ROLE OF SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE 1 (SGK1) IN MORPHINE AND COCAINE REWARD BEHAVIORS

By

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ABSTRACT

ROLE OF SERUM- AND GLUCOCORTICOID-REGULATED KINASE 1 (SGK1)
IN MORPHINE AND COCAINE REWARD BEHAVIORS

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The mechanisms by which drugs of abuse, such as morphine and cocaine, induce long-term neuroadaptations that underlie addiction have not yet been fully defined. One promising brain region for study is the dopamine-rich ventral tegmental area (VTA), known to play a critical role in reward. Given recent evidence that chronic drug exposure increases VTA expression of serum- and glucocorticoid-inducible kinase 1 (SGK1), we wanted to investigate whether chronic drug exposure also increases SGK1 kinase activity and if altering VTA SGK1 activity affects drug reward behaviors. We found that chronic, but not acute, morphine or cocaine administration increased phosphorylation of SGK1 at S78 as well as phosphorylation of an SGK1 substrate, NDRG. To then investigate whether VTA SGK1 activity influences drug reward behaviors, we generated herpes simplex virus (HSV) constructs for local, short-term overexpression of SGK1 mutants. We found VTA expression of catalytically inactive SGK1-K127Q significantly lowered voluntary morphine consumption compared to constitutively active SGK1-S422D. We next examined cocaine locomotor activity and conditioned place preference (CPP) behaviors. Both HSV-GFP and HSV-K127Q mice exhibited locomotor sensitization to cocaine and robust CPP; however, there were no significant differences between the two groups. Given the complexity in these results, future studies will be needed to more fully understand the role of VTA SGK1 activity in reward behavior, including the specific role of S78 phosphorylation. We hope that such studies may identify molecular mechanisms underlying drug dependence that might serve as novel targets for therapeutic intervention in addiction.

Dedicated to my parents,
Patricia Leahy Fallon and Peter Barry Fallon,
for their selfless sacrifices, love, and encouragement.

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shelves, and being himself. Ken's remarkable, sunny attitude often made my day and helped me keep perspective. I thank A.J.'s post-doc, Andrew Eagle, for troubleshooting CPP, for always being happy to teach, and for chatting about outreach and law enforcement issues. To my fellow graduate students, Paula Gajewski, Sophia Kaska, Sarah Cooper, and Liz Williams: thank you all for making graduate school a more hilarious and tolerable place. I thank our exchange student, Julia Brückner, and undergrads Megan Kechner, Tyler Ellsworth, and Devon Pratt for reminding me how much fun college was. In particular I want to thank our undergrad Mackenzie Thibault for the heroic job she did with helping me slice mouse brains. I also want to thank whomever in our lab came up with the nickname "Special Agent BWoww," which I will one day have to put on a name plate. Labs like ours are special places, and I wish my labmates great success in their careers and happiness in their lives.

I am a firm believer that nobody gets anywhere in life alone. Without my closet friend from Michigan State, fellow forensics student Ashley Doran, there would be no sanity or laughter in East Lansing, MI. If not for Laura Fox, my best friend and wifey from Cornell University, occasionally checking in to screw my head back on straight, I could not have accomplished as much as I have, in school and in life, while staying sane. I warmly thank Brad Abramson, who has never stopped reminding me to live a little. Most importantly, my family has provided every tool I've ever needed to succeed, and saying "thank you" could never be enough. Aunt Nini, thank you for taking me under your wing. You always remind me of who I am and where I came from. Dad, I can't believe we've finally reached the first of two graduate degrees. You've been there to challenge and support every decision when I've needed it the most. To my late mom, who taught me everything I know about being conscientious, candid, and compassionate: your water girl did it. Now onward, to the next adventure and next degree!

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KEY TO ABBREVIATIONS

AAV	Adeno-associated virus
ANOVA	Analysis of variance
BMK1	Big mitogen-activated protein kinase 1
bp	Base pairs
CDC	Center for Disease Control and Prevention
CPP	Conditioned place preference
DA	Dopamine
DAT	Dopamine transporter
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ENaC	Epithelial sodium channel
ERK	Extracellular signal-regulated kinase
FKHRL1	Forkhead transcription factor like 1
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GHB	γ-hydroxybutyric acid
GSK3	Glycogen synthase kinase 3
HA	Hemagglutinin
HSV	Herpes simplex virus
i.p	Intraperitoneal
kDa	Kilodaltons

KO	Knock-out
L-DOPA	l-3,4-dihydroxyphenylalanine
LSD	Lysergic acid diethlamide
MAPK	Mitogen-activated protein kinase
MIT	Massachusetts Institute of Technology
μOR	μ-opioid receptor
MS	Mass spectrometry
MSU	Michigan State University
mTORC2	Mammalian target of rapamycin complex 2
NAc	Nucleus accumbens
Nedd4-2	Neural precursor cell expressed, developmentally down-regulated 4-2
NFDM	Non-fat dry milk
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Tween-20
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PVDF	polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RO	Reverse osmosis

RT-PCR	Real-time PCR
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SGK	Serum- and glucocorticoid-inducible kinase
TH	Tyrosine hydroxylase
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

CHAPTER 1: Introduction

The dopaminergic reward system

The neurotransmitter dopamine (DA) plays a crucial role in a wide range of central nervous system functions. For example, the four major dopaminergic pathways in the brain mediate reward and motivated behavior (mesolimbic pathway), emotional affect (mesocortical pathway), voluntary motor control (nigrostriatal pathway), and secretion of prolactin from the anterior pituitary (tuberoinfundibular pathway; reviewed in (Arias-Carrion et al. 2010, Bannon & Roth 1983, Chakravarthy et al. 2010, Fitzgerald & Dinan 2008)). Moreover, in the periphery DA mediates vasodilation, reduces lymphocyte activation, facilitates kidney function, and inhibits gastrointestinal motility (reviewed in (Beaulieu & Gainetdinov 2011)). DA belongs to the catecholaminergic family of neurotransmitters which also includes norepinephrine and epinephrine. In the brain it is synthesized in nerve terminals in a two-step process whereby the amino acid l-tyrosine is first oxidized by the enzyme tyrosine hydroxylase (TH) into l-3,4-dihydroxyphenylalanine (L-DOPA); L-aromatic amino acid decarboxylase then converts L-DOPA to DA. Specialized transporter proteins facilitate the storage and clearance of DA at the presynaptic terminal. The vesicular monoamine transporter (VMAT) which packages DA into synaptic vesicles is one such protein. Another is the presynaptic DA transporter (DAT) that is necessary for reuptake of DA following synaptic release and is a critical mediator of the strength and duration of the neurotransmitter signal (Giros et al. 1991, Kilty et al. 1991, Shimada et al. 1991). G-protein coupled DA receptors mediate DA signaling and are located on both pre- and post-synaptic neurons. DA receptors are grouped into two families: the D1-like family includes the D1 and D5 receptors which are coupled to stimulatory G_S proteins and the D2-like family

includes the D2, D3, and D4 receptor subtypes which are coupled to inhibitory $G_{i/o}$ proteins (reviewed in (Beaulieu & Gainetdinov 2011, Missale et al. 1998)). Thus, DA modulates the neuronal excitability via second messenger signals such as adenylate cyclase and downstream kinases and phosphatases.

Neurons that produce DA are located in discreet structures within the brain. Given DA's essential role in movement and reward, the best-studied populations are the substantia nigra (A10) and ventral tegmental area (VTA; A9) regions, respectively (reviewed in (Bjorklund & Dunnett 2007)). Additional groups of DA neurons include those in the retina and olfactory bulb, along with the more poorly characterized A11-A15 neurons of the diencephalon and the A9 retrorubral field (Yamamoto & Vernier 2011). As a consequence of our focus on drugs of abuse and reward, we will focus on the VTA neurons for the remainder of this introduction.

The mesocorticolimbic pathway is essential for reward and motivation and consists of VTA DA neurons that project to the nucleus accumbens (NAc) and prefrontal cortex (PFC). These VTA DA neurons exhibit tonic and phasic firing activity to regulate this circuit. While stimuli such as drugs of abuse can affect tonic firing of VTA DA neurons, in general it is their regulation of phasic or "burst" firing that leads to alteration in DA levels in output structures (such as the NAc and PFC) and contributes to reward (Floresco et al. 2003, Grace 1991, Grace 1995, Grace et al. 2007). Although the majority of VTA neurons are dopaminergic (60-65%), there is a significant contribution of interneurons that produce γ-aminobutyric acid (GABA, 30-35%) as well as a small (<5%) population of glutamatergic neurons (Nair-Roberts et al. 2008, Sesack & Grace 2010, Swanson 1982). The GABA interneurons play an important role in maintaining DA tone through local inhibition of VTA DA neurons; thus altering GABAergic activity can have a profound effect on mesocorticolimbic output. For example, opiate drugs such

as morphine bind to μ-opioid receptors (μOR) on GABA interneurons to inhibit GABA release. GABAergic inhibition leads to disinhibition of VTA DA neurons, increasing DA release in the NAc and other output structures (Di Chiara & Imperato 1988, Johnson & North 1992). This release of DA in the NAc, either by direct activation of VTA DA neurons or disinhibition, produces feelings of reward (Di Chiara & Imperato 1988). The importance of increased VTA DA activity for reward behavior was demonstrated in the 1950s in a study where rats learned to repeatedly press a lever to electrically stimulate the mesolimbic reward pathway (Olds & Milner 1954). Release of DA in the NAc is also a critical for reward, as evidenced by the decrease in self-administration of heroin, cocaine, and the D1 and D2 receptor agonist apomorphine in rats when the NAc DA terminals are lesioned using 6-hydroxydopamine (Zito et al. 1984). In addition to mediating the pleasurable effects produced by natural rewards such as food and sex, most drugs of abuse hijack this mesolimbic reward circuit by greatly increasing output from the VTA to the NAc to exert their addictive properties.

Addiction as a public health concern

Drugs of abuse contribute to the heavy economic, personal, and social burdens of addiction, which we define here as continued drug-seeking behaviors despite adverse consequences. Drug abuse is a highly prevalent public health concern: the National Survey on Drug Use and Health found in 2012 (the most recent year for which data are available) that 8.5% of Americans, or 22.2 million people over the age of 12, had a substance dependence or abuse disorder (Substance Abuse and Mental Health Services Administration 2013). The federal government estimates that drug abuse costs $300 billion annually in crime, health care, and lost productivity (Manchikanti et al. 2010). While the economic costs are large, even more

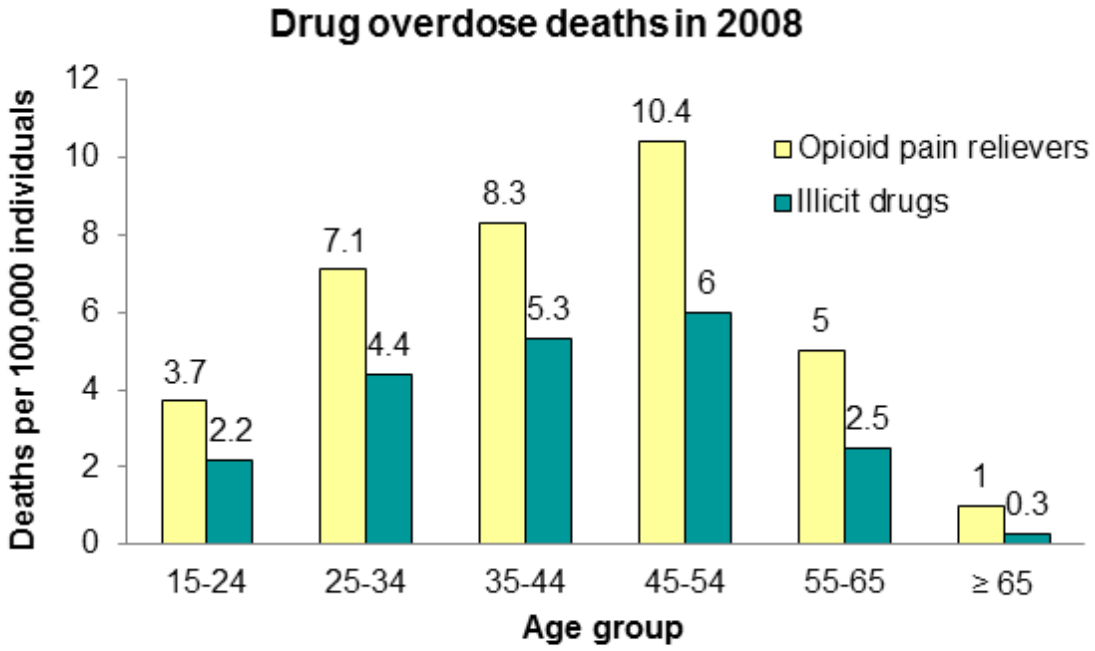

Figure 1. Deaths from opioid pain reliever and illicit drug overdoses. Adapted from the CDC Morbidity and Mortality Weekly Report, 60(43): 1489, 2011. In 2008, more overdose deaths were attributable to opioid pain relievers alone than all other illicit drugs combined.

concerning are the lives lost to overdoses. In 2007, deaths from opioid analgesics exceeded those from heroin and cocaine combined (Center for Disease Control and Prevention 2012) and in the following year deaths from opioid pain relievers surpassed the number of deaths from all other illegal drugs combined (**Figure 1**; Center for Disease Control and Prevention 2011, National Institute on Drug Abuse 2013). In spite of the grave economic and human costs, few effective treatment options for drug addiction are available. Behavioral therapy for generalized addiction has produced mixed results (reviewed in (Carroll & Onken 2005)). And while there are several approved drugs that offer modest effects to treat opiate addiction (including methadone,

buprenorphine, and naltrexone; reviewed in (Kirchmayer et al. 2002, Mattick et al. 2014)), there are no approved pharmacological treatments available for cocaine addiction. The prevalence of opiate and cocaine addiction combined with the dearth of effective treatment options underscore the need to improve our understanding of addiction with the hopes of developing novel pharmacological interventions for this disease.

Neurobiology of opiate and cocaine addiction

In order for more effective treatments to be developed, our understanding of the drug-induced neurobiological adaptations must improve. Both opiates and cocaine increase synaptic DA in the mesolimbic reward system to produce feelings of reward (Di Chiara & Imperato 1988). Pharmacologically, opiate drugs such as morphine elicit reward by binding to μORs on VTA GABAergic interneurons, which then disinhibit the VTA DA neurons, increasing DA release. This is supported by electrophysiological studies demonstrating increased VTA DA neuron firing in response to morphine (Matthews & German 1984). In contrast, cocaine, a stimulant, physically blocks DAT. This prevents DA reuptake from the synapse and consequently prolongs stimulation of DA receptors in the NAc to produce feelings of pleasure (Kuhar et al. 1991). Thus, while cocaine acts locally in the NAc, VTA DA neurons still play a crucial role, as the VTA-NAc projection is necessary for cocaine's pharmacological effect (Roberts & Koob 1982). As illustrated in **Figure 2**, both disinhibition by opiates and disruption of DAT-mediated DA reuptake are sufficient to increase DA in the NAc and PFC (Di Chiara & Imperato 1988, Di Chiara & North 1992). These results demonstrate that DA produced by the VTA and released in the NAc is necessary for the pharmacological actions of morphine and cocaine.

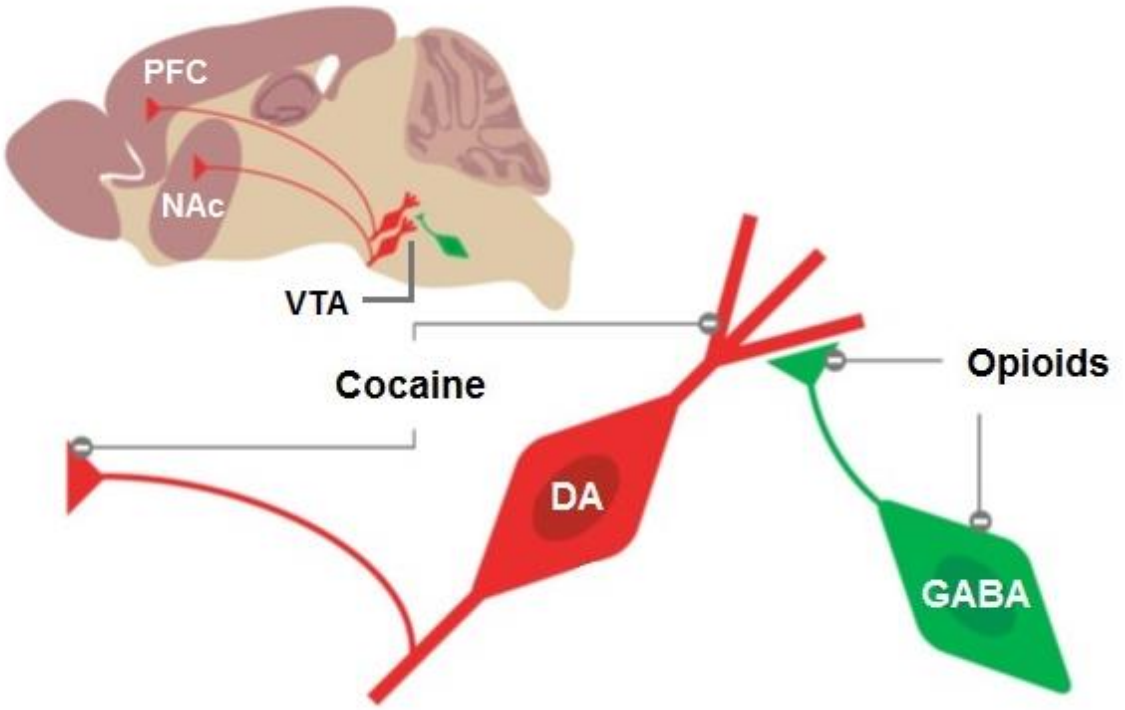

Figure 2. Schematic illustration of the actions of opiates and cocaine in the reward circuit. This sagittal slice illustrates the projections from VTA GABA interneurons (green) and DA neurons (red) to the NAc and PFC. Below, opiates such as morphine elicit feelings of reward by binding to μORs on VTA GABA interneurons, resulting in VTA DA neuron disinhibition and increased DA release in the NAc. In contrast, stimulants such as cocaine block DAT, prolonging the presence of synaptic DA to produce pleasurable feelings. Modified from Luscher & Malenka 2011.

The behavioral effects of opiates and cocaine, which also rely on increased VTA-NAc DA signaling, can be measured experimentally. One such behavior is self-administration: rats voluntarily self-administer intravenous morphine (Headlee et al. 1955) as well as cocaine ((Pickens & Thompson 1968), reviewed in (Schuster & Thompson 1969)). More specifically, rats will also repeatedly press a lever to self-administer morphine directly into the VTA, and this response is attenuated by treatment with the μOR antagonist naloxone (Bozarth & Wise 1981).

Intravenous cocaine self-administration in rats is similarly inhibited by the DA receptor antagonists spiroperidol and SCH23390 (Maldonado et al. 1992, Phillips et al. 1983). The highly addictive and rewarding nature of cocaine is further demonstrated by monkeys that self-administer the drug intravenously to the point of exhaustion, convulsions, or death (Deneau et al. 1969). Despite the different mechanisms of morphine and cocaine action, both drugs are clear reinforcers of drug-acquiring behavior. In a parallel fashion, oral consumption paradigms involving the preference to drink drug solutions versus pharmacologically inactive control solutions have also been developed as another measure of self-administration. Mice prefer drinking morphine to water or quinine control solutions (Belknap et al. 1989, Ferraro et al. 2005, Forgie et al. 1988, Horowitz et al. 1977), while rats can be trained to prefer cocaine to water or glucose solutions (Falk et al. 1996, Falk et al. 1990). Thus, there exist multiple types of voluntary self-administration paradigms that we can take advantage of in order to study drug reward and motivation.

In addition to self-administration, enhanced locomotor activity is another drug-induced behavior amenable to study in the laboratory. While not a reward behavior per se, locomotor activity in response to repeated opiate or cocaine exposures can be examined for sensitization, a phenomenon in which drug-induced movement is increased after multiple drug injections compared to a single exposure (Babbibi & Davis 1972, Robinson & Berridge 1993, van Rossum et al. 1962). Cross-sensitization can also occur, as morphine pre-treatment has been shown to enhance locomotor sensitization to intra-NAc cocaine infusions (Cunningham et al. 1997). Locomotor sensitization is believed to reflect drug-induced plasticity changes; specifically, the alteration of NAc dendritic spine morphology (Robinson et al. 1999, Robinson & Kolb 1999).

Together these results suggest that drug-induced neuroadaptations in the reward circuit contribute to long-term behavioral changes such as locomotor sensitization.

Lastly, cocaine conditioned place preference (CPP) offers a measure of drug reward through comparison of how much time an animal chooses to spend in a chamber which they associate with the injection of a pleasure-inducing drug versus a chamber associated with saline injection. Not only do intravenous morphine and cocaine induce a preference for the drug-paired chamber in rats (Mucha et al. 1982, Rossi & Reid 1976), but specific, direct injection of morphine into the VTA also increases CPP (Phillips & LePiane 1980), emphasizing the critical role of the VTA in this reward behavior. While it has long been clear that the VTA mediates numerous drug-elicited behaviors, the neuroadaptations induced in the reward circuit by chronic drug exposure are not yet well understood.

Serum- and glucocorticoid-inducible kinase 1 (SGK1)

While it is well-established that increased activity of mesolimbic DA circuit is a critical mediator of the rewarding aspect of drug abuse, more recent studies have attempted to uncover the molecular mechanisms underlying this process. In genomic screens, McClung et al. and Heller et al. separately identified an upregulation of serum- and glucocorticoid-inducible kinase 1 (SGK1) mRNA in the VTA in response to chronic morphine administration (Heller et al. in press, McClung et al. 2005). Additionally, SGK1 mRNA was upregulated in the VTA in response to chronic cocaine injections and was one of only a few genes whose expression was similarly regulated by both drugs (Heller et al. in press). Given this similar regulation, SGK1 has been hypothesized to play a role in the neuroadaptations that occur in response to chronic drug exposure and warrants closer examination.

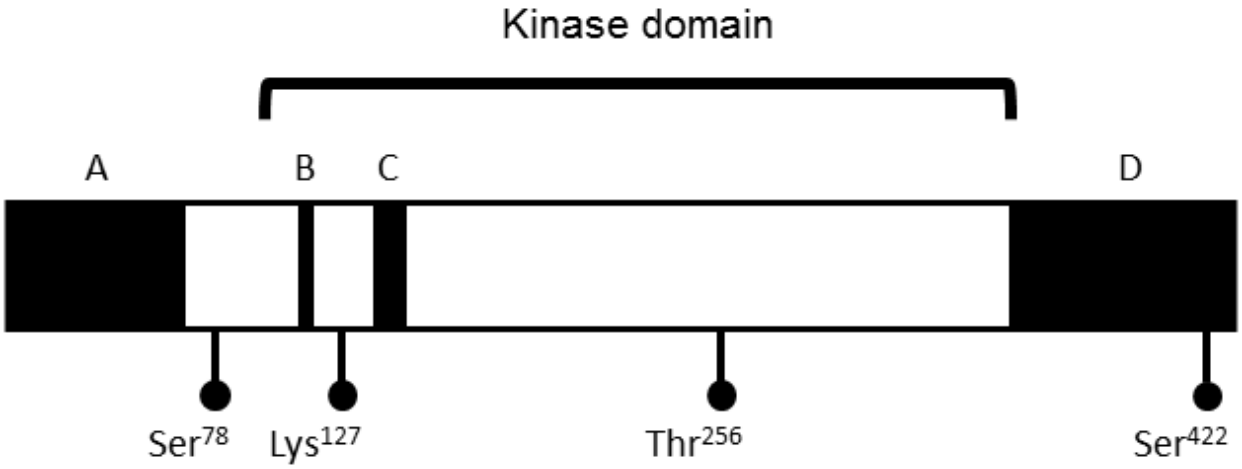

Figure 3. Schematic of rat SGK1 regulatory sites. Phosphorylation at S78, T256, and S422 modulate catalytic activity. Residue K127 is essential for binding of ATP. The bracket denotes the AGC kinase domain. The boxes represent domains suggested by sequence homology and the letters indicate the following sequence features: A = mitochondrial localization sequence, B = ATP-binding, C = nuclear localization sequence, D = AGC kinase C-terminal domain. Modified from the National Center for Biotechnology Information Uniprot database entry Q06226.

SGK1 is a 431 amino acid, 50 kDa serine/threonine protein kinase that is part of the AGC kinase family which also includes protein kinase B (PKB, also known as Akt), protein kinase A, protein kinase C, and protein kinase G (see **Figure 3** for schematic). SGK1 was originally identified as an immediate-early gene induced in response to serum and glucocorticoids (Webster et al. 1993a, Webster et al. 1993b). The structure of SGK1 includes a two-lobed kinase fold (Bossemeyer et al. 1993, Hanks et al. 1988) in which the N- and C-terminal lobes contain a β-strand and α-helix domain, respectively. The catalytic domain partially lies in the hinge area between the two lobes (Zhao et al. 2007). There is an ATP-binding domain, in which the residue K127 is critical for ATP binding (**Figure 3**). Mutations at this residue (K127Q or K127M) render

the kinase catalytically inactive (Park et al. 1999), a consequence with useful applications that will be exploited in the present study.

Within the cell, SGK1 can be found in the nucleus, cytoplasm, or associated with the plasma membrane, with each location having unique functional consequences (reviewed in (Firestone et al. 2003)), and accordingly there is a nuclear localization sequence between residues 131 and 141 (Maiyar et al. 2003). Nuclear localization of SGK1 coincides with pro-proliferative signaling, while activity in the cytoplasm corresponds with terminally differentiated and cell cycle-arrested states (Alliston et al. 2000, Buse et al. 1999, Maiyar et al. 2003). Shuttling of SGK1 between the nucleus and cytoplasm is a pro-proliferative signal (Brunet et al. 2001). Polyubiquitination at the N-terminus results in localization predominantly at the plasma membrane, resulting in subsequent degradation by the 26S proteasome (Brickley et al. 2002); this suggests a mechanism for rapid inactivation after stimulus-induced activation.

In addition to being regulated by its subcellular localization, SGK1 function also seems to depend on distribution in different tissue types. Its most well-characterized role concerns osmoregulation and regulation of sodium transport in the kidneys. SGK1 phosphorylates the ubiquitin ligase Nedd4-2 (neural precursor cell expressed, developmentally down-regulated 4-2), and interrupts its interaction with epithelial sodium channels (ENaC), promoting sodium retention by increasing cell surface ENaC expression (Debonneville et al. 2001, Kamynina & Staub 2002, Wulff et al. 2002). In spite of these seemingly essential functions, SGK1 knockout (KO) mice are viable and only display salt retention deficiencies in response to low-salt diets (Faresse et al. 2012, Fejes-Toth et al. 2008, Wulff et al. 2002), suggesting that the osmoregulatory actions of SGK1 are part of a redundant system. In addition to this well-defined renal activity, SGK1 in heart tissue, blood vessels, and lung buds reveals a potential role for the

development of the heart and blood vessels ((Lee et al. 2001), reviewed in (Firestone et al. 2003)), while SGK1 in ovaries may have multiple roles in controlling cell cycle progression and differentiation (Alliston et al. 2000). Additionally, SGK1 has been found in the brain, eye, liver, pancreas, and intestines (reviewed in (Loffing et al. 2006)), although its function in these tissues is not yet well-understood.

Interestingly, several other isoforms of SGK have been identified, each with different tissue distribution. Most notably, SGK1.1 is a brain-specific isoform that results from alternative splicing of the SGK1 gene (Arteaga et al. 2008). *In situ* hybridization specifically localizes SGK1.1 to the hippocampus, dentate gyrus, cerebral cortex, cerebellar Purkinje cells, and the cerebellar granule layer in mice, overlapping with most areas of SGK1 expression in the brain (Arteaga et al. 2008). In contrast to SGK1, SGK1.1 activity does not appear to regulate ENaC expression in the brain, instead regulating neuronal excitability via modulation of the M-current, a voltage-dependent non-inactivation potassium current mediated by Kv7.2 and Kv7.3 potassium channels (Miranda et al. 2013). In addition to SGK1.1, SGK2 and SGK3 are two other splice variants. Both have approximately 80% sequence homology to the SGK1 catalytic domain and somewhat different tissue distribution (Kobayashi et al. 1999), but little work has been done concerning their function.

In addition to chronic morphine and cocaine, increased SGK1 gene expression is triggered by a variety of stressful stimuli. The following is a non-exhaustive list of other stimuli that can induce SGK1 expression: activators of extracellular signal-regulated kinase (ERK) signaling pathways (epidermal growth factor (EGF), fibroblast growth factor, platelet-derived growth factor, and tissue plasminogen activator), mechanical lesion to the cortex, ischemic injury to the brain, DNA damaging agents, osmotic changes, cell shrinkage or volume decrease, and

numerous others (reviewed in (Firestone et al. 2003)). The SGK1 promoter contains many transcription factor binding sites which may mediate gene expression induced by the aforementioned stimuli. However, only a few transcription factors have been validated *in vitro* or *in vivo*; these include glucocorticoids (Webster et al. 1993b), follicle stimulating hormone (Alliston et al. 2000), and sorbitol-induced hyperosmotic stress (Bell et al. 2000). Thus, SGK1 mRNA is often induced in response to stressful stimuli, suggesting that this kinase plays a role in responding and adapting to such circumstances.

After mRNA has been translated into protein, SGK1 activity is regulated by post-translational phosphorylation as part of two signaling pathways. S422 and T256 are canonical phosphorylation sites that are required for catalytic activity; they are located in the C-terminal hydrophobic motif and activation loop, respectively (**Figure 3** and (Garcia-Martinez & Alessi 2008)). As illustrated in **Figure 4**, the mammalian target of rapamycin complex 2 (mTORC2) first phosphorylates S422 (Garcia-Martinez & Alessi 2008), potentiating phosphorylation at T256 by phosphoinositide-dependent kinase 1 (PDK1, (Park et al. 1999)). Serum-induced stimulation of PI3K results in phosphorylation and activation of PDK1, but the mediators of serum-induced mTORC2 activity are not yet known. An additional phosphorylation site, S78, has been identified outside the catalytic domain (**Figure 3**). Its phosphorylation is mediated by the mitogen-activated protein kinase (MAPK)/ERK pathway and has been shown to be phosphorylated by big mitogen-activated kinase 1 (BMK1)/ERK5 (Hayashi et al. 2001, Lee et al. 2006). Importantly, activation of SGK1 by phosphorylation at S422/T256 versus S78 has been shown to mediate different behavioral effects, which will be discussed in detail below (Lee et al. 2007, Tsai et al. 2002).

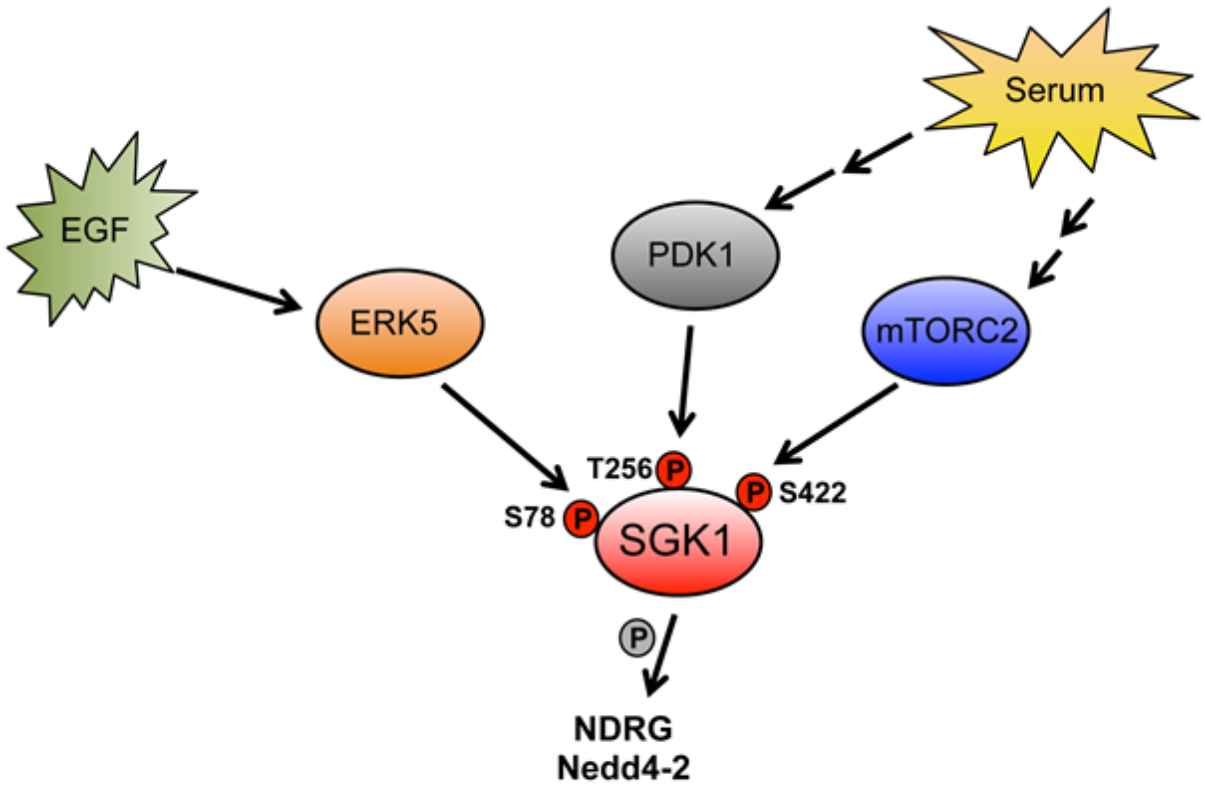

Figure 4. Proposed model for regulation of SGK1 phosphorylation and activity. Serum stimulation of mTORC2 and PDK1 phosphorylation of SGK1 Ser422 and T256, respectively. Activation of the MAPK/ERK pathway by EGF results in S78 phosphorylation by BMK1/ERK5. Activated SGK1 can then phosphorylate substrates such as NDRG and Nedd4-2. Reproduced with permission from M. S. Mazei-Robison.

Although increased SGK1 mRNA has been observed in response to many drugs of abuse (Gonzalez-Nicolini & McGinty 2002, Hassan et al. 2010, Kerns et al. 2005, McClung et al. 2005, Nichols & Sanders-Bush 2002, Piechota et al. 2010), no studies to date have examined drug-induced changes in SGK1 activity. Instead, evidence supporting a role for SGK1 activity in the brain is from studies of learning and memory in the hippocampus. One such study has directly compared hippocampal regulation of SGK1 at S78 versus T256; this was achieved by overexpressing SGK1 mutants capable of blocking phosphorylation at each site. While the

T256A mutant impaired spatial memory formation, the S78A mutant had no effect on spatial learning (Lee et al. 2006), suggesting for the first time that SGK1 regulation via these two separate activation sites leads to behaviorally distinct outcomes. Additional studies similarly support a role for SGK1 activity in hippocampal-mediated learning: Tsai and colleagues showed that wild-type SGK1 injection into the hippocampus reduced the time necessary to navigate a water maze, whereas injection of a kinase deficient S422A mutant inhibited maze performance (Tsai et al. 2002). In another study, transient hippocampal overexpression of an S78A mutant inhibited, while a phospho-mimetic S78D mutant enhanced, retention of a learned fear response (Lee et al. 2007). While these studies illustrate that S78-, S422-, and T256-mediated hippocampal SGK1 activation are important for learning and memory, they also suggest the specific manner in which SGK1 activity is regulated is important, a finding that may be relevant to our drug-induced changes in the VTA. In order to describe phosphorylation-dependent regulation of SGK1 activity, however, there must exist a measure of SGK1 activity; one such readout is the phosphorylation of SGK1 substrates.

Substrates of SGK1

Only a few substrates of SGK1 have been identified, and the functions of these substrates are not yet fully elucidated. Those which have been studied and whose function has been at least partially categorized include Nedd4-2 (Debonneville et al. 2001, Kamynina & Staub 2002, Snyder et al. 2002), glycogen synthase kinase 3 (GSK3) (Kobayashi et al. 1999), forkhead transcription factor like 1 (FKHRL1)/forkhead homeobox type O (Brunet et al. 2001), B-Raf kinase (Zhang et al. 2001), and N-myc downstream regulated gene (NDRG) (Murray et al. 2004). Importantly, all of these proteins except NDRG are also phosphorylated by PKB/Akt

(Murray et al. 2005). Consequently, phosphorylation of NDRG has been used as a specific readout of SGK1 activity (Burgon et al. 2014, Heller et al. in press, Murray et al. 2005). There are four known NDRG isoforms: NDRG1, NDRG2, NDRG3, and NDRG4. They have roughly 60% sequence homology to each other and vary in size from 40 kDa to 42 kDa (Qu et al. 2002, Zhao et al. 2001, Zhou et al. 2001). While not yet fully characterized, the four isoforms have different yet overlapping distributions and functions in the nervous system (reviewed in (Melotte et al. 2010)). SGK1 phosphorylates NDRG1 and NDRG2 at multiple sites including T330, S332, and T348, and indeed this triple phosphorylated state is most commonly reported (Murray et al. 2004). SGK1 phosphorylation of NDRG1 also primes the latter for subsequent phosphorylation by GSK3 (Murray et al. 2005). Unfortunately the consequences of NDRG hyperphosphorylation are not well-described. However, both NDRG1 and NDRG2 have been implicated in neurological diseases, including Charcot-Marie-Tooth demyelination disease of peripheral nerves (Okuda et al. 2004) and Alzheimer's disease (Mitchelmore et al. 2004). Despite the dearth of knowledge about the function of NDRG, its phosphorylation currently stands as the best measure of SGK1 activity.

SGK1 and drugs of abuse

While no studies have been published on the effects of SGK1 kinase activity on drug behaviors, there are many observations that SGK1 gene expression increases in response to drug exposure. Most relevant are results from a DNA microarray study that showed SGK1 mRNA expression is induced in the VTA of rodents treated with chronic morphine (McClung et al. 2005). A similar result has been reported in the extended amygdala: SGK1 transcription only increases in response to chronic, not acute, morphine administration, and this effect depends on

the presence of the μ-opioid receptor (Befort et al. 2008). Chronic treatment with oxycodone, another opioid, similarly elevates SGK1 transcription in whole brain lysates (Hassan et al. 2010). There is also evidence that acute drug administration can induce SGK expression. For example, in the striatum SGK1 transcription increases in response to acute administration of amphetamine (Gonzalez-Nicolini & McGinty 2002), methamphetamine, ethanol, morphine, and heroin (Piechota et al. 2010); while in the prefrontal cortex acute ethanol (Kerns et al. 2005) and lysergic acid diethylamide (LSD) (Nichols & Sanders-Bush 2002) increase SGK1 mRNA. Despite the evidence that SGK1 expression is altered in response to acute or chronic administration of a wide variety of drugs, the ability of SGK1 kinase activity to mediate drug responses has not yet been investigated.

Aims of the present study

Currently addiction is defined by behavioral measures. Our goal is to characterize the neuronal molecular adaptations induced by chronic drug exposure that may mediate these behavioral changes. Out hypothesis is that VTA activity of SGK1 mediates morphine and cocaine drug reward. Therefore in this study, we examined whether modulating SGK1 activity in the VTA would affect drug reward behaviors. This was achieved by performing behavioral assays in mice with VTA-specific overexpression of SGK1 mutants, accomplished via the generation of herpes simplex virus (HSV) constructs. The SGK1 mutants included an S78A phospho-deficient mutant, an S78D phospho-mimetic mutant, and a K127Q catalytically inactive mutant. We used both a morphine two-bottle choice paradigm as well as a cocaine CPP test as behavioral measures of drug reward. In this way, our experiments offer the first evidence of a role for VTA SGK1 activity in drug-elicited behaviors.

CHAPTER 2: Materials and Methods

Laboratory animals

Pathogen-free male C57Bl/6J mice (8-9 weeks of age, Jackson Laboratory, Bar Harbor, Maine) were used in these studies. Mice were group housed in standard cages until stereotaxic surgery. Mice were provided with free access to food and water. Temperature and humidity were maintained at constant levels and rooms followed a 12 hour light-dark cycle beginning at 7:00 A.M. Animals were housed in Michigan State University (MSU) facilities overseen by the MSU Institutional Animal Care and Use Committee according to National Institute of Health Guide for the Care and Use of Laboratory Animals. Procedures were performed according to AUF# 02/13-017-00.

Drug treatment

Morphine sulfate (Sigma) and cocaine hydrochloride (Sigma) were dissolved in 0.9% sterile saline for intraperitoneal (i.p.) injections. A dose of 15 mg/kg was used for single or repeated (7 days) daily injections to generate tissue for Western blot analysis.

Western blot analysis

Western blot analyses were completed as described in Mazei-Robison et al. (2011). Briefly, brains were removed and sliced into 1 mm thick sections with a brain matrix. VTA punches (1.25 mm) were collected and stored at -80°C. Tissue was thawed on ice, sonicated in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100,

1% sodium deoxycholate) containing protease and phosphatase inhibitors (Sigma), and centrifuged 15 minutes at 20,000 RCF. Supernatants were removed and protein concentration was determined by Lowry assay (BioRad). Samples (5-20 μg total protein) were electrophoresed on 4-15% precast SDS gels (BioRad), transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% non-fat dry milk (NFDM) in PBS with 0.1% Tween-20 (PBST) for 1 hour at room temperature. Blots were incubated in primary antibodies overnight, shaking at 4°C, then washed three times for 10 minutes in PBST and incubated in secondary antibody conjugated to horseradish peroxidase (VectorLabs) in 5% NFDM in PBST for 1 hour shaking at room temperature, and finally washed again three times for 10 minutes in PBST. Bands were then visualized using enhanced chemoluminescence. In some instances membranes were stripped for 30 minutes in Restore™ PLUS Western Blot Stripping Buffer (Thermo), blocked again and incubated in additional primary antibodies. Primary antibodies were purchased from Millipore: SGK1 (07-315), Cell Signaling Technology: phospho-SGK1 (5599), phospho-NDRG (3217), NDRG (5196), hemagglutinin (HA, 3724), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 2118), and Sigma: TH (T1299). Western blot data were normalized to the amount of total protein by either GAPDH or Swift Stain (G-Biosciences) quantification.

Generation of SGK1 mutant cDNA and subcloning

Agilent's QuikChange Site-Directed Mutagenesis Kit (#200518) was used to generate the S78A, S78D, and K127Q mutations on a pFLAG-CMV-4 plasmid containing HA-tagged rat SGK1 cDNA (obtained from M. Greenberg, Harvard) according to the manufacturer's instructions. Primers for each mutant were designed and purchased from IDT.

S78A forward: 5'-CCTCACCTCCTCCAGCTCCCTCTCAACAAATC-3'

and reverse: 5’- GATTTGTTGAGAGGGAGCTGGAGGAGGTGAGG-3’

S78D forward: 5'-CCCTCACCTCCTCCAGATCCCTCTCAACAAATC-3'

and reverse: 5'-GATTTGTTGAGAGGGATCTGGAGGAGGTGAGGG-3'

K127Q forward: 5'-GCATTCTATGCCGTCCAAGTTTTGCAGAAGAAAGC-3'

and reverse: 5'-GCTTTCTTCTGCAAAACTTGGACGGCATAGAATGC-3'.

Briefly, polymerase chain reaction (PCR) using Pfu Turbo DNA polymerase and the pFLAG-CMV-4-SGK1 vector and primers was utilized to generate the new mutant vectors. Dpn1 digestion removed the parent template, leaving behind only the new, mutated plasmids. Bacteria were transformed with the mutant plasmids which were then mini-prepped (Qiagen). BamHI and KpnI restriction endonucleases (New England Biosystems) were used to digest the SGK1 insert from the pFLAG-CMV-4 vector. The S78A, S78D, and K127Q cDNAs were each ligated into the p1005 HSV expression vector (Mazei-Robison et al. 2011). Dideoxysequencing was performed to verify that the desired mutations were present and no others had been introduced (ACGT, Inc.) Plasmids were then sent to Dr. Rachael Neve at the Viral Genome Core of the Massachusetts Institute of Technology (MIT) for viral packaging. HSVs were validated by Western blotting for GFP, HA, and SGK1 after stereotaxic injection into the VTA.

Stereotaxic surgery and viral-mediated overexpression

Mice were anesthetized with an i.p. injection of ketamine (100 mg/kg)/xylazine (10 mg/kg). The VTA was targeted using the coordinates -3.2 mm anterior/posterior, +1.0 medial/lateral, and -4.6 mm dorsal/ventral. Bilateral infusions of 0.5 μl each of HSV-GFP, HSV-SGK1-S78A, HSV-SGK1-S78D, HSV-SGK1-K127Q, or constitutively active (CA) HSV-SGK1-S422D (hereafter HSV-S78A, HSV-S78D, HSV-K127Q, and HSV-CA, respectively)

were administered through 33 g Hamilton syringes at a rate of 0.1 μl/min. All HSVs carrying SGK1 also expressed GFP from a separate promoter. Mice were allowed to recover for 24 hours before beginning behavioral experiments. The HSV vectors encoding GFP and SGK1-CA had been previously used and validated (Heller et al. in press, Mazei-Robison et al. 2011).

Two-bottle choice test

After stereotaxic surgery mice were singly housed in standard cages. Two bottles each containing 40 ml of reverse osmosis (RO) water were provided for the 24 hours following surgery. For the next 3 days, mice had a choice between two bottles containing 40 ml of either morphine sulfate or quinine sulfate prepared in 0.2% sucrose-RO water. Multiple concentrations of morphine and quinine were tested in order to establish an optimal concentration for SGK1 mutant experiments. Experiment 1: HSV-GFP mice were provided with bottles containing 0.3 mg/ml morphine and 0.06 mg/ml quinine, 0.2 mg/ml morphine and 0.04 mg/ml quinine, or 0.1 mg/ml morphine and 0.02 mg/ml quinine. Experiment 2: HSV-GFP mice were provided with bottles containing 0.1 mg/ml morphine and 0.02 mg/ml quinine, 0.05 mg/ml morphine and 0.01 mg/ml quinine, or 0.01 mg/ml morphine and 0.002 mg/ml quinine. Experiments 3 and 4: HSV-GFP, HSV-SGK1-CA, or HSV-SGK1-K127Q (Experiment 3) and HSV-GFP or HSV-SGK1-S78A mice (Experiment 4) were provided with bottles of 0.03 mg/ml morphine and 0.006 mg/ml quinine.

Bottle positions were switched every 24 hours to detect potential bias for a given side. Animals were sacrificed by cervical dislocation on the fourth day after surgery and whole brains were preserved in 4% paraformaldehyde (PFA) for 3 days before a PBS rinse and cryoprotection in 30% sucrose in PBS-0.1% sodium azide for sectioning to verify correct targeting.

Preference for morphine was calculated as (total morphine consumed in ml) ÷ (total fluid consumed in ml) × 100%. Preference was calculated for each of the three choice days (daily preference) and as the averaged preference over the three choice days (average preference). Average daily fluid intake during the three choice days was also measured to detect gross changes in fluid consumption.

Cocaine locomotor activity and CPP

Standard CPP conditions and methods were utilized in this study with slight modifications (Heller et al. in press, Kelz et al. 1999). Three-chambered CPP boxes were used (San Diego Instruments) and chambers were distinguished by unique floor textures, wall patterns, and lighting intensities. The central chamber featured metal bar floors, white walls, and maximum intensity lighting; one pairing chamber featured a smooth plastic floor, gray walls, and low lighting; and the second pairing chamber featured a roughly textured plastic floor, black and white diagonally striped walls, and low lighting. Prior to surgery, mice were pre-tested in the three-chambered CPP box for 20 minutes to ensure there was no chamber preference. Mice underwent stereotaxic surgery and were then allowed to recover for 24 hours in standard cages. On the second and third days after surgery mice were placed in one chamber for 30 minutes in the morning (saline injection) and the opposite chamber in the afternoon (20 mg/kg cocaine). Locomotor activity was collected during pairing sessions. On the fourth day after surgery mice were placed in the center chamber and allowed to freely explore all three areas for a 20 minute post-test. Locomotor activity is expressed as number of ambulatory motion counts as measured by breaks of adjacent beams in the CPP chambers ("counts"). CPP data are expressed as time spent in the drug-paired – time spent in the saline-paired chamber (seconds) during the post-test.

Mice were sacrificed by cervical dislocation after the post-test and whole brains were preserved in 4% PFA for 3 days before a PBS rinse and cryoprotection in 30% sucrose in PBS-0.1% sodium azide for sectioning to verify correct targeting.

Statistics

All data are presented as mean ± standard error of the mean (SEM). Unpaired Student's t-tests were used for the analysis of two experimental groups (Experiment 4 and CPP). One-way analysis of variance (ANOVA) was used for analysis of three or more experimental groups (Western blot analysis, Experiments 1-3, and locomotion), followed by a Tukey's or Bonferroni multiple comparison test when appropriate. Two-way repeated measure ANOVA was performed for daily drinking preferences. Effects were considered significant at $p < 0.05$.

CHAPTER 3: Results

Assessment of drug-induced SGK1 signaling changes in the VTA

Mice were given daily i.p. injections of morphine or cocaine to assess if SGK1 signaling in the VTA differed in response to acute (1 day) versus chronic (7 day) drug administration. Chronic injection of morphine or cocaine increased pSGK1 and pNDRG levels in the VTA compared to saline-injected controls (**Figure 5A**; one-way ANOVAs, pSGK1: $F(2, 21) = 5.92$ and $p < 0.01$, pNDRG: $F(2,21) = 11.3$ and $p < 0.001$, post-hoc Tukey's multiple comparison test). No differences were observed in total levels of SGK1 or NDRG (**Figure 5A**, one-way ANOVAs). To determine if an acute injection of drug could also increase SGK1 and NDRG phosphorylation, we examined protein levels in the VTA one hour after a single drug injection. No differences were observed in either the phosphorylated or total amounts of SGK1 or NDRG proteins (**Figure 5B**, one-way ANOVAs). These data suggest that chronic administration of drug is necessary to produce changes in SGK1 signaling in the VTA.

Generation and validation of HSV-SGK1 constructs

To investigate whether drug-induced changes in VTA SGK1 signaling mediate drug-related behaviors, several HSV viral vectors were created to allow direct and selective manipulation of SGK1 activity in the VTA. The HSV-GFP, HSV-SGK1 wild type, and HSV-CA vectors were readily available and previously validated in the lab (Heller et al. in press, Mazei-Robison et al. 2011). The HSV-CA vector contains a mutation at SGK1's canonical activation site, S422D, which mimics phosphorylation and produces constitutively active SGK1 protein. To explore the role of phosphorylation at S78, which we observed is robustly increased in the VTA

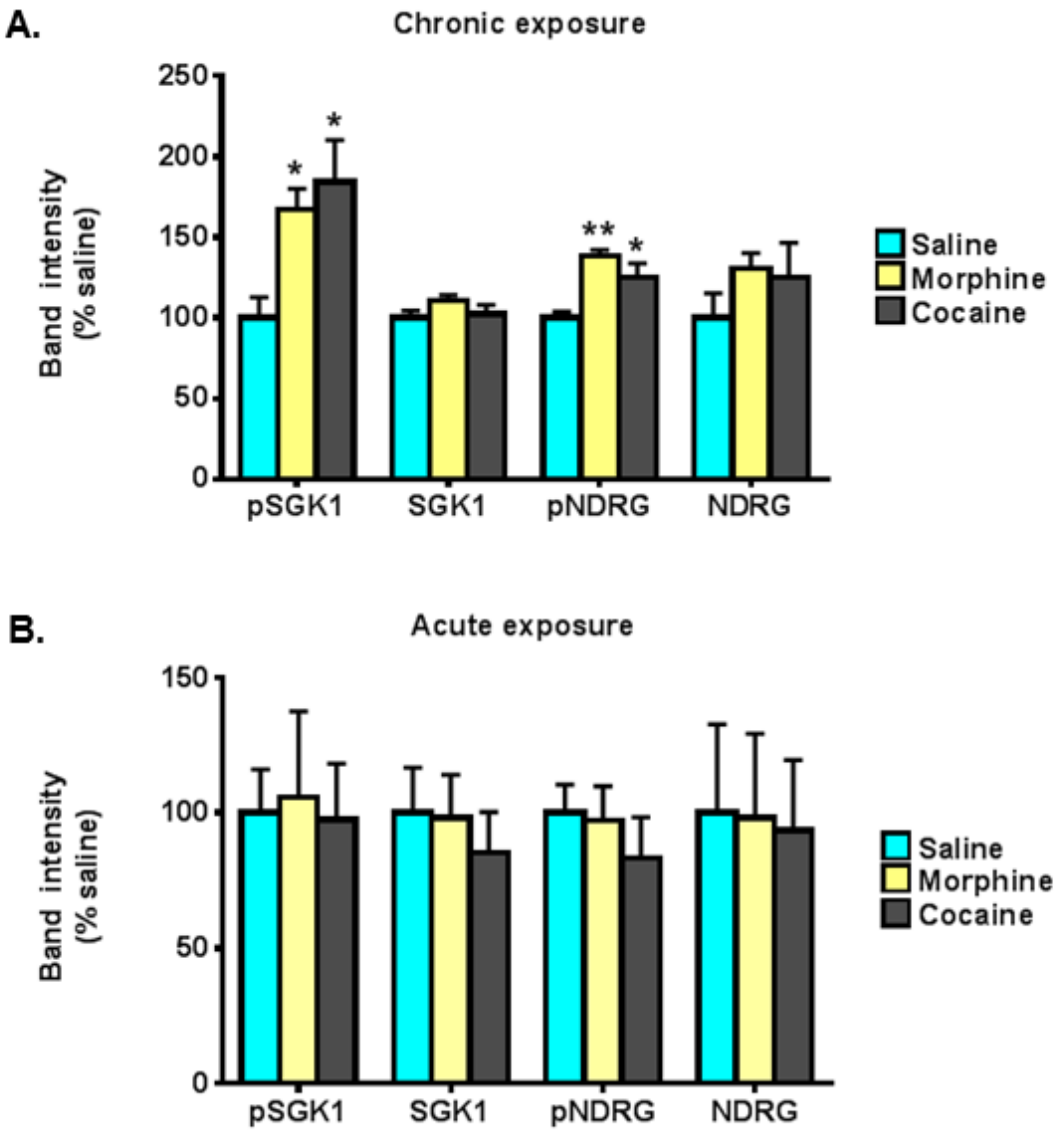

Figure 5. SGK1 phosphorylation and activity in the VTA are increased after repeated, but not single, morphine and cocaine injections. Mice were injected once or daily for seven days with saline, morphine, or cocaine (15 mg/kg). VTA was dissected one hour after the last injection and tissue was processed for Western blot analysis. Data are expressed as mean ± SEM, n = 8 mice per group. **A.** Seven day morphine and cocaine administration increases phosphorylation of SGK1 S78 and the SGK1 substrate NDRG compared to saline control (one-way ANOVAs, * = $p < 0.05$ or ** = $p < 0.01$, post-hoc Tukey's multiple comparison). **B.** No significant differences in protein expression were observed one hour after a single drug administration (one-way ANOVAs).

in response to chronic drug administration (**Figure 5A**), we created additional SGK1 mutant viral vectors. Specifically, S78A and S78D mutants were generated to assess effects resulting from preventing or mimicking phosphorylation at S78, since modifying activation at this site might elicit different behavioral changes than the CA (S422D) mutant. We also produced a catalytically inactive (K127Q) SGK1 mutant to investigate whether preventing SGK1 activation altered drug-reward behaviors. To generate the additional S78A, S78D, and K127Q mutants for behavioral experiments we performed site-directed mutagenesis of a pFLAG-CMV-4 plasmid containing HA-tagged rat SGK1 cDNA. Once cloned into the p1005 vector, correct insertion was verified by dideoxysequencing. Control and mutant viruses were packaged by Dr. Rachael Neve (MIT).

To validate viral expression *in vivo*, HSV-GFP, HSV-S78A, HSV-S78D, or HSV-K127Q was infused into the mouse VTA via stereotaxic surgery and levels of HA, SGK1, and GFP were measured by Western blot analysis. First, we assessed whether or not the HA tag was present to confirm viral-mediated overexpression of the SGK1 mutants, as HA is not endogenously expressed in the mouse. The samples from HSV-S78A and HSV-K127Q mice produced detectable HA signals whereas the HSV-GFP and HSV-S78D samples did not (**Figure 6A**). While this was expected for HSV-GFP samples since the HSV-GFP construct does not express HA tag, the absence of HA in the HSV-S78D was not expected. The sequencing results were re-examined to ensure that no additional mutations were introduced that might have led to an inadvertent stop codon or protein degradation, and efforts are currently underway to obtain a new preparation of the HSV-S78D virus to use in future studies.

Surprisingly, the level of SGK1 protein was not increased by viral treatments, although K127Q samples exhibited a non-significant increase in SGK1 expression (**Figures 6B and 6D**,

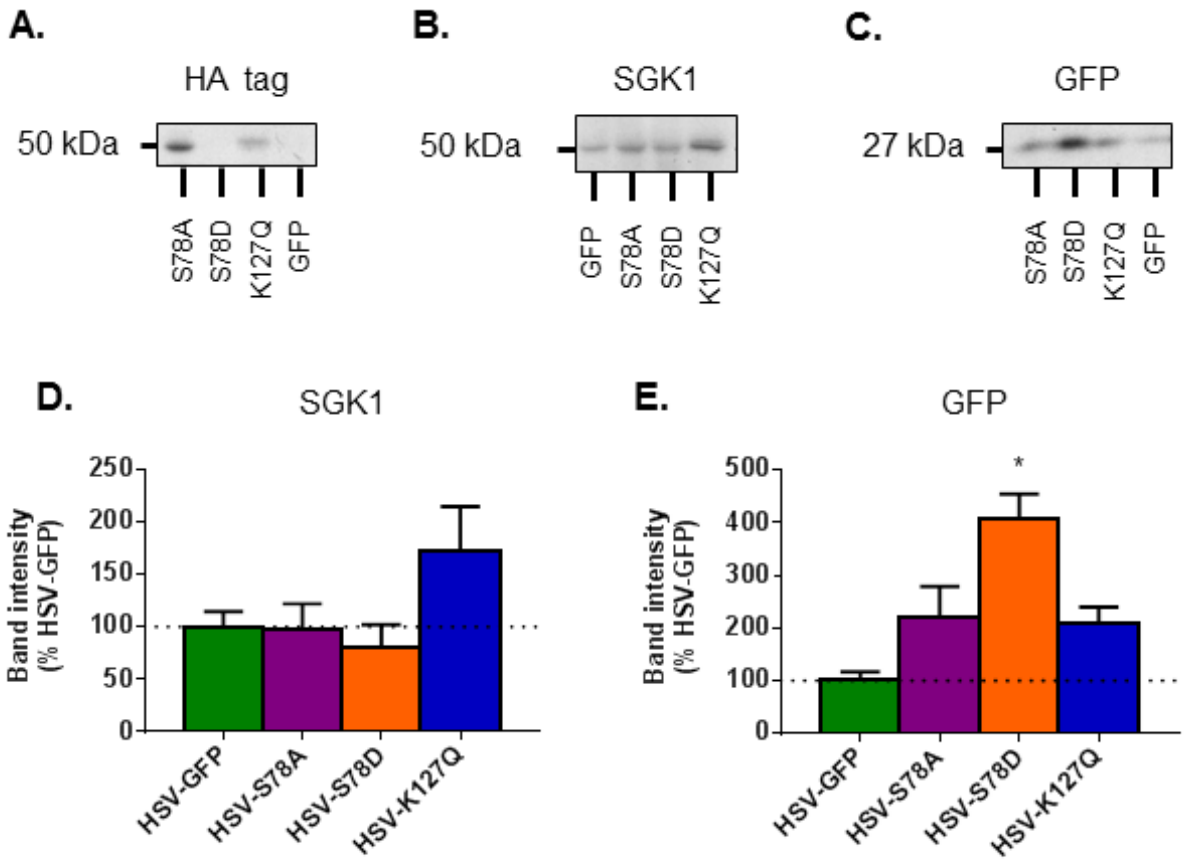

Figure 6. Validation of HSV viral vectors. Mice received bilateral VTA injections of HSV-GFP, HSV-S78A, HSV-S78D, or HSV-K127Q. Mice were sacrificed three days later, VTA was dissected, and tissue was processed for Western blot analysis. Data are expressed as mean ± SEM, n = 3 mice for HSV-GFP, n = 4 mice for SGK1 viruses. **A-C.** Western blots of HA tag (30 s), SGK1 (30 s), and GFP (5 s). **D.** No significant differences were observed in SGK1 expression among the different viral treatments (one-way ANOVA). **E.** GFP expression is significantly increased in HSV-S78D samples (one-way ANOVA, * = $p < 0.05$, post-hoc Tukey's multiple comparison).

one-way ANOVA). The lack of statistical significance may be due to a small sample size (n = 3 for HSV-GFP, n = 4 for SGK1 viruses) in this validation experiment. Another possibility is that the total level of SGK1 protein is tightly regulated in the VTA, an idea we more fully address in the discussion. Despite not seeing an increase in total SGK1 protein, SGK1-S78A and SGK-K127Q are likely producing viral-mediated SGK1 protein expression. We base this conclusion

on the grounds that the HA signal produces a more accurate measure of transgene expression since it distinguishes between endogenous and exogenous SGK1

Finally, GFP was measured as a general marker of viral infusion. While GFP expression was observed in all samples, expression in S78D mice was surprisingly higher than in the other mice (**Figures 6C and 6E**; one-way ANOVA, $F(3,11) = 7.88$, $p < 0.05$, post-hoc Tukey's multiple comparison). Additionally, there was a trend for the S78A and K127Q viruses to express more GFP than the GFP virus. This may have been due in part to the HSV-GFP preparation used, which was much older than the freshly prepared SGK1 viral vectors. Ultimately we relied on the data from HA to demonstrate successful expression of the S78A and K127Q mutants, and these were chosen for further analysis in behavioral experiments.

Establishment of morphine dose in a voluntary two-bottle choice assay

Experiment 1: In the two-bottle choice assay, mice are presented with unlimited access to two bottles of fluid (one each of morphine and quinine solutions), and their preference for morphine was used as a measure of reward behavior. Quinine was used as a control for the bitterness of morphine so that the two solutions would have a similar taste. Additionally, the ratio of morphine to quinine was held constant so that only morphine, not bitterness, contributed to the preference level. While determining the appropriate morphine and quinine concentrations for the two-bottle choice assay, we limited our analysis to control mice (HSV-GFP) that only overexpress GFP in the VTA.

In the initial experiment we sought to establish concentrations that produced approximately 70% preference for morphine so that any potential increase or decrease in preference induced by altered SGK1 activity could be detected. Prior data obtained in the

laboratory from animals that had not undergone surgery found that 0.3 mg/ml morphine and 0.06 mg/ml quinine resulted in approximately 70% preference (data not shown). However, we found that HSV-GFP mice demonstrated near maximal preference not only at the 0.3 mg/ml dose originally used, but also with the lower morphine doses tested (0.2 mg/ml and 0.1 mg/ml). Averaged over the three choice days, the observed preference levels were 81.0 ± 2.3%, 75.2 ± 4.7%, and 75.7 ± 4.5% for the mice receiving 0.3 mg/ml, 0.2 mg/ml, and 0.1 mg/ml morphine, respectively, and no significant differences were detected (**Figure 7A**, one-way ANOVA). This maximal preference was more evident in the day-by-day analysis, where all morphine doses produced ~90% preference on days 2 and 3 of testing (**Figure 7B**). The observation of increased morphine preference over time was confirmed statistically, where we observed a significant main effect for the day factor but no significant difference for the dose factor or the day-dose interaction (two-way repeated measures ANOVA, day factor: $F(2, 18) = 23.1$ and $p < 0.0001$, dose factor: $F(2, 9) = 0.83$, and interaction: $F(4, 18) = 0.28$). To ensure that the presence of morphine or quinine did not impair normal fluid intake, we also analyzed the mean daily intake volume. Importantly, varying the concentrations of morphine and quinine had only minor effects on general fluid intake, with mice exposed to the highest dose of morphine (0.3 mg/ml) actually drinking significantly more total fluid than mice exposed to the lowest dose (0.1 mg/ml; 7.25 ± 0.46 ml vs. 5.94 ± 0.25 ml total fluid; **Figure 7C**; one-way ANOVA, $F(2, 12) = 4.45$, $p < 0.05$, post-hoc Tukey's multiple comparison test). Neither group imbibed significantly more or less fluid than mice receiving 0.2 mg/ml morphine (6.37 ± 0.15 ml total fluid, **Figure 7C**). No significant differences in either morphine or quinine intake volumes were observed between the three dose groups (Morphine: 5.00 ± 0.61 ml vs. 4.59 ± 0.40 ml vs. 4.45 ± 0.30 ml, and quinine: 2.25 ± 0.65 ml vs. 1.78 ± 0.31 ml vs. 1.49 ± 0.13 ml for the mice receiving 0.3 mg/ml, 0.2

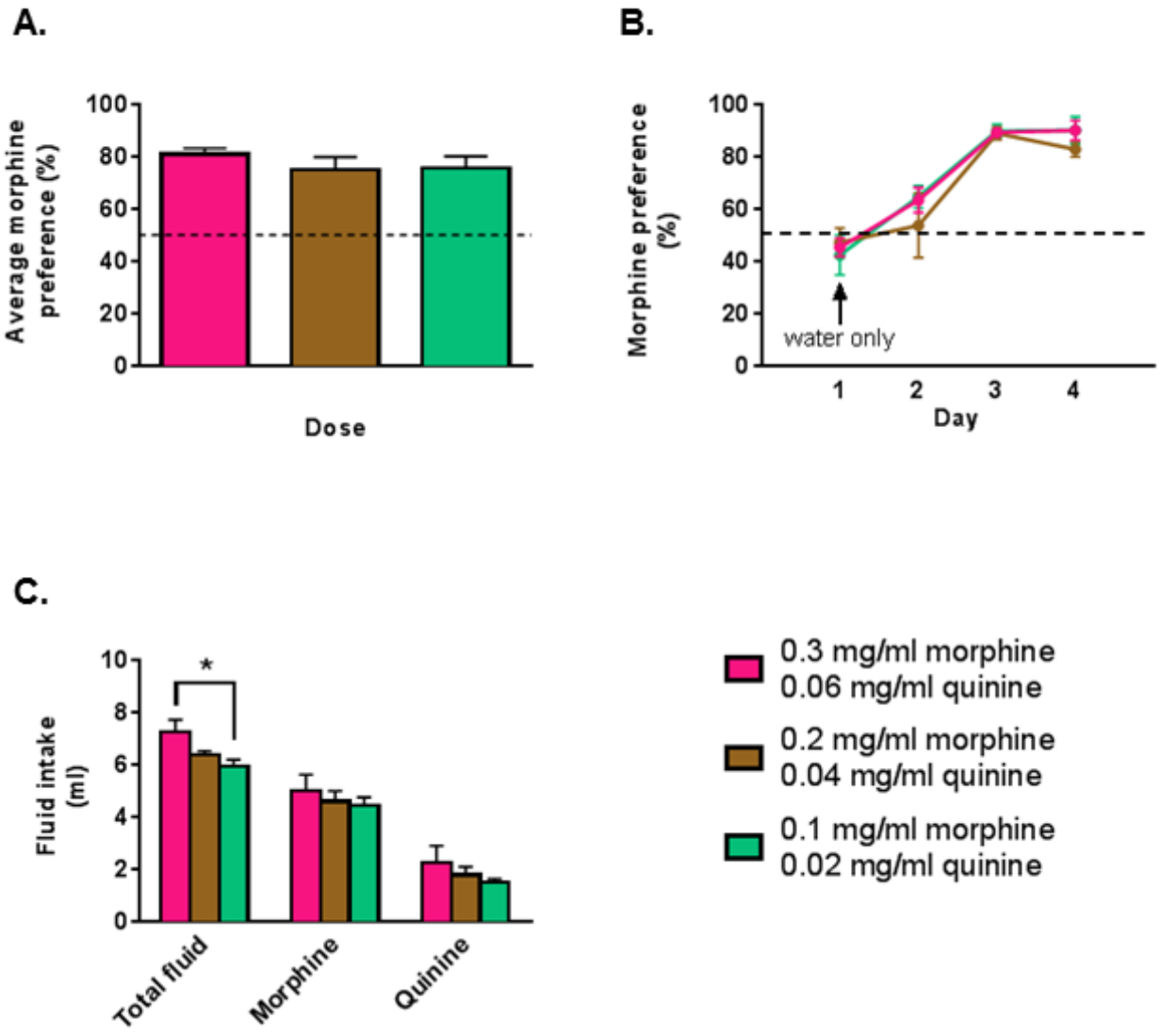

Figure 7. Establishing a morphine dose for the two-bottle choice assay (Experiment 1). HSV-GFP was bilaterally infused into the VTA. On day 1 after surgery mice received two bottles containing just water; on days 2-4 bottles contained either morphine or quinine in 0.2% sucrose. All data are expressed as mean ± SEM, n = 3-5 mice per group. **A.** Altering morphine concentration did not affect the average three-day morphine preference (one-way ANOVA). **B.** Morphine preference significantly increased over time for all groups (two-way repeated measures ANOVA, day factor: $p < 0.0001$). **C.** Average daily intake of total fluid, morphine, or quinine was only modestly influenced by morphine dose. Mice receiving 0.3 mg/ml morphine drank significantly more total fluid than mice receiving 0.1 mg/ml morphine (one-way ANOVA, * = $p < 0.05$, post-hoc Tukey's multiple comparison).

mg/ml, and 0.1 mg/ml morphine, respectively; **Figure 7C**; one-way ANOVAs).

Experiment 2: Given the near maximal response to the lowest dose tested in Experiment 1, in the next experiment it was therefore necessary to lower the concentrations of morphine and quinine. Thus, we next tested morphine doses of 0.1 mg/ml, 0.05 mg/ml, and 0.01 mg/ml. We observed a very similar average three-day morphine preference for the 0.1 mg/ml morphine dose as in Experiment 1 (75.7 ± 4.5% for Experiment 1 vs. 74.7 ± 2.8% for Experiment 2; **Figures 7A and 8A**). Surprisingly, the 0.05 mg/ml dose produced a similar preference (73.3 ± 5.6%) as the higher 0.1 mg/ml dose, while the lowest dose (0.01 mg/ml) produced no preference for morphine (49.4 ± 4.9%), a statistically significant effect (**Figure 8A**, one-way ANOVA, $F(2, 11) = 10.64$, $p < 0.005$, post-hoc Tukey's multiple comparison test). When we examined daily morphine preference, we found that by the final choice day the higher dose (0.1 mg/ml and 0.05 mg/ml) cohorts displayed greater preference for morphine than was desired (**Figure 8B**). Similar to the three-day average morphine preference, a significant dose effect was observed in the analysis of daily morphine preference (two-way repeated measures ANOVA, dose factor: $F(2, 11) = 10.64$ and $p < 0.005$, day factor: $F(2, 22) = 3.23$, and interaction: $F(4, 22) = 0.33$). Tukey's post-hoc multiple comparisons revealed that both mice receiving 0.1 mg/ml morphine (days 2-3) and mice receiving 0.05 mg/ml morphine (day 4) exhibited increased morphine preference compared to mice receiving 0.01 mg/ml morphine. Somewhat surprisingly, modest effects on fluid intake were observed. Mice receiving 0.1 mg/ml morphine drank less total fluid (6.68 ± 0.02 ml) than mice receiving 0.01 mg/ml morphine (7.51 ± 0.16 ml), though neither group drank significantly more or less total fluid from the mice receiving 0.05 mg/ml morphine (5.15 ± 0.22 ml; **Figure 8C**; one-way ANOVA, $F(2, 10) = 5.21$, $p < 0.05$, post-hoc Tukey's multiple comparison test). This is in contrast to Experiment 1, where the highest morphine dose (0.3 mg/ml) produced

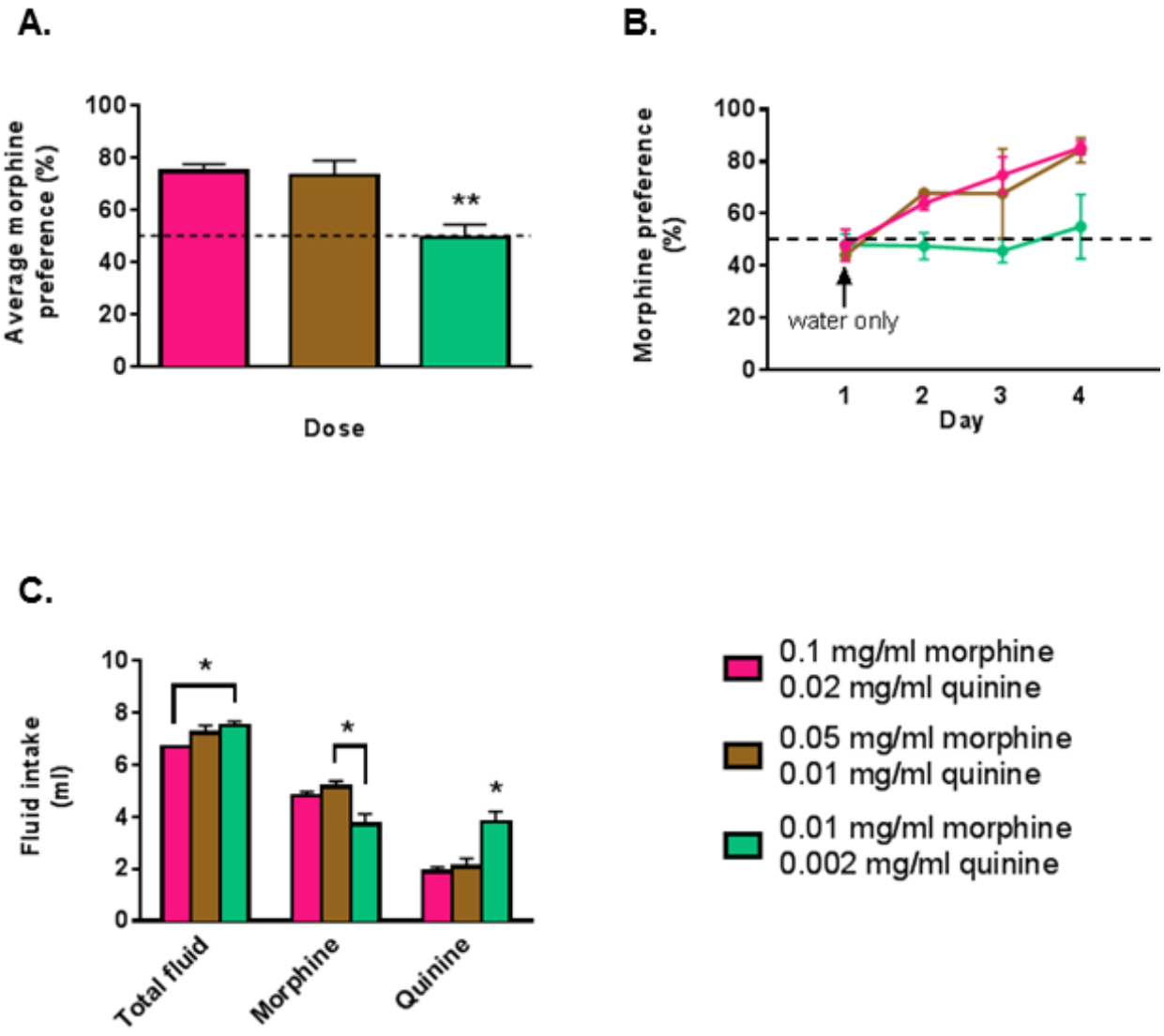

Figure 8. Establishing a morphine dose for the two-bottle choice assay (Experiment 2). HSV-GFP was bilaterally infused into the VTA. On day 1 after surgery mice received two bottles containing just water; on days 2-4 bottles contained either morphine or quinine in 0.2% sucrose. All data are expressed as mean ± SEM, n = 4-5 mice per group. **A.** No differences were observed in the three-day average morphine preference between the two highest morphine doses tested. However, both were significantly greater than the lowest dose (one way ANOVA, ** = $p < 0.01$, post-hoc Tukey's multiple comparison). **B.** The lowest morphine dose also exhibited decreased daily morphine preference (two-way repeated measures ANOVA, dose factor: $p < 0.005$). **C.** Average daily intake of total fluid, morphine, or quinine was modestly influenced by morphine dose. Mice receiving the lowest morphine dose exhibited increased total fluid and quinine intake and decreased morphine intake (one-way ANOVAs, * = $p < 0.05$, post-hoc Tukey's multiple comparison).

a slightly greater intake than the lowest (0.1 mg/ml) dose, and suggests a complex relationship between morphine dose and total fluid intake. Differences in morphine intake were also observed: mice receiving 0.05 mg/ml morphine drank more morphine (5.15 ± 0.22 ml) than mice receiving 0.01 mg/ml morphine (3.71 ± 0.40 ml), though neither group was significantly different than mice receiving 0.1 mg/ml morphine (4.79 ± 0.17 ml, **Figure 8C**, one-way ANOVA, $F(2, 10) = 6.31$, $p < 0.05$, post-hoc Tukey's multiple comparison). Finally, quinine intake was elevated in the mice receiving 0.01 mg/ml morphine (3.80 ± 0.40 ml) compared to the other two groups (1.89 ± 0.18 ml for the 0.1 mg/ml group and 2.07 ± 0.33 ml for the 0.05 mg/ml group), explaining the absence of morphine preference in these mice (**Figure 8C**, one-way ANOVA, $F(2, 10) = 10.24$, $p < 0.005$). Since 0.05 mg/ml and 0.01 mg/ml morphine produced preferences that were too high and too low, respectively, ultimately an intermediate dosing paradigm of 0.03 mg/ml morphine and 0.006 mg/ml quinine was selected for subsequent experiments. Ideally this paradigm would produce a morphine preference of ~75% by the final choice day, allowing us to determine if enhancing or inhibiting SGK1 activity in the VTA affects morphine preference.

Effects of modulating VTA SGK1 activity on voluntary morphine consumption

Experiment 3: Using the 0.03 mg/ml morphine and 0.006 mg/ml quinine dose established above, voluntary morphine consumption was tested in mice that received intra-VTA infusions of HSV-GFP, HSV-CA, or HSV-K127Q to determine whether enhancing or inhibiting SGK1 activity promotes or reduces voluntary morphine consumption, respectively. We observed that expression of HSV-K127Q in the VTA significantly decreased average morphine preference compared to HSV-CA (55.8 ± 1.2% for HSV-K127Q vs 64.9 ± 2.6% for HSV-CA; **Figure 9A**;

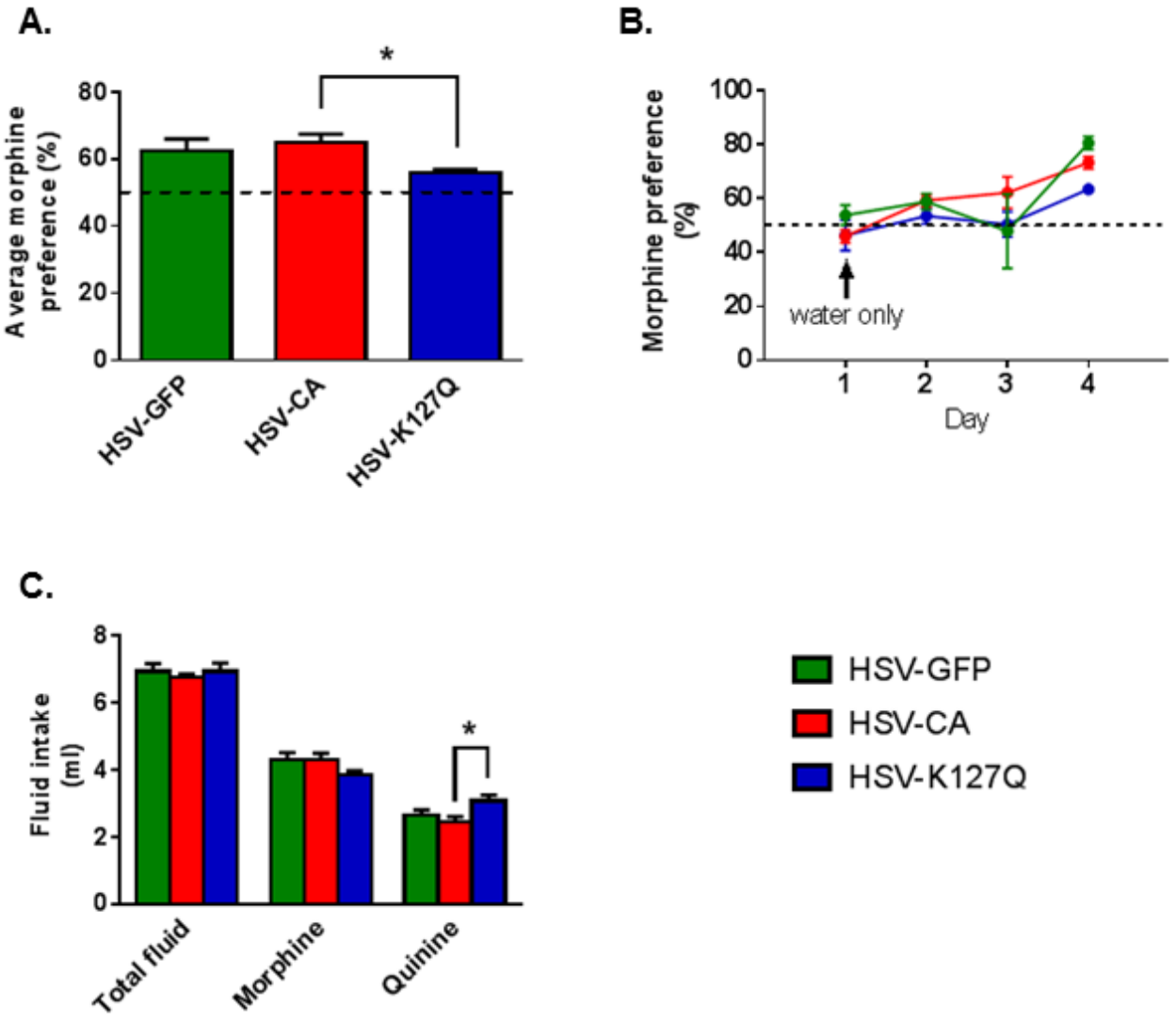

Figure 9. Overexpression of SGK1-K127Q in the VTA decreases morphine preference (Experiment 3). HSV-GFP, HSV-CA, or HSV-K127Q was bilaterally infused into the VTA. On day 1 after surgery mice received two bottles containing just water; on days 2-4 bottles contained either morphine or quinine in 0.2% sucrose. All data are expressed as mean ± SEM, n = 5-6 mice per group. **A.** K127Q mice exhibited a lower three-day average morphine preference than CA mice (one-way ANOVA, * = $p < 0.05$, post-hoc Tukey's multiple comparison). **B.** Daily morphine preference was significantly influenced by both viral treatment and day (two-way repeated measures ANOVA, day factor: $p < 0.001$, virus factor: $p < 0.05$). **C.** Average intake of total fluid, morphine, and quinine over three choice days. Quinine intake was significantly greater in K127Q mice than in CA mice (one-way ANOVA, * = $p < 0.05$, post-hoc Tukey's multiple comparison).

one-way ANOVA, $F(2, 14) = 3.75$, $p < 0.05$, post-hoc Tukey's multiple comparison test). While neither SGK1 mutant exhibited a significantly different preference from HSV-GFP, mice expressing catalytically inactive SGK1 displayed a strong trend for lower morphine preference than HSV-GFP mice, a difference that may become significant when the experiment is repeated with an increased sample size. The decreased preference of K127Q mice is also clear in the daily preference data, where we observed significant main effects for day and viral treatment, (**Figure 9B**; two-way repeated measures ANOVA, day factor: $F(2, 28) = 9.69$ and $p < 0.001$, virus factor: $F(2, 14) = 3.75$ and $p < 0.05$, and interaction: $F(14, 28) = 0.60$). Importantly, overexpression of SGK1 in the VTA did not affect total fluid intake among treatment groups (**Figure 9C**, one-way ANOVA). Instead, the decrease in average morphine preference of HSV-K127Q mice can be attributed to significantly higher quinine intake and a trend toward lower morphine intake compared to HSV-CA mice (**Figure 9C**; one-way ANOVAs, quinine: $F (2, 14) = 4.07$, $p < 0.05$, post-hoc Tukey's multiple comparison).

Experiment 4: Given that we detected increased SGK1 S78 phosphorylation in mice chronically treated with morphine, we wanted to next determine if blocking phosphorylation of this residue would also negatively affect morphine preference, similarly to the catalytically inactive K127Q mutant. However, we did not observe any difference in the average morphine preference between the HSV-GFP and HSV-K127Q mice (**Figure 10A**; unpaired Student's t test, two-tailed). Similarly, daily morphine preference did not statistically differ between the groups (**Figure 10B**, two-way repeated measures ANOVA). While we observed no significant difference in daily preference, we noticed a trend for S78A mice to have increased morphine preference (See days 2 and 3, **Figure 10B**) which we did not expect, as we had predicted that eliminating S78 phosphorylation would decrease morphine preference. Indeed, when we

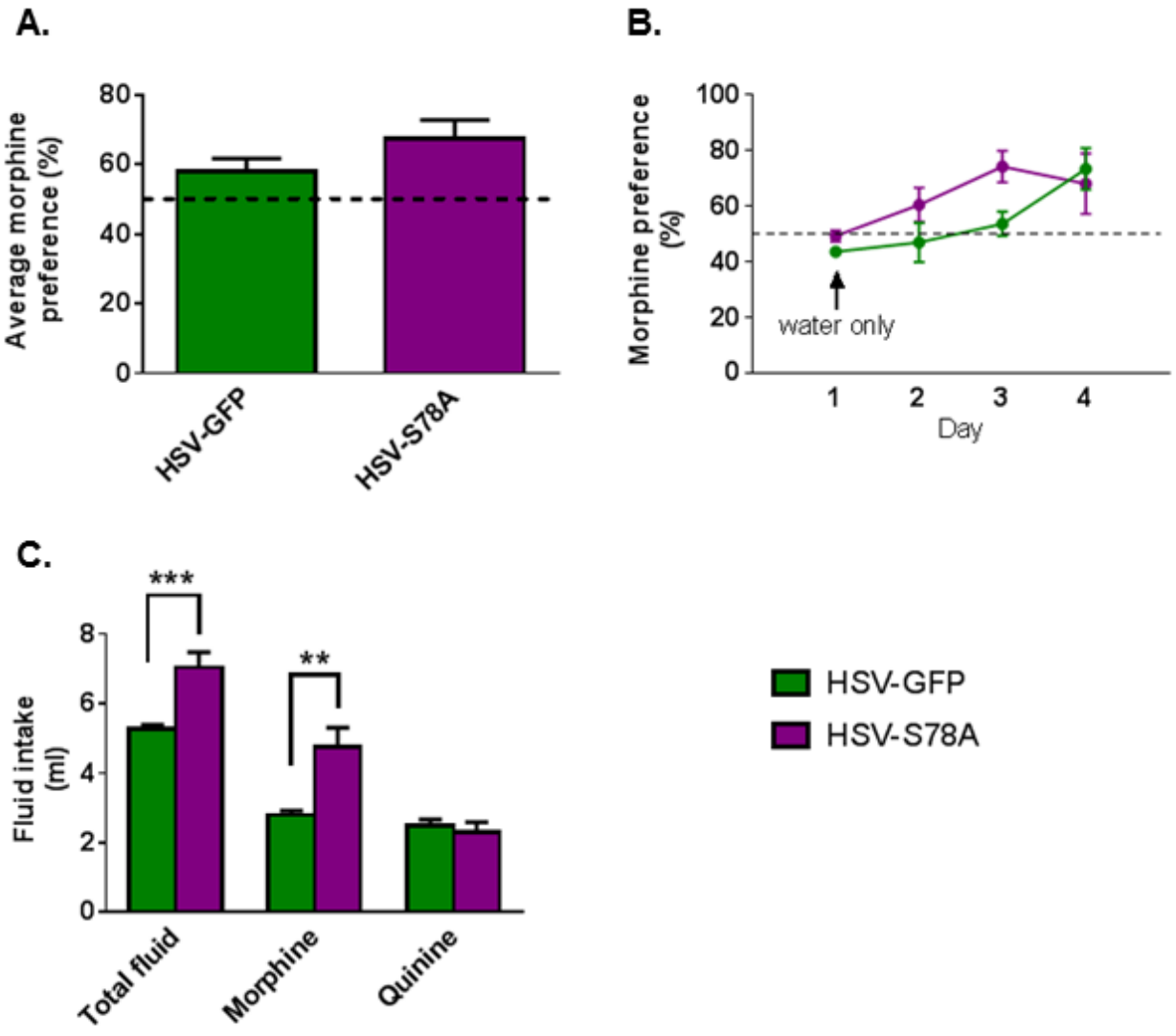

Figure 10. Overexpression of SGK1-S78A does not affect average morphine preference, but does increase total fluid and morphine intake (Experiment 4). HSV-GFP or HSV-S78A was bilaterally infused into the VTA. On day 1 after surgery mice received two bottles containing just water; on days 2-4 bottles contained either morphine or quinine in 0.2%-sucrose. All data are expressed as mean ± SEM, n = 7-8 mice per group. **A.** S78A and GFP mice exhibited similar three-day average morphine preferences (unpaired Student's t test). **B.** Daily morphine preference was not significantly influenced by the day, viral treatment, or day-virus interaction (two-way repeated measures ANOVA). **C.** Average intake of total fluid, morphine, and quinine over three choice days. S78A mice drank significantly more total fluid and morphine than control mice (unpaired Student's t tests, two-tailed, df = 13, total fluid: *** = $p < 0.005$, morphine: ** = $p < 0.01$).

examined average fluid intake we found that HSV-S78A mice drank significantly more morphine and total fluid than HSV-GFP mice while quinine consumption was similar for both groups (**Figure 10C**, unpaired Student's t tests, two t-tailed, df = 13, morphine consumption: $t = 3.19$, $p < 0.01$ and total fluid: $t = 3.69$, $p < 0.005$). It will be worthwhile to determine if this unexpected trend becomes significant in future replications with a larger sample size.

Effects of modulating VTA SGK1 activity on cocaine locomotor activity and CPP

In order to determine the effects of modifying VTA SGK1 activity on another measure of reward behavior, we next completed cocaine CPP. We first evaluated the K127Q mutant as it significantly decreased voluntary morphine preference. Consistent with expectations, ambulatory locomotion, which counted breaks of adjacent beams in the CPP chambers, was significantly higher when mice were injected with cocaine compared to saline. Averaged over the two conditioning days, mice infused with HSV-GFP had increased locomotor activity when injected with cocaine as opposed to saline (**Figure 11A**; one-way ANOVA, $F(3, 24) = 22.2$, $p < 0.0001$, post-hoc Tukey's multiple comparison). This effect was also clearly demonstrated in mice infused with HSV-K127Q, as cocaine-induced locomotor activity was again greater than that of saline (**Figure 11A**). However, there were no significant differences in locomotor activity observed between HSV-GFP and HSV-K127Q mice in response to either saline or cocaine (**Figure 11A**).

Additionally, we observed cocaine-induced locomotor sensitization in both HSV-GFP and HSV-K127Q mice, such that counts of ambulatory motion were higher on day 2 than on day 1 (**Figure 11B**; two-way repeated measures ANOVA, **GFP**: $n = 8$, drug factor: $F(1, 14) = 46.0$ and $p < 0.0001$, day factor: $F(1, 14) = 12.7$ and $p < 0.005$, and interaction: $F(1, 14) = 1.23$, post-

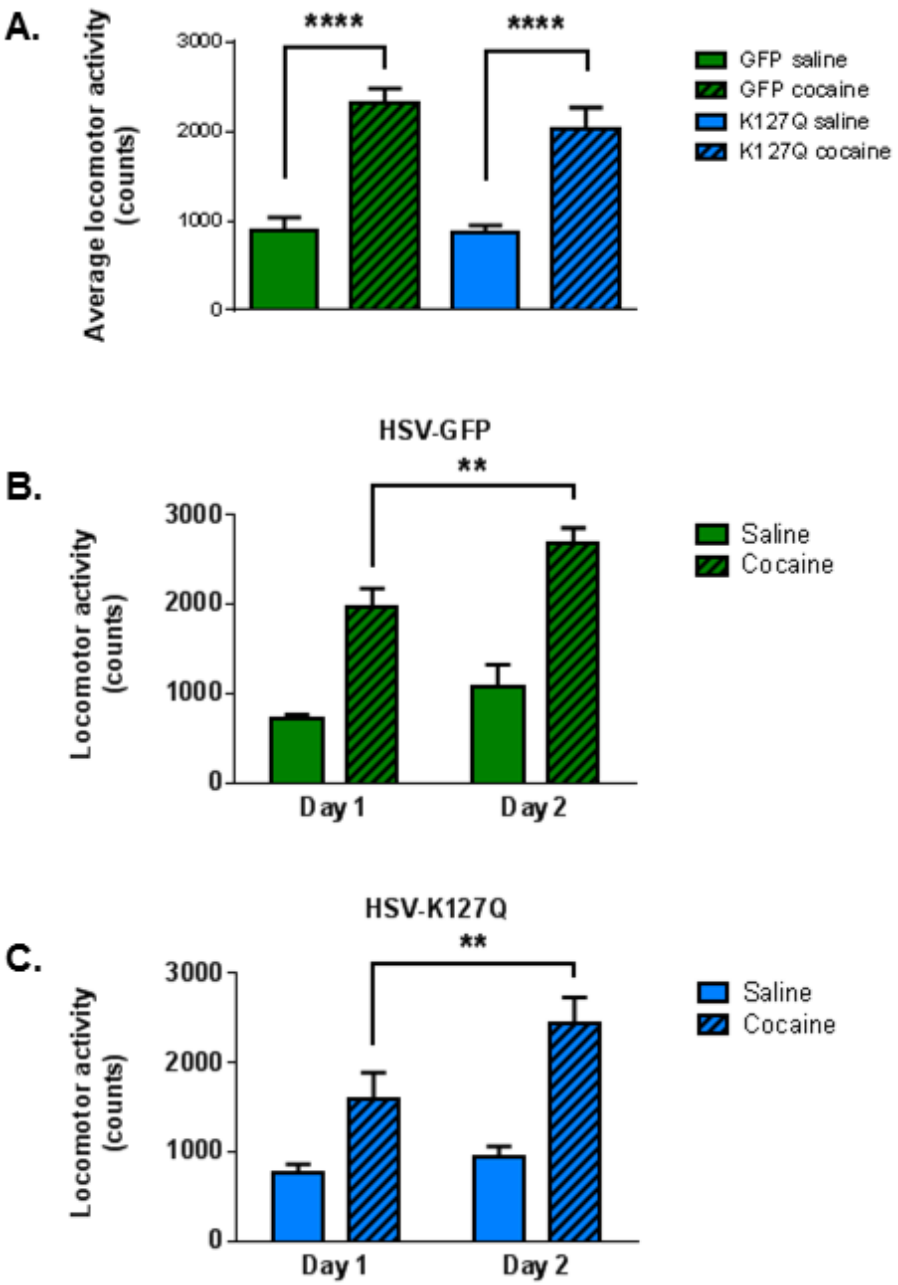

Figure 11. Decreasing VTA SGK1 activity does not affect cocaine-induced locomotor activity. HSV-GFP and HSV-K127Q mice were injected with saline or cocaine (20 mg/kg) and locomotor activity (number of adjacent beam breaks) was recorded for 20 min. All data are reported as mean ± SEM, n = 6 or 8 mice per group. **A.** Cocaine increased average two-day locomotor activity in both HSV-GFP and HSV-K127Q mice (one-way ANOVA, **** = $p < 0.001$, post-hoc Tukey's multiple comparison). **B.** Cocaine induced locomotor sensitization in HSV-GFP mice with significant day and drug effects (two-way repeated measures ANOVA, day factor: $p < 0.005$, drug factor: $p < 0.0001$, post-hoc Bonferroni multiple comparison, ** = $p < 0.01$). **C.** Cocaine also induced locomotor sensitization in HSV-K127Q mice with significant day and drug effects (two-way repeated measures ANOVA, day factor: $p < 0.05$, drug factor: $p < 0.005$, post-hoc Bonferroni multiple comparison, ** = $p < 0.01$).

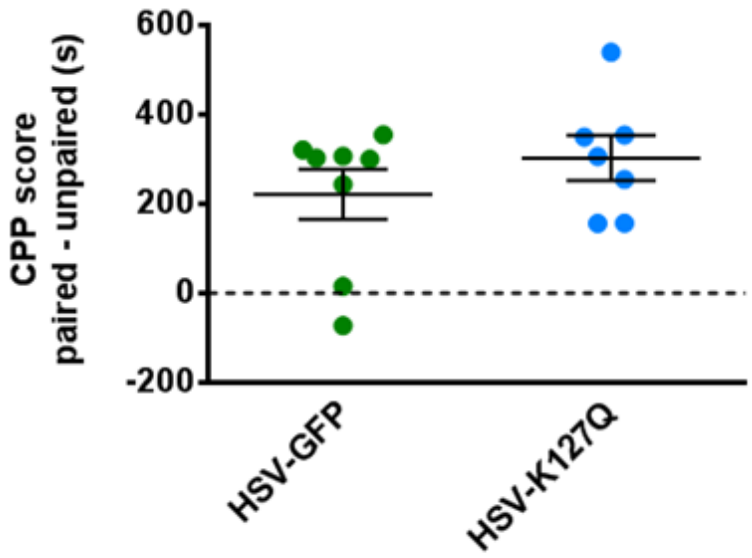

Figure 12. Decreasing VTA SGK1 activity does not affect cocaine CPP. HSV-GFP and HSV-K127Q mice underwent two days of CPP pairing (20 mg/kg cocaine) followed by a 20 min post-test. CPP score was calculated as the time spent in the paired chamber – time spent in the unpaired chamber during the post-test. Both groups demonstrated a robust preference for the cocaine-paired chamber, but CPP score did not significantly differ between the two groups. Data are reported as mean ± SEM, n = 7 or 8 mice per group.

hoc Bonferroni's multiple comparison; **Figure 11C**; **K127Q**: n = 6, drug factor: $F(1, 10) = 20.4$ and $p < 0.005$, day factor: $F(1, 10) = 9.44$ and $p < 0.05$, and interaction: $F(1, 10) = 4.26$, post-hoc Bonferroni's multiple comparison). These data suggest that decreasing SGK1 activity in the VTA does not affect cocaine-induced locomotor activation.

The final reward behavior that we assessed was cocaine CPP. We found that both HSV-GFP and HSV-K127Q exhibited a strong preference for the chamber paired with a 20 mg/ml cocaine injection; however there was no difference in preference between the two groups (**Figure 12**, unpaired Student's t-test, two-tailed). We had predicted that overexpression of SGK1-K127Q would decrease cocaine CPP, since it had decreased voluntary morphine

preference. One possible reason for not observing a larger difference in CPP is the dose of cocaine used. Specifically, 20 mg/kg of cocaine is a relatively high dose of cocaine for CPP, and it is plausible that a lower dose would be more appropriate to detect differences induced by decreased VTA SGK1 activity (Kelz et al. 1999, Koo et al. 2012).

CHAPTER 4: Discussion

The data described herein establish that chronic morphine or cocaine exposure markedly increases SGK1 activity in the VTA. While other studies have investigated expression of SGK1 mRNA, this study is one of the first to explore activation of SGK1 signaling and its behavioral consequences in response to drugs of abuse. McClung et al. were the first to identify the increase in rat SGK1 gene expression in the VTA in response to morphine in a DNA microarray study (McClung et al. 2005). This was recently replicated by Heller et al., in which RNA sequencing demonstrated significant increases in SGK1 mRNA in the VTA of chronic morphine- or cocaine-treated mice twenty-four hours after the last drug injection (Heller et al. in press). While these prior studies examined changes in the VTA, Befort et al. determined that SGK1 mRNA in the extended amygdala was also significantly increased after a chronic, escalating dose morphine paradigm in wild-type mice (Befort et al. 2008). This change did not occur after a single morphine exposure or in μ-opioid receptor KO mice. These results are significant because upregulation of SGK1 gene expression depended not just on morphine binding to the μ-opioid receptor but also on chronic, repeated opioid administration.

Despite these consistent findings that repeated morphine administration increases SGK1 gene expression, when we examined SGK1 protein levels following repeated morphine or cocaine injections we did not observe any difference from the saline-injected controls (**Figure 5A**). One consideration is that mRNA expression does not always correlate with protein translation. For example, SGK1 mRNA is upregulated four hours after a single morphine injection in the striatum, but the total amount of SGK1 protein was decreased at this time point (Piechota et al. 2010). The observation that total SGK1 protein levels do not change in the VTA,

regardless of exposure frequency (**Figure 5** and (Heller et al. in press)), suggests the existence of a feedback mechanism to maintain relatively constant levels of SGK1 in the face of external stimuli; i.e. drug-induced increases in mRNA are countered by decreased protein translation and/or increased turnover.

Another factor that may contribute to differences between gene and protein expression is the drug administration protocol used in each of these studies. Morphine pellets only modestly elevate morphine blood levels but this increase is stable and persists for days, whereas i.p. injection of morphine result in large, rapid increases in blood concentration (about three times the peak concentration caused by pellets) that also returns to baseline rapidly (within 12 hours) (Fischer et al. 2008). McClung et al. (2005) implemented pellets while the present study used daily 15 mg/kg injections of morphine or cocaine similar to those used by Heller et al. (in press). Finally, Befort et al. (2008) used a slightly different injection paradigm that utilizes twice-daily injections with escalating morphine doses that produces a more consistent blood morphine level than the normal daily injection paradigm and is known to produce opiate dependence (Befort et al. 2008). While single injections provide useful information about acute drug exposure, morphine pellets and repeated daily injections, especially the escalating dose paradigm, more closely approximate the drug concentrations that might be observed in the blood of an addicted individual seeking to maintain a perpetual, low-level high and avoid harsh withdrawal symptoms (Fischer et al. 2008).

While we don't observe an increase in SGK1 protein following chronic drug administration, we do detect increased SGK1 phosphorylation at the S78 site (**Figure 5A**). S78 phosphorylation is of interest because it is known to be a substrate for a different upstream kinase than the canonical S422 and T256 phosphorylation sites, and its phosphorylation has been

shown to have biological consequences (Hayashi et al. 2001). Our observation that S78 phosphorylation in the VTA is increased by chronic drug exposure suggested that SGK1 kinase activity itself may be increased. Indeed, we found increased phosphorylation of the SGK1 substrate NDRG following chronic drug treatment (**Figure 5A**). To examine whether acute exposure to drugs also induced an increase in VTA SGK1 activity, mice were given a single injection of saline, morphine, or cocaine and then sacrificed one hour later. VTA expression of total and phosphorylated SGK1 and NDRG remained unchanged (**Figure 5B**), implying that acute drug exposure is insufficient to alter activity in this pathway. Taken together, these results for the first time indicate that SGK1 activity, in addition to expression, is increased in the VTA by chronic exposure to two common drugs of abuse.

To explore if increased SGK1 activity mediated by S78 phosphorylation affects drug reward, we designed HSV-SGK1 mutant constructs for direct injection and overexpression in the VTA. The purpose of the first mutant, S78A, was to have an SGK1 mutant that is incapable of being phosphorylated and activated at this site. By still permitting phosphorylation of the canonical S422 and T256 sites, we aimed to distinguish between SGK1 activity in response to phosphorylation at S78 versus the canonical sites. The second mutant, S78D, was designed to mimic constitutive phosphorylation, and therefore activation, at the S78 site. Finally, we generated a kinase dead mutant (K127Q) to decrease SGK1 activity and potentially prevent any drug-induced changes in the VTA. With these viruses in hand, we hoped to characterize the role of S78 phosphorylation and SGK1 activity in drug reward behavior.

In order to validate the viruses, expression of HA, SGK1, and GFP was measured by Western blot analysis three days after infusion, a time point consistent with maximal HSV-mediated transgene expression (Barrot et al. 2002, Carlezon Jr 1998). HA was measured to

confirm that the SGK1 mutants were being correctly expressed. We observed that HA was present in the S78A and K127Q samples but undetectable in the GFP and S78D samples (**Figure 6A**). While the absence of HA in the HSV-GFP samples was expected, its absence in the HSV-S78D samples was not. It may be possible that the S78D mutant was indeed expressed, but the mutation caused a misfolding of the protein that triggered degradation. However, we think this is unlikely as others have been able to successfully overexpress a SGK1 S78D mutant in the hippocampus (Lee et al. 2007). We injected multiple aliquots of the HSV-S78D preparation with the same effect and reaffirmed via sequencing that no additional mutations in the SGK1 sequence were introduced. We plan to test our HSV-S78D construct in a cell culture model to determine whether the problem lies in our expression vector or in the preparation of the virus. It is possible that the HSV-S78D viral preparation was not active, or that the preparation that we received is not actually SGK1-S78D. To this end, a new viral preparation has been ordered; we hope to validate this new preparation for use in the behavioral studies in the future.

Next, we examined SGK1 expression. We might have expected to see increased SGK1 protein in samples from mice infused with the SGK1 viruses. However, we found similar SGK1 expression for all constructs (**Figures 6B and 6D**), which may be explained by the detection of endogenous as well as virally-expressed SGK1. It is possible that there is tight regulation of the total amount of SGK1 protein, such that expression of the virally-expressed mutant results in downregulated expression of the endogenous protein. Moreover, the antibody for total SGK1 protein is not very robust, and therefore it might not have detected smaller but significant changes in the amount of SGK1. For example, previous work from the lab has shown a lack of correlation between HSV-mediated RNA and protein overexpression, where there was a relatively small increase (~50%) in Rictor protein but a large increase (more than 150-fold) in

Rictor mRNA (Mazei-Robison et al. 2011). We plan to measure SGK1 RNA levels following HSV infusion in our next set of experiments. Finally, the small sample size (n = 3-4 mice per group) likely increased variability; future validation experiments will involve a larger cohort of mice. In conclusion, because the HA tag was the most accurate measure of expression of the SGK1 mutants from the HSVs and there was no evidence of SGK1 overexpression in the S78D samples, this virus was excluded from future behavioral studies until we receive and validate the new batch of virus.

Finally, GFP was also measured as part of our validation of the SGK1 mutant HSVs. As our HSV construct is a dual cistronic vector, all cells that express our SGK1 transgene (driven by an IE 4/5 promoter) also express GFP (via a CMV promoter). While we had expected similar GFP expression from all viruses, there are a few possible explanations for why we saw a trend for increased GFP in samples from the SGK1 viruses and a significant increase in GFP in the S78D samples (**Figures 6C and 6E**). One possible explanation is that the GFP virus used in this experiment was an older preparation compared to the recently produced SGK1 viruses. Consequently the new viral preps may be of a higher titer and therefore transduce more cells in the VTA. Further, variation in the GFP signal may be partially attributed to the precise brain area that was collected during dissection. Tissue was dissected by collecting small punches of VTA identified by eye, and may have been subject to punches slightly off-center from true VTA tissue or small differences in the number of total cells collected. One remedy to improve the accuracy of tissue collection would be to use a fluorescence dissecting microscope to visually identify the GFP-infected cells to guide the punches. This is, in fact, one of the most useful aspects of GFP expression from the HSVs. This approach was not utilized for this study however because such an instrument was not readily available for use by the laboratory. Another method to check

transduction efficiency would be to use immunohistochemical techniques to identify and then count GFP-labeled cells, keeping in mind that increased GFP as measured by Western blot (as for the S78D samples) could be due either to an increased number of cells infected with GFP or increased GFP expression per cell. As mentioned above, an additional experiment that could validate viral expression is real time (RT)-PCR to examine RNA levels of GFP, HA, and SGK1 in the VTA. Regardless of the relative differences in GFP exhibited by the different groups, it was concluded that all viruses were indeed capable of expressing functional GFP protein. This suggests that the HSV constructs were able to transduce cells in the VTA and that we could correctly target this brain region.

One caveat of using HSVs is that viral expression of SGK1 mutants does not eliminate endogenous SGK1 activity. Brunet et al. showed that overexpressing a catalytically inactive K127Q mutant or a T256A/S422A double mutant exhibited reduced phosphorylation of an SGK1 substrate, FKHRL1 (Brunet et al. 2001). Thus, overexpression of the inactive S78A and K127Q mutants could be expected to reduce phosphorylation of NDRG and other SGK1 substrates. Indeed, Heller et al. demonstrated that HSV-mediated overexpression of the S422D SGK1-CA mutant increased phosphorylation of NDRG compared to wild-type SGK1 (Heller et al. in press), and measuring phosphorylation of NDRG after transduction would provide an alternate means to validate the HSVs. One approach to eliminate the contribution of endogenous SGK1 activity would be to use SGK1 KO mice. However, SGK1 KO mice are not ideal for this purpose because of SGK1's functions in other organs (reviewed in (Lang et al. 2009, Lang et al. 2006, Lang et al. 2010, Loffing et al. 2006)). While neither systemic nor an inducible, kidney-specific KO of SGK1 is lethal, these mice have sodium retention deficiencies (Faresse et al. 2012, Fejes-Toth et al. 2008, Wulff et al. 2002). Using HSV-mediated expression provides

several benefits over KO mice: results can be obtained in weeks instead of waiting months or years to breed mice, expression can be targeted to a specific brain region of interest, and developmental effects are avoided by using adult mice. In light of these advantages, we chose HSVs for more targeted alteration of SGK1 activity.

Following validation of the S78A and K127Q viruses, we next sought to determine whether alteration of SGK1 activity in the VTA affected drug reward behavior. We first elected to examine morphine preference using a two-bottle choice drinking assay. The purpose of our first experiments was to determine concentrations of morphine and quinine necessary to produce a preference of about 70% in control mice. Quinine is used as a control for the bitter taste of morphine, which C57Bl6 mice otherwise avoid when given a choice between a morphine solution and water (Horowitz et al. 1977). All tested dose pairs maintained the same morphine:quinine ratio to eliminate differences in taste as a factor in preference. Additionally, both quinine and morphine solutions were prepared in 0.2% sucrose to mitigate some of the bitterness and maintain normal intake volumes (Forgie et al. 1988).

Preliminary results in our laboratory demonstrated that mice who had never undergone stereotaxic surgery demonstrated 70-75% preference for morphine using a 0.3 mg/ml morphine, 0.06 mg/ml quinine pairing. A 70-75% preference level was desired for these studies because it would allow us to see increases or decreases in morphine preference. However, we observed that stereotaxic surgery appeared to sensitize the morphine preference response in mice, such that in Experiment 1 they demonstrated near maximal preference (**Figures 7A-B**, 75-80% preference for the three-day average and ~90% for the final two choice days) for all three doses tested (0.3 mg/ml, 0.2 mg/ml, and 0.1 mg/ml morphine). Since this strong preference for morphine would prohibit us from identifying whether any of the SGK1 mutants are able to increase preference,

we chose lower doses in Experiment 2 in an attempt to find a pair of doses that would result in 70-75% preference by the final choice day. Of the doses tested, both 0.1 mg/ml and 0.05 mg/ml morphine demonstrated strong morphine preference on the final two choice days (**Figure 8B**). In contrast, mice given 0.01 mg/ml morphine exhibited no morphine preference. Thus, an intermediate dose pair of 0.03 mg/ml morphine and 0.006 mg/ml quinine was elected for use in the behavioral evaluation of the SGK1 mutants. It is important to note that this dose was one-tenth the original concentration validated for use in the laboratory with naïve animals. We believe that while in recovery from the invasive stereotaxic surgery, the mice were more motivated to drink the morphine solutions to help alleviate discomfort. We hypothesize that the mice continued to prefer morphine at lower concentrations because they may have derived an analgesic effect from the drug. While this might be considered a confounding factor in evaluating the rewarding properties of morphine, we do not expect any differences in analgesic potency between our HSV surgery groups. Thus, we chose the 0.03 mg/ml morphine dose to evaluate changes in preference following alteration of SGK1 activity in the VTA.

Once an appropriate dose was established for the two-bottle choice study, Experiment 3 aimed to determine whether altering SGK1 activity in the VTA affected morphine preference. Since we did not have the S78D mutant to increase SGK1 kinase activity, we chose to use the phospho-mimetic S422D CA for this purpose, along with the catalytically inactive K127Q mutant to decrease SGK1 activity in the VTA. Given that chronic morphine administration increased SGK1 activity in the VTA, we hypothesized that increasing SGK1 activity (via overexpression of SGK1-S422D) might prime the mice for drug exposure and increase morphine preference, and conversely, decreasing SGK1 activity (via overexpression of SGK1-K127Q) would decrease preference. As we predicted, we found that decreasing VTA SGK1 activity

significantly decreased morphine preference (**Figure 9A**). In contrast, the CA mutant did not increase morphine preference, as we found a similar preference between HSV-CA and HSV-GFP mice (**Figure 9A**). These results suggest that while decreasing VTA SGK1 activity is sufficient to alter reward, increasing SGK1 activity, at least through the canonical S422 activation site, does not affect morphine preference.

We next wanted to more specifically probe the effect of decreasing S78-mediated SGK1 activity by overexpressing SGK1-S78A in the VTA to determine whether a *global* decrease in VTA SGK1 activity or a more *targeted* decrease in S78-mediated catalytic activity was responsible for the decrease in morphine preference that we observed in Experiment 3. In contrast to SGK1-K127Q overexpression, infusion of HSV-S78A into the VTA did not decrease morphine preference. Instead, contrary to our expectation, we observed a trend for increased morphine preference in HSV-S78A mice and significantly higher morphine intake than HSV-GFP mice (**Figures 10A and 10C**). These results suggest that SGK1 S78 phosphorylation may normally act to limit morphine reward, so it will be essential to perform drinking experiments with HSV-S78D to help elucidate the role of S78 phosphorylation in voluntary morphine consumption.

While there is ample evidence that SGK1 gene expression is induced in multiple brain regions by a variety of drugs of abuse (Gonzalez-Nicolini & McGinty 2002, Heller et al. in press, Kerns et al. 2005, McClung et al. 2005, Nichols & Sanders-Bush 2002, Piechota et al. 2010), little work has been done to determine what role its catalytic activity plays in drug-related behaviors. To date, most of what we know about SGK1 neuronal activity and S78 phosphorylation is from the rat hippocampus, and consequently pertains to learning and memory (Lee et al. 2007, Lee et al. 2006, Tsai et al. 2002). Specifically, hippocampal S78

phosphorylation increased thirty minutes, one hour, and three hours after contextual fear conditioning, a task in which rats learn to anticipate aversive effects when they are placed in an environment they associate with the fearful stimulus (Lee et al. 2007). Importantly, transient hippocampal expression of a mutant preventing S78 phosphorylation (S78A) reduced the contextual fear retention after the initial training period, whereas expression of a phosphor-mimetic mutant (S78D) enhanced fear conditioning. Another study found that hippocampal overexpression of an S422A mutant (which blocks phosphorylation at the canonical site) impaired spatial working memory, whereas overexpressing SGK1-S78A did not cause such an impairment (Lee et al. 2006). These results implicate a role for hippocampal SGK1 in memory formation and suggest that altering SGK1 S78 phosphorylation *in vivo* has behavioral consequences. As is the case in the hippocampus, we think that VTA SGK1 activity mediated by S78 versus S422 phosphorylation may differentially affect behavior. In particular this could explain why the S422D (CA) mutant did not affect voluntary morphine consumption while the S78A mutant did; clearly it will be critical to determine the effect of S78D overexpression.

A caveat to the design of the drinking studies was the short time frame over which measurements were collected. This was a necessity due to the use of the HSVs, which display maximal transgene expression within 12 hours that then persists for approximately five days before declining (Neve et al. 2005). An alternate approach would be to use adeno-associated viruses (AAVs) to facilitate SGK1 overexpression in the VTA, which take longer to achieve maximal expression but expression is then virtually permanent (Kaspar et al. 2002). Using an AAV expression system would permit the SGK1 mutant mice to fully recover from surgery before commencing the drinking studies. Not only would this reduce or eliminate confounding effects from morphine drinking to alleviate post-operation discomfort but it might also reduce

variation in average preference, as preference may stabilize over a longer time. One issue when using AAVs for overexpression, however, is the limited length of DNA sequence that can be packaged into an AAV (reviewed in (Choi et al. 2014)). At approximately 1300 bp, the SGK1 gene may suffer from inefficient expression due to its size. While new methods of increasing the size of transgenes that can be expressed by AAVs are explored (Choi et al. 2014) it may still be possible to produce efficient SGK1 expression from AAVs.

While the morphine consumption results were intriguing and permitted us to explore how SGK1 activity alters self-administration of an addictive drug, we wanted to determine whether these findings extended to other drug-related behaviors. CPP was next examined to measure how often mice sought an environment which they associate with the pleasurable effects of cocaine; it is worth recalling the obvious parallel to the definition of addiction, which focuses on drug-*seeking* behavior. Because CPP is one of the most commonly assessed measures of drug reward and because SGK1 phosphorylation was increased by both morphine and cocaine, we elected to next determine whether alteration of SGK1 VTA activity could affect cocaine CPP. We chose to start our analysis with the K127Q kinase-dead mutant since it produced the strongest and most consistent effect on morphine preference, where it decreased morphine consumption.

We found that in both HSV-GFP and HSV-K127Q mice cocaine increased locomotor activity and that locomotor sensitization, defined as an increased cocaine locomotor response on day 2 compared to day 1, occurred (**Figure 11A**). This result differs from that of Heller et al., where they showed that expression of SGK1-CA in the VTA impaired cocaine-induced locomotor sensitization at a dose of 6 mg/kg (Heller et al. in press). One interpretation for the absence of sensitization in the Heller experiment is that the CA mutant had greater locomotor activation in response to the initial cocaine injection than GFP controls, and that may have

obstructed the sensitizing effects of cocaine. At the higher cocaine dose (20 mg/kg) used in our study, we do not see any evidence for a difference in the initial cocaine locomotor responses between GFP and K127Q mice; however, the protocols were very different between the two studies. Heller et al. (in press) used a lower dose of cocaine and activity was monitored for a more extended time-course in a home-cage setting, typical of traditional behavioral sensitization experiments. Here, we only examined two days of cocaine-induced locomotion in the CPP apparatus, a novel environment for the mice. It will be worth comparing locomotion of HSV-GFP and HSV-K127Q mice with a lower dose of cocaine and a more appropriate sensitization protocol to determine whether decreasing VTA SGK1 activity truly has no effect on cocaine sensitization. In addition, we plan to examine morphine-induced locomotor activity, as SGK1-CA was observed to enhance locomotor sensitization to morphine. This divergence between the morphine and cocaine response suggests that despite the similar ability of cocaine and morphine to increase SGK1 gene expression and S78 phosphorylation, the behavioral outcomes of altered SGK1 activity may be drug-specific.

As expected from the cocaine-induced increase in locomotor activity, robust conditioning was observed as both HSV-GFP and HSV-K127Q mice demonstrated a preference for the cocaine-paired chamber (**Figure 12**). However, we did not observe any differences in the magnitude of CPP scores between the two groups. This result suggests that decreasing SGK1 activity in the VTA is not sufficient to alter cocaine reward. Nevertheless, to solidify this conclusion we would like to repeat this CPP experiment using a lower dose of cocaine. We used a relatively high dose of cocaine (20 mg/kg) initially to ensure that we achieved demonstrable CPP with our protocol, as we were validating the CPP equipment that had just arrived in the laboratory. Although cocaine has been repeatedly proven to produce CPP over a range of doses

including 20 mg/kg (Kelz et al. 1999, Koo et al. 2012), it has also been shown to produce adverse effects at higher doses (Brackett et al. 2000, Hearn et al. 1991). This dose may have been sufficient to cause unpleasant side effects in some mice, leading to an aversion to the drug-paired chamber in two of the HSV-GFP mice and potentially artificially lowering the CPP score of the GFP group (**Figure 12**).

Another potential consequence of using a high dose of cocaine is that we may have produced maximal preference, or a ceiling effect, for the cocaine-paired chamber. As such, even if VTA SGK1 activity partially mediates reward, knocking down SGK1 activity with the K127Q mutant may have been insufficient to diminish the reward produced by 20 mg/kg of cocaine, thereby explaining why we did not see differences in CPP scores. As such, in the future we would like to repeat this experiment with lower doses of cocaine. Additionally, we elected to test only the K127Q virus because we were hoping to see the most dramatic effect possible on reward behavior by preventing SGK1 activation via phosphorylation at either the S78 or T256 and S422 canonical sites. Once the dosing regimen is optimized, we would like to repeat CPP testing using the S78A and S78D viruses, which we would predict to increase and decrease CPP scores, respectively. Furthermore, morphine CPP could be attempted with the SGK1 HSVs that have already been validated, although the use of AAVs is another attractive option. If AAVs expressing the SGK1 mutants could be created and produce sufficient expression, they would be ideal for re-evaluating the impact of altering SGK1 activity once SGK1 expression had stabilized. This would permit additional pairing days to strengthen association of the drug to the chamber and possibly increase the chance of observing significant differences in CPP scores among the different viral treatment groups.

In summary, we propose a number of future experiments. Most pressingly we are awaiting the arrival of a new batch of HSV-S78D virus for validation. As discussed above, there are multiple ways to approach validation of these viruses in addition to the Western blots used for this study, i.e. immunohistochemistry to detect GFP in infected cells, RT-PCR to detect transgene overexpression, and tissue collection guided by a fluorescence dissecting microscope. The next steps would be to use the SGK1 mutants in two-bottle choice studies for morphine, in particular comparing the effects of the S78A and S78D mutants to judge whether affecting activation at the S78 site affected self-administration reward behavior. To determine if the conclusions about the effects of SGK1 S78-regulated activity on self-administration are more broadly applicable to other classes of drugs, we could design a similar two-bottle choice study for cocaine, which has also proven amenable to oral intake measures (Falk et al. 1996, Falk et al. 1990). Similarly, we wish to use these same viruses in a morphine CPP paradigm to determine if activation or inhibition of S78 phosphorylation-mediated signaling promotes or diminishes the pleasurable effects of morphine reward. The behavioral experiments would also be repeated with larger cohorts of mice in order to increase statistical power. These studies, which would be relatively easy to accomplish, would round out the existing data described herein.

Additionally, there are numerous experimental approaches not undertaken in this work that could be pursued to elucidate the behavioral effects of SGK1 S78 regulation. A literature search revealed that there are no studies where SGK1 has been knocked-out in any part of the brain (aside from systemic KO mice, previously discussed). However, we could take advantage of floxed SGK1 mice (Fejes-Toth et al. 2008), where the SGK1 gene is flanked by loxP sites, for two types of experiments. First, floxed SGK1 mice could be bred to mice that express Cre recombinase under the control of the DAT promoter (DAT-Cre mice) to generate a mouse with

SGK1 selectively knocked out of DA neurons; DAT-Cre mice have been used extensively (Zhuang et al. 2005). The offspring mice lacking SGK1 specifically in DA neurons could then be used in concert with HSV overexpression of SGK1 mutants in the VTA to study drug-induced behaviors such as voluntary drug consumption, locomotor activity, and CPP. One disadvantage of this combined genetic and viral-mediated approach, however, is that SGK1 would be absent from all DA neurons, not just the VTA. For example, SGK1 would be knocked out of substantia nigra DA neurons, which help regulate voluntary muscle control, so it would be essential to ensure that removing SGK1 from all DA neurons does not produce unintended locomotor abnormalities. Additionally, as this breeding would generate a developmental KO of SGK1, there may be other unintended developmental effects. The second type of experiment that could be performed would be to infuse the VTA of adult floxed-SGK1 mice with an AAV or HSV that carries Cre recombinase (also readily available; (Kaspar et al. 2002, Rinaldi et al. 1999)). This approach would have the distinct advantage of knocking out SGK1 only at the site of the injection in the VTA and also avoids unintended consequences of a developmental KO. In addition to examining potential behavioral effects of SGK1's absence, these mice could then undergo a second surgery to receive infusions of HSVs or AAVs carrying the SGK1 mutants described in this study. The HSV-SGK1 mutants are ready for use and could be used to repeat the experiments described herein, this time ensuring that behavioral effects would not be confounded by endogenous SGK1 activity. More powerful still would be expression of AAV-SGK1 mutants. As expression is effectively permanent, behavioral experiments with both drugs could be performed in mice that are only expressing the SGK1 mutants in the VTA, something that could not be achieved with HSVs due to their shorter expression time. This would allow us to obtain a more accurate profile of the behavioral consequences of S78 activation without the

confounding factors of endogenous SGK1 activity. An alternate approach to measure behavioral and signaling changes due to SGK1 mutants would be to use siRNA to reduce the contributions of endogenous SGK1. In concert with HSV-SGK1 mutant infusions, siRNA knock-down of endogenous SGK1 would help to isolate the consequences of inhibiting or mimicking activation with the S78A and S78D viruses or completely blocking kinase activity with the K127Q virus.

If the experiments described above provide evidence that modulating SGK1 activity by altering S78 phosphorylation impacts multiple reward behaviors, our next steps would attempt to uncover the upstream and downstream mediators of this activity. It is already known different kinases mediate SGK1 phosphorylation at the different activation sites: BMK1 (ERK5), ERK2, and p38 MAPK phosphorylate S78 (Hayashi et al. 2001, Lee et al. 2006, Meng et al. 2005); mTORC2 phosphorylates S422 (Garcia-Martinez & Alessi 2008); and PDK1 phosphorylates T256 (Park et al. 1999). Thus, we could determine whether activity of these upstream kinases in the VTA is influenced by chronic administration of morphine or cocaine. Importantly, it is also plausible that activation at these different sites induces a preference for phosphorylating different downstream substrates. Indeed, this has been shown for AKT, another kinase in the same AGC family as SGK1 (reviewed in (Liao & Hung 2010)). One method to identify differences in substrate phosphorylation after expression of the different SGK1 mutants is to utilize a phosphopeptide enrichment protocol. Briefly, protein would be extracted from VTA samples and digested with an enzyme such as trypsin. Samples would then be enriched for phosphopeptides (including those from proteins phosphorylated by SGK1) using titanium dioxide columns (commercially available from Thermo Scientific) and liquid chromatography-mass spectrometry (MS)/MS analysis. This would allow us to identify different phosphopeptides (and therefore by extension the proteins) present in the samples from various SGK1 mutant treatments. For

example, we should be able to determine if S78 mutants prefer to phosphorylate different or the same substrates as S422 mutants, clarifying the role that phosphorylation of these two SGK1 regulatory sites has on downstream mediators. Our laboratory is well-positioned to take advantage of this technique as we have a working relationship with the Keck Center at Yale University, which specializes in these types of proteomic analyses. Results from these experiments would provide an important step forward in understanding the molecular pathways that mediate drug-induced reward behaviors.

The results described herein are some of the first to explore a role for SGK1 activity in neuronal adaptations induced by chronic morphine and cocaine, two highly addictive and frequently abused drugs. Numerous other studies have shown that VTA SGK1 gene expression is increased not only in response to chronic morphine (Heller et al. in press, McClung et al. 2005) and cocaine (Heller et al. in press) but also in whole brain lysates after chronic oxycodone (Hassan et al. 2010), the striatum after acute amphetamine (Gonzalez-Nicolini & McGinty 2002), ethanol, morphine, heroin, or methamphetamine (Piechota et al. 2010), and in the prefrontal cortex after acute ethanol (Kerns et al. 2005) and LSD (Nichols & Sanders-Bush 2002). However, these studies only evaluated changes in SGK1 gene expression and did not attempt to measure SGK1 levels or alter SGK1 activity to determine a causative role for SGK1 in drug-induced behavioral changes. By investigating the upstream activators and downstream mediators of site-specific regulation of SGK1 activity we will contribute to the growing body of knowledge of how reward pathway signaling is altered by drug use and abuse. Our data provide some of the first evidence that SGK1 kinase activity, not just its action as an immediate-early gene, is regulated by chronic drug exposure. Moreover, the effects on morphine drinking preference demonstrate that changes in SGK1 activity in the VTA are behaviorally relevant.

With continued experimentation, including behavioral assays such as voluntary consumption and CPP, as well as more sophisticated biochemical studies, we hope to further characterize the role of SGK1 signaling in the rewarding effects of drugs of abuse. These studies may shed light on how this kinase is involved in the neuroadaptations that underlie drug addiction and dependence and might lead to the development of novel targets for therapeutic intervention in addiction.

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