Con Coc	p>0.9999
					Day x Drug	F (1, 10) = 0.05914	p=0.8128		Day 1:K127Q Coc vs. Day 7:K127Q Coc	p>0.9999
					Day x Virus	F (1, 10) = 0.2766	p=0.6104		Day 1:Con Sal vs. Day 1:K127Q Sal	p=0.9999
					Drug x Virus	F (1, 10) = 0.1893	p=0.6728		Day 1:Con Coc vs. Day 1:K127Q Coc	p>0.9999
					Day x Drug x Virus	F (1, 10) = 3.936e-005	p=0.9951		Day 7:Con Sal vs. Day 7:K127Q Sal	p=0.997
									Day 7:Con Coc vs. Day 7:K127Q Coc	p>0.9999
									Day 1:Con M Sal vs. Day 1:Con Coc	p=0.7618
									Day 1:K127Q Sal vs. Day 1:K127Q Coc	p=0.9585
									Day 7:Con Sal vs. Day 7:Con Coc	p=0.8936
									Day 7:K127Q Sal vs. Day 7:K127Q Coc	p=0.993
Figure 21 - Reduced catalytic activity in VTA DA neurons does not impair conditioned cociane reward at a low dose.										
Fig 21A	DA K127Q	Male	Con: 8	Unpaired t-test		t=0.8570, df=18	p=0.4027			
	CPP posttest scores		K127Q: 12							
	DA K127Q	Female	Con: 9	Unpaired t-test		t=0.2827, df=18	p=0.7806			
	CPP posttest scores		K127Q: 11							
Fig 21B	DA K127Q	Male	Con: 8	Two-way ANOVA	Interaction	F (1, 36) = 1.172	p=0.2861		Saline:Control vs. Saline:K127Q	p=0.3997

Table 3. (cont'd).

	CPP conditioning locomotor activity		K127Q: 12		Drug	F (1, 36) = 4.804	p=0.0349	Tukey	Saline:Control vs. Cociane:Control p=0.8901
					Virus	F (1, 36) = 11.05	p=0.0020		Saline:Control vs. Cociane:K127Q p=0.0022
									Saline:K127Q vs. Cociane:Control p=0.8536
									Saline:K127Q vs. Cociane:K127Q p=0.0633
									Cociane:Control vs. Cociane:K127Q p=0.018
	DA K127Q	Female	Con: 9	Two-way ANOVA	Interaction	F (1, 36) = 0.01738	p=0.8959		Saline:Control vs. Saline:K127Q p=0.9943
	CPP conditioning locomotor activity		K127Q: 11		Drug	F (1, 36) = 6.228	p=0.0173	Tukey	Saline:Control vs. Cociane:Control p=0.3949
					Virus	F (1, 36) = 0.05060	p=0.8233		Saline:Control vs. Cociane:K127Q p=0.3884
									Saline:K127Q vs. Cociane:Control p=0.2363
									Saline:K127Q vs. Cociane:K127Q p=0.2226
									Cociane:Control vs. Cociane:K127Q p=0.9999
Fig 21C	DA K127Q	Male	Con: 8	Unpaired t-test		t=2.366, df=18	p=0.0294		
	Pretest locomotor activity		K127Q: 12						
	DA K127Q	Male	Con: 8	Unpaired t-test		t=3.054, df=18	p=0.0068		
	Posttest locomotor activity		K127Q: 12						
	DA K127Q	Female	Con: 9	Unpaired t-test		t=1.913, df=18	p=0.0718		
	Pretest locomotor activity		K127Q: 11						
	DA K127Q	Female	Con: 9	Unpaired t-test		t=2.351, df=18	p=0.0303		
	Posttest locomotor activity		K127Q: 11						
Figure 22 - Morphine preference is not changed by decreased VTA DA SGK1 catalytic activity.									
Fig 22A	DA K127Q	Male	Con: 10	Two-way ANOVA with RM	Time x Virus	F (3, 75) = 0.5897	p=0.6237		
	Daily morphine preference		K127Q: 17		Time	F (3, 75) = 1.548	p=0.2091		
					Virus	F (1, 25) = 0.9607	p=0.3364		
					Subject	F (25, 75) = 3.645	p<0.0001		
	DA K127Q	Male	Con: 10	Unpaired t-test		t=0.9801, df=25	p=0.3364		
	Average morphine preference		K127Q: 17						
Fig 22B	DA K127Q	Male	Con: 10	Unpaired t-test		t=0.6112, df=25	p=0.5466		
	Average total fluid intake		K127Q: 17						
	Average morphine intake	Male	Con: 10	Unpaired t-test		t=1.134, df=25	p=0.2677		
			K127Q: 17						
	Average quinine intake	Male	Con: 10	Unpaired t-test		t=0.4866, df=25	p=0.6308		
			K127Q: 17						

Table 3. (cont'd).

Fig 22C	DA K127Q Daily quinine preference	Male	Con: 4 K127Q: 5	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (3, 21) = 1.362 F (1.771, 12.39) = 2.025 F (1, 7) = 0.07271 F (7, 21) = 1.788	p=0.2817 p=0.1758 p=0.7952 p=0.1428
	DA K127Q Average quinine preference	Male	Con: 4 K127Q: 5	Unpaired t-test		t=0.2695, df=7	p=0.7953
Fig 22D	DA K127Q Average total fluid intake	Male	Con: 4 K127Q: 5	Unpaired t-test		t=0.1603, df=7	p=0.8772
	DA K127Q Average quinine intake	Male	Con: 4 K127Q: 5	Unpaired t-test		t=0.2951, df=7	p=0.7765
	DA K127Q Average water intake	Male	Con: 4 K127Q: 5	Unpaired t-test		t=0.5009, df=7	p=0.6318
Figure 23 - Decreased VTA SGK1 catalytic activity does not alter sucrose preference.							
Fig 23A	VTA K127Q Daily sucrose preference	Male	GFP: 6 K127Q: 7	Two-way ANOVA with RM	Day x Virus Day Virus Subject	F (3, 33) = 1.826 F (1.397, 15.37) = 3.271 F (1, 11) = 3.454 F (11, 33) = 2.092	p=0.1616 p=0.0792 p=0.0900 p=0.0502
	VTA K127Q Average sucrose preference	Male	GFP: 6 K127Q: 7	Unpaired t-test		t=1.954, df=11	p=0.0766
Fig 23B	VTA K127Q Average total fluid intake	Male	GFP: 6 K127Q: 7	Unpaired t-test		t=0.8238, df=11	p=0.4276
	Average sucrose intake	Male	GFP: 6 K127Q: 7	Unpaired t-test		t=2.160, df=11	p=0.0537
	Average water intake	Male	GFP: 6 K127Q: 7	Unpaired t-test		t=1.379, df=11	p=0.1952
Fig 23C	DA K127Q Daily sucrose preference	Male	Con: 12 K127Q: 15	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (3, 75) = 0.9562 F (2.020, 50.50) = 48.00 F (1, 25) = 1.429 F (25, 75) = 4.202	p=0.4180 p<0.0001 p=0.2431 p<0.0001
	DA K127Q Average sucrose preference	Male	Con: 12 K127Q: 15	Unpaired t-test		t=1.085, df=25	p=0.2884

Table 3. (cont'd).

	DA K127Q Daily sucrose preference	Female	Con: 14 K127Q: 18	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (3, 90) = 0.8882 F (3, 90) = 7.227 F (1, 30) = 1.549 F (30, 90) = 2.937	p=0.4504 p=0.0002 p=0.2229 p<0.0001
	DA K127Q Average sucrose preference	Female	Con: 14 K127Q: 18	Unpaired t-test		t=1.244, df=30	p=0.2231
Fig 23D	DA K127Q Average total fluid intake	Male	Con: 12 K127Q: 15	Unpaired t-test		t=0.9286, df=25	p=0.3620
	Average sucrose intake	Male	Con: 12 K127Q: 15	Unpaired t-test		t=0.08217, df=25	p=0.9352
	Average water intake	Male	Con: 12 K127Q: 15	Unpaired t-test		t=1.681, df=25	p=0.1052
	DA K127Q Average total fluid intake	Female	Con: 14 K127Q: 18	Unpaired t-test		t=0.4965, df=30	p=0.6231
	Average sucrose intake	Female	Con: 14 K127Q: 18	Unpaired t-test		t=1.106, df=30	p=0.2774
	Average water intake	Female	Con: 14 K127Q: 18	Unpaired t-test		t=0.9933, df=30	p=0.3285
Figure 24 - Cocaine intake and motivation to earn a reward are not mediated by VTA DA SGK1 catalytic activity.							
Fig 24A	DA K127Q Sucrose acquisition of all mice	Males	Con: 22 K127Q: 20	Log-rank (Mantel-Cox) test		Chi square = 0.2112	p=0.6458
Fig 24B	DA K127Q Sucrose rewards earned	Males	Con: 11 K127Q: 10	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (6, 114) = 0.9070 F (3.085, 58.62) = 48.99 F (1, 19) = 3.340 F (19, 114) = 4.496	p=0.4926 p<0.0001 p=0.0834 p<0.0001
Fig 24C	DA K127Q Days to sucrose acquisition	Males	Con: 11 K127Q: 10	Unpaired t-test		t=0.9845, df=19	p=0.3372
Fig 24D	DA K127Q Cocaine rewards earned	Males	Con: 11 K127Q: 10	Two-way ANOVA with RM	Time x Virus Time Virus Subject	F (6, 114) = 1.044 F (2.672, 50.77) = 9.178 F (1, 19) = 1.527 F (19, 114) = 5.997	p=0.4006 p=0.0001 p=0.2316 p<0.0001

Table 3. (cont'd).

Fig 24E	DA K127Q Progressive ratio breakpoint	Males	Con: 6 K127Q: 5	Unpaired t-test		t=0.1738, df=9	p=0.8659			
Fig 24F	DA K127Q Cocaine dose-response curve	Males	Con: 7 K127Q: 8	Two-way ANOVA with RM	Dose x Virus Dose Virus Subject	F (5, 65) = 0.4041 F (2.649, 34.44) = 23.07 F (1, 13) = 1.514 F (13, 65) = 4.737	p=0.8443 p<0.0001 p=0.2404 p<0.0001			
Figure 25 - K127Q expression in VTA DA neurons lacking endogenous SGK1 expression does not alter cocaine CPP.										
Fig 25A	VTA DA K127Q rescue CPP posttest scores	Male	Con: 8 DA KO: 6 Rescue: 6	One-way AVOVA		F (2, 17) = 0.4350	p=0.6543			
	VTA DA K127Q rescue CPP posttest scores	Female	Con: 8 DA KO: 5 Rescue: 5	One-way AVOVA		F (2, 15) = 1.235	p=0.3189			
Fig 25B	VTA DA K127Q rescue CPP conditioning locomotor activity	Male	Con: 8 DA KO: 6 Rescue: 6	Two-way AVOVA	Interaction Drug Virus	F (2, 34) = 0.03965 F (1, 34) = 12.39 F (2, 34) = 0.04558	p=0.9612 p=0.0012 p=0.9555	Tukey	Saline:Control vs. Saline:DA SGK1 KO Saline:Control vs. Saline:Rescue Saline:Control vs. Cocaine:Control Saline:Control vs. Cocaine:DA SGK1 KO Saline:Control vs. Cocaine:Rescue Saline:DA SGK1 KO vs. Saline:Rescue Saline:DA SGK1 KO vs. Cocaine:Control Saline:DA SGK1 KO vs. Cocaine:DA SGK1 KO Saline:DA SGK1 KO vs. Cocaine:Rescue Saline:Rescue vs. Cocaine:Control Saline:Rescue vs. Cocaine:DA SGK1 KO Saline:Rescue vs. Cocaine:Rescue Cocaine:Control vs. Cocaine:DA SGK1 KO Cocaine:Control vs. Cocaine:Rescue Cocaine:DA SGK1 KO vs. Cocaine:Rescue	p>0.9999 p=0.9996 p=0.2443 p=0.4614 p=0.373 p=0.9998 p=0.3027 p=0.5117 p=0.4252 p=0.1882 p=0.362 p=0.2893 p=0.9999 p>0.9999 p>0.9999
	VTA DA K127Q rescue CPP conditioning locomotor activity	Female	Con: 8 DA KO: 5 Rescue: 5	Two-way AVOVA	Interaction Drug Virus	F (2, 30) = 2.351 F (1, 30) = 31.51 F (2, 30) = 4.390	p=0.1126 p<0.0001 p=0.0213	Tukey	Saline:Control vs. Saline:DA SGK1 KO Saline:Control vs. Saline:Rescue Saline:Control vs. Cocaine:Control Saline:Control vs. Cocaine:DA SGK1 KO Saline:Control vs. Cocaine:Rescue Saline:DA SGK1 KO vs. Saline:Rescue Saline:DA SGK1 KO vs. Cocaine:Control Saline:DA SGK1 KO vs. Cocaine:DA SGK1 KO Saline:DA SGK1 KO vs. Cocaine:Rescue Saline:Rescue vs. Cocaine:Control	p=0.9977 p=0.9941 p=0.284 p=0.0109 p<0.0001 p>0.9999 p=0.6927 p=0.0665 p=0.001 p=0.7492

Table 3. (cont'd).

								Saline:Rescue vs. Cocaine:DA SGK1 KO	p=0.0803
								Saline:Rescue vs. Cocaine:Rescue	p=0.0013
								Cocaine:Control vs. Cocaine:DA SGK1 KO	p=0.4957
								Cocaine:Control vs. Cocaine:Rescue	p=0.0131
								Cocaine:DA SGK1 KO vs. Cocaine:Rescue	p=0.5778
Fig 25C	VTA DA K127Q rescue CPP pretest locomotor activity	Male	Con: 8 DA KO: 6 Rescue: 6	One-way AVOVA	F (2, 17) = 0.8293	p=0.4533			
	VTA DA K127Q rescue CPP posttest locomotor activity	Male	Con: 8 DA KO: 6 Rescue: 6	One-way AVOVA	F (2, 17) = 2.097	p=0.1535			
	VTA DA K127Q rescue CPP pretest locomotor activity	Female	Con: 8 DA KO: 5 Rescue: 5	One-way AVOVA	F (2, 15) = 4.579	p=0.0280	Tukey	Control vs. DA SGK1 KO	p=0.0411
								Control vs. K127Q Rescue	p=0.0867
								DA SGK1 KO vs. K127Q Rescue	p=0.9309
	VTA DA K127Q rescue CPP posttest locomotor activity	Female	Con: 8 DA KO: 5 Rescue: 5	One-way AVOVA	F (2, 15) = 2.300	p=0.1346			

Chapter 4. Conclusions and future directions

Dissertation summary

The abuse of illicit drugs is a substantial problem in the United States (NIDA, 2020a, b; SAMHSA, 2019). Despite the prevalence and significant negative impact of substance use disorders, treatments remain inadequate. This issue stems, in part, from our limited understanding of drug-induced changes in the mesocorticolimbic dopamine (DA) system. Within this circuit, the ventral tegmental area (VTA) is a critical mediator of drug reward (Volkow and Morales, 2015), and drug-induced changes in VTA gene expression and neuronal activity have been linked to maladaptive behaviors in mice (Lammel et al., 2014; Volkow and Morales, 2015). We previously identified serum- and glucocorticoid-inducible kinase 1 (SGK1) as one of only five genes transcriptionally upregulated in the VTA following chronic cocaine and morphine administration. Expanding on this transcriptional regulation, induction of VTA SGK1 catalytic activity and phosphorylation at Ser78 were found following chronic drug administration (Heller et al., 2015). Supported by SGK1-regulation of stress- and learning-induced behaviors (Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008; Yuen et al., 2011), changes in phosphorylation and activity of VTA SGK1 were proposed as modulators of drug-related behaviors. Previous work had demonstrated that VTA overexpression of a constitutively active SGK1 mutant (S422D) was capable of altering locomotor sensitization to injections of cocaine or morphine but with no significant effects on drug conditioned place preference (CPP) (Heller et al., 2015). However, these studies only focused on increased VTA SGK1 catalytic activity, leaving half of the story untold. To expand on these early findings, the work outlined in this thesis sought to characterize

the impact of decreased VTA SGK1 mRNA expression, catalytic activity, and phosphorylation on cocaine and morphine reward-related behaviors.

Experiments performed in Chapter 2 aimed to identify the role of SGK1 expression in reward behaviors. Specifically, a FlxSGK1 mouse line was utilized to assess reward behaviors following deletion of SGK1 within the VTA or DA neurons. VTA-specific knockdown of SGK1 did not alter morphine preference as measured by a two-bottle choice (TBC) task and, similarly, did not change cocaine CPP. Together, these findings indicate that VTA SGK1 expression is not required for drug reward behaviors. To next manipulate SGK1 in a cell type-specific manner, a mouse line lacking SGK1 in all DA neurons was generated. As this is a developmental deletion, basic physiological measurements related to DA were taken throughout early adulthood. Male and female DA SGK1 knockout (KO) mice showed a modest but significant reduction in body weight, thought to be driven by a decrease in body length. Additionally, male mice exhibited increased locomotor activity in an open field task. While mice did have altered physiology, DA SGK1 KO did not alter drug reward behaviors, consistent with VTA knockdown of SGK1. In all, this work demonstrated for the first time that while SGK1 is regulated by drugs of abuse, the expression of SGK1 itself is not required for drug reward behaviors.

Though no changes in reward behavior were observed following SGK1 gene deletion, it is possible that other AGC kinases could be engaging in opportunistic compensation (Burchfield et al., 2004; Di Cristofano, 2017; Pearce et al., 2010), obscuring the potential impact of decreased SGK1 catalytic activity. To circumvent compensation caused by SGK1 deletion, a viral-mediated approach to altered SGK1 function was taken. Utilizing an SGK1 mutant lacking the ability to bind ATP (K127Q), and therefore rendered

catalytically inactive, the experiments outlined in Chapter 3 sought to characterize the impact of decreased SGK1 catalytic activity on drug reward behaviors. Early work showed that decreased VTA SGK1 catalytic activity was capable of impairing cocaine CPP and modulated morphine preference. VTA overexpression of wild-type SGK1 did not alter CPP behavior, supporting that reward effects were not due to a general overexpression of SGK1 protein in the VTA, but were specific for SGK1 constructs with impaired catalytic activity.

As the VTA is a heterogenous brain region (Morales and Margolis, 2017), we generated a Cre-dependent version of the K127Q virus in order to drive expression of our SGK1 mutant in a cell type-specific manner. With this tool, experiments next characterized catalytic activity in VTA DA, but not GABA, neurons a critical mediator of conditioned cocaine reward. Additionally, cocaine-induced psychomotor activity was robustly increased in mice with VTA DA neuron K127Q expression, while no difference was observed following a GABA-specific manipulation. However, effects of SGK1 activity on drug reward are more complicated than originally predicted. This idea is supported by data demonstrating that decreased VTA DA SGK1 activity did not alter cocaine self-administration, seemingly contradicting CPP results. Similarly, though decreased VTA SGK1 catalytic activity was capable of modulating morphine reward, DA neuron-specific K127Q expression within the VTA did not have an effect, suggesting that SGK1 function in this neuronal population did not drive our previous decrease in morphine reward. Future experiments characterizing SGK1's impact on VTA DA cellular plasticity and electrophysiological properties will be crucial in understanding these differences in drug-related behaviors.

The research conducted as part of this thesis has expanded knowledge of SGK1 function in the central nervous system. Previous work had indicated that SGK1 catalytic activity is capable of modulating stress- and learning-related behaviors (Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008; Yuen et al., 2011), but the relationship between SGK1 catalytic activity and drug reward was unknown. In short, preventing the normal drug-induced upregulation of VTA SGK1 catalytic activity is sufficient to disrupt cocaine and morphine reward, and it is catalytic activity specifically in DA neurons that drives the observed effects on cocaine CPP. Though this work answers questions critical to evaluating SGK1 as a potential therapeutic agent for the treatment of substance use disorders, alternative approaches could have been taken to manipulate SGK1 catalytic activity and to identify the VTA neuronal population in which SGK1 catalytic activity is regulated by drugs of abuse.

Alternative approaches

Pharmacological inhibition of endogenous SGK1

While viral-mediated overexpression of SGK1 mutants has been a critical tool for understanding the behavioral impact of changes in SGK1 catalytic activity (Heller et al., 2015; Lee et al., 2006; Lee et al., 2003; Licznerski et al., 2015; Tsai et al., 2002; Tyan et al., 2008), one caveat with this approach is that endogenous SGK1 protein remains expressed in infected cells and may contribute to altered behavior. Alternatively, reducing SGK1 function using a FlxSGK1 mouse line eliminates this potential confound, however, deletion of endogenous protein may result in opportunistic compensation by other AGK family proteins (Burchfield et al., 2004; Di Cristofano, 2017; Pearce et al., 2010; Sommer

et al., 2013). In order to overcome these potential limitations, pharmacological inhibition of endogenous VTA SGK1 is a promising future avenue to pursue.

SGK1 inhibitors have been generated. The most widely used SGK1 inhibitor is GSK650394, a competitive antagonist that was originally developed as a potential therapeutic for prostate cancer (Sherk et al., 2008). GSK650394 has been used most extensively in cell culture models; however, *in vivo* activity has been assessed, namely in cancerous tissues with delivery via direct injection into tumors (Berdel et al., 2014). The use of GSK650394 is also now being explored in the central nervous system, primarily in stroke and pain models. In animal models of stroke, intracerebroventricular infusion of GSK650394 decreased volume of tissue damage when administered at the time of ischemic insult (Inoue et al., 2016). Moreover, inhibition of spinal cord SGK1 via intrathecal (i.t.) GSK650394 delivery blunted the expression of pain behaviors in both inflammatory and neuropathic pain models, highlighting the ability of GSK650394 to alter behavior (Peng et al., 2012; Peng et al., 2013). Similarly, i.t. GSK650394 reduced antinociception following chronic morphine treatment (Xiao et al., 2019), suggesting that SGK1 inhibition is also capable of modulating drug-associated behaviors. Though these studies show effects of inhibitor application on behavior, biochemical studies explicitly demonstrating decreased SGK1 catalytic activity were not performed. These additional studies are necessary for providing evidence of successful use in the central nervous system.

While these studies suggest a promising use for this SGK1 inhibitor, there are limitations to the interpretation of these studies. Notably, poor cell permeability has been reported (Di Cristofano, 2017) and GSK650394 inhibits SGK2 as well as SGK1 (Sherk et

al., 2008). Cell permeability has been a chief concern of our lab, as GSK650394 works well in our hands when used in a Neuro2A cell culture model, a mouse neuroblastoma cell line, but has not been successful *in vivo* using i.p. or intra-VTA applications (unpublished, Vedrana Bali). This may be explained by the fact that cancerous cells can have different membrane permeability (Anghileri et al., 1988). Additionally, ischemic damage may also increase membrane permeability (Ames and Nesbett, 1983), explaining observable effects of GSK650394 application in stroke models. EMD638683 is another available inhibitor with greater selectivity for SGK1; however, *in vivo* studies utilizing oral consumption of the inhibitor require high doses, suggesting poor bioavailability (Ackermann et al., 2011). To combat these issues in specificity, permeability, and bioavailability, novel inhibitors are currently in development (D'Antona et al., 2015; Halland et al., 2015). Recent work characterized the lipophilic area within SGK1's ATP binding pocket as 47% larger that of AKT, likely accounting for inhibitor specificity for SGK1 over other AGC family kinases (Ortuso et al., 2014). Similarly, identification of unique segments of SGK1's structure suggests that it is possible to generate highly specific inhibitors (Zhao et al., 2007), which are needed to better understand the function of SGK1 in the central nervous system.

Characterization of drug-induced VTA SGK1 catalytic activity and pSer78

The work in Chapter 3 used viral-mediated overexpression of a catalytically inactive SGK1 mutant (K127Q) to demonstrate that catalytic activity in VTA DA, but not GABA, neurons plays a critical role in conditioned cocaine reward. Because decreased catalytic activity in VTA DA neurons disrupted cocaine CPP, this suggests that DA

neurons would show an increase in SGK1 catalytic activity following chronic drug treatment; however, this prediction remains unproven. Moreover, SGK1 is expressed in many cell types, and morphine has been shown to induce glial SGK1 regulation in the striatum (Arteaga et al., 2008; Avey et al., 2018; Slezak et al., 2013), highlighting the need to characterize VTA SGK1 regulation in a cell type-specific manner . Immunohistochemistry (IHC) presents the best method for identifying which cell types in the VTA increase SGK1 catalytic activity and phosphorylation at Ser78 following chronic drug administration. Again, given the effect of neuronal SGK1 manipulation on drug reward behavior (Chapter 3), VTA DA and GABA neurons are of particular interest, though glial induction cannot be discounted. While originally proposed as part of this thesis, this work could not be completed due to antibody limitations. Though IHC protocols using primary antibodies against pSer78 and pNDRG, a measure of SGK1 catalytic activity, were tested using multiple antigen retrieval techniques, a measurable signal could not be obtained above background signal or without considerable concern for antibody specificity. Antibodies against phosphorylated SGK1 at Thr256 and Ser422 would provide an indirect measurement of catalytic activity, but similar issues have been reported with these antibodies, as they are less reliable even in western blot (Garcia-Martinez and Alessi, 2008). As commercially available antibodies have been inconsistent, development of a new antibodies against pSer78 and pNDRG may be the best solution to address this important question.

Identification of VTA cell types that upregulate SGK1 mRNA expression in response to drug administration using *in situ* hybridization represents a second approach that could have been taken. While this remains an interesting idea, mRNA expression

does not directly parallel protein regulation. SGK1 was initially selected as a protein of interest based on its transcriptional upregulation following chronic cocaine or morphine treatment; however, total SGK1 levels are not increased in the VTA by repeated drug administration (Heller et al., 2015). Additionally, the time course for mRNA vs protein level regulation does not directly overlap. Following 7 days of i.p. cocaine or morphine administration, SGK1 mRNA induction was observed 24 hours after the last injection but protein regulation is not different from saline controls at this timepoint (Heller et al., 2015). These data highlight that VTA SGK1 mRNA is differently regulated from its catalytic activity and phosphorylation. Further, manipulation of SGK1 gene expression in knockout mouse models did not affect behavior (Chapter 2) but protein alteration through viral-mediated overexpression of SGK1 mutants was capable to disrupting drug reward (Chapter 3). Therefore, while *in situ* hybridization would better characterize SGK1 transcriptional regulation, this may not provide insight into protein-level changes.

Future directions

Role of SGK1 activity on VTA DA-glutamatergic plasticity

The findings in this thesis have characterized the behavioral effects of altered SGK1 expression and catalytic activity on drug reward behavior; however, future work should explore potential mechanisms through which these behaviors are modulated. Findings in Chapter 3 demonstrated that decreased catalytic activity in VTA DA neurons via overexpression of a catalytically inactive mutant (K127Q) is sufficient to decrease cocaine CPP. It is presumed that this occurs through disrupted SGK1 signaling, and, therefore, identification of targets downstream of SGK1 would ultimately provide a

mechanism for disrupted cocaine reward behavior.

Chronic cocaine and morphine are both known to alter VTA DA-glutamatergic plasticity (Doyle and Mazei-Robison, 2020; Francis et al., 2019), and VTA glutamatergic signaling is necessary for the formation of drug CPP (Harris and Aston-Jones, 2003; Harris et al., 2004). Evidence from the literature shows that SGK1 is capable of modulating AMPAR synaptic expression, excitatory currents, and LTP in association with altered stress- or learning-related behaviors (Licznerski et al., 2015; Ma et al., 2006; Yuen et al., 2011). Therefore, a promising line of future investigation includes the impact of SGK1 catalytic activity on VTA DA-glutamatergic plasticity, partially focusing on receptor trafficking and AMPA/NMDA ratio.

As drug administration results in increased VTA DA GluA1 receptor trafficking to the synapse (Lane et al., 2008; Lane et al., 2011), SGK1 regulation of these receptors and their resulting changes in excitatory postsynaptic currents (EPSCs) are of future interest. To date, there is evidence for SGK1 modulation of these events. As previously described in the PFC, stress-induced SGK1 activity is capable of increasing GluA1 membrane insertion though a proposed SGK1−GDI−Rab4 signaling cascade, resulting in increased mEPSC amplitude (Figure 9) (Liu et al., 2010; Yuen et al., 2011). Similarly, in the dorsal horn of the spinal cord, inflammatory pain results in increased GluA1 insertion through an SGK1−glutamate receptor interacting protein (GRIP)-associated protein 1 (GRASP1)−Rab4 signaling cascade (Peng et al., 2012), though these results were not followed up with electrophysiological studies. Finally, in primary cell culture of rat PFC neurons, viral-mediated overexpression of an SGK1 mutant with decreased catalytic activity (S422A) decreased amplitude and frequency of miniature EPSCs (mEPSCs)

(Licznerski et al., 2015). The behavioral studies performed in this thesis characterized impaired drug reward as a consequence of decreased VTA SGK1 catalytic activity. This behavioral measure may be a result of disrupted of GluA1 membrane insertion, leading to altered glutamatergic transmission. This hypothesis could be tested biochemically by fractioning virally infected VTA tissue to probe for membrane vs. cytoplasmic expression of GluA1 in mice with decreased VTA DA catalytic activity (K127Q) and GFP control mice. To follow up this work, *ex vivo* slice electrophysiology could be used to assess altered mEPSC amplitude and frequency in VTA DA neurons of control vs. K127Q mutant expressing mice. For both of these proposed studies, it would be informative to assess changes both at baseline as well as following morphine or cocaine exposure. While much of the literature has linked SGK1 regulation of glutamatergic plasticity to AMPARs, there is further support of SGK1 regulation of glutamatergic transmission though kainite and NMDA receptors (Inoue et al., 2016; Strutz-Seebohm et al., 2005), indicating that regulation of these receptors may also be of future interest.

Drug administration is also well characterized in its ability to increase AMPA/NMDA ratio of VTA DA neurons (Authement et al., 2016; Baimel and Borgland, 2015; Borgland et al., 2004; Saal et al., 2003; Ungless et al., 2001). While research demonstrating SGK1-mediated AMAPR trafficking does not directly address potential changes in AMPA/NMDA ratio, increased AMPAR current contributes to the cocaine-induced increase in VTA DA AMPA/NMDA ratio (Borgland et al., 2004). *Ex vivo* slice electrophysiology could be used to assess altered drug-induced AMPA/NMDA ratio in virally infected VTA DA neurons. Further, both increased GluA1 membrane expression and AMPA/NMDA ratio are correlates on enhanced long-term potentiation (LTP). In the hippocampus, viral-mediated

overexpression of an SGK1 mutant with reduced catalytic activity (S422A) impairs the late phase of LTP (Ma et al., 2006). Given the prediction that SGK1 is capable of modulating GluA1 insertion and AMPA/NMDA ratio, decreased VTA DA catalytic activity may also disrupt LTP and is a worthy area of future study. In combination, these future studies are critical in assessing the role of SGK1 in the potentiation of VTA DA-glutamatergic signaling following drug administration.

As an additional measure of VTA DA-glutamatergic plasticity, analysis of SGK1 mediation of dendritic spine structure and density is of particular interest. Spines are often used as a readout of structural plasticity, and changes in spine density have been correlated with altered cocaine locomotor sensitization and CPP (Maze et al., 2010; Pulipparacharuvil et al., 2008; Russo et al., 2009). Further, drug-induced changes in spine density and structure are well documented in other brain regions, including the nucleus accumbens (NAc), prefrontal cortex (PFC), and hippocampus (Robinson et al., 2002; Robinson and Kolb, 1999; Robinson and Kolb, 2004; Russo et al., 2010). This hypothesis linking SGK1 activity to dendritic spines is supported by research demonstrating that SGK1 knockdown via shRNA decreased spine density in cultured hippocampal and cortical neurons (Piechota et al., 2010). Similarly, in the PFC, overexpression of a kinase deficient SGK1 mutant (S422A) decreased spine density (Licznerski et al., 2015). Further support can be found in the expression of PSD-95, a driver of synaptic assembly and maturation (El-Husseini et al., 2000). In the hippocampus, overexpression of constitutively active SGK1 mutant (S422D) increased PSD-95 expression (Ma et al., 2006), suggesting a relationship between SGK1 catalytic activity and dendritic spines. As mice with decreased VTA DA SGK1 catalytic activity showed impaired cocaine CPP (Chapter 3),

future work should assess K127Q-induced changes in spine density and structure. Ideally, future work would compare spine properties of K127Q vs. GFP expressing controls following drug administration; however, given the well-known difficulties with measuring VTA DA spines, biochemical measurements of PSD-95 may act as a realistic proxy for spine alterations. Interestingly, in the hippocampus K127Q overexpression increased total, stubby, and mushroom spine density in the dorsal CA1 (Steffke et al., 2020). While these results are unexpected, they support the use of viral mediated overexpression to study changes in spine density and structure.

SGK1 in VTA DA electrophysiological membrane properties and DA output

Work in the periphery and limited research in the central nervous system has defined SGK1 as a mediator of a variety of ion channels, transporters, and transcription factors (Lang et al., 2006; Lang et al., 2010). Therefore, it is unlikely that SGK1 regulation is limited to glutamatergic plasticity. A shared effect of chronic cocaine and morphine treatment is increased VTA DA firing and decreased DA output in the NAc. As SGK1 catalytic activity is upregulated at the time of these homeostatic changes, SGK1 could be proposed as a mediator of these events.

Ion channels mediate VTA DA intrinsic excitability and firing properties and many are modulated by drug administration (Francis et al., 2019; Gantz et al., 2018; Morikawa and Morrisett, 2010). Knowledge of altered VTA DA intrinsic excitability is critical to understanding the mechanism by which decreased VTA DA SGK1 catalytic activity alters drug reward. Future experiments should utilize *ex vivo* slice electrophysiology to analyze VTA DA neurons expressing catalytically inactive SGK1 (K127Q) vs. GFP controls

following cocaine or morphine administration. Specifically, measurements of spontaneous firing rate, resting membrane potential, isolated potassium-mediated currents, and neuronal excitability in response to voltage steps would address questions surrounding altered ion channel regulation and cell excitability. In addition to increased VTA DA firing rate, chronic cocaine and morphine administration decrease DA output in the NAc. One experiment to provide support for altered output in K127Q-expressing mice is measurement of extracellular DA in the NAc using fast-scan cyclic voltammetry. Altered DA output in mice with decreased VTA DA SGK1 catalytic activity would build on findings showing decreased CPP (Chapter 3) and contribute to a mechanism behind this disrupted behavior.

Projection-specific SGK1 manipulation on behavior

Experiments in Chapter 3 demonstrated that SGK1 activity in VTA DA, but not GABA, neurons drove a decrease in cocaine CPP behavior. However, recent work in the VTA has illustrated the impressive complexity of this brain region (Lammel et al., 2014; Margolis et al., 2006; Morales and Margolis, 2017). It is now well known that VTA DA projections to different brain regions underlie distinct behaviors (de Jong et al., 2019; Lammel et al., 2014; Tang et al., 2020). Moreover, VTA DA projections have unique properties and responses to drug administration (Lammel et al., 2008; Lammel et al., 2011). The studies performed in this thesis allowed for the manipulation of VTA SGK1 catalytic activity specifically within VTA and GABA neurons; however, DA projection-specific manipulation of SGK1 catalytic activity would allow for further assessment of VTA DA SGK1 catalytic activity in drug reward behaviors. Additionally, retrograde labeling of

VTA DA projections in combination with IHC for pNDRG and pSer78 would allow for the identification of the specific circuits that drive SGK1 catalytic activity and phosphorylation following chronic cocaine or morphine administration. Given the critical role of NAc DA signaling in drug reward behavior, VTA DA neurons projecting to the NAc are a key neuronal population to investigate for further characterization of SGK1 regulation.

Function of VTA SGK1 pSer78 in drug-reward behaviors

In addition to increased VTA SGK1 catalytic activity, chronic cocaine and morphine administration increase phosphorylation of SGK1 at Ser78 (Heller et al., 2015); however, little is known about this phosphorylation event. Work in the hippocampus has demonstrated that contextual fear conditioning increases pSer78 (Lee et al., 2007), and this is behaviorally relevant, as hippocampal overexpression of phosphodeficient SGK1 mutant (S78A) is sufficient to impair fear retention (Lee et al., 2007). These data provide precedent for the study of SGK1 Ser78 phosphorylation in the context of drug administration.

To test the effect of SGK1 Ser78 phosphorylation state on drug reward behavior, the VTA of adult male C57Bl/6J mice were bilaterally injected with phosphodeficient (HSV-HA-SGK1-S78A-GFP), phosphomimetic (HSV-HA-SGK1-S78D-GFP), or control GFP viruses. Using cocaine CPP (7.5 mg/kg) to assess cocaine reward, mice with decreased Ser78 phosphorylation (S78A) showed a significant decrease in time spent in the drug paired chamber compared to GFP controls (Figure 26A), indicative of decreased cocaine reward. Mice overexpressing the S78D mutant were not different from controls (Figure 26A). These data suggest that while decreased VTA SGK1 pSer78 is sufficient to

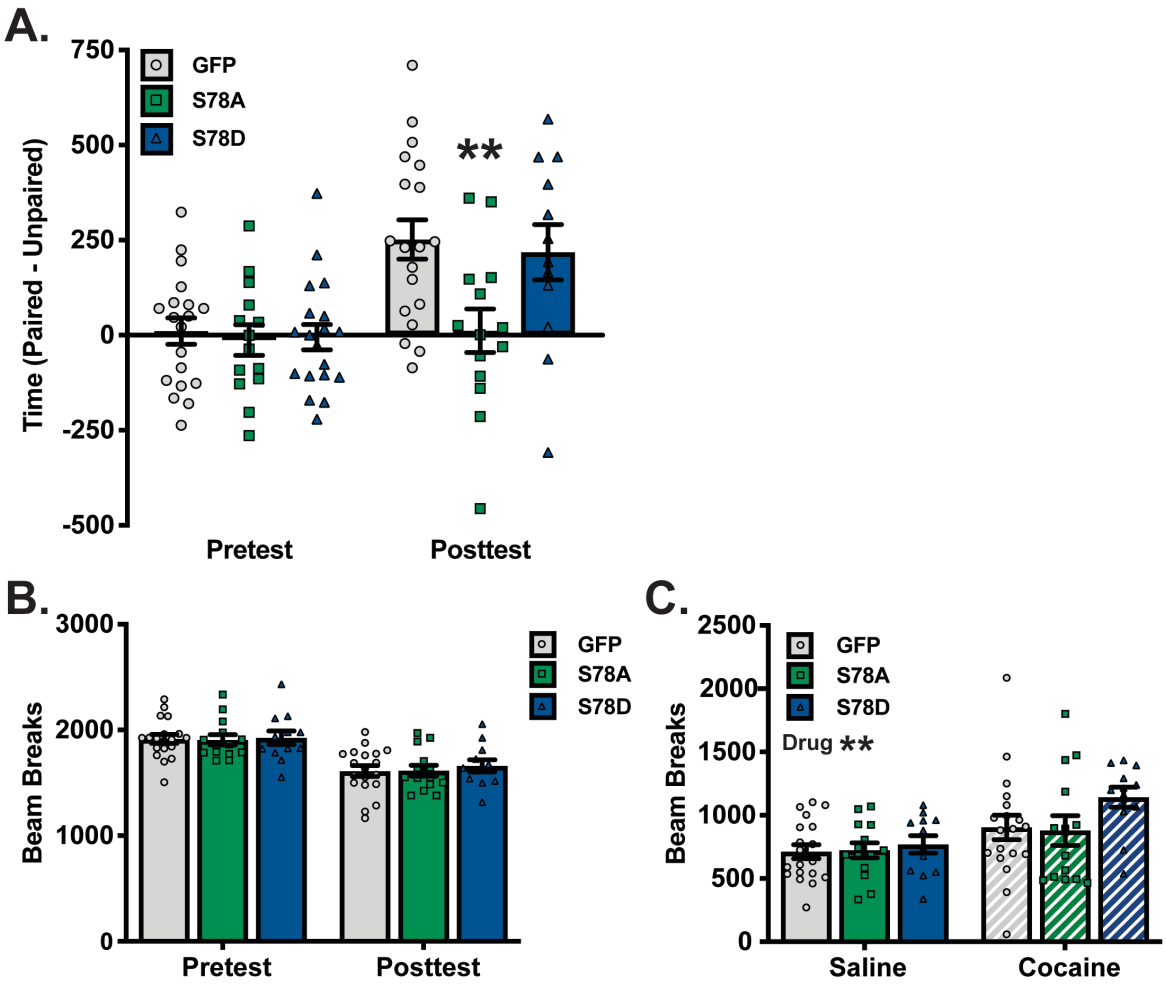

Figure 26. Decreased phosphorylation of VTA SGK1 at Ser78 is sufficient to impair cocaine reward.

A. VTA overexpression of a phosphodeficient SGK1 Ser78 mutant (S78A) was sufficient to decrease cocaine reward (7.5 mg/kg) while a phosphomimetic mutant (S78D) had no effect (n=12-19, one-way ANOVA followed by a Dunnett post-hoc test, p=0.009).

B. Locomotor activity measured during CPP pretest and posttest sessions was unaltered by Ser78 mutant overexpression (n=12-19, one-way ANOVA).

C. During cocaine CPP conditioning sessions, cocaine increased locomotor activity but VTA pSer78 manipulations did not affect locomotor activity (n=12-19, two-way ANOVA, main effect of drug, p<0.001).

impair cocaine reward, mimicking these events does not further increase reward. Neither viral manipulation altered locomotor behavior during CPP testing or conditioning compared to GFP controls (Figure 26B-C). Since chronic morphine similarly increases VTA SGK1 pSer78 (Heller et al., 2015), a second cohort of mice were run in a TBC task to measure morphine preference (0.05 mg/mL). Overexpression of S78A significantly reduced daily morphine preference compared to GFP controls while S78D mice did not differ from controls (Figure 27A), though there was a non-significant decrease in preference observed in the S78A group when the 4 days were averaged together (Figure 27B). This effect was driven by a significant decrease in morphine intake in S78A-expressing mice, with S78D mice drinking significantly less quinine than controls (Figure 27C). Paralleling cocaine reward, these data demonstrate that decreasing VTA SGK1 pSer78 is capable of disrupting morphine reward, though mimicking this phosphorylation does not further increase reward. These observed effects are consistent with viral-mediated manipulation of SGK1 catalytic activity. Decreased VTA SGK1 catalytic activity (K127Q) was sufficient to impair cocaine CPP and morphine preference (Chapter 3) while constitutively active SGK1 (S422D) does not alter cocaine or morphine CPP (Heller et al., 2015). Together, these preliminary studies demonstrate the behavioral relevance of VTA SGK1 pSer78 following chronic drug administration.

Future work would seek to further expand these behavioral findings. Studies determining the cell type of regulation are of chief interest. IHC quantification of neuronal cell types with induced pSer78 following chronic drug administration and behavioral measures of drug reward following cell type-specific overexpression of Ser78 mutants would be the first steps, paralleling VTA SGK1 catalytic activity studies. However, little is

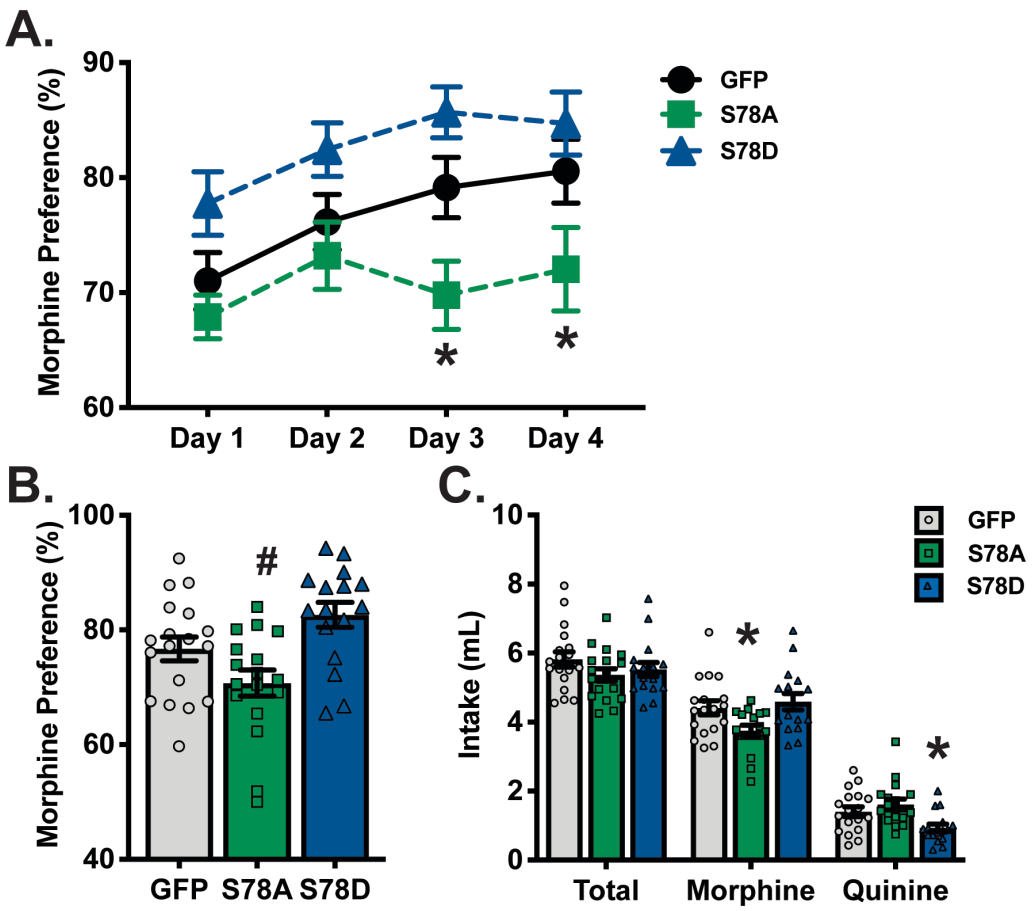

Figure 27. Morphine preference is reduced by a viral-mediated decrease in VTA SGK1 pSer78.

A. Morphine preference (0.05 mg/mL) in a two-bottle choice task was reduced by VTA overexpression of phosphodeficient SGK1 mutant (S78A), but phosphomimetic SGK1 (S78D) overexpression showed no difference in preference from GFP controls (n=16-18, two-way ANOVA followed by a Dunnett post-hoc test, main effect of virus, $p<0.05$).

B. When the 4 days of morphine preference were averaged together, there was a non-significant trend toward decreased preference in mice overexpressing VTA SGK1 S78A and no change to S78D expressing mice (n=16-18, one-way ANOVA followed by a Dunnett post-hoc test, $p=0.0977$).

C. VTA Ser78 manipulation did not alter total fluid intake, but decreased pSer78 (S78A) significantly reduced average morphine intake and increased pSer78 (S78D) significantly reduced quinine intake (n=16-18, one-way ANOVA followed by a Dunnett post-hoc test, $p<0.05$).

known about the functional relevance of phosphorylation at this reside, exposing a critical gap in knowledge. Current *in vitro* pharmacological studies in our lab explore the relationship between SGK1 catalytic activity and pSer78. In a Neuro2A cell model, decreased SGK1 inhibition drove a decrease in pSer78 but decreased pSer78 did not alter catalytic activity (Vedrana Bali, unpublished). Work continues on this relationship and potential mediators of these events (Hayashi et al., 2001). In addition to the potential role of S78 phosphorylation on catalytic activity, effects on protein localization are unknown. Potential changes in subcellular localization, such as trafficking to the nucleus or plasma membrane, following phosphorylation would provide critical insight into the function of SGK1. Overall, understanding the functional implications of phosphorylation at Ser78 will allow for a better understanding of SGK1 in not only the context of addiction but other pathophysiology as well.

Therapeutic potential

The ultimate goal of this research was to assess the impact of decreased SGK1 catalytic activity on reward behaviors, contributing to the evaluation of SGK1 as a potential therapeutic target. Substance use disorder is a prevalent issue in the United States (NIDA, 2020a, b; SAMHSA, 2019), and opioids and cocaine have the highest risk of harm of all drugs of abuse (Morgan et al., 2013; Nutt et al., 2007). Moreover, a majority of individuals are polysubstance users, a particularly vulnerable population (Butelman and Kreek, 2017; Leeman et al., 2016; Leri et al., 2003; Liu et al., 2018b). Though imperfect, pharmacological interventions do exist for opioid use disorder (Strang et al., 2020); however, stimulant use disorder relies entirely on behavioral therapies, which boast only

moderate success (Kampman, 2019). Moreover, existing treatments for substance use disorders focus on a single substance, while instead, the most efficacious therapies may require treatments to span drug classes. Because VTA SGK1 is similarly regulated by opioids (morphine) and stimulants (cocaine), it is poised as a novel therapeutic target for both opioid and stimulant use disorders.

The research performed in this thesis is necessary for our understanding of SGK1 signaling in regulating drug reward behavior. Results demonstrating reduced cocaine and morphine reward following decreased VTA SGK1 activity support SGK1 inhibition as an area of future study. Further, a lack of effect on natural reward behaviors is encouraging, as these results may suggest that inhibition of SGK1 is specific to drug reward without producing anhedonia. An SGK1 inhibitor has been developed for the treatment of prostate cancer (Sherk et al., 2008), though a new generation of inhibitors may address issues with membrane permeability and specificity (D'Antona et al., 2015; Halland et al., 2015). Additionally, as SGK1 is expressed ubiquitously in the body, identification for SGK1 downstream targets regulated by chronic drug administration may allow for the selection of more specific, isolated targets. Altogether, the research presented in this thesis helps build the foundation for novel mechanisms contributing to opioid and stimulant use disorders, the understanding of which is necessary for the development of better pharmaceutical treatments for substance use disorders.

Table 4. Statistics table for SGK1 Ser78 behavioral data.

	Description	Sex	Group size	Statistical test	Main effect	t- or f-statistic	p-value	Post-hoc test	Post-hoc comparison	p-value
Figure 26. Decreased phosphorylation of VTA SGK1 at Ser78 is sufficient to impair cocaine reward.										
Fig 26A	HSV S78A/S78D CPP posttest scores	Male	GFP: 19 S78A: 14 S78D: 12	One-way ANOVA		F (2, 42) = 4.834	p=0.0129	Dunnett	GFP vs. S78A GFP vs. S78D	p=0.0093 p=0.896
Fig 26B	HSV S78A/S78D CPP pretest locomotor activity	Male	GFP: 19 S78A: 14 S78D: 12	One-way ANOVA		F (2, 42) = 0.02935	p=0.9711			
	HSV S78A/S78D CPP posttest locomotor activity	Male	GFP: 19 S78A: 14 S78D: 12	One-way ANOVA		F (2, 42) = 0.2443	p=0.7844			
Fig 26C	HSV S78A/S78D CPP conditioning locomotor activity	Male	GFP: 19 S78A: 14 S78D: 12	Two-way ANOVA	Interaction Drug Virus	F (2, 84) = 0.8270 F (1, 84) = 11.84 F (2, 84) = 1.909	p=0.4409 p=0.0009 p=0.1546			
Figure 27. Morphine preference is reduced by a viral-mediated decrease in VTA SGK1 pSer78.										
Fig 27A	HSV S78A/S78D Daily morphine preference	Male	GFP: 18 S78A: 17 S78D: 16	Two-way ANOVA with RM	Day x Virus Day Virus Subject	F (6, 144) = 1.141 F (3, 144) = 8.590 F (2, 48) = 7.301 F (48, 144) = 5.710	p=0.3414 p<0.0001 p=0.0017 p<0.0001	Dunnett	Day 1: GFP vs. S78A Day 1: GFP vs. S78D Day 2: GFP vs. S78A Day 2: GFP vs. S78D Day 3: GFP vs. S78A Day 3: GFP vs. S78D Day 4: GFP vs. S78A Day 4: GFP vs. S78D	p=0.6165 p=0.1386 p=0.652 p=0.1752 p=0.0252 p=0.1535 p=0.0444 p=0.4494
Fig 27B	HSV S78A/S78D Average morphine preference	Male	GFP: 18 S78A: 17 S78D: 16	One-way ANOVA		F (2, 48) = 7.301	p=0.0017	Dunnett	GFP vs. S78A GFP vs. S78D	p=0.0977 p=0.1077
Fig 27C	HSV S78A/S78D Average total fluid intake	Male	GFP: 18 S78A: 17 S78D: 16	One-way ANOVA		F (2, 48) = 1.296	p=0.2830			
	HSV S78A/S78D Average morphine intake	Male	GFP: 18 S78A: 17 S78D: 16	One-way ANOVA		F (2, 48) = 4.885	p=0.0117	Dunnett	GFP vs. S78A GFP vs. S78D	p=0.0391 p=0.7570
	HSV S78A/S78D Average quinine intake	Male	GFP: 18 S78A: 17 S78D: 16	One-way ANOVA		F (2, 48) = 5.938	p=0.0050	Dunnett	GFP vs. S78A GFP vs. S78D	p=0.4640 p=0.0414

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