

**Inhibitory signaling and reward: the role of GIRK channels in the
mesocorticolimbic dopamine system**

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Dedication

I would like to dedicate this to my family, without whom I could not have accomplished this. Your unwavering support and encouragement has been the cornerstone of my life. I can't thank you enough!

To my Carini Family, Mary Carini and Jeff Martocci, for being exceptional role models and showing me the importance of life-long learning. Thank you for your love and support.

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Abstract

Drug abuse, including the illicit use of opioids and cocaine, is a critical global problem with high social and economic costs. Given the current lack of selective and efficacious pharmacotherapies to treat addiction, it is vital that we gain a better understanding of the cellular and molecular targets of drugs of abuse, and how modulation of these targets leads to the development of addiction. Although different abused drugs work through different mechanisms of action, addiction is thought to have a common pathway, the mesocorticolimbic DA system, which consists of the ventral tegmental area (VTA) and reciprocal connections with downstream targets including the prefrontal cortex (PFC) and nucleus accumbens (NAc). Various drugs of abuse, such as opioids and cocaine, induce adaptations to excitatory and inhibitory signaling within the mesocorticolimbic DA system. Although drug-induced adaptations to excitatory signaling within the mesocorticolimbic DA system have been well characterized, the adaptations and importance of inhibitory signaling requires further investigation. G protein-coupled receptors (GPCRs) and downstream effectors/second messengers represent a major target for drug-induced changes in mesocorticolimbic signaling. G protein-gated inwardly rectifying K⁺ (GIRK) channels are a key downstream target of inhibitory GPCRs, including the mu opioid receptor (MOR), gamma-aminobutyric acid receptor (GABA_BR), and the dopamine type 2 receptor (D₂R). GIRK channels have been shown to mediate the inhibitory effects of many neurotransmitters in the central nervous system, and dysregulation of GPCR-GIRK signaling has been identified in a number of disorders, including addiction. This dissertation focuses on the role of GIRK-dependent inhibitory signaling throughout the mesocorticolimbic DA system, and how this form of

signaling contributes to the cellular and reward-related behavioral effect of drugs of abuse.

The importance of GIRK-dependent signaling to drug addiction is supported by the fact that several drugs of abuse can produce adaptations to the GPCR-GIRK signaling cascade. GIRK-dependent signaling was also believed to be a key regulatory of opioid effects within the mesocorticolimbic DA system, thought to ultimately contribute to opioid reward. Here, we challenged the opioid signaling “dogma” and show that GIRK-dependent signaling in midbrain GABA neurons is not required for disinhibition of VTA DA neurons and subsequent opioid-induced motor-stimulation. Interestingly, it appears the unique GIRK2/GIRK3 channel found in VTA DA neurons can modulate the behavioral sensitivity to opioids. In addition, we found that this GIRK2/GIRK3 channel can also modulate D₂R autoinhibitory feedback signaling of VTA DA neurons and cocaine locomotor sensitivity, suggesting it could be a shared mechanism of other drugs of abuse as well. Taken together, these findings demonstrate that GPCR-GIRK inhibitory signaling in VTA DA neurons is critical to the molecular and behavioral effects of drugs of abuse. These findings in VTA DA neurons also suggest that the discovery of novel compounds that modulate GIRK channel function in a subunit-dependent manner could help us prevent and/or treat addiction.

Table of Contents

Dedication	i
Acknowledgements	ii
Abstract	iii
Table of Contents	v
List of Tables	viii
List of Figures	ix
List of Abbreviations	x
CHAPTER 1. INTRODUCTION	1
I. DRUG ADDICTION	2
II. DRUGS OF ABUSE	4
i. Opioids	4
ii. Psychostimulants: Cocaine	6
III. NEUROCIRCUITRY OF REWARD: MESOCORTICOLIMBIC DA SYSTEM	9
i. Ventral Tegmental Area (VTA)	11
ii. Prefrontal Cortex (PFC)	11
iii. Drug-induced Adaptations	13
IV. INHIBITORY SIGNALING: GPCR-GIRK	13
i. G Protein Coupled Receptors	13
A. Structure	13
B. Function	14

ii. G Protein-Gated Inwardly Rectifying Potassium (GIRK) Channels	15
A. Function and Pharmacology	15
B. Subunits	15
C. Expression	16
D. GIRK channels and Addiction	19
V. DRUG-INDUCED ALTERATIONS TO INHIBITORY GPCR-GIRK SIGNALING	20
i. Opioids: MOR-GIRK	20
A. Pharmacology	20
B. Opioid Signaling in the VTA	21
ii. Cocaine: GABA _B R-GIRK and D ₂ R-GIRK	24
A. Cocaine: Elevated Dopamine	24
B. Inhibitory Signaling: GABA _B R	24
a. VTA	25
b. mPFC	25
C. Inhibitory Signaling: D ₂ R	26
a. VTA	27
VI. PURPOSE OF STUDIES	27
Chapter 2 Author Contributions	30
CHAPTER 2. GIRK channels modulate opioid-induced motor activity in a cell type- and subunit-dependent manner	31
Introduction	32

Materials and Methods	33
Results	41
Discussion	60
Chapter 3 Author Contributions	65
CHAPTER 3. GIRK channels in VTA DA neurons regulate the	
sensitivity of the mesocorticolimbic DA system to cocaine	66
Introduction	67
Materials and Methods	69
Results	72
Discussion	82
CHAPTER 4. DISCUSSION	85
General summary	86
The relevance of GIRK channels to the cellular and reward-related	
behavioral effects of opioids	87
The relevance of VTA DA neuron GIRK-dependent signaling to	
the cellular and reward-related behavioral effects of cocaine	92
Future directions	96
Concluding Thoughts	100
REFERENCES	102

List of Tables

Table 3.1	Electrophysiological properties of VTA DA neurons in DATCre(+/-): <i>Girk2</i> ^{flox/flox} mice	75
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List of Figures

Figure 1.1	The mesocorticolimbic DA system.	10
Figure 1.2	Inhibitory GPCR and GIRK channel subunit expression in the mesocorticolimbic DA system.	18
Figure 1.3	Opioid-induced disinhibition of VTA DA neurons hypothesis.	23
Figure 2.1	Constitutive <i>Girk</i> subunit ablation and morphine-induced motor activity.	42
Figure 2.2	Generation of <i>Girk2</i> ^{lox/lox} mice.	44
Figure 2.3	GABA neuron-specific ablation of GIRK channels and morphine-induced motor activity.	47
Figure 2.4	Characterization of the GIRK channel in VTA GABA neurons.	49
Figure 2.5	Intracranial DAMGO-induced motor activity.	51
Figure 2.6	Direct activation of GIRK1-containing GIRK channels and motor activity.	53
Figure 2.7	DA neuron-specific ablation of GIRK channels and systemic morphine-induced motor activity.	56
Figure 2.8	<i>Girk3</i> ablation and rescue: morphine-induced motor activity.	59
Figure 3.1	DA neuron-specific ablation of GIRK channels reduces D ₂ R-dependent signaling.	74
Figure 3.2	DA neuron-specific ablation of GIRK channels enhances acute cocaine-induced motor stimulation.	77
Figure 3.3	DA neuron-specific ablation of GIRK channels alters cocaine sensitization.	79
Figure 3.4	DA neuron-specific ablation of GIRK channels does not impact cocaine reward.	81

List of Abbreviations

AC	adenylyl cyclase
Ba ²⁺	barium
Ca ²⁺	calcium
CNS	central nervous system
CPP	conditioned place preference
DA	dopamine
DAT	dopamine reuptake transporter
DOR	delta opioid receptor
D ₂ R	dopamine type 2 receptor
GABA	γ-aminobutyric acid
GABA _B R	γ-aminobutyric acid type B receptor
GDP	guanosine diphosphate
GHB	gamma-Hydroxybutyrate
GIRK	G protein-gated inwardly-rectifying K ⁺ channel
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HPC	hippocampus
I _h	hyperpolarization-activated current
I _{KACH}	cardiac K ⁺ channel, GIRK1/GIRK4 tetramer
K ⁺	potassium
Kir	inwardly-rectifying K ⁺ channel
KOR	kappa opioid receptor
PFC	prefrontal cortex
PLC	phospholipase C
mPFC	medial prefrontal cortex
MOR	mu opioid receptor
MSN	medium spiny neuron
MT	medial terminal nucleus of the accessory optical tract
NAc	nucleus accumbens
RGS	regulator of G protein signaling
RMTg	rostromedial tegmental nucleus
SA	self-administration
SN	substantia nigra
SNX27	sorting nexin 27
TM	transmembrane
TPN	tertiapin
VTA	ventral tegmental area

CHAPTER 1

INTRODUCTION

I. DRUG ADDICTION

Drug Abuse refers to inappropriate use of both illicit and legal drugs. This can include repeated use of drugs to produce pleasure, alleviate stress, to avoid reality, and using prescription drugs in ways other than prescribed or using someone else's prescription. Drug addiction corresponds to the DSM definition of substance use disorder, which is diagnosed when an individual presents with 2-3/11 symptomatic criteria that span four major groupings: impaired control, social impairment, risky use, and pharmacological criteria [1]. Illicit use of drugs, such as cannabis, cocaine, heroin, and prescription psychotherapeutics, is a critical problem in the United States and worldwide. In 2012, an estimated 23.9 million Americans, 9.2% of the population, 12 years of age or older were current illicit drug users [2]. Worldwide, an estimated 155-250 million people 15-64 years of age used illicit substances in 2008, with opioid and cocaine abuse among the most common [3, 4].

Significant individual and public health concerns arise from the abuse of illicit and legal substances. Drug abuse can cause or contribute to more than 70 medical conditions, such as heart disease, pregnancy complications, infectious diseases, or accidents and trauma, which require hospitalization and/or long term medical care [5]. The high prevalence of infectious diseases, like HIV and hepatitis, with IV drug use has become an increasing public health concern and complicates individual long term care options [6]. Drug abuse also has a high co-occurrence with many psychological disorders, which can complicate treatment options. The financial burden is also high, with approximately 2% of GDP in the United States (more than \$700 billion a year) spent

on health care costs, crime, and lost productivity due to drug use [6, 7]. On average, 16% of any state's budget is devoted to the costs of addiction and risky drug use, but for every dollar spent on addiction and substance abuse, only 2 cents of that goes to prevention and treatment [5, 8]. One out of every five Medicaid dollars is spent on hospital care attributable to substance abuse [8]. Given the social and economic consequences associated with illicit drug use, it is imperative that we gain a better understanding of the biological mechanisms that ultimately lead to addiction.

Drug addiction is a chronic, relapsing brain disorder that is defined as compulsive drug seeking and use despite negative consequences to both the user and loved ones [1] [9, 10]. Drugs with high abuse liability, such as opioids and cocaine, produce an initial state of well-being or euphoria, "rewarding effects", by acting on the reward center of the brain [11-16]. Use of the drug continues in an attempt to recreate the euphoric experience and/or avoid undesirable feelings of withdrawal, in turn resulting in a pattern of repeated drug use. This repeated drug exposure leads to a variety of neurochemical adaptations in the brain that can result in physical dependence, and ultimately, the development of addiction. Current treatment approaches include medication and behavioral therapy, and have been shown to be most effective when combined. Treatment plans usually begin with monitored detoxification, treatment, and continual care to prevent relapse; however it is important to note that no single treatment plan is appropriate for everyone. Behavioral therapy can include individual and group based therapy to address mental health and personal issues that may be contributing to the substance abuse [17]. To date there are few pharmacologic options that can be used for addiction therapy/treatment. Currently available pharmacotherapies are mainly used to

help ease withdrawal or to help prevent relapse and diminish cravings; however, their effectiveness is not ideal with a relapse rate of 40-60% [10, 18, 19]. Opioid addicts have the highest relapse rate with nearly 80-90% using within the first year following abstinence [20]. Inpatient treatment for cocaine addiction has a much higher success rate, with around 20-30% relapse rate in the first year following abstinence [21]. With relapse rates remaining between 40-60%, vast improvements are needed in our understanding of the mechanisms through which drugs with high abuse liability produce their rewarding effects in an effort to improve prevention and/or treatment of drug addiction.

II. DRUGS OF ABUSE

i. Opioids

The term ‘opiate’, refers to any preparation made from the natural alkaloids of opium poppy resin, *Papaver somniferum* [22, 23]. In contrast, ‘opioid’ refers to compounds produced naturally by the opium poppy, as well as compounds that mimic the pharmacological effect of opiates. This includes all natural products derived from the opium poppy, semi-synthetic and synthetic products, and endogenous opioid peptides [24, 25]. Opioids have been used for centuries to treat pain [26-28], but seem to have an equally long history of abuse. Records indicate that opium was used by Greeks and Romans as far back as the third century B.C. for its analgesic as well as euphoric properties [22, 23, 29]. This historical use of opium dating back centuries for pain management and anesthesia demonstrates just how effective an analgesic it is, and how even with the discovery of other pharmacotherapies to manage pain, opioids remain the gold standard of pain relief. Today the prevalence of opioid use for non-medical

purposes, including heroin and prescription opioids, is estimated at >1% of the world's population 15-64 years of age [6]. Moreover, in 2010, the Substance Abuse and Mental Health Services Administration reported that approximately 2 million Americans were using prescription painkillers non-medically [2], and the number of unintentional overdose deaths has more than tripled from 140,000 to 540,000 between 1999 and 2011 [30].

Morphine is the most widely used opioid to treat both acute and chronic pain [24, 31-33]. It was first isolated from the opium poppy in 1805 by pharmacist Friedrich Wilhelm Adam Sertürner and marketed commercially by Merck Pharmaceuticals in 1827 [34]. Since its isolation, drug companies have created numerous semi-synthetic (hydrocodone, oxycodone, heroin, buprenorphine, etc.) and synthetic (tramadol, fentanyl, methadone, etc.) opioid compounds for pain relief, anesthesia, and the treatment of drug dependence [24, 25]. Although opioids remain the most effective drugs for the relief of pain, many side effects accompany the use of opioid-based drugs. For example, acute use can cause constipation, itching, nausea and vomiting, drowsiness/sedation, and respiratory depression [24, 35-40]. Chronic use of opioids can result in hyperalgesia, hormonal effects, immunosuppression, the development of tolerance and abuse, and ultimately addiction [40-48]. To date, our understanding of and ability to disentangle the beneficial outcomes associated with opioids (analgesia) from the undesirable side effects (tolerance and addiction) is highly limited.

Opioid dependence and addiction are managed currently with a combination of psychosocial therapy and opioid substitution pharmacotherapies, such as methadone and buprenorphine, or opioid antagonists, such as naltrexone and naloxone [3, 17, 49].

These medications can be used individually or in combination to detoxify the addict, gradually taper the amount of opioid used, and to help suppress withdrawal symptoms [50]. Optimal results are achieved through office-based opioid treatment and psychosocial treatment. Although effective pharmacotherapies, these medications come with their own set of challenges and limitations [51]. Methadone can have a side effect profile similar to opioid abuse, including adverse drug interactions and abuse liability [3, 49]. An additional challenge is that methadone therapy requires daily or regular clinic visits leading to lack of compliance and relapse rates of 80-90% [3, 10, 20, 52]. As opioid abuse continues to rise, costing society approximately \$72 billion per year [53, 54], it seems imperative that we gain a better understanding of the mechanisms that mediate the analgesic versus the addictive effects of opioids, to in turn better prevent or better treat these conditions.

ii. Psychostimulants: Cocaine

Cocaine has been used for centuries for its euphoric and energizing properties. Records indicate that the tribes of Peru, Bolivia, and Columbia have chewed and brewed coca leaves for centuries to fight fatigue and hunger as far back as 3000 B.C. [55-57]. Cocaine consumption did not become a problem until around 1884, when it was coined a “miracle drug” and used in an attempt to treat various disorders [57, 58]. Even with the discovery in the 1900s that cocaine holds little therapeutic efficacy, cocaine use has continued to remain a global critical problem. The United Nations World Drug Report stated that 14.3 million people worldwide reported using cocaine between 2005 and 2006, and 1.6 million people in the United States, 0.6% of the population, used cocaine in 2012

[2, 4]. Further, cocaine use and abuse continues to be a costly economic and public health burden [5-8].

Cocaine is a 'psychostimulant', which refers to a class of drugs that produce a transient increase in psychomotor activity with antidepressant or mood-elevating properties [59]. Psychostimulants include cocaine, amphetamine, amphetamine derivatives, caffeine, and nicotine [60]. The active ingredient in coca leaves, cocaine, was isolated by Albert Niemann in 1859 [58]. Following its isolation, cocaine was used in numerous tonics and wines that were heavily endorsed by medical professionals from 1860 to early 1900s [57, 58]. Cocaine was also present in the initial formula of Coca-Cola. In the late 1800s, cocaine was used in Western medicine as a local anesthetic and minor pain reliever [57, 58]. Once it became clear in the early 1900s that cocaine offered little efficacy in treating various disorders and possessed high addictive properties, it was removed from all products and labeled as an illicit substance [57, 58, 61]. However, the damage was already done and cocaine was here to stay. Intranasal use of cocaine powder became popular again in the 1970s for social-recreational use. The development of free base cocaine, or 'crack', in 1974 which could be smoked for a more rapid "high", resulted in an explosion of cocaine abuse and addiction [55, 57, 58]. The 'crack' epidemic among other illicit substance use caused the United States to wage a "War on Drugs" in the 1990s. Despite these efforts, cocaine use remains an issue today [2].

Cocaine and amphetamine have very limited therapeutic use, but are commonly abused due to their euphoric, energizing, decreased appetite, and increased alertness effects [62]. Other less desirable effects include increased muscle tension, tachypnea, hyperpyrexia, tremors, diaphoresis, myocardial infarction and stroke [62-64]. Repeated

cocaine use can result in restlessness, irritability, stereotypic behaviors, insomnia, and overdose [64-66]. Cocaine overdose can result in serious life-threatening medical conditions such as convulsions/seizures, cerebral hemorrhage, and cardiac or respiratory failure [67, 68].

Cocaine dependence and addiction, unlike opioid addiction, is often treated with behavioral interventions such as cognitive-behavioral therapy. There are currently no pharmacotherapies that have met the criteria for regulatory approval or acceptance in the medical community for the treatment of psychostimulant addiction [62]. However, currently there are a number of pharmacologic treatments for psychostimulant-use disorders, as defined by the DMSV, in clinical trials [62]. These pharmacotherapies include three main categories: naltrexone, disulfiram/anatabuse, and agonist-like replacement therapies [69-73]. Although some of these clinical trials seem promising, we still have a long way to go toward effective and safe pharmacotherapies for the treatment of psychostimulant addiction. It is important to note that, as we see with other substance abuse disorders, treatment of psychostimulant addiction is often most effective when combined with the treatment of comorbid psychosocial disorders and/or withdrawal symptoms. Due to the lack of pharmacotherapies available to treat psychostimulant addiction, it seems imperative that we gain a better understanding of the mechanisms that mediate the “rewarding” effects of cocaine, to in turn better prevent or better treat addiction.

III. NEUROCIRCUITRY OF REWARD: MESOCORTICOLIMBIC DA SYSTEM

Although various abused drugs work through different mechanisms of action, addiction is thought to have a common anatomic substrate, the mesocorticolimbic DA system [74, 75]. The mesocorticolimbic DA system is comprised of dopamine (DA) neurons originating from the ventral tegmental area (VTA) with axons projecting to limbic (nucleus accumbens (NAc)) and cortical (prefrontal cortex (PFC)) nuclei depicted in Figure 1.1. These DA neurons respond to natural rewards important for survival and reproduction, such as food, water, and sex, by becoming more active and releasing more DA at the synapses of downstream targets [76, 77]. Dysfunction of the mesocorticolimbic DA system is associated with addiction and mental health disorders, including major depression and schizophrenia [78, 79]. Drugs of abuse, including opioids and cocaine, are thought to exert their euphoric effects by hijacking the signaling in the brain's natural "reward circuit", leading to alterations in DA and other neurochemical signaling pathways within this circuit. These drug-induced alterations in "reward circuit" cellular activity can result in various physiological and behavioral changes centered on escalating and unregulated intake of the drug, which characterizes addiction [79].

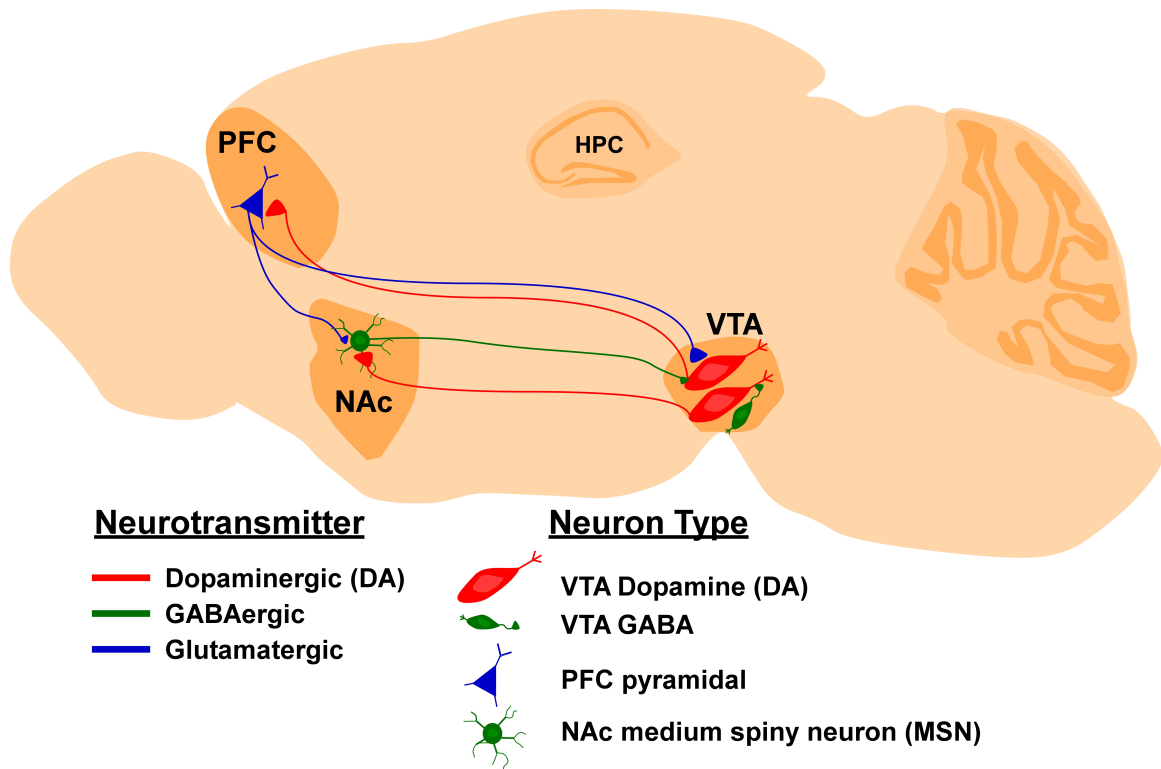


Figure 1.1. The mesocorticolimbic DA system. Cartoon depiction of key players in the mesocorticolimbic DA system as discussed here. VTA DA neurons project to downstream targets such as glutamatergic pyramidal neurons of the PFC and GABAergic medium spiny neurons (MSN) of the NAc. The PFC and NAc send reciprocal glutamatergic and GABAergic projections back to the VTA. Abbreviations: Ventral tegmental Area (VTA), prefrontal cortex (PFC), nucleus accumbens (NAc), hippocampus (HPC).

i. Ventral Tegmental Area (VTA)

The activity of the VTA has been shown to play a key role in the rewarding properties of drugs of abuse, with various alterations in VTA signaling depending of the class of drug [80]. The VTA, often thought of as the origin of the “reward circuit”, is a small nucleus located along the ventral midline of the brain, above the brainstem. It is also referred to as the A10 region, referring to the A10 dopaminergic neuron population, which projects to the NAc [81]. However, the VTA is a heterogeneous population of neurons consisting of (65%) DA, (30%) gamma-aminobutyric acid (GABA), and (5%) glutamate neurons [82-87].

The relevance of the VTA to drug-induced, reward-related behavior is highlighted by several experiments incorporating intracranial lesioning or pharmacological manipulations. For example, infusion of opioids into the VTA can evoke reward-related behaviors, such as motor-stimulation and conditioned place preference [88-90]. Rodents will also work to self-administer opioids directly into the VTA, while cocaine self-administration can be disrupted by intra-VTA infusion of DA type 1 receptor (D₁R) antagonists or GABA_BR agonist (baclofen) [14, 91-94]. In addition, lesions that disrupt signaling between the VTA and NAc disrupted cocaine and heroin self-administration and heroin-induced locomotor activity [95, 96]. Thus, the VTA is a critical component of the intrinsic reward pathway and a neuronal substrate for drug-induced, reward-related behaviors.

ii. Prefrontal Cortex (PFC)

The PFC is the most rostral portion of the frontal lobe, which is located in the anterior portion of the brain. It is often thought of as an input association cortex,

responsible for decision-making, goal-directed behavior, and processing reward-related information [97, 98]. The PFC is critical for executive functions, attention, memory, intelligence, and language [97]. Damage to the PFC can produce drastic alterations in personality, memory, and emotional state [99-102]. One subregion of importance is the medial prefrontal cortex (mPFC), a highly organized structure comprised of glutamatergic pyramidal neurons and GABAergic interneurons [97, 103, 104]. The mPFC plays a key role in modulating the mesocorticolimbic DA system. In particular, mPFC provides excitatory input that helps regulate the activity of VTA and NAc neurons. Within the VTA, mPFC glutamatergic neurons synapse on both of the predominant cell types, VTA GABA and DA neurons. In turn, DA neurons of the VTA project back to the mPFC, while VTA GABA neurons project to the NAc, creating a feedback loop to regulate mesocorticolimbic cellular excitability [105-107].

mPFC activation is vital to cognitive functions, including the integration and processing of reward-related information [108, 109]. Imaging studies in human cocaine addicts show that mPFC function is altered, represented as decreased cerebral blood flow and glucose metabolism, during cocaine withdrawal and exposure to cocaine-paired cues [110-113]. Rodent studies, involving lesioning or pharmacologic manipulation of the PFC, support the contention that the mPFC mediates several aspects of reward-related behaviors, including locomotor sensitization, drug-taking behavior, and cue- or drug-induced relapse [114-119]. For example, infusions of DA receptor antagonists into the mPFC decreased drug-primed reinstatement [120, 121]. The glutamatergic output of the mPFC to the VTA stimulates DA release to the NAc, triggering a number of drug-induced adaptations to the VTA and NAc that ultimately underlie the drug-induced

alterations in reward-related behavior [122-125]. Thus, the PFC is a critical component of the intrinsic reward pathway and a neuronal substrate for drug-induced, reward-related behaviors.

iii. Drug-Induced Adaptations

Exposure to various drugs of abuse, such as opioids and cocaine, triggers drug-induced adaptations to excitatory and inhibitory signaling within the mesocorticolimbic DA system [75, 80, 126, 127]. Drug exposure triggers an increase in excitatory signaling, via increased strength of excitatory synapses in VTA DA neurons [128-130]. Drug-induced adaptations to excitatory signaling within the mesocorticolimbic DA system have been well characterized [75, 80, 90, 126, 127, 131-133]. However, the adaptations and importance of inhibitory signaling requires further investigation, including the mechanisms of drug-induced changes to inhibitory signaling in the VTA and mPFC, and the role they play in the cellular and behavioral effects of drugs of abuse.

IV. INHIBITORY SIGNALING: GPCR-GIRK

i. G Protein-Coupled Receptors

A. Structure

G protein-coupled receptors (GPCRs) form a superfamily of proteins that play a vital role in regulating physiology, such as cell growth and development, cardiac function, immune function, neurological function, and metabolism [134]. This receptor superfamily represents a prominent therapeutic target, with nearly 45-50% of the drugs on the market targeting a GPCR [135, 136]. While the GPCR superfamily is highly diverse, all members exhibit seven α -helical transmembrane (TM) regions with an

extracellular amino-terminus and intracellular carboxy-terminus [137, 138]. GPCRs are activated by extracellular binding of various ligands including hormones, proteins, lipids, and neurotransmitters [138-142]. Key inhibitory GPCRs relevant to the work discussed here include the mu opioid receptor (MOR), gamma-aminobutyric acid receptor (GABA_BR), and the dopamine type 2 receptor (D₂R).

B. Function

The G protein signaling cascade, comprised of a GPCR, G protein, and downstream effectors/second messengers, is a major target for drug-induced changes in mesocorticolimbic signaling. G proteins are comprised of three subunits, G α , G β and G γ that are bound together with the GPCR in the inactive state. The G protein signaling cascade is activated by ligand binding to the GPCR. Ligand binding triggers a conformational change in the GPCR, allowing it to serve as a guanine nucleotide exchange factor for the associated G protein [143]. Specifically, the activated GPCR catalyzes the exchange of guanosine diphosphate (GDP) bound to the G α protein for guanosine triphosphate (GTP). The GTP-bound G α subunit then dissociates from the G $\beta\gamma$ subunit, allowing each subunit to bind to various downstream effectors. The intrinsic GTPase activity of the G α subunit hydrolyzes the bound GTP into GDP, promoting the re-association of the G α and G $\beta\gamma$ subunits and inactivation of the G protein signaling cascade [143, 144]. Following the re-association of the GPCR and G protein, the GPCR can be activated again in the presence of an agonist.

The effect of G proteins on neuronal activity depends on subunit identities and downstream intracellular targets [145]. Specifically, G α_s and G α_q promote excitatory effects within the neuron through the activation of downstream enzymes, adenylyl

cyclase (AC) and phospholipase C (PLC), and their subsequent production of second messengers. In contrast, $G\alpha_{i/o}$ promotes inhibitory changes via inhibition of adenylyl cyclase [146-148]. The $G\beta\gamma$ subunit can also modulate neuronal activity through direct inhibition of voltage-gated Ca^{2+} channels and activation of G protein-gated inwardly rectifying potassium (K⁺) (GIRK/ K_{ir3}) channels [149-156].

ii. G protein-gated inwardly rectifying potassium (GIRK) channels

A. Function and Pharmacology

Proper functioning of the nervous system requires a balance of excitatory and inhibitory signaling, and GIRK channels serve as an important regulator of inhibitory control. GIRK channels are expressed throughout the central nervous system (CNS) and act as downstream targets for various $G\alpha_{i/o}$ -linked GPCRs, such as MOR, GABA_BR and D₂R [157-163]. GIRK channels are activated by the direct binding of $G\beta\gamma$ following GPCR activation [149]. Activation of GIRK channels produces a postsynaptic efflux of potassium from the cell, responsible for hyperpolarization/inhibition of the neuron [164-166].

B. Subunits

Throughout the CNS, GIRK channels serve as an important regulator of cellular activity. Four mammalian GIRK channel subunits have been identified, GIRK1 (Kir3.1/Kcnj3), GIRK2 (Kir3.2/Kcnj6), GIRK3 (Kir3.3/Kcnj9) and GIRK4 (Kir3.4/Kcnj5), and have distinct but overlapping expression patterns [164, 165, 167]. GIRK1, GIRK2, and GIRK3 are widely expressed throughout the nervous system and interact to form homotetrameric (homomeric) and heterotetrameric (heteromeric) GIRK channels [157, 167]. GIRK4 is primarily found in the heart and a few other neuronal

populations. In the heart GIRK4 interacts with GIRK1 to form the cardiac GIRK channel ($I_{K_{ACh}}$) [157, 167]. The molecular differences between the various subunits regulate differences in expression and trafficking to the plasma membrane [167]. Only GIRK2 and GIRK4 can form homomeric GIRK channels, as they contain an endoplasmic reticulum export motif [168-170]. GIRK1 and GIRK3 lack this motif so they must interact with GIRK2 or GIRK4 to leave the endoplasmic reticulum and form a functional channel at the plasma membrane [154, 168, 169, 171-174]. GIRK3 and GIRK2c, a splice variant of GIRK2, also contain a class I PDZ binding motif that allows them to interact with other proteins that contain a PDZ domain, such as sorting nexin 27 (SNX27) [175, 176]. GIRK3 also contains a lysosomal targeting sequence, which reduces GIRK3-containing channel expression at the plasma membrane and total protein levels [168].

C. Expression

Neuronal GIRK channels are mainly expressed somatodendritically [177-179]. Somatodendritic expression refers to GIRK channels found in perisynaptic regions of the soma and dendrites as well as in the dendritic shaft and spines, as demonstrated in various *in situ* hybridization and immunoelectron microscopic labeling experiments [160, 180-182]. Although mostly postsynaptic, some presynaptic expression of GIRK channels has been observed in certain neuron populations [179, 183]. The three main mammalian GIRK subunits identified (GIRK1, GIRK2, and GIRK3) are expressed in a variety of brain regions. Heteromeric channels comprised of GIRK1 and GIRK2 are the prominent GIRK channel in the brain [181, 184]. However, GIRK subunit expression depends on the brain region and cell type of interest [167, 185]. For example, in brain regions such as the mPFC and hippocampus GIRK1, GIRK2, and GIRK3 are expressed, making

several GIRK channel subunit combinations possible [185-187] (Figure 1.2). DA neurons of the substantia nigra (SN) are unique in that they only express GIRK2 homomeric channels [155, 185]. The two main cell types of the VTA have different GIRK subunit expression. VTA GABA neurons express GIRK1, GIRK2, and GIRK3, making several GIRK channel subunit combinations possible [185-187] (Figure 1.2). However, DA neurons of the VTA only express GIRK2 and GIRK3 forming GIRK2/GIRK3 heteromeric channels [180, 188]. It has also been shown that in the absence of GIRK3 expression (*Girk3*^{-/-}), functional GIRK2 homomeric channels can form (Figure 1.2). Different GIRK channel subunit compositions have been linked to differences in GIRK channel functional properties, such as sensitivity to GPCR stimulation [185, 188, 189], but whether subunit composition was relevant to drug-induced reward-related behavior required further investigation.

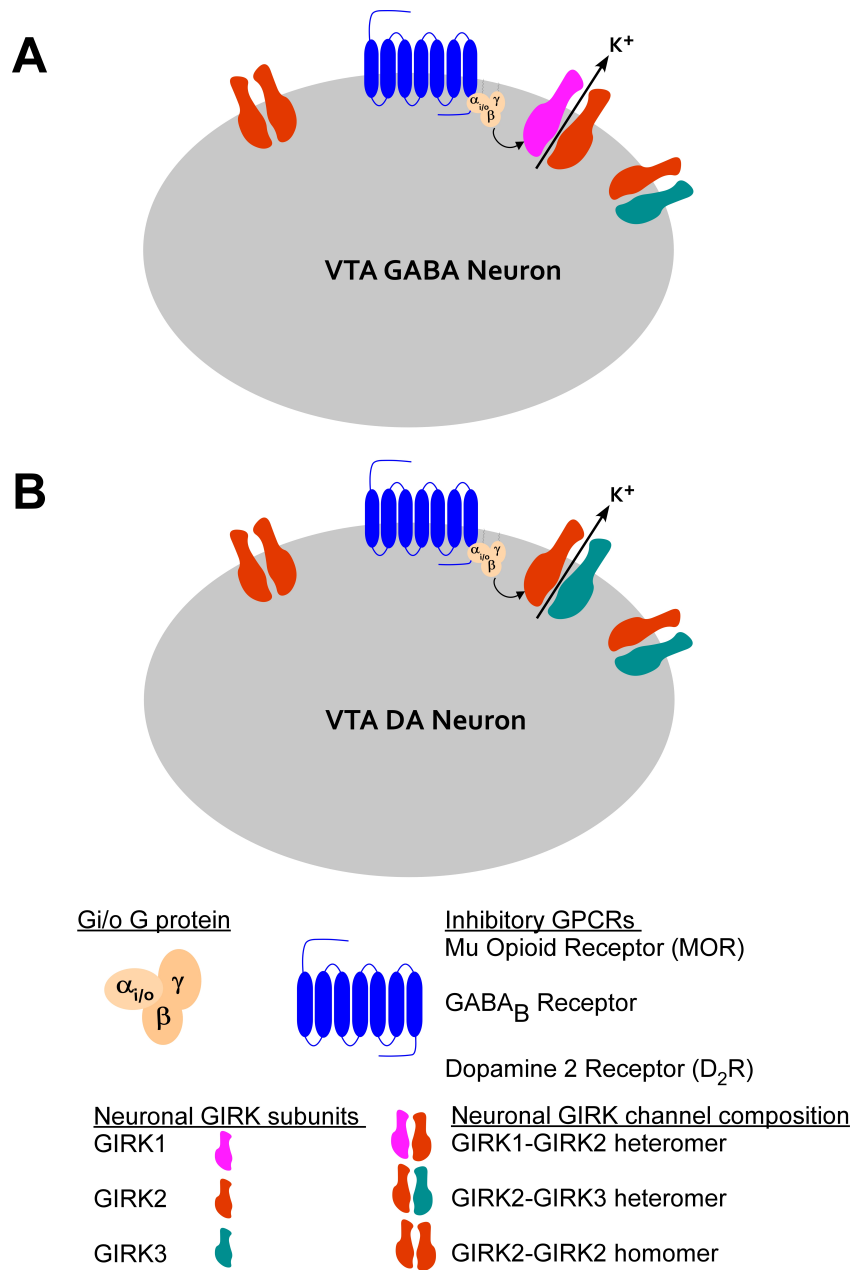


Figure 1.2. Inhibitory GPCR and GIRK channel subunit expression in the mesocorticolimbic DA system. Inhibitory GPCR-GIRK signaling plays an important role in controlling cellular excitability of VTA DA and GABA neurons. GABA_BR are found on both neuron populations (**A**, **B**). D₂Rs are expressed on VTA DA neurons (**B**), while MORs are expressed on both DA and GABA neurons (**A**, **B**) of the VTA. VTA GABA neurons (**A**) express GIRK1, GIRK2, and GIRK3 subunits, with the predominant GIRK channel likely containing GIRK1 and GIRK2 (GIRK1/GIRK2 heteromer). VTA DA neurons express a GIRK2/GIRK3 heteromeric channel (**B**). Of note, both VTA neuron populations express GIRK2, allowing for the expression of GIRK2/GIRK2 homomeric channels.

D. GIRK Channels and Addiction

GIRK channel activity is a key regulator of many central nervous system functions, such as processing of reward and pain signals, motor control, and cognition. Dysregulation of GIRK-dependent signaling is implicated in drug addiction, hyperalgesia, Parkinson's disease and epilepsy, Down Syndrome, as well as depression, anxiety, attention deficit hyperactivity disorder (ADHD), and schizophrenia [165, 186, 187, 190-194]. The relevance of GIRK-dependent signaling to drug addiction is supported by the fact that GPCR-GIRK signaling has been shown to play a role in both the cellular and behavioral effect of several drugs of abuse [195]. Different drugs of abuse work on a variety of molecular and cellular targets, yet most evoke overlapping behaviors, such as motor-stimulation, escalated drug-taking, and cue- or drug-induced relapse [74, 79, 165, 196]. Similarities in the drug-induced reward-related behaviors suggest a common neuronal/molecular pathway through which drugs of abuse work to produce reward and ultimately addiction [197, 198]. As GIRK channels regulate the cellular and reward-related behaviors associated with multiple drugs of abuse (e.g. gamma-Hydroxybutyrate (GHB), cocaine, opioids), they are one potential candidate [188, 195, 199, 200]. In this context, the exact mechanism(s) through which various drugs of abuse modulate GIRK activity and the relevance of GIRK channel subunit composition to the reward-related behavioral effect of drugs of abuse remains unclear. With further insights into the nuances of drug-induced adaptations of GPCR-GIRK signaling in the VTA, the development of new potential therapeutic targets for treating addiction may be possible.

V. DRUG-INDUCED ALTERATIONS TO INHIBITORY GPCR-GIRK SIGNALING

i. Opioids: MOR-GIRK

A. Pharmacology

Opioid receptors are part of the GPCR family and can be divided into three subtypes mu (MOR), delta (DOR), and kappa (KOR) [201]. MORs are expressed in regions of the mesocorticolimbic DA system, the dorsal horn of the spinal cord, periaqueductal gray, and intestinal tract, and can be activated by many agonists including morphine, other prescription opioid pain relievers, and endogenous opioid compounds [202]. Opioid activation of MOR and subsequent modulation of downstream targets are thought to be responsible for producing both their desired analgesic effect as well as their undesired side effects, including tolerance and addiction [203]. These reward-related effects linked to activation of MOR are thought to arise from alterations to cellular signaling within the mesocorticolimbic DA system. While the mechanisms through which opioids produce analgesia are well understood [204], further work is needed to improve our understanding of how opioid-induced changes in signaling within the mesocorticolimbic DA system contribute to opioid reward.

MORs couple to the $G\alpha_{i/o}$ class of G proteins and modulate a number of downstream effectors [205]. The activated $G\alpha_{i/o}$ inhibits adenylyl cyclase, while free $G\beta\gamma$ inhibits voltage-gated Ca^{2+} channels and activates GIRK channels [205]. MORs are located at both presynaptic terminals and somatodendritic sites of various neurons. Presynaptic MOR activation inhibits voltage gated Ca^{2+} channels, leading to reduced Ca^{2+} entry and reduced neurotransmitter release [206-208]. Somatodendritic MOR

activation is thought to activate GIRK channels, resulting in hyperpolarization of the neuron [178, 209, 210]. Currently the importance of presynaptic versus somatodendritic MOR activation to the rewarding properties of opioids is currently not well-understood.

B. Opioid Signaling in the VTA

The VTA has been established as a critical anatomic substrate of opioid reward-related behavior through studies involving intracranial lesion or pharmacology. For example, chemical lesion of the VTA-NAc connection using 6-OHDA disrupts DA signaling between the VTA and NAc to successfully block heroin self-administration and opioid-induced locomotor activity [96]. Pharmacology studies show that rodents will work to self-administer opioid agonists directly to multiple brain regions, but self-administration to the VTA can be established at lower doses than to adjacent mesocorticolimbic regions [93, 211]. In addition, opioid agonists infused into the VTA establish reward-related behaviors such as locomotor activity and conditioned place preference (CPP) [89, 212]. Thus, the VTA is critical to opioid-induced reward.

Dogma in the opioid field has maintained that opioids exert their rewarding effects through alterations in VTA neuronal activity, and specifically enhanced activity of the VTA DA neurons [213]. This idea was substantiated by the work of Johnson and North in the early 1990s. Their studies suggested that activation of MORs on VTA GABA neurons was responsible for the opioid-induced increase in extracellular DA and subsequent reward-related effects of opioids [83, 214]. More specifically, under normal conditions, the tonic release of GABA from VTA GABA neurons is able to inhibit VTA DA neurons and maintain a basal level of DA released to downstream targets, like the NAc. However, when an opioid, such as morphine, is present there are a number of

changes to VTA neuronal activity. When morphine is present, it activates MORs on VTA GABA neurons, resulting in the hyperpolarization of these neurons [214]. The MOR-induced activation of somatodendritic GIRK channels is thought to underlie this opioid-induced cellular hyperpolarization. These hyperpolarized GABA neurons then release less GABA on VTA DA neurons, resulting in the disinhibition of VTA DA neurons [215, 216]. The disinhibition of the VTA DA neurons then increases firing activity, leading to increased levels of DA release in downstream targets [217, 218]. Although these changes in signaling have been shown, there are still many unanswered questions regarding the mechanism(s) of opioid actions within the VTA, specifically the contribution of MOR-GIRK signaling in VTA GABA neurons. Furthermore, the relevance of MOR-GIRK signaling in VTA GABA neurons to opioid-induced reward-related behaviors remains poorly understood.

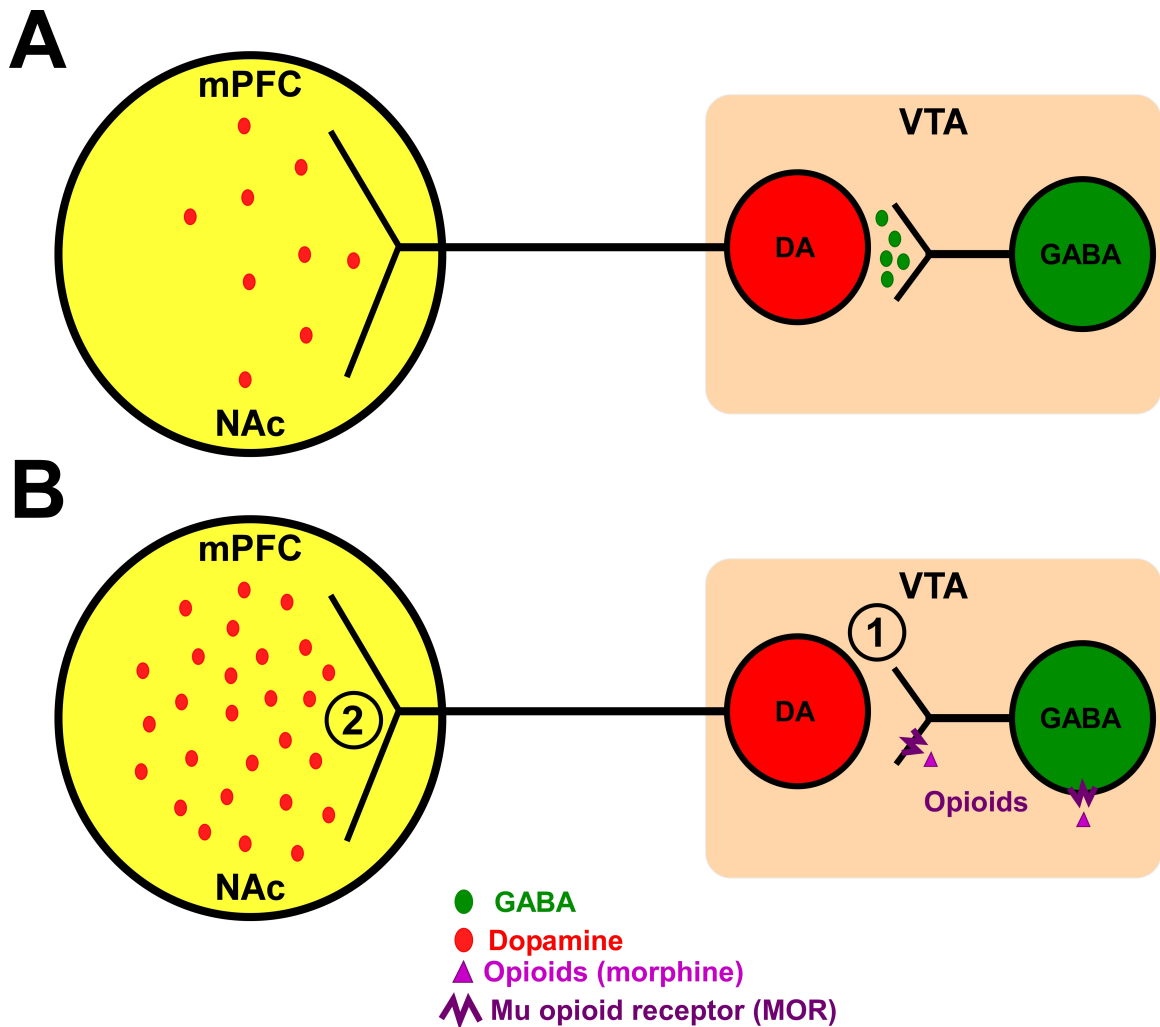


Figure 1.3. Opioid-induced disinhibition of VTA DA neurons hypothesis. The proposed model of opioid effects in the VTA suggested that under normal conditions **(A)** tonic VTA GABA neuron activity maintain a basal level of VTA DA neuron activity and DA release to downstream targets (mPFC/NAc). **(B)** When opioids are present they activate MORs on VTA GABA neurons resulting in (1) VTA GABA neuron inhibition. Reduced GABA input results in (2) disinhibition of VTA DA neurons and increased DA levels in downstream targets.

ii. Cocaine: GABA_BR-GIRK and D₂R-GIRK

A. Cocaine: Elevated Dopamine

Cocaine produces its euphoric and addictive effects mainly through its actions in the mesocorticolimbic DA system [219-221]. Following exposure, cocaine crosses the blood-brain barrier and produces a rapid increase in DA levels within the mesocorticolimbic DA system [219-221]. This increase in DA is achieved by cocaine binding to and blocking the DA reuptake transporter (DAT) found on DA neurons [222-224]. By preventing DAT to transport DA out of the synaptic cleft and back into the presynaptic DA neuron terminal, more DA is present in the synapse allowing for continued activation of DA receptors [90, 225, 226]. In addition to elevated DA levels, cocaine exposure has been shown to trigger adaptations to both excitatory and inhibitory signaling within the mesocorticolimbic DA system. Much of the recent research has focused on the acute alterations or persistent synaptic adaptations drugs of abuse produce in excitatory neurotransmission [80, 227]. Thus, there is still much to be learned about the molecular and functional mechanisms of drug-induced adaptations to inhibitory signaling, including signaling via GABA_BR and D₂R, within the mesocorticolimbic DA system and the relevance of these adaptations to the reward-related behavior effects of cocaine.

B. Inhibitory Signaling: GABA_BR

The GABA_BR is a Gα_{i/o}-coupled GPCR that plays a key role in inhibitory neurotransmission. Agonists for GABA_BR include the endogenous neurotransmitter GABA and drugs such as baclofen and GHB. GABA_BRs are expressed throughout the CNS, including the VTA and mPFC. Somatodendritic GABA_BR activation results in

cellular hyperpolarization through activation of GIRK channels throughout the CNS [178]. Expression and activity of the GABA_BR can be altered by exposure to drugs of abuse, such as psychostimulants [187, 199, 228].

a. VTA

Cocaine-induced adaptations in GABA_BR-mediated inhibitory signaling can be found in both VTA GABA and DA neurons. For example, a single injection of cocaine is sufficient to trigger the suppression of GABA_BR-GIRK signaling in VTA DA and GABA neurons [199, 229]. In VTA DA neurons, this adaptation persists up to 4-7 days following cocaine exposure and is correlated with enhanced AMPAR-mediated current [128, 199]. Accompanying this cocaine-induced reduction in inhibitory signaling is a reduction in GIRK2 expression at the plasma membrane [199]. Repeated cocaine exposure results in a similar reduction in GABA_BR-GIRK signaling in VTA DA neurons, as well as cocaine-induced behavioral sensitization [199]. This data suggests that acute and repeated cocaine exposure triggers a reduction in GABA_BR-GIRK signaling in VTA DA neurons, and that GABA_BR-GIRK signaling in VTA DA neurons plays a role in the motor-stimulatory effects of cocaine.

b. mPFC

GABA_BR-GIRK signaling in the mPFC plays a key role in modulating reward-related behavior and is reduced by repeated cocaine exposure [187]. Accompanying this cocaine-induced reduction in GABA_BR-GIRK signaling is a reduction in GABA_BR and GIRK2 expression at the plasma membrane. Infusion of the GABA_BR agonist baclofen into the mPFC prior to cocaine exposure completely blocks the ability of cocaine to increase locomotor activity and induces behavioral sensitization [230]. Interestingly,

prior experience with repeated cocaine prevents these effects of baclofen on reward-related behavior [231, 232]. Additionally, baclofen infusion into the mPFC of cocaine-treated animals enhances glutamate release to the VTA and NAc, with opposite effects observed in drug-naïve animals [231]. Importantly, such increases in mPFC output to the VTA and NAc are thought to be responsible for the effects of psychostimulants and other drugs of abuse on reward-related behaviors, including locomotor sensitization, drug-taking behavior, and cue- or drug-induced relapse [80, 114, 118]. Collectively, these findings suggest that cocaine impairs GABA_BR-dependent signaling in the VTA and mPFC, and these changes contribute to the cocaine-induced reward-related behaviors.

C. Inhibitory Signaling: D₂R

The D₂R is a G $\alpha_{i/o}$ G protein GPCR that plays a key role in inhibitory neurotransmission [150]. Agonists for D₂R include the endogenous neurotransmitter DA and drugs, such as quinpirole. D₂Rs are expressed throughout the CNS and are found on neurons within the VTA, both at presynaptic and somatodendritic locations. Somatodendritic D₂R activation results in neuronal hyperpolarization through activation of GIRK channels [233, 234]. Similar to GABA_BR, the expression and activity of the D₂R can be altered by exposure to drugs of abuse, such as psychostimulants including cocaine. DA activation of the somatodendritic D₂Rs on VTA DA neurons represents a key autoinhibitory feedback pathway that attempts to restore the activity of VTA DA neurons and extracellular DA concentration [233]. Genetic ablation or pharmacologic inhibition of this D₂R autoinhibitory feedback pathway results in altered cellular and behavioral responses to cocaine [235-237].

a. VTA

D₂Rs are located somatodendritically, on the soma and dendrite, on VTA DA neurons and are activated by cocaine-induced extracellular release of DA in the VTA [238, 239]. Cocaine produces bidirectional changes in the firing rate of VTA DA neurons based on acute or repeated cocaine exposure. These somatodendritic D₂Rs work to decrease DA neuron firing rates as an autoinhibitory pathway to counteract the increase of DA caused by acute cocaine, likely through activation of GIRK channels [150, 199, 239]. In contrast, repeated cocaine exposure causes a temporary increase in firing rate that can reset shortly (<10 days) following withdrawal [240-242]. This persistent cocaine-induced increase in mesocorticolimbic DA transmission is correlated with a reduction in D₂R autoreceptor function on VTA DA neurons. Acute pharmacologic stimulation of D₂R in the VTA also reduces D₂R autoreceptor function on VTA DA neurons [243]. In addition to these *in vitro* studies showing D₂R-dependent signaling is weakened by repeated cocaine, imaging studies have shown that human cocaine addicts have reduced D₂R expression in parts of their mesocorticolimbic DA system [244]. Collectively, these findings suggest that D₂R signaling in VTA DA neurons is vital to the molecular adaptations triggered by cocaine exposure, and that cocaine-induced changes in D₂R-dependent signaling in the VTA may contribute to cocaine-induced reward-related behaviors.

VI. Purpose of Studies

Drug addiction represents a costly financial and social burden to society. Current available treatments for substance abuse are limited, largely ineffective, and produce

many undesirable side effects. In order to identify novel therapeutic targets and improve pharmacotherapy, a better understanding of the molecular and cellular processes involved in addiction is required. While drug induced adaptations to excitatory signaling within the mesocorticolimbic DA system have been well characterized, the adaptations and importance of inhibitory signaling requires further investigation. The goal of these dissertation studies was to enhance our understanding of the role of G protein dependent inhibitory signaling throughout the mesocorticolimbic DA system, and how it mediates the cellular and behavioral effect of drugs of abuse.

In this work, I investigated the role of GIRK channels in the VTA using a number of pharmacologic and genetic tools. It is important to note, that although several compounds directly modulate the activity of GIRK channels, they lack specificity and act on several other types of potassium channels. Barium (Ba^{2+}) and tertiapin (TPN) are two widely used GIRK channel antagonists [245-247]. However, the low potency and lack of selectivity make results of studies utilizing these compounds difficult to interpret [166, 245, 248, 249]. This lack of selective pharmacology has pushed the majority of GIRK channel research to utilize genetic approaches, such as knockout mice and targeted viral manipulations [190, 209, 250]. The generation of *Girk*^{-/-} mice involved the removal of the N-terminus, membrane-spanning domains, the pore regions, and most of the C-terminus, resulting in a constitutive loss of the specific GIRK subunit expression. One limitation of constitutive ablation of a gene is that compensatory genetic adaptations can occur, such as upregulation/downregulation of other genes. These compensatory adaptations can mask phenotypes and make results difficult to interpret.

To gain a more refined understanding of the importance of GIRK-dependent signaling in drug-induced reward-related behavior, and the associated underlying cellular mechanisms, we generated a conditional knockout mouse line targeting GIRK2 (*Girk2*^{fllox/fllox}). The “floxing” of a gene allows for Cre recombinase to selectively remove the gene of interest [251]. The generation of the *Girk2*^{fllox/fllox} line gave us the ability to manipulate GIRK2-dependent signaling in specific neuron populations by crossing this line with various neuron-specific Cre-driver lines (DA neurons (DATCre) and GABA neurons (GADCre)). This novel approach used throughout my dissertation research afforded selective and conditional knockdown of GIRK-dependent signaling, in turn providing valuable insight into the relevance of GPCR-GIRK signaling to the molecular and behavioral effects of drugs of abuse. The work in Chapter 2 is focused on the cellular and behavioral effects of opioids on signaling within the VTA and the relevance of GIRK channel function to opioid-induced locomotor activity. Chapter 3 is focused on G protein-dependent inhibitory signaling in VTA DA neurons and its relevance to the reward-related behavioral effects linked to cocaine. Together the findings of these studies demonstrate that inhibitory signaling through GPCR-GIRK signaling in neurons of the VTA is critical to the molecular and behavioral effects of drugs of abuse.

Author Contributions for Chapter 2:

Kotecki L: Responsible for the majority of electrophysiological and behavioral data presented in Chapter 2. Contributions included development of study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, and critical revision.

Hearing MC: Contributed to validation of novel *Girk2*^{lox/lox} line in Figure 2.2 and provided some input into development of study conception and design.

McCall NM: Contributed some electrophysiological data characterizing DATCre:*Girk2*^{lox/lox} mice in Figure 2.7D/E.

Marron Fernandez de Velasco E and Xia Z: Validated novel *Girk2*^{lox/lox} line in Figure 2.2.

Pravetoni M: Contributed some early behavioral data of constitutive *Girk*^{-/-} response to opioid-induced locomotor activity in Figure 2.1.

Arora D: Contributed electrophysiological data in Figure 2.4B/C with early validation of GIRK subunit contribution to GABA_BR-GIRK signaling in putative VTA GABA neurons.

Victoria NC: Assisted with IHC validation for loss of GIRK2 protein levels in the midbrain of DATCre(+):*Girk2*^{lox/lox} mice in Figure 2.7A.

Slesinger PA and Munoz MB: Created and provided GIRK3 lentivirus used in Figure 2.8.

Weaver CD: Provided ML297 used in Figure 2.6.

Wickman K: Contributions included development of study conception and design, interpretation of data, drafting of manuscript, and critical revision.

CHAPTER 2

GIRK channels modulate opioid-induced motor activity in a cell type- and subunit-dependent manner

Kotecki L, Hearing MC, McCall NM, Marron Fernandez de Velasco E, Pravetoni M, Arora D, Victoria NC, Munoz MB, Xia Z, Slesinger PA, Weaver CD, Wickman K. GIRK channels modulate opioid-induced motor activity in a cell type- and subunit-dependent manner. J Neurosci. 2015; 35(18):7131-42.

Introduction

The dopamine (DA) projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is a critical anatomic substrate for drug-induced exploratory/seeking behavior (motor activation) and an associated anticipatory state comparable to ‘wanting’ [225, 252, 253]. Neuroadaptations within this pathway underlie the development and expression of behavioral sensitization and drug seeking associated with repeated drug administration [252, 254, 255]. Opioids enhance VTA DA neuron activity [216], leading to increased DA levels and neuronal activation in the NAc, which in turn augments motor activity [256-259]. The relevance of the opioid-induced increase in DA neurotransmission is underscored by the inhibitory impact of DA receptor antagonists and intra-NAc DA depletion on opioid-induced motor activity [88, 260, 261], and the lack of morphine-induced motor activation in DA-deficient mice [262].

The motor-stimulatory and associated neurochemical effects of opioids are dependent on the mu opioid receptor (MOR) [218, 263-265]. MOR activation has been proposed to inhibit GABA neurons that dampen the output of VTA DA neurons (disinhibition), leading to increased DA neurotransmission [214]. Indeed, VTA DA neurons in the rat receive opioid-sensitive input from GABA neurons in the VTA and rostromedial tegmental nucleus (RMTg) [266, 267]. Both pre- and postsynaptic mechanisms have been proposed to mediate the inhibitory impact of opioids on midbrain GABA neurotransmission [214, 266-268], though their relative significance to the cellular and behavioral effects of opioids remains unclear.

The postsynaptic inhibitory effect of opioids on GABA neurons is thought to involve activation of G protein-gated inwardly-rectifying K⁺ (GIRK/Kir3) channels [196, 214]. In

support of this contention, loss of GIRK1 or GIRK2 correlates with diminished opioid inhibition of locus coeruleus and spinal cord neurons [209, 269], and blunted intrathecal morphine and DAMGO analgesia [270, 271]. Moreover, loss of GIRK2 correlates with diminished systemic morphine-induced analgesia [191], and ablation of GIRK channels in the locus coeruleus precludes the induction of opioid dependence [186].

Here, we sought to determine whether and how GIRK channels contribute to opioid-induced motor activation. Our working hypothesis was that opioids inhibit midbrain GABA neurons in a GIRK-dependent manner, leading to disinhibition of VTA DA neurons and an associated increase in motor activity. Using novel mutant mice and pharmacological tools, however, we found that GIRK channel activation in GABA neurons plays little role in opioid-induced motor activity. Instead, our data suggest that the GIRK channel in VTA DA neurons modulates the sensitivity to the motor-stimulatory effects of opioids in mice.

Materials and Methods

Animals.

Animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Generation of constitutive *Girk*^{-/-} mice was described previously [190, 209, 250]. GAD67-eGFP mice [272] and Pitx3-eGFP mice [273] were generously provided by Drs. T. Kaneko and Meng Li, respectively. *Gad2*^{tm2(cre)Zjh}/J (GAD-Cre), B6.SJL-Slc6a3^{tm1.1c(cre)Bkmn}/J (DAT-Cre), and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Girk2^{flox/flox} mice were generated under contract by InGenious Targeting Labs (Ronkonkoma, NY).

Drugs.

DAMGO, naloxone, and baclofen were purchased from Sigma (St. Louis, MO). CGP54626 was purchased from Tocris (Bristol, UK). rTertiapin-Q (TPN) and GIRK2 antibody were purchased from Alomone Labs (Jerusalem, Israel). Morphine was purchased from Boynton Pharmacy (University of Minnesota). ML297 was synthesized in the Vanderbilt Institute of Chemical Biology Chemical Synthesis Core and Vanderbilt MLPCN Specialized Chemistry Center.

Generation of *Girk2*^{flox/flox} mice.

The mouse *Girk2* gene (also known as *Kcnj6*; Gene ID 16522) consists of at least 7 exons and spans ~250 kbp on the reverse strand of chromosome 16 (94,749,266-94,997,696). Several alternative splice variants have been identified that utilize 1 of 2 potential translation start sites [274]. Exon 4 was targeted, as it is present in all known *Girk2* splice variants, and because it contains coding sequence for key functional domains of the GIRK2 protein. A C57BL/6 BAC clone (RP23: 155F22) containing *Girk2* exon 4 and surrounding sequence was identified and used to generate the targeting construct. The targeting construct was validated by sequencing, linearized, and electroporated into iTL BA1 (C57BL/6 x 129/SvEv) hybrid embryonic stem cells. Neomycin-resistant clones (400) were screened by PCR, which identified 5 candidate clones that were amplified, re-analyzed by PCR, and evaluated by Southern blotting for proper integration of the targeting construct (including both 5' and 3' homology arms); 3 of these 5 clones harbored a properly-targeted *Girk2* allele. Two ES clones (#152 and

#364) were then micro-injected into C57BL/6 blastocysts, resulting in 7 chimeras exhibiting a high percentage of *agouti* coat color. These mice were mated to C57BL/6N flippase (Flp) deleter mice (C57BL/6-Tg(ACTFLPe)) to remove the NEO cassette. Two F1 offspring (deriving from ES clone #364) were identified that lacked the NEO cassette, but did contain intact 5' and 3' homology arms and *loxP* sites (as assessed by PCR and DNA sequencing). These mice, which were heterozygous for the conditional mutant allele (*Girk2*^{fl^{ox}/wt}), were then backcrossed with C57BL/6J mice to amplify the colony. Resulting *Girk2*^{fl^{ox}/wt} offspring were then inbred to produce mice homozygous for the *Girk2*^{fl^{ox}} allele (*Girk2*^{fl^{ox}/fl^{ox}} mice).

Quantitative RT-PCR.

Punches (2-mm diameter, 2-mm thick) from the cortex and hippocampus, and half of the cerebellum, were taken from wild-type and *Girk2*^{fl^{ox}/fl^{ox}} siblings (7-10 wk). Punches were frozen on crushed dry ice and stored at -80°C. Total RNA of cortex and hippocampus was isolated using the RNeasy Plus MicroTM Kit (Qiagen; Valencia, CA). Total RNA from the cerebellum was isolated using TRIzol (InvitrogenTM, Life Technologies; Grand Island, NY) according to manufacturer's recommendations, and treated with recombinant DNase I (Roche; Indianapolis, IN). Reverse transcription was performed using an iScriptTM cDNA synthesis kit (Bio-Rad; Hercules, CA). Quantitative PCR was performed on a LightCycler® 480 II system in a final volume of 20 µL with a LightCycler® 480 SYBR Green I Master kit (Roche). After pre-incubation at 95°C for 5 min, amplification consisted of 45 cycles of denaturation for 10 s at 95°C, followed by annealing for 30 s at 60°C and extension for 10 s at 72°C. qRT-PCR oligonucleotide sequences for GIRK2 are as follows: GIRK2(forward) – 5'-

CGTGGAGTGAATTATTGAATCT-3' and GIRK(reverse) - 5'-
GTCATTTCTTCTTTGTGCTTTT-3'. GAPDH mRNA levels were used for
normalization purposes using GAPDH QuantiTect oligonucleotides (Qiagen). Samples
were analyzed using the $2^{\Delta\Delta Ct}$ method [275].

Quantitative immunoblotting.

Punches (2-mm diameter, 2-mm thick) of defined brain regions were taken from
wild-type and *Girk2^{lox/lox}* siblings (7-10 wk). Samples were sonicated in 1% SDS lysis
buffer containing a Halt phosphatase and protease inhibitor cocktail (Thermo Fisher
Scientific Inc.; Waltham, MA), heated at 85°C for 10 min, and centrifuged at 4°C for 20
min at 16,000xg. Protein samples (40 µg) were then separated by SDS-PAGE and
transferred to nitrocellulose membranes. Membranes were blocked in 5% milk/PBS,
incubated overnight at 4°C with antibodies against GIRK2 (1:200; Alomone Labs) or β-
actin (1:10,000; Abcam; Cambridge, MA), and diluted in 5% milk/PBS/0.1% Tween-20.
Membranes were washed with PBS/0.1% Tween-20 and incubated with donkey anti-
rabbit (926-68072; 1:5000; LI-COR Biosciences; Lincoln, NE) or donkey anti-mouse
(926-32212; 1:1000-5000; LI-COR) secondary antibodies. Blots were developed using
the Odyssey infrared imaging system (LI-COR), and the integrated density of each band
was measured using ImageJ software (NIH; Bethesda, MD).

Immunohistochemistry.

Mice (7-10 wk) were given a euthanizing dose of ketamine/xylazine (250 and 25
mg/kg i.p., respectively) and perfused transcardially with Ca²⁺-free Tyrode's solution
followed by 4% paraformaldehyde containing 14% picric acid. Brains were post-fixed
overnight in Sorenson's Buffer containing 10% sucrose at 4°C. Tissue was sectioned

coronally at 12 μm by cryostat and mounted to slides. Sections containing the VTA were washed in TBS for 10 min, and then placed in boiling citric acid (10 mM; 30 min). Slides were then washed in TBS (3 x 5 min), and incubated for 1 h in TBS blocking solution containing casein, Tween-20, and Triton X-100 (all at 0.2%). Sections were exposed to rabbit anti-GIRK2 antibody (1:1000; Alomone labs) diluted in TBS solution containing casein and Tween-20 (both 0.2%) overnight, at room temperature in a humidity chamber. The following day, sections were washed in TBS (3 x 20 min) and then incubated for 2 h in donkey anti-rabbit IgG Cy3 secondary antibody (1:500; Jackson Immunoresearch Laboratories, Inc.; West Grove, PA) diluted in TBS containing casein and Tween-20 (both at 0.2%). Sections were washed in TBS (3 x 20 min), dehydrated through a series of graded alcohols, cleared in xylene, and coverslipped with DPX (Thermo Fisher Scientific Inc.). Fluorescence was visualized with an IX81ZDC2 Olympus microscope with disk-scanning unit and imaged with MetaMorph Advanced software. Digital images were colorized with ImageJ 64 software and prepared for micrographic presentation in Adobe Illustrator and Photoshop 6 (Adobe Systems, Inc.); no adjustments were made to saturation, brightness or contrast.

Slice electrophysiology.

Coronal and horizontal slices from wild-type and mutant mice (4-10 wk) were prepared as described [199]. VTA neurons found in the lateral aspect of the VTA, near the medial terminal nucleus of the accessory optic tract, were targeted for analysis. Upon achieving whole-cell access, the current response to a 1-s voltage ramp (-60 to -120 mV) was used to measure I_h presence and amplitude and spontaneous activity ($I=0$) was assessed. For rheobase measurements, cells were held in current-clamp at 0 pA to obtain

resting membrane potential and then progressively-increasing current pulses (1-s duration) were applied until the cell fired an action potential. Changes in somatodendritic holding current evoked by baclofen, DAMGO, or ML297 were measured at a holding potential of -60 mV, as described [199].

Some studies involved targeted evaluation of VTA DA and GABA neurons, as defined by eGFP expression in VTA neurons from Pitx3-eGFP and GAD67-eGFP mice, respectively [272, 273]. For other studies, we relied on morphological and functional criteria to identify “putative” VTA GABA and DA neurons. Specifically, small (apparent capacitance <40 pF) VTA neurons exhibiting little or no I_h current (<35 pA) were identified as putative GABA neurons, as these properties were typical of eGFP-positive VTA neurons from GAD67-eGFP mice [180]. Larger (apparent capacitance >50 pF) VTA neurons exhibiting substantial I_h current (>100 pA) were identified as putative DA neurons, as these properties correlated well with tyrosine hydroxylase expression [188, 199], and because this was a typical profile for eGFP-positive VTA neurons in slices from Pitx3-eGFP mice [180].

Morphine-induced motor activity.

Systemic morphine-induced locomotor activity assessments were performed as described [276]. Subjects (6-12 wk) were handled and acclimated to the testing room for 1 h prior, followed by habituation to activity chambers and systemic injections (saline, i.p.), on each of the 3 days preceding testing. Total distance traveled during the 60-min period following saline injection on the last acclimation day was defined as the response to 0 mg/kg morphine. Subjects were then given an ascending series of systemic morphine doses (3, 10, 30 mg/kg i.p.), with doses separated by 3-d intervals.

Intracranial manipulations.

Mice (6-8 wk) were anesthetized with ketamine and xylazine (100 and 10 mg/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA). For intracranial pharmacology studies, bilateral stainless steel guide cannulae (Plastics One Inc.; Roanoke, VA) targeting the VTA (-3.08 mm AP, \pm 0.5 mm ML, -3.5 mm DV) or RMTg (-3.90 mm AP, \pm 0.5 mm ML, -3.4 mm DV) were implanted 1 mm above the infusion target. Cannulae were held in place by stainless steel mounting screws (0-80 x 3/32; Plastics One) and dental acrylate (Henry Schein; Melville, NY), with stylets inserted following surgery to prevent blockage. Mice were allowed to recover for 7-9 d prior to behavioral testing.

On day 1 of testing, mice were gently restrained (mimicking the injection procedure) and placed in the open-field chamber. On day 2, mice were restrained, and then dummy cannulae were removed and replaced with injection cannulae attached to the delivery system. On day 3, mice were given infusions (0.5 μ L/side/min) of vehicle (saline or saline/DMSO) or drug (DAMGO or ML297) using a KSD230 multi-channel syringe pump controller (KD Scientific Inc.; Holliston, MA). Injection cannulae consisted of 20-mm 33-GA stainless steel hypodermic tubes inserted into a 9-mm 26-GA segment in order to protrude 1 mm beyond the tip of the 10-mm guide cannula. Drug was delivered via catheter lines connected to a 10 μ L Hamilton syringe. After infusion, injection cannulae were held in place for an additional 1-2 min to ensure proper drug diffusion. Thereafter, subjects were placed in the motor activity monitoring units for 60 min. Guide cannulae placement was assessed by *post-hoc* histology.

For the viral GIRK3 reconstitution study, cDNAs for yellow fluorescent protein (YFP) fused to mouse GIRK3, or green fluorescent protein (GFP) alone as a control, were sub-cloned into a CaMKII-pBOB 3rd-generation self-inactivating lentiviral vector (kindly provided by Dr. Inder Verma). Lentivirus was produced by the Gene Transfer, Targeting, and Therapeutics Core at The Salk Institute for Biological Studies. Viral titer was $\sim 8 \times 10^9$ TU/mL as determined by real-time PCR of infected 293T cells. GIRK3-YFP or control lentivirus were injected into the VTA (-3.08 mm AP, ± 0.5 mm ML, -4.4 mm DV) of male mice (6-7 wk) using a 5 μ L Hamilton syringe. Following infusion (0.5 μ L/site; 0.5 μ L/min), the syringe was left in place for 10 min to reduce backflow. Systemic morphine-induced motor activity was evaluated 21 d after viral infusion, and only data from mice with confirmed viral expression in the VTA were analyzed.

Statistical analysis.

Data are presented throughout as the mean \pm SEM. Statistical analyses were performed using Prism 6 (GraphPad Software, Inc.; La Jolla, CA) and SigmaPlot 11.0 (Systat Software, Inc.; San Jose, CA). Unless specifically noted, male and female mice were used for all studies. No sex differences were observed for any measured parameter, and thus, data from male and female mice were pooled to increase statistical power. Electrophysiological data and motor activity data were analyzed with a Student's *t* test, one-way or two-way ANOVA, or two-way repeated measures ANOVA followed by individual comparisons using the Bonferroni post-test method. For all statistical comparisons, differences were considered significant if $P < 0.05$.

Results

Morphine-induced motor activity in $Girk^{-/-}$ mice

We began by evaluating the impact of constitutive *Girk* subunit ablation on systemic morphine-induced motor activity. Since ablation of either *Girk1* or *Girk2* correlated with diminished opioid analgesia [191, 270, 271], and since *Girk2* ablation correlated with decreased opioid-induced hypothermia [277], we predicted that mice lacking GIRK1 and/or GIRK2 would exhibit diminished systemic morphine-induced motor activity. Surprisingly, however, the motor-stimulatory effect of systemic morphine was enhanced in *Girk2^{-/-}* mice at the higher doses tested (10 and 30 mg/kg; **Fig. 2.1**). *Girk1^{-/-}* mice also showed increased morphine-induced activity, but only at the highest morphine dose. Thus, GIRK channels modulate systemic morphine-induced motor activity, but in a manner inconsistent with their predicted role as proximal mediators of the underlying opioid-induced signaling.

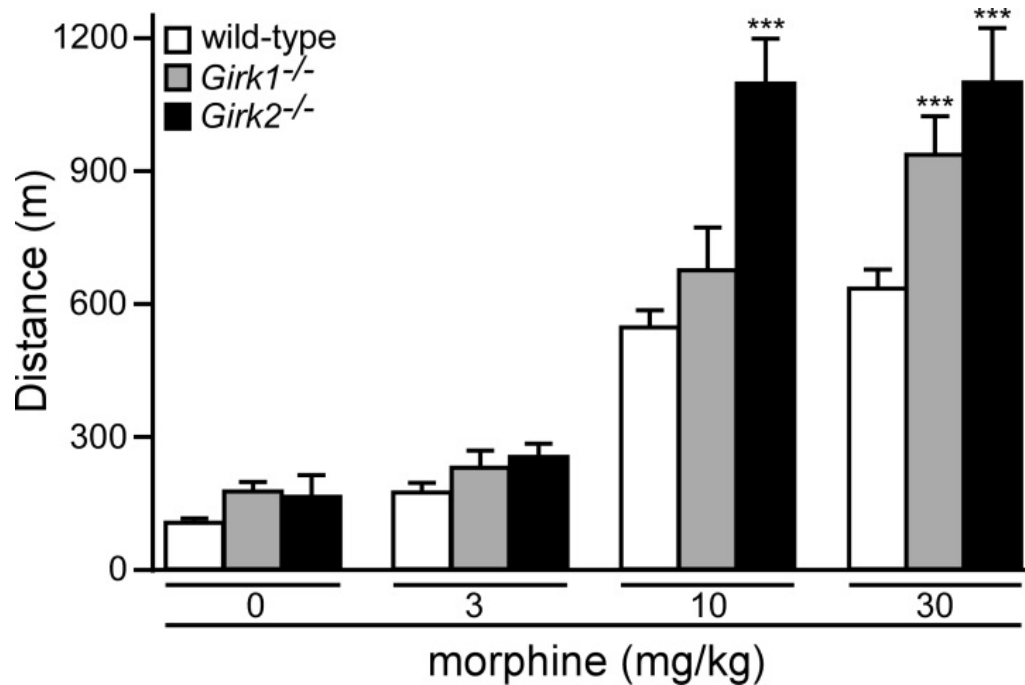


Figure 2.1. Constitutive *Girk* subunit ablation and morphine-induced motor activity. Total distance traveled by adult male wild-type (white; n=26/dose), *Girk1*^{-/-} (gray; n=18/dose), and *Girk2*^{-/-} (black, n=7/dose) mice during a 60-min test following systemic administration of saline (0) or morphine (3, 10, 30 mg/kg i.p.). Main effects of genotype ($F_{(2,48)}=12.8$, $P < 0.0001$) and dose ($F_{(3,144)}=133.6$, $P < 0.0001$) were observed, as well as a significant interaction between genotype and dose ($F_{(6,144)}=5.4$, $P < 0.0001$). Symbol: *** $P < 0.001$ vs. wild-type (within dose).

Morphine-induced motor activity in mice lacking GIRK channels in GABA neurons

GIRK channel activation in GABA neurons has been implicated in the opioid-induced disinhibition of VTA DA neurons and associated behavior [196, 214]. Global and constitutive GIRK subunit ablation, however, may impact excitability in multiple cell types that exert opposing influence on morphine-induced motor activity [228]. To evaluate the impact of selective ablation of GIRK channels in GABA neurons on morphine-induced motor activity, we generated conditional *Girk2*^{-/-} (*Girk2*^{fllox/fllox}) mice (**Fig. 2.2A-C**). After determining that *Girk2*^{fllox/fllox} mice exhibited normal levels of GIRK2 mRNA and protein in multiple brain regions (**Fig. 2.2D,E**), we crossed *Girk2*^{fllox/fllox} mice with *Gad2*^{tm2(cre)Zjh/J} (GAD-Cre) mice, which express Cre recombinase under the control of the *Gad2* promoter. Cre recombinase-driven reporter expression in GAD-Cre mice is observed throughout the brain, but is restricted to GABA neurons [278].

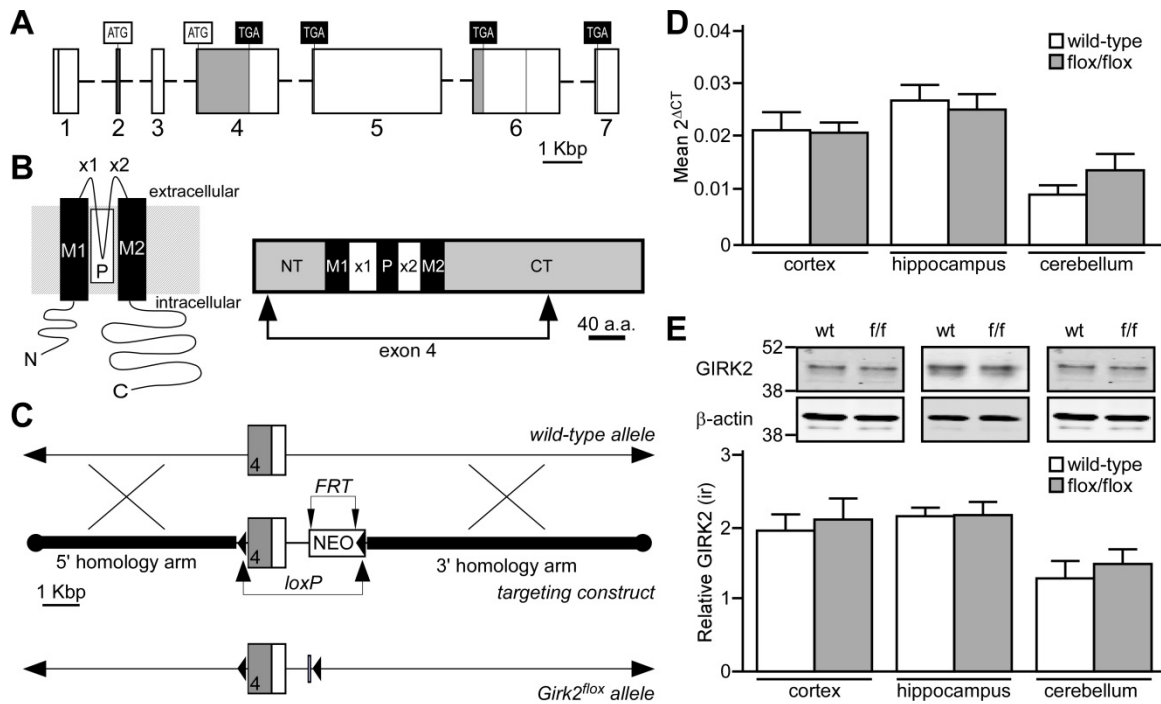


Figure 2.2. Generation of *Girk2*^{flox/flox} mice. (A) Depiction of the mouse *Girk2/Kcnj6* gene, highlighting the 7 known exons (rectangles with corresponding numbers below) [274]. While several alternative splice variants have been identified, utilizing two distinct translation initiation codons (ATG) and four distinct translation stop codons (TGA), all known variants contain exon 4. Shaded regions of exons denote protein-coding sequence. Some exons (1, 4, and 6) contain internal splice acceptor sites, denoted by vertical lines. (B) Schematic depictions of a GIRK2 subunit. The image on the left shows the membrane topology of a GIRK2 subunit. Each subunit contains intracellular N- and C-terminal domains, 2 membrane spanning domains (M1 and M2), 2 extracellular loops (x1 and x2), and a pore domain (P). The schematic on the right is a linear depiction of the GIRK2a subunit, a prominent isoform in the brain [274]. The region encoded by exon 4, which includes most of the N-terminus/NT, both membrane-spanning domains and extracellular loops, the pore domain, and much of the C-terminus/CT, is denoted by arrows. (C) The *Girk2* targeting strategy included engineering a *loxP* site just upstream (214 bp) of exon 4 and incorporating a neomycin (NEO) resistance cassette containing flippase (Flp) recognition target (*FRT*) sites and a second *loxP* site; this cassette was positioned 795 bp downstream from exon 4. In total, the target region spanned 1.93 Kbp, and was flanked by a 5' homology arm (extending ~5.4 Kbp upstream from exon 4), and a 3' homology arm (a ~7 Kbp fragment found immediately downstream from the target

region). The NEO cassette was removed crossing selected chimeras with C57BL/6N Flp deleter mice, yielding the *Girk2^{lox}* allele. **(D)** Tissue from the cortex (n=9-10/genotype; $t_{(17)}=0.09$, $P=0.9$), hippocampus (n=6-7/genotype; $t_{11}=0.4$, $P=0.7$), and cerebellum (n=7-8/genotype; $t_{(13)}=1.3$, $P=0.2$) of wild-type and *Girk2^{lox/lox}* (f/f) mice were evaluated for GIRK2 mRNA levels using quantitative RT-PCR. **(E)** GIRK2 protein immunoreactivity (ir) relative to β -actin in samples from the cortex (n=6-7/genotype; $t_{(11)}=0.4$, $P=0.7$), hippocampus (n=5-7/genotype; $t_{(10)}=0.05$, $P=1.0$), and cerebellum (n=4-7/genotype; $t_{(9)}=0.6$, $P=0.6$) of wild-type and *Girk2^{lox/lox}* (f/f) mice were assessed using quantitative immunoblotting. The level of GIRK2, which is seen as a doublet (top panel), was compared to the level of β -actin control (bottom panel) in each sample. Gel images of GIRK2 and β -actin were cropped and aligned with a molecular weight scale showing the markers for 52 and 38 kDa.

To assess the functional impact and cell-type specificity of GIRK channel ablation in GAD-Cre:*Girk2*^{lox/lox} mice, we measured somatodendritic currents evoked by the GABA_B receptor agonist baclofen in putative GABA and DA neurons of the VTA. Previous work has shown that baclofen-induced currents in these two neuron populations are mediated in large part by GIRK2-containing channels [180, 188]. As predicted, baclofen-induced currents in putative VTA DA neurons were comparable in slices from GAD-Cre(+):*Girk2*^{lox/lox} and GAD-Cre(-):*Girk2*^{lox/lox} mice (**Fig. 2.3A,B**). In contrast, currents in putative VTA GABA neurons were significantly smaller in slices from GAD-Cre(+):*Girk2*^{lox/lox} mice as compared to GAD-Cre(-):*Girk2*^{lox/lox} controls (**Fig. 2.3C,D**). Despite the loss of GIRK channels in putative GABA neurons in GAD-Cre(+):*Girk2*^{lox/lox} mice, no difference in systemic morphine-induced motor activity was observed between these mice and GAD-Cre(-):*Girk2*^{lox/lox} controls (**Fig. 2.3E**).

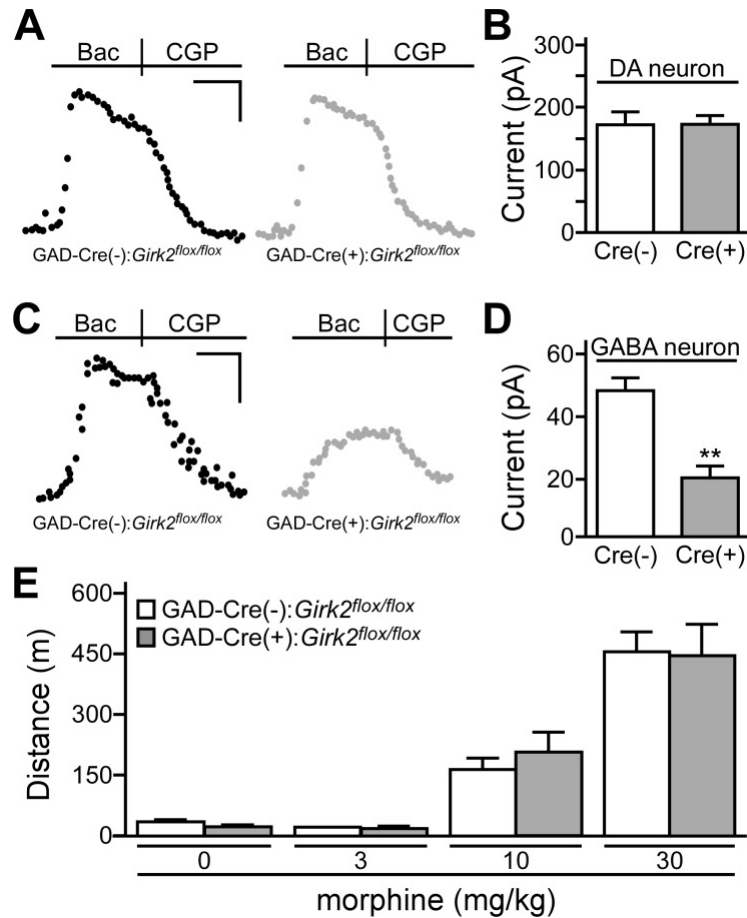


Figure 2.3. GABA neuron-specific ablation of GIRK channels and morphine-induced motor activity. (A) Representative traces of currents evoked by baclofen (Bac, 200 μ M) in putative VTA DA neurons from GAD-Cre(-):*Girk2*^{lox/lox} (black) and GAD-Cre(+):*Girk2*^{lox/lox} (gray) mice. Currents were reversed by the GABA_B receptor antagonist CGP54626 (CGP, 2 μ M). Scale bars: 60 pA/200 s. (B) Summary plot of baclofen-induced currents in putative VTA DA neurons from GAD-Cre(-):*Girk2*^{lox/lox} (white; n=7) and GAD-Cre(+):*Girk2*^{lox/lox} (gray; n=8) mice. No genotype-dependent difference was observed ($t_{(13)}=0.09$, $P=0.9$). (C) Representative traces of currents evoked by baclofen (Bac, 200 μ M) in putative VTA GABA neurons from GAD-Cre(-):*Girk2*^{lox/lox} (left, black) and GAD-Cre(+):*Girk2*^{lox/lox} (right, gray) mice. Scale bars: 20 pA/200 s. (D) Summary plot of baclofen-induced currents in putative VTA GABA neurons from GAD-Cre(-):*Girk2*^{lox/lox} (white; n=6) and GAD-Cre(+):*Girk2*^{lox/lox} (gray; n=5) mice. A significant genotype-dependent difference was observed ($t_{(9)}=4.1$, $P<0.01$). (E) Total distance traveled (in m) by GAD-Cre(-):*Girk2*^{lox/lox} (white; n=14) and GAD-Cre(+):*Girk2*^{lox/lox} (gray; n=7) mice during a 60-min period following systemic (i.p.) administration of saline (0) or morphine (3, 10, 30 mg/kg). A main effect of dose ($F_{(3,57)}=76.6$, $P<0.0001$) was observed, but there was no main effect of genotype ($F_{(1,19)}=0.02$, $P=0.9$), nor was there an interaction between genotype and dose ($F_{(3,57)}=0.3$, $P=0.8$).

Impact of GIRK channel manipulations on intracranial DAMGO-induced activity

VTA DA neurons express a unique GIRK channel subtype (GIRK2/GIRK3 heteromer), while single-cell RT-PCR analysis suggests that VTA GABA neurons may express GIRK1, GIRK2, and GIRK3 (**Fig. 2.4A**) [180, 188]. To test this proposal directly, we characterized GIRK channel activity in VTA GABA neurons. In slices from wild-type mice, baclofen (200 μ M) reliably evoked an outward (inhibitory) current in putative VTA GABA neurons (**Fig. 2.4B**). Bath application of the non-selective K⁺ channel blocker Ba²⁺ (0.3 mM) or the GIRK channel blocker rTertiapin-Q (TPN, 200 nM) blunted baclofen-induced currents (50-60%) in these neurons (**Fig. 2.4C**). Baclofen-induced currents in putative VTA GABA neurons from *Girk1*^{-/-} or *Girk2*^{-/-} mice (but not *Girk3*^{-/-} mice) were attenuated relative to controls, and were comparable in magnitude to currents measured in wild-type neurons in the presence of GIRK channel blockers (**Fig. 2.4C**).

To test whether GIRK1-containing channels mediate the MOR-dependent inhibition of VTA GABA neurons, we measured somatodendritic currents evoked by the MOR agonist DAMGO. Outward currents evoked by DAMGO (3 μ M) and reversed by naloxone (10 μ M) were observed in only a subset (4/15) of VTA GABA neurons (**Fig. 2.4D,E**). None of the putative VTA GABA neurons from *Girk1*^{-/-} mice (0/13) displayed an evident DAMGO-induced current (**Fig. 2.4E**), supporting the contention that GIRK1-containing GIRK channels mediate the direct inhibitory effect of MOR agonists on a subset of VTA GABA neurons.

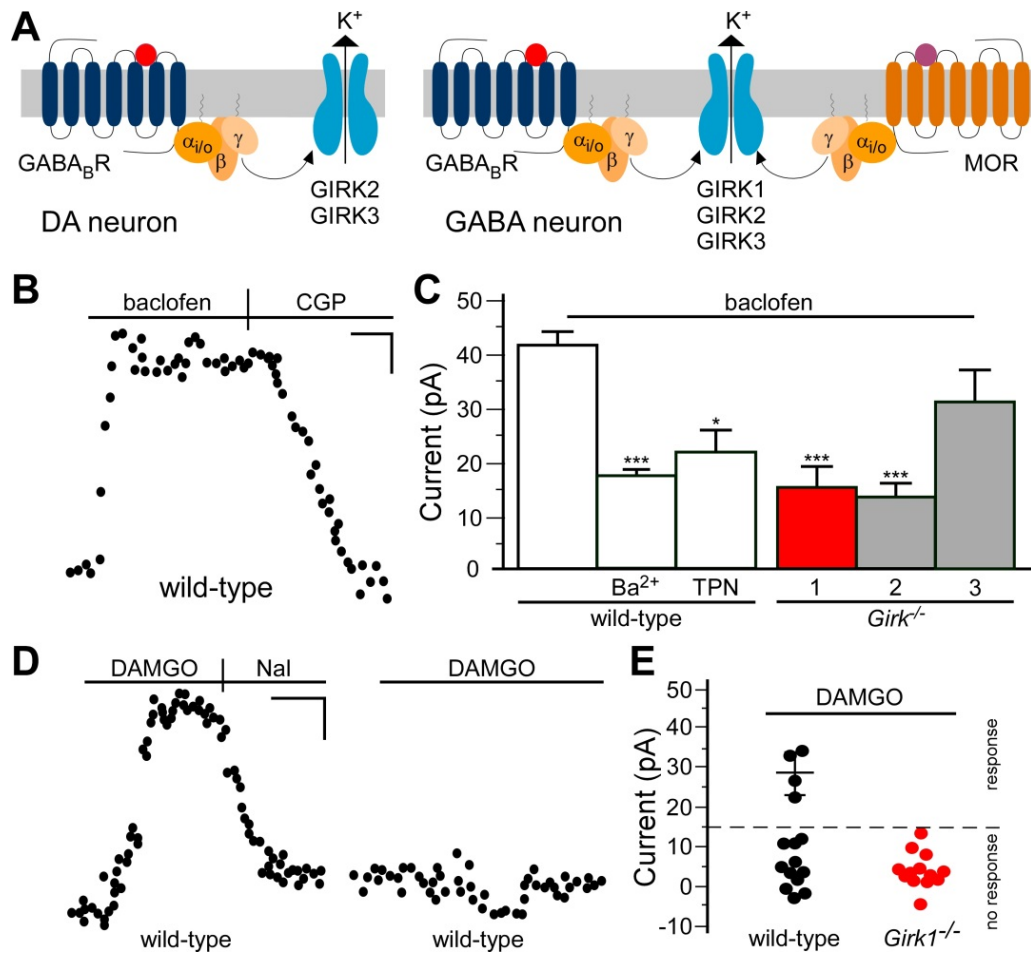


Figure 2.4. Characterization of the GIRK channel in VTA GABA neurons. (A) Proposed GIRK subunit and receptor expression patterns in VTA DA and GABA neurons. (B) Typical current induced by baclofen (200 μ M) and reversed by CGP54626 (CGP, 2 μ M) in a putative VTA GABA neuron from a wild-type mouse. Scale bars: 5 pA/120 s. (C) The impact of pharmacologic and genetic ablation of GIRK channels on baclofen-induced currents in putative VTA GABA neurons ($F_{(5,58)}=12.5$; $P<0.0001$). Currents are plotted for putative VTA GABA neurons from wild-type mice in the absence (control; $n=21$) or presence of external Ba^{2+} (0.3 mM; $n=8$) or rTertiapin-Q (TPN, 200 nM; $n=4$), and in neurons from *Girk1*^{-/-} (red; $n=9$), *Girk2*^{-/-} (gray; $n=14$), and *Girk3*^{-/-} mice (gray; $n=8$). Symbols: **** $P<0.05$ and 0.001, respectively, vs. control. (D) Responses of two VTA GABA neurons (eGFP-positive VTA neurons in slices from GAD67-eGFP mice) to a saturating concentration of DAMGO (3 μ M). The response observed in the neuron shown on the left was reversed by naloxone (Nal, 10 μ M). Scale bars: 5 pA/120 s. (E) Summary of DAMGO-induced currents in putative VTA GABA neurons from wild-type (4/15 responders; 28.6 ± 2.7 pA) and *Girk1*^{-/-} (0/13 responders) mice. Approximately one-third (4/15) of putative VTA GABA neurons evaluated exhibited a response to DAMGO (defined as a naloxone (Nal)-reversible current of >15 pA); none of the neurons in slices from *Girk1*^{-/-} mice exhibited a response to DAMGO.

Since *Girk1* ablation should alter GIRK channel activity in VTA GABA but not VTA DA neurons, we next compared the motor activity evoked by infusion of DAMGO into the VTA of wild-type and *Girk1*^{-/-} mice. Intra-VTA DAMGO increased motor activity in wild-type mice, with the maximal response observed at 1 nmol (**Fig. 2.5A,B**). The motor-stimulatory effect of intra-VTA DAMGO was completely preserved in *Girk1*^{-/-} mice. Administration of DAMGO to the adjacent RMTg also evoked increased motor activity in wild-type mice, in a dose-dependent manner, with peak response observed at the 0.1 nmol dose (**Fig. 2.5C,D**). Intra-RMTg opioid-induced motor activity was not reduced in *Girk1*^{-/-} mice. Rather, elevated motor activity was seen in *Girk1*^{-/-} mice at the two highest doses tested. These findings argue that GIRK channel activation in midbrain GABA neurons is not required for opioid-induced motor activity.

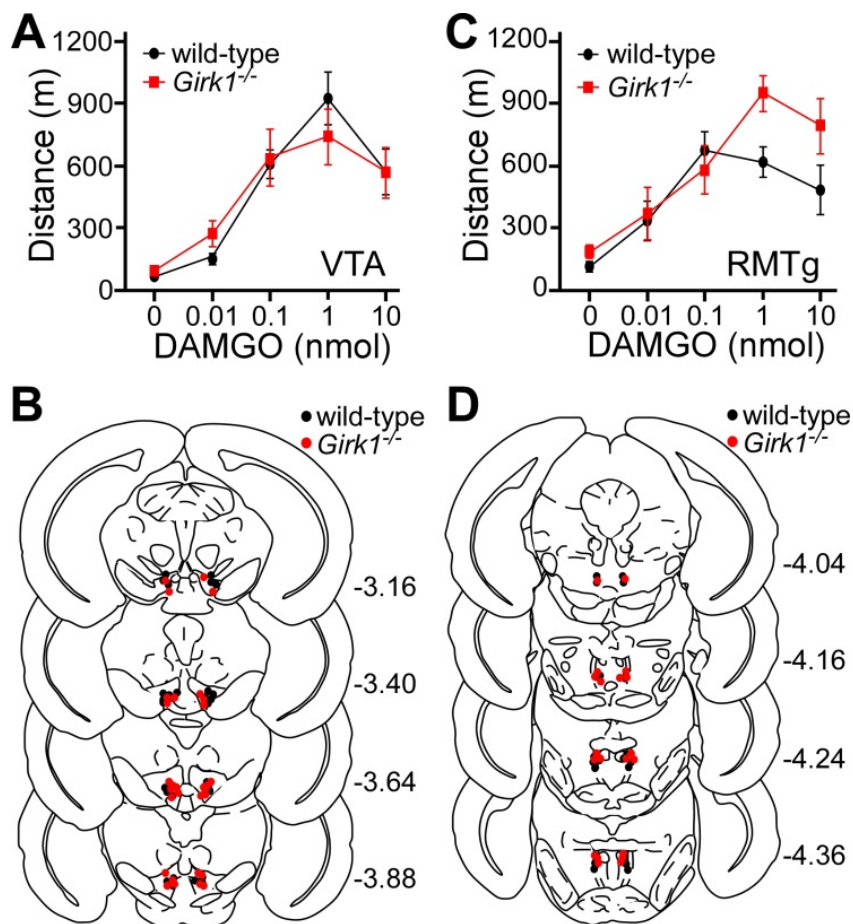


Figure 2.5. Intracranial DAMGO-induced motor activity. (A) Open-field motor activity of adult male wild-type (black; n=15) and congenic *Girk1*^{-/-} (red; n=15) mice during a 60-min period following bilateral intra-VTA infusion of 0 (saline; n=15/genotype), 0.01 (n=5-6/genotype), 0.1 (6-7/genotype), 1 (5-8/genotype), and 10 (5-8/genotype) nmol DAMGO. A main effect of DAMGO dose was observed ($F_{(4,70)}=36.2$, $P<0.001$), but there was no effect of genotype ($F_{(1,70)}=0.005$, $P=1.0$) or genotype/concentration interaction ($F_{(4,70)}=0.8$, $P=0.5$). (B) Schematic of coronal sections (from bregma) depicting cannulae placements in mice used for intra-VTA experiments. (C) Open-field motor activity of male wild-type (black; n=11) and congenic *Girk1*^{-/-} (red; n=10) mice during a 60-min test following bilateral intra-RMTg infusion of 0 (saline; n=10-11/genotype), 0.01 (n=5-7/genotype), 0.1 (5-7/genotype), 1 (6-10/genotype), and 10 (6-10/genotype) nmol DAMGO. A main effect of DAMGO dose ($F_{(4,67)}=18.2$, $P<0.001$) and genotype ($F_{(1,67)}=5.2$, $P<0.05$) was observed, but there was no genotype/concentration interaction ($F_{(4,67)}=2.0$, $P=0.1$). (D) Schematic of cannulae placements in mice evaluated in the intra-RMTg DAMGO study.

To test whether GIRK channel activation in GABA neurons is sufficient to enhance motor activity, we employed a novel direct-acting agonist (ML297) of GIRK1-containing GIRK channels [279]. In VTA slices, ML297 induced an outward current in putative GABA neurons from wild-type mice, but not in putative GABA neurons from *Girk1*^{-/-} mice (**Fig. 2.6A,B**). ML297 also evoked no response in VTA DA neurons (**Fig. 2.6A**), suggesting that intracranial infusion of ML297 should selectively inhibit neurons expressing GIRK1-containing channels (*e.g.*, VTA GABA neurons) without affecting neurons expressing GIRK1-lacking channels (*e.g.*, VTA DA neurons). When administered directly to the VTA or RMTg of wild-type mice, ML297 failed to increase motor activity (**Fig. 2.6C-E**). Thus, direct activation of GIRK1-containing channels in the VTA and RMTg is insufficient to trigger motor activation.

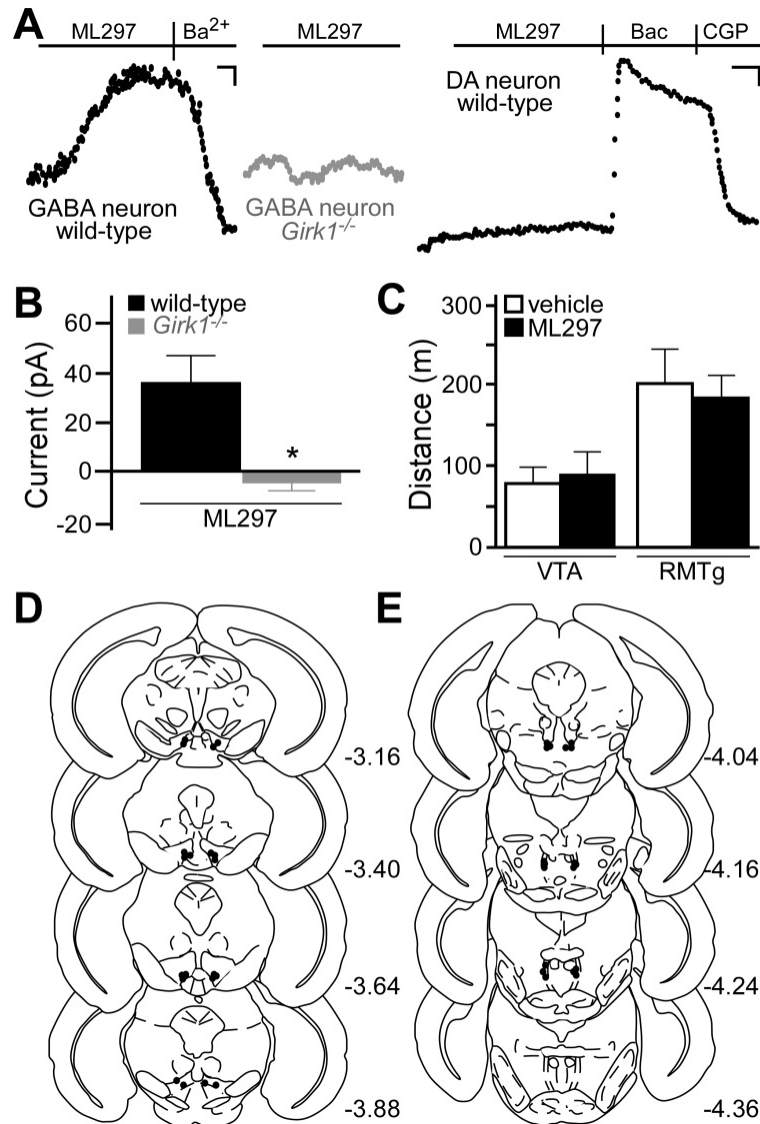


Figure 2.6. Direct activation of GIRK1-containing GIRK channels and motor activity. (A) ML297 (10 μ M) evoked a Ba²⁺-sensitive (0.3 mM) outward current in putative VTA GABA neurons from wild-type (left, black) but not *Girk1*^{-/-} (middle, gray) mice; scale bars, 15 pA/180 s. ML297 did not evoke an outward current in VTA DA neurons (right, black; n=6), as shown in this recording from an eGFP-positive neuron from a Pitx3-eGFP mouse; scale bars, 30 pA/200 s. (B) Summary of ML297-induced currents in putative VTA GABA neurons from wild-type (n=6) and *Girk1*^{-/-} (n=5) mice ($t_{(9)}=2.9$, $P<0.05$). (C) Open-field motor activity of male wild-type mice during a 60-min test following bilateral infusion of vehicle (0.1% DMSO, white) or ML297 (10 nmol, black) into the VTA (n=10/group; $t_{(18)}=0.4$, $P=0.7$) or RMTg (n=9/group; $t_{(16)}=0.3$, $P=0.7$). (D,E) Schematic of coronal sections (from bregma) depicting cannulae placements in mice used for the intra-VTA and intra-RMTg ML297 studies.

Morphine-induced activity in mice lacking GIRK channels in DA neurons

Collectively, the data presented above argue that GIRK channel activation in GABA neurons is not required for opioid-induced motor activation. To test whether loss of GIRK channels in DA neurons modulates opioid-induced motor activity, *Girk2*^{lox/lox} mice were crossed with B6.SJL-Slc6a3^{tm1.1c(cre)Bkmn}/J (DAT-Cre) mice, which express Cre recombinase under the control of the DA transporter (DAT) promoter. Cre recombinase-driven reporter expression in DAT-Cre mice is restricted to DA neurons [280]. Immunohistochemical analysis revealed a striking reduction in GIRK2 immunoreactivity in the VTA from DAT-Cre(+):*Girk2*^{lox/lox} mice (**Fig. 2.7A**).

To assess the functional impact and cell-type specificity of GIRK channel ablation in DAT-Cre(+):*Girk2*^{lox/lox} mice, we measured baclofen-induced GIRK currents in VTA DA neurons. To permit direct electrophysiological characterization of DA and non-DA VTA neurons, DAT-Cre:*Girk2*^{lox/lox} mice were crossed with mice expressing eGFP under the control of the promoter for the DA neuron-specific transcription factor, Pitx3 [273]. As predicted, baclofen-induced currents in small eGFP-negative neurons lacking I_h current (putative VTA GABA neurons) were comparable in slices from Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} and Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} mice (**Fig. 2.7B,C**). In contrast, baclofen-induced currents in eGFP-positive (DA) VTA neurons were dramatically-reduced in slices from Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} mice as compared to controls (**Fig. 2.7D,E**). Importantly, like constitutive *Girk2*^{-/-} mice, DAT-Cre(+):*Girk2*^{lox/lox} mice were more sensitive to the motor-stimulatory effect of systemic morphine than DAT-Cre(-):*Girk2*^{lox/lox} controls (**Fig. 2.7F**).

Since some VTA DA neurons are directly inhibited by MOR agonists [281-284], we evaluated the impact of the DA neuron-specific loss of GIRK2 on DAMGO-induced currents in VTA DA neurons. DAMGO (3 μ M) evoked naloxone-sensitive outward currents in a subset (5/25) of VTA DA neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} mice (**Fig. 2.7G,H**). Though a similar fraction of VTA DA neurons from Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} mice exhibited DAMGO-induced outward currents, current amplitudes were significantly blunted relative to controls, indicating that activation of GIRK2-containing channels contributes to the direct inhibitory effect of DAMGO in this subset of VTA DA neurons.

We also asked whether there were differences in the excitability of VTA DA neurons from Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} and Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} mice. VTA DA neurons from both genotypes exhibited tonic spontaneous firing patterns (not shown), with no difference in firing rate (Cre(+): 2.2 \pm 0.3 vs. Cre(-): 2.0 \pm 0.2 Hz; n=31-33/group; *P*=0.6). Similarly, there was no difference in rheobase (Cre(+): -12 \pm 5.0 vs. Cre(-):-13.9 \pm 3.6 pA; n=26-30/group; *P*=0.8) or holding current (V_{hold} = -60 mV; Cre(+): 10.82 \pm 17.9 vs. Cre(-): 44.35 \pm 20.6 pA; n=26-27/group; *P*=0.1) between Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} and control mice, respectively. Thus, the cell-specific loss of GIRK2 did not significantly alter key measures of excitability in VTA DA neurons.

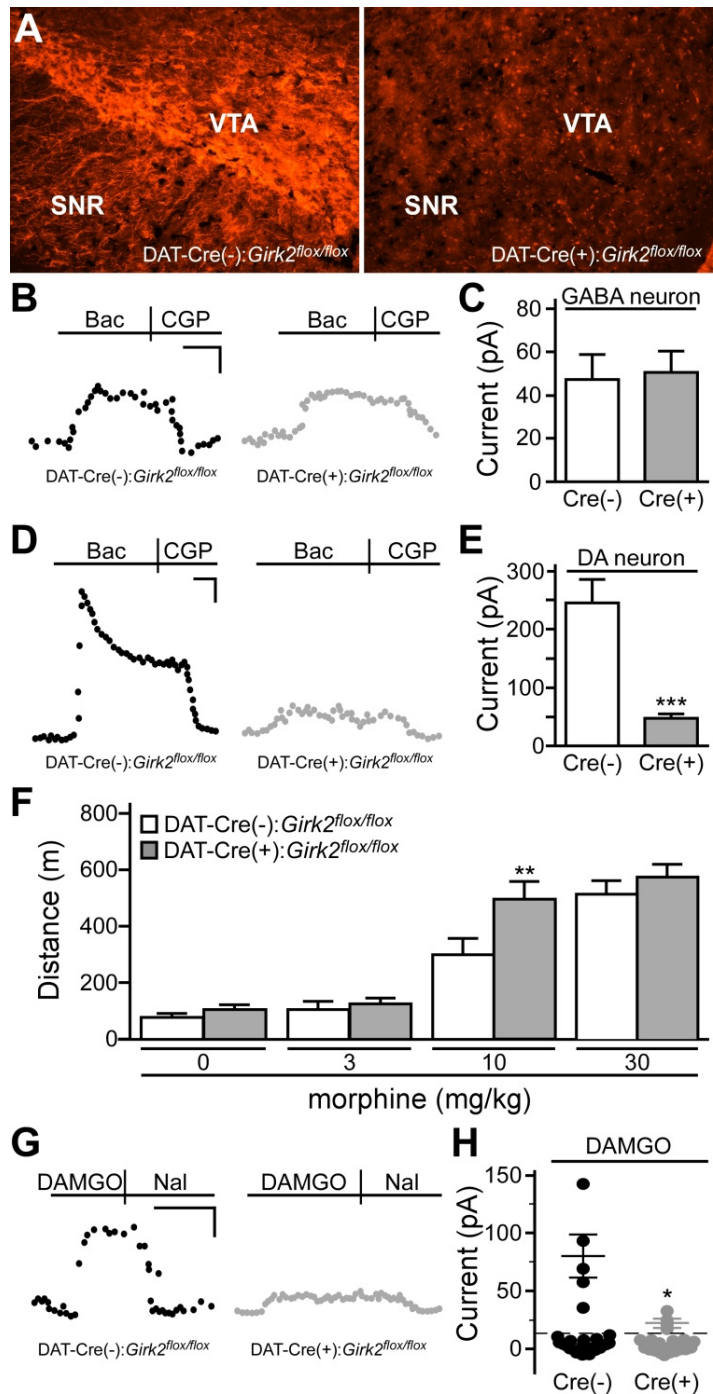


Figure 2.7. DA neuron-specific ablation of GIRK channels and systemic morphine-induced motor activity. (A) Girk2 immunolabeling in midbrain sections from DAT-Cre(-):*Girk2^{fllox/fllox}* and DAT-Cre(+):*Girk2^{fllox/fllox}* mice. Images shown were taken from slices -2.98 mm relative to bregma. Abbreviations: VTA, ventral tegmental area; SNR, substantia nigra pars reticulata. (B) Representative traces evoked by baclofen (Bac, 200 μ M) in putative VTA GABA (eGFP-negative) neurons from Pitx3-eGFP(+)/DAT-Cre(-

); *Girk2*^{lox/lox} (left, black) and Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} (right, gray) mice. Scale bars: 40 pA/200 s. (C) Summary of baclofen-induced currents in putative VTA GABA (eGFP-negative) neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} (white; n=10) and Pitx3-eGFP(+)/GADCre(+):*Girk2*^{lox/lox} (gray; n=7) mice ($t_{(15)}=0.2$, $P=0.8$). (D) Representative currents evoked by baclofen (200 μ M) in VTA DA (eGFP-positive) neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} (left, black) and Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} (right, gray) mice. Scale bars: 60 pA/200 s. (E) Summary of baclofen-induced currents in VTA DA (eGFP-positive) neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{lox/lox} (white; n=10) and Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{lox/lox} (gray; n=12) mice ($t_{(20)}=5.2$, $P<0.0001$). (F) Open-field motor activity of DAT-Cre(-):*Girk2*^{lox/lox} (white; n=6/dose) and DAT-Cre(+):*Girk2*^{lox/lox} (gray; n=12/dose) mice during a 60-min period following systemic administration of saline (0) or morphine (3, 10, 30 mg/kg). Main effects of dose ($F_{(3,48)}=85.3$, $P<0.0001$) and genotype ($F_{(1,16)}=2.9$, $P=0.1$) were observed, along with a dose/genotype interaction ($F_{(3,48)}=2.9$, $P<0.05$). Symbols: ** $P<0.01$ vs. DAT-Cre(-):*Girk2*^{lox/lox} mice. (G) Responses of DA neurons from DAT-Cre(-):*Girk2*^{lox/lox} and DAT-Cre(+):*Girk2*^{lox/lox} mice to a saturating concentration of DAMGO (3 μ M). DAMGO-induced currents were reversed by naloxone (Nal, 10 μ M). Scale bars: 40 pA/200 s. (H) Summary of DAMGO-induced currents in VTA DA neurons from DAT-Cre(-):*Girk2*^{lox/lox} (5/25 responders; 80.1 \pm 18.4 pA) and DAT-Cre(+):*Girk2*^{lox/lox} (4/20 responders; 22.7 \pm 3.4 pA) mice. Symbols: * $P<0.05$ vs. DAT-Cre(-):*Girk2*^{lox/lox} mice.

Morphine-induced activity in $Girk3^{-/-}$ mice

Data from DAT-Cre(+):*Girk2*^{fllox/fllox} mice suggested that loss of GIRK channels in VTA DA neurons contributes in part to the increased sensitivity of constitutive *Girk2*^{-/-} mice to systemic morphine-induced motor activity, and suggests that enhanced GIRK channel activity in these neurons might yield diminished opioid sensitivity. Since prior work has shown that the residual GIRK channel in VTA DA neurons from *Girk3*^{-/-} mice (GIRK2 homomer) is more sensitive to GABA_BR-dependent stimulation than its wild-type counterpart (GIRK2/GIRK3 heteromer) [188], we next evaluated the impact of *Girk3* ablation on systemic morphine-induced motor activity. Constitutive *Girk3*^{-/-} mice were significantly less sensitive to morphine-induced motor activity than wild-type counterparts (**Fig. 2.8A**). To determine whether the loss of GIRK3 in the VTA underlies the diminished sensitivity of *Girk3*^{-/-} mice to systemic morphine-induced motor activity, we restored GIRK3 expression in the VTA of *Girk3*^{-/-} mice using a recombinant lentivirus. Viral expression of GIRK3 in the VTA of *Girk3*^{-/-} mice restored normal sensitivity to the motor-stimulatory effect of morphine (**Fig. 2.8A-C**).

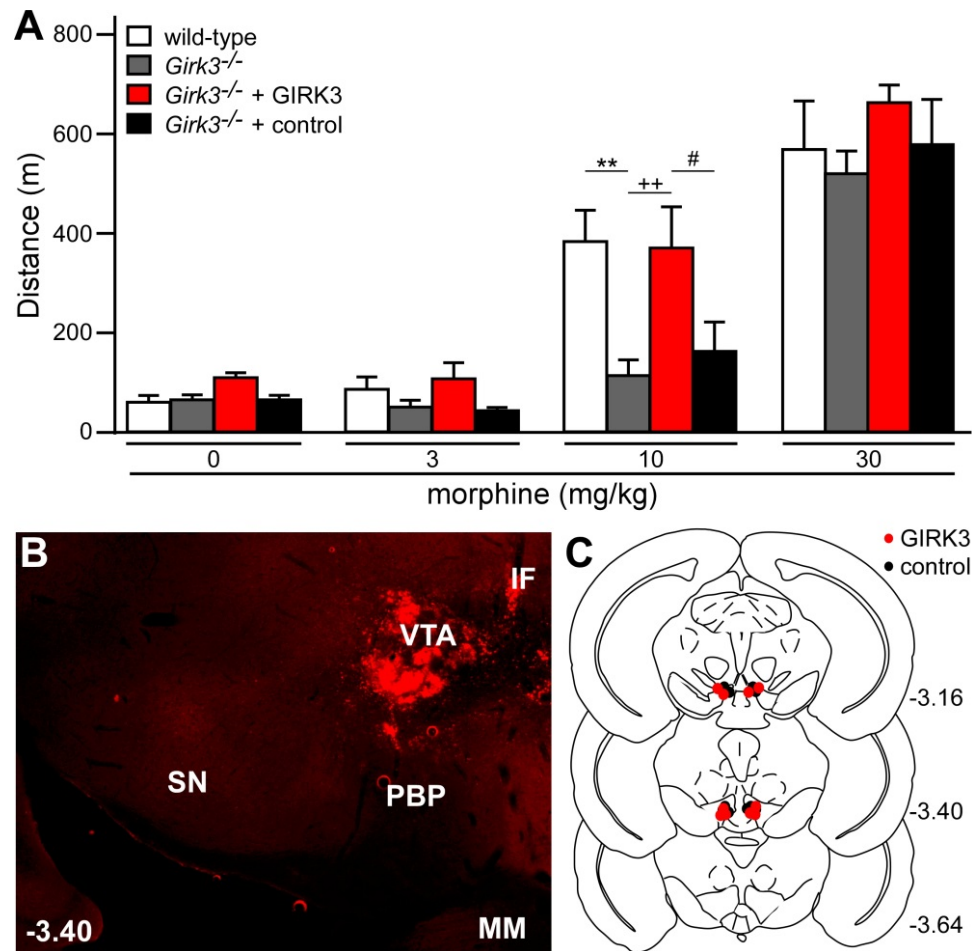


Figure 2.8. *Girk3* ablation and rescue: morphine-induced motor activity. (A) Total distance traveled by wild-type (white; n=6) and *Girk3*^{-/-} (gray; n=6) mice, as well as *Girk3*^{-/-} mice given intra-VTA GIRK3 (red; n=5) or control (black; n=5) lentivirus, during a 60-min period following systemic administration of saline (0) or morphine (3, 10, 30 mg/kg). A main effect of dose ($F_{(3,54)}=103.4$, $P<0.0001$) and genotype ($F_{(3,18)}=3.2$, $P<0.05$) was observed, but there was no dose/genotype interaction ($F_{(9,54)}=1.8$, $P=0.1$). Symbols: ** $P<0.01$ vs. wild-type, ++ $P<0.01$ vs. *Girk3*^{-/-}, # $P<0.05$ vs. *Girk3*^{-/-} + control. (B) Representative image showing lentiviral-driven GIRK3-YFP (YFP expression shown in red) expression in the VTA of a *Girk3*^{-/-} mouse. Abbreviations: SN, substantia nigra; PBP, parabrachial pigmented nucleus; IF, interfascicular nucleus; MM, medial mammillary nucleus. (C) Schematic of coronal sections (from bregma) depicting viral expression in mice included in this study.

Discussion

Opioid-induced motor-activation is an unconditioned behavioral response that involves increased DA neurotransmission in the mesolimbic (VTA-NAc) pathway [88, 260]. GIRK channels have been proposed to mediate the direct inhibitory effects of opioids on midbrain GABA neurons that suppress VTA DA neuron output, and thus, we predicted that loss of GIRK signaling would suppress opioid-induced motor activity. Instead, we found that GIRK channel activation in GABA neurons is not required for opioid-induced motor activation in mice, nor is it sufficient to evoke an increase in motor activity. GIRK channels in VTA DA neurons are, however, a key determinant of sensitivity to the motor-stimulatory effect of opioids.

The opioid-induced disinhibition of VTA DA neurons was proposed initially to involve the direct inhibition of local (VTA) GABA neurons [214]. In support of this model, MOR expression within the VTA is most prominent in GABA neurons [285], VTA GABA neurons synapse onto VTA DA neurons [267, 286, 287], opioids hyperpolarize and/or inhibit the spontaneous activity of VTA GABA neurons [214, 281, 288, 289], and input to VTA DA neurons from VTA GABA neurons is opioid-sensitive [267]. Moreover, inhibition of VTA GABA neurons leads to activation of VTA DA neurons [286]. Recent evidence, however, suggests that the disinhibition of VTA DA neurons reflects the opioid-induced inhibition of GABAergic input from the RMTg [290, 291]. Indeed, most midbrain (VTA) DA neurons in rodents and non-human primates are innervated and inhibited by RMTg GABA projections [290, 292], and this comparably dense input may be better poised to regulate the excitability of VTA DA neurons than the more sparse input from VTA GABA neurons [287, 293, 294].

Whether the opioid-induced disinhibition of VTA DA neurons is attributable to the inhibition of VTA or RMTg GABA neurons, or reflects a broad suppression of GABA input to VTA DA neurons from these and other structures, including the NAc and ventral pallidum [295, 296], our data argue that the opioid-induced inhibition of GABAergic input to VTA DA neurons involves a presynaptic mechanism and/or a non-GIRK somatodendritic mechanism(s), at least in the mouse. Consistent with the former possibility, MOR labeling in the VTA is found on axon terminals of GABA neurons [285], and opioid inhibition of GABAergic neurotransmission in several brain regions, including the VTA, is mediated by a reduced probability of presynaptic neurotransmitter release [266-268]. Furthermore, pharmacological inactivation of the RMTg blocked the ability of intra-VTA morphine to activate VTA DA neurons [293], arguing that the disinhibitory effect of intra-VTA opioids on VTA DA neurons is due to inhibition of RMTg GABA afferents.

Non-GIRK postsynaptic mechanisms may also contribute to the opioid-induced disinhibition of VTA DA neurons. Notably, the opioid-induced hyperpolarization and inhibition of spontaneous activity in VTA and RMTg GABA neurons has been observed by multiple groups [266, 288, 294]. Indeed, DAMGO suppressed the spontaneous activity of 92% of eGFP-positive (GABA) VTA neurons from GAD67-eGFP mice [288]. Using this same mouse line, we show here that most VTA GABA neurons lack an evident DAMGO-induced inhibitory current, reminiscent of findings in the rat [297]. Similarly, only one-third of the RMTg neurons projecting to the ventral midbrain (VTA and substantia nigra) were hyperpolarized by MOR agonists [266]. Our finding that intra-RMTg DAMGO stimulated motor activity, however, argues that the direct

inhibition of RMTg neurons is sufficient to stimulate motor activity. Interestingly, morphine was reported to suppress excitatory input to RMTg neurons via a GIRK-independent postsynaptic mechanism [294]. Thus, MOR may couple preferentially to a non-GIRK somatodendritic effector(s) in midbrain GABA neurons. At present, the behavioral relevance of the opioid-induced, GIRK-dependent and GIRK-independent inhibition of midbrain GABA neurons is unclear.

Some VTA DA neurons express MOR, and both direct excitatory and inhibitory effects of MOR activation on VTA DA neurons have been reported [283]. Consistent with previous reports [281-283], we found that ~20% of mouse VTA DA neurons were inhibited by DAMGO. Moreover, we show that GIRK channel activation mediates the direct inhibitory effect of DAMGO on this subset of VTA DA neurons. Disruption of MOR-GIRK signaling in these neurons, however, seems unlikely to explain the enhanced morphine-induced motor activity observed in *Girk2*^{-/-} or DAT-Cre(+):*Girk2*^{flox/flox} mice. Indeed, if MOR-GIRK signaling in these neurons underlies the motor-stimulatory effect of systemic or intra-VTA opioids, then the loss of GIRK channels should diminish and not enhance the opioid-induced behavior. It is possible, however, that this VTA DA neuron subpopulation normally tempers opioid-induced motor stimulation, perhaps akin to a negative feedback pathway. In this scenario, diminished MOR-GIRK signaling could result in enhanced opioid-induced motor stimulation.

The output of VTA DA neurons is tempered by “long-loop” (GABA_BR-dependent) and D₂ DA autoreceptor (D₂R)-mediated negative feedback mechanisms that are engaged by *in vivo* administration of drugs of abuse [298, 299]. The GIRK2/GIRK3 channel mediates the inhibitory effect of GABA_BR and D₂R activation on VTA DA neurons [188,

239]. The enhanced morphine-induced motor activity seen in DAT-Cre(+):*Girk2*^{lox/lox} mice may reflect, therefore, the loss of these feedback mechanisms. In support of this contention, key excitability measures in the absence of drug were comparable in VTA DA neurons from DAT-Cre(+):*Girk2*^{lox/lox} and DAT-Cre(-):*Girk2*^{lox/lox} mice. Furthermore, our observations with *Girk3*^{-/-} mice and viral reconstitution of GIRK3 in the VTA, together with previous work showing that *Girk3* ablation correlates with enhanced sensitivity of the residual GIRK channel to GPCR-dependent activation [180], argues that the sensitivity of the VTA DA neuron GIRK channel to GPCR stimulation is a critical determinant of morphine-induced motor activity.

Acute and repeated psychostimulant exposure suppresses GABA_B and D₂ DA receptor-dependent inhibitory signaling in VTA DA neurons [150]. A single injection of cocaine, for example, transiently suppressed GABA_BR-GIRK signaling in VTA DA neurons, by triggering a subcellular redistribution of GIRK channels from the membrane to intracellular sites [199]. As decreased GIRK-dependent signaling in VTA DA neurons should correlate with enhanced opioid-induced motor activity, our findings suggest a plausible explanation for behavioral cross-sensitization observed between opioids and psychostimulants [300-305].

While the vast majority of neurons in the mouse VTA are dopaminergic or GABAergic [288], some glutamatergic neurons are present as well [84, 306]. Most glutamatergic neurons are found in the medial aspect of the VTA [84], including some that co-express VGlut2 and TH [85, 307]. We targeted the lateral aspect of the VTA to minimize the potential impact of this small neuron population on our study. While many of our experiments utilized transgenic mice with fluorescently-tagged GABA and DA

neurons, some experiments relied on a combination of cell size (apparent capacitance) and I_h current size to identify putative VTA GABA and DA neurons. This combination of features, as well as the amplitude of the baclofen-induced somatodendritic current, has proven useful for distinguishing between DA and GABA neurons in the mouse VTA [180, 188, 199]. We cannot exclude the possibility, however, that some putative VTA DA and GABA neurons evaluated in this study were glutamatergic neurons. At present, the expression of GIRK channels and MOR in VTA glutamatergic neurons, and their relevance to the motor-stimulatory effects of systemic and intracranial opioids, are unclear.

Opioids such as morphine are the most commonly-administered drugs for pain, despite possessing an unfavorable side effect profile and significant addictive liability [204]. Given the increase in abuse of prescription opioids [308], it is particularly important that we understand the circuitry, cell types, and mechanisms mediating both the beneficial and problematic effects of opioids. These insights may suggest novel approaches to eliciting the beneficial outcome associated with opioids (analgesia) while avoiding undesirable side effects (addiction). Data described herein suggest that the unique GIRK channel subtype (GIRK2/GIRK3 heteromer) in VTA DA neurons sets the sensitivity of the mesolimbic circuit to morphine. Given that previous work has implicated this channel in circuit-level and behavioral effects of other drugs of abuse [188, 309], these findings suggest that therapeutic approaches targeting the GIRK2/GIRK3 channel might prove useful for treating some aspects of drug addiction.

Author Contributions for Chapter 3:

McCall NM: Responsible for electrophysiology data presented in Chapter 3- Figure 3.1 C/D. Contributions included development of study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, and critical revision.

Kotecki L: Responsible for IHC (Figure 3.1B) and behavioral data presented in Chapter 3-Figure 3.2, 3.3, 3.4. Contributions included development of study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, and critical revision.

Carlbom N: Assisted with the collection of behavioral data of DATCre:*Girk2^{flox/flox}* mice locomotor response to acute and repeated cocaine in Figure 3.2 and 3.3.

Wickman K: Contributions included development of study conception and design, interpretation of data, and drafting of manuscript.

CHAPTER 3

Loss of GIRK channels in dopamine neurons blunts D₂R autoinhibitory feedback and enhances reward-related behavioral response to cocaine

McCall NM, Kotecki L, Carlblom N, Wickman K. Loss of GIRK channels in dopamine neurons blunts D₂R autoinhibitory feedback and enhances reward-related behavioral response to cocaine. Manuscript in preparation.

Introduction

The use of illicit substances, such as cocaine, continues to be a global problem, with the World Health Organization estimating that in 2008, 155 to 250 million people (~4.5% of the world's population age 15-64) used illicit substances [4]. Drug addiction is a chronic relapsing disorder defined by compulsive drug seeking and taking despite the presence of negative consequences [1, 310]. Many of the current pharmacotherapies for addiction are targeted toward the receptor of interest, but are not very effective in treating addiction and often have undesirable off target effects [135]. To more effectively treat and potentially prevent addiction, a fundamental understanding of the underlying cellular mechanisms of addiction is needed. The mesocorticolimbic dopamine (DA) system, which consists of the ventral tegmental area (VTA) and its reciprocal connections with downstream targets such as the nucleus accumbens (NAc) and prefrontal cortex (PFC), is a critical cellular substrate for addiction. Indeed, both acute and repeated cocaine exposure can alter excitatory and inhibitory signaling within the mesocorticolimbic DA system [150]. Cocaine enhances the presence of DA in synapses through the blockade of the DA transporter (DAT) [256, 310]. This increase in extracellular DA levels in the mesocorticolimbic pathway is a critical component of drug-induced reward-related behaviors, such as exploration (motor activity) and drug seeking/wanting [252, 256]. While much of the research on the underlying cellular mechanisms of cocaine addiction has focused on drug-induced adaptations in excitatory neurotransmission in the mesocorticolimbic DA system [122, 131, 310, 311], recent work has shown that cocaine exposure also triggers modifications to inhibitory signaling [91, 123, 298, 299, 312-317].

In addition to increasing DA levels in downstream targets, cocaine increases extracellular levels of DA in the VTA, where DA transporters are also present. This extracellular DA in the VTA binds to somatodendritic inhibitory $G_{i/o}$ G-protein coupled DA receptors (Type 2 DA receptors; D_2R) [233]. D_2R receptor activation results in neuronal inhibition through the activation of G protein gated inwardly rectifying K^+ (GIRK/Kir3) channels, which serves as a key autoinhibitory mechanism designed to temper VTA-mediated DA neurotransmission [150, 298, 299, 318, 319]. GIRK channels have been shown to mediate the postsynaptic inhibitory effects of many neurotransmitters within the CNS, including DA and GABA [164, 165, 239]. VTA DA neurons express GIRK2/GIRK3 heteromeric channels [180, 188], and DA neurons of the VTA appear to be the only cell type that exclusively expresses this unique GIRK channel.

The importance of GIRK channel activity to DA-dependent reward-related behaviors is indicated by the observation that constitutive *Girk*^{-/-} mice exhibit altered baseline and drug-induced reward-related behaviors. Constitutive *Girk2*^{-/-} mice exhibit baseline hyperactivity in their home cage and open field test [276, 320], phenotypes that can be normalized by systemic administration of SCH 23390, a D_1R antagonist [320]. Constitutive *Girk2*^{-/-} mice also show enhanced motor activation following both systemic morphine and cocaine administration; however, *Girk3*^{-/-} mice exhibit attenuated motor activation to these drugs [228, 321]. Constitutive *Girk2*^{-/-} and *Girk3*^{-/-} mice also exhibit decreased cocaine self-administration [322]. These results, along with prior work showing that the GIRK channel in VTA DA neurons of *Girk3*^{-/-} mice (GIRK2 homomer) is more sensitive to $GABA_B$ R-dependent stimulation than its wild-type counterpart (GIRK2/GIRK3 heteromer) [188], suggest that the loss of GIRK2 in VTA DA neurons

increases the sensitivity of these animals to the cellular and behavioral effects of drugs of abuse. In the present study, we utilized a DA neuron-specific *Girk2*^{-/-} mouse line (DATCre:*Girk2*^{lox/lox}) [321], to probe the relevance of GIRK channels in VTA DA neurons on D₂R autoinhibitory signaling and cocaine-induced reward-related behavior. Using these novel mutant mice and pharmacological tools, we found that the loss of GIRK channels in DA neurons blunts D₂R autoinhibitory feedback and enhances the motor and reward-related behavioral responses of these mice to cocaine.

Materials and Methods

Animals.

Animal use was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. The generation of *Girk2*^{lox/lox} mice in partnership with InGenious Laboratory was previously described in Chapter 2 [321]. Pitx3-eGFP mice were provided by Dr. Meng Li. B6.SJL-slc6a3^{tm1.1c\cre}Bk^{mn}/J (DATCre; #006660) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12 h light/dark cycle, with food and water available *ad libitum*.

Drugs.

Cocaine hydrochloride, sulpiride, quinpirole, and tetrodotoxin (TTX) were purchased from Sigma (St. Louis, MO).

Motor activity.

Systemic cocaine-induced locomotor activity assessments in DATCre(+/-):*Girk2*^{lox/lox} mice were performed in automated open-field activity chambers (Med-Associates Inc.; St Albans, VT), as described [276]. In brief, male and female subjects (7-

10 wk) were handled and acclimated to the testing room for 1 h prior to test day, followed by habituation to activity chambers and systemic injections (saline i.p.) over 2 daily sessions. Total distance traveled during the 60-min period following the second day of saline injection was defined as the response to 0 mg/kg cocaine. For acute cocaine-induced activity studies, subjects were given 1 of 3 cocaine doses (3, 15, 30 mg/kg i.p.). For repeated cocaine-induced activity (sensitization) studies, subjects received 15 mg/kg cocaine for 5 consecutive days, and a 15 mg/kg cocaine challenge administered 10-12 days after the last injection. Cocaine sensitization occurred if the activity response was higher on cocaine day 5 (C5) than cocaine day 1 (C1) or challenge (ES) > C1.

Conditioned Place Preference (CPP).

The CPP test was conducted in an apparatus divided into two compartments with a guillotine-style door as previously described (Med Associates Inc.; St Albans, VT) [323]. The floor of one compartment consisted of an array of stainless steel rods (grid) and the other compartment consisted of thin chicken wire (mesh). During the preconditioning test, mice were placed in the apparatus and allowed free exploration for 15 min; time spent in the “grid” and “mesh” chambers was recorded. Mice were then assigned to drug treatment (15 mg/kg cocaine) or vehicle (saline) treatment groups and paired with the least preferred compartment (CS⁺). The other chamber (CS⁻) was paired with a saline treatment. During the AM conditioning trials, subjects were injected with saline and confined (30 min) to the corresponding CS⁻ chamber. During the PM conditioning trials, subjects were injected with saline or cocaine and confined (30 min) to the corresponding CS⁺ chamber. A total of 3 training sessions was performed. Side preferences were evaluated after the last day of training and place preference was determined by

calculating the difference in time spent in the CS⁺ and CS⁻ chambers during the test sessions; “preference” is defined as (CS⁺ – CS⁻) within a test session.

Slice Electrophysiology.

Horizontal slices (225 μm) from male and female Pitx3-eGFP(+)/DATCre(+/-):*Girk2*^{fllox/fllox} mice (5-6 wk) were prepared as previously described in Chapter 2 [321]. Studies involved targeted evaluation of VTA DA neurons, as defined by eGFP expression in VTA neurons from Pitx3-eGFP [272]. Upon achieving whole-cell access, I_h was measured in voltage-clamp mode using a 1-s voltage ramp (-60 to -120 mV). Subsequently, spontaneous activity was measured in current-clamp mode (I=0) for 1 min. Following a 3 to 5-min stabilization period (V_{hold} = -60 mV), changes in holding current evoked by quinpirole were measured, as described [199]. Other cell parameters, including series resistance, capacitance, and input resistance, were tracked throughout the experiment. All command potentials factored in junction potential of -15 mV, predicted using JPCalc software (Molecular Devices).

Immunohistochemistry.

Pitx3-eGFP mice (7-10 wk) were given a euthanizing dose of ketamine/xylazine (250 and 25 mg/kg i.p., respectively) and perfused transcardially with Ca²⁺-free Tyrode's solution followed by 4% paraformaldehyde containing 14% picric acid. Brains were post-fixed overnight in Sorenson's Buffer containing 10% sucrose at 4°C. Tissue was sectioned coronally at 12 μm by cryostat and mounted to slides. Staining was performed as described [321]. Sections were exposed to sheep anti-Tyrosine Hydroxylase antibody (1:1000; Alomone labs) and donkey anti-sheep IgG Cy3 secondary antibody (1:500; Jackson Immunoresearch Laboratories, Inc.; West Grove, PA)

Data analysis.

Data are presented throughout as the mean \pm SEM. Statistical analyses were performed using Prism 6 (GraphPad Software, Inc.; La Jolla, CA) and SigmaPlot 11.0 (Systat Software, Inc.; San Jose, CA). Electrophysiological and behavioral data were analyzed with a Student's t test, one-way ANOVA, or two-way repeated measures ANOVA followed by individual comparisons using the Bonferroni post-test method, as appropriate. Where no sex differences were observed, data were pooled to increase statistical power. For all statistical comparisons, differences were considered significant if $P < 0.05$.

Results

D₂R-dependent signaling is reduced in VTA DA neurons from DATCre(+):Girk2^{flox/flox} mice

Previous work suggests that the GABA_BR- and D₂R-dependent inhibition of midbrain DA neurons is mediated in large part by activation of GIRK channels containing GIRK2 [188, 239]. Described in detail in Chapter 2, we reported the creation of a DA neuron-specific conditional *Girk2* knockout mouse line, DAT-Cre:*Girk2*^{flox/flox} (Fig 3.1A). We demonstrated that DAT-Cre(+):*Girk2*^{flox/flox} mice showed a striking reduction in GIRK2 immunoreactivity in the VTA and significantly attenuated GABA_BR-dependent GIRK currents [321]. To evaluate the impact of the DA neuron-specific loss of *Girk2* on D₂R-dependent inhibition, we measured somatodendritic currents evoked by the D₂R agonist quinpirole in VTA DA neurons in DATCre(+/-):*Girk2*^{flox/flox} mice. To permit direct electrophysiological characterization of DA and non-DA VTA neurons, DAT-

Cre:*Girk2*^{flx/flx} mice were crossed with mice expressing eGFP under the control of the promoter for Pitx3, which is a DA neuron-specific transcription factor [273]. eGFP labeling in this line correlates well with DA neuron markers [180], including tyrosine hydroxylase (TH, **Fig. 3.1B**). VTA DA neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{flx/flx} mice displayed outward currents in response to bath application of quinpirole that was reversed by the D₂R antagonist sulpiride. As predicted, quinpirole-induced current amplitudes in eGFP-positive (DA) VTA neurons were dramatically reduced in slices from Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{flx/flx} mice as compared to Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{flx/flx} controls (**Fig. 3.1C,D**). We did not observe any sex difference in quinpirole-induced current amplitude (data not shown). We also observed no significant effect of sex or genotype on other properties of VTA DA neurons (**Table 3.1**).

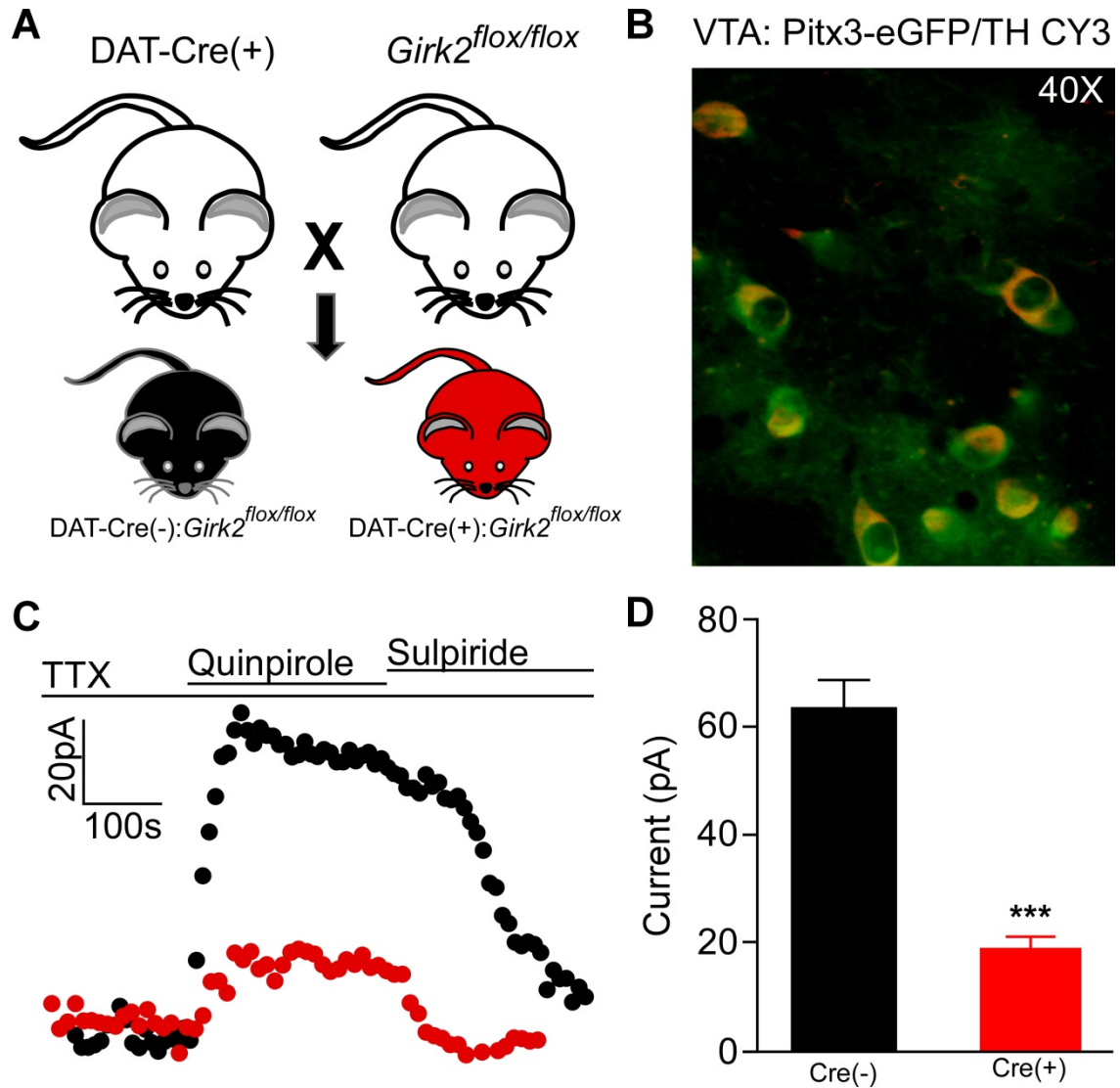


Figure 3.1. DA neuron-specific ablation of GIRK channels reduces D₂R-dependent signaling. (A) Schematic depicting how DA neuron-specific *Girk2* KO mouse line (DATCre(+/-):*Girk2*^{flox/flox}) was generated. (B) Immunohistochemistry showing Pitx3-eGFP labeling and tyrosine hydroxylase (TH) overlap in VTA DA neurons in sections taken from a Pitx3-eGFP (+) mouse. (C) Representative currents ($V_{\text{hold}} = -60$ mV) evoked by quinpirole (20 μM) in VTA DA neurons from DATCre(+):*Girk2*^{flox/flox} (red) and DATCre(-):*Girk2*^{flox/flox} (black) control mice. Currents were reversed by the D₂R antagonist sulpiride (5 μM). (D) Summary of peak quinpirole-induced currents in VTA DA neurons from DATCre(+):*Girk2*^{flox/flox} (red) and DATCre(-):*Girk2*^{flox/flox} (black) control mice. Symbols (***) P < 0.001 (n=19-23 recordings/genotype).

Parameter	DATCre(-): <i>Girk2</i> ^{flox/flox}	DATCre(+): <i>Girk2</i> ^{flox/flox}
N (n)	23 (11)	19 (10)
Mouse age (d)	40	40
C _M (pF)	49 ± 4	44 ± 3
RMP (mV)	-38 ± 1	-38 ± 2
I _h amplitude (pA)	-165 ± 49	-68 ± 20

Table 3.1. Electrophysiological properties of VTA DA neurons from DATCre(+/-):*Girk2*^{flox/flox} mice (related to Figure 1). Summary of intrinsic and active electrophysiological properties of VTA DA neurons from Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{flox/flox} and Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{flox/flox} mice. N (n), number of recordings and number of animals; mouse age (d); C_M, apparent membrane capacitance; RMP, resting membrane potential; I_h, hyperpolarization-activated cation current. No differences were found in C_M, RMP, or I_h, between Pitx3-eGFP(+)/DAT-Cre(-):*Girk2*^{flox/flox} and Pitx3-eGFP(+)/DAT-Cre(+):*Girk2*^{flox/flox} mice.

GIRK signaling in VTA DA neurons and cocaine-induced behavior

Exposure to acute cocaine produces an unconditioned response of motor stimulation. Repeated exposure to cocaine results in behavioral sensitization, which is characterized by an enhanced response to subsequent cocaine exposure. Cocaine behavioral sensitization has been likened to drug craving and is thought to be a key component of drug relapse [150, 324, 325]. We have previously reported that constitutive *Girk2*^{-/-} mice exhibited increased sensitivity to the motor-stimulatory effects of acute and repeated systemic cocaine [228, 322]. To test whether this enhanced sensitivity to cocaine in *Girk2*^{-/-} mice was attributable to a loss of GIRK-dependent signaling in VTA DA neurons, we evaluated the impact of DA neuron-specific GIRK ablation on acute and repeated systemic cocaine-induced motor activity.

DATCre(+/-):*Girk2*^{fllox/fllox} mice were acclimated for 3 days (d) to handling and the injection protocol prior to acute cocaine exposure (**Fig 3.2A**). While *Girk2*^{-/-} mice had elevated levels of baseline motor activity [228], DATCre(+):*Girk2*^{fllox/fllox} mice exhibited slightly elevated baseline motor activity (saline day 2, S2), but this difference was not statistically significant. Since global ablation of *Girk2* correlated with increased sensitivity to cocaine in this task, we predicted that DATCre(+):*Girk2*^{fllox/fllox} mice would exhibit enhanced acute systemic cocaine-induced motor activity. Both DATCre(-):*Girk2*^{fllox/fllox} and DATCre(+):*Girk2*^{fllox/fllox} mice exhibited a robust dose-dependent increase in motor activity following acute systemic cocaine injection (**Fig 3.2B**). As predicted, DATCre(+):*Girk2*^{fllox/fllox} mice exhibited enhanced motor activity response at all cocaine doses, with significant genotype differences observed at the two higher doses of cocaine (15 and 30 mg/kg), similar to cocaine responses in constitutive *Girk2*^{-/-} mice.

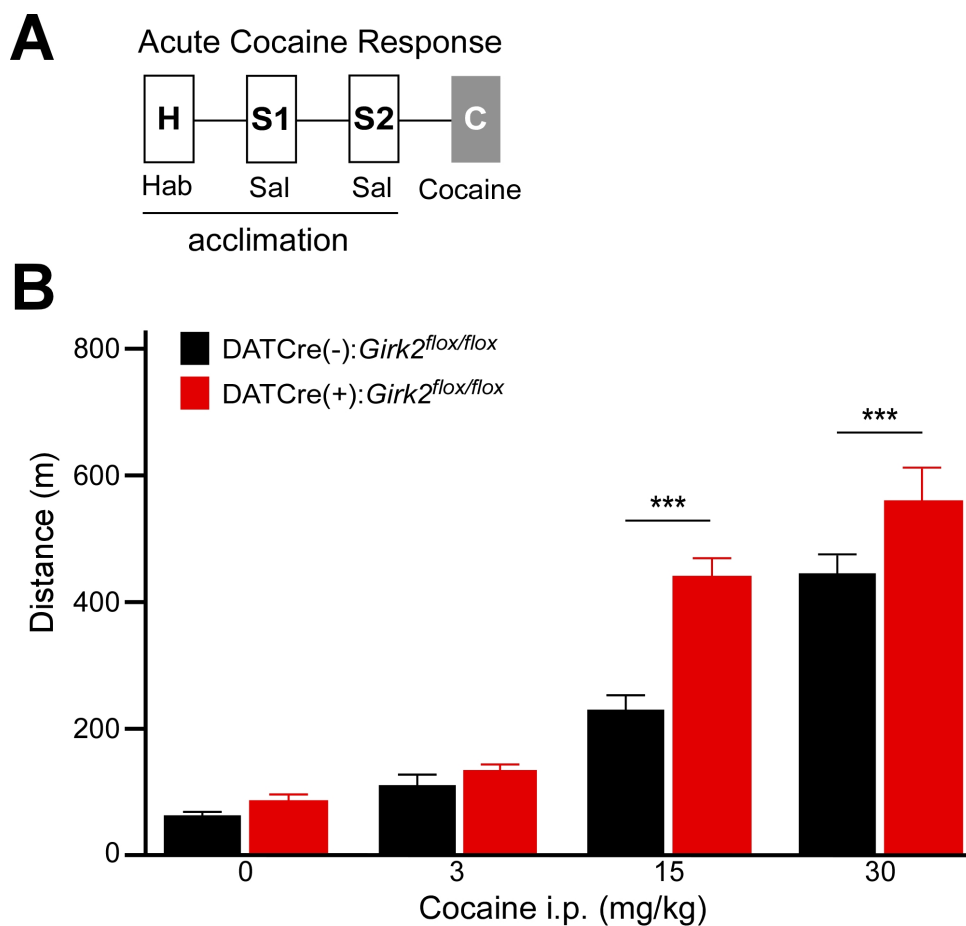


Figure 3.2. DA neuron-specific ablation of GIRK channels enhances acute cocaine-induced motor stimulation. (A) The acute cocaine treatment study included three acclimation days (H, S1, S2) followed by single cocaine injection (3, 15, 30 mg/kg i.p.). (B) Distance traveled (m) following saline (0) injection on the 2nd saline day (S2), and after cocaine injection (n=11-19 per treatment/genotype). The DATCre(+):*Girk2*^{lox/lox} mice showed enhanced locomotor responses to 15 and 30 mg/kg cocaine compared to DATCre(-):*Girk2*^{lox/lox} controls. Symbols (***) P<0.001).

To measure cocaine behavioral sensitization, mice were acclimated to the handling and injection protocol for 3d prior to 5d of repeated 15 mg/kg cocaine exposure (**Fig 3.3A**). DATCre(+):*Girk2*^{lox/lox} mice exhibited an enhanced motor activity response to repeated cocaine administration, as compared to controls (**Fig 3.3B**). DATCre(+):*Girk2*^{lox/lox} mice showed elevated cocaine-induced activity levels on every day of cocaine exposure (C1-C5). Both DATCre(-):*Girk2*^{lox/lox} and DATCre(+):*Girk2*^{lox/lox} mice displayed sensitization to the repeated cocaine dosing paradigm. However, DATCre(+):*Girk2*^{lox/lox} mice showed a reduced magnitude of sensitization (ratio C5:C1 and ES:C1) compared to DATCre(-):*Girk2*^{lox/lox} controls, suggesting that DATCre(+):*Girk2*^{lox/lox} mice show some level of pre-sensitization to the behavioral effects of cocaine (**Fig 3.3C**). These results argue that the loss of GIRK-dependent signaling in VTA DA neurons alters the sensitivity to cocaine-induced locomotor activity.

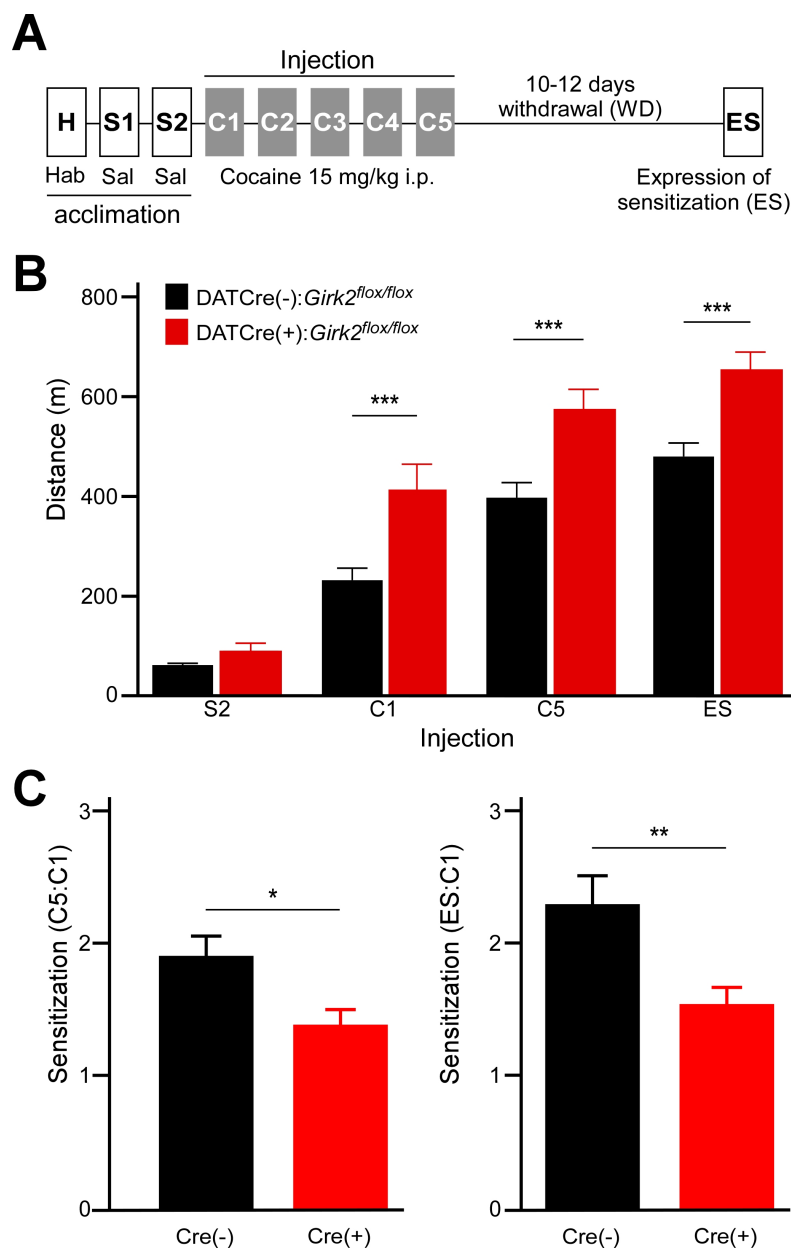


Figure 3.3. DA neuron-specific ablation of GIRK channels alters cocaine sensitization. (A) Schematic of repeated cocaine testing protocol. (B) Distance traveled (m) following saline (S2) injection, and after the 1st (C1), 5th (C5), and challenge injection of cocaine (ES) (n=17-19 per treatment/genotype). The DATCre(+):*Girk2*^{lox/lox} mice showed enhanced locomotor responses compared to DATCre(-):*Girk2*^{lox/lox} controls throughout the test. (C) Both groups showed sensitization: C5 response > C1 response and ES challenge response > C1 response. DATCre(+):*Girk2*^{lox/lox} mice showed a reduced magnitude of sensitization (ratio C5:C1 and ES:C1) to DATCre(-):*Girk2*^{lox/lox} mice, suggesting a pre-sensitized state. Symbols (* P<0.05, ** P<0.01, *** P<0.001).

We next assessed the effect that the loss of GIRK-dependent signaling in VTA DA neurons had on the rewarding effect of cocaine. We used a 3d-conditioning session conditioned place preference test for 15 mg/kg cocaine [323], a dose that showed genotype differences in locomotor sensitivity to both acute and repeated cocaine exposure (**Fig 3.4A**). Both DATCre(-):*Girk2*^{lox/lox} and DATCre(+):*Girk2*^{lox/lox} mice established a conditioned place preference for cocaine using this protocol, however there was no difference between genotypes with respect to preference magnitude (**Fig 3.4B**). While these results do not support the contention that GIRK-dependent signaling in VTA DA neurons is important to the rewarding effect of cocaine, it is possible that 15 mg/kg is a saturating dose of cocaine. Repeated studies with a lower dose of cocaine may yield a genotype difference, as was the case in similar cocaine CPP studies done with DA neuron-specific autoreceptor *D₂R*^{-/-} (AutoDrd2KO) mice [235].

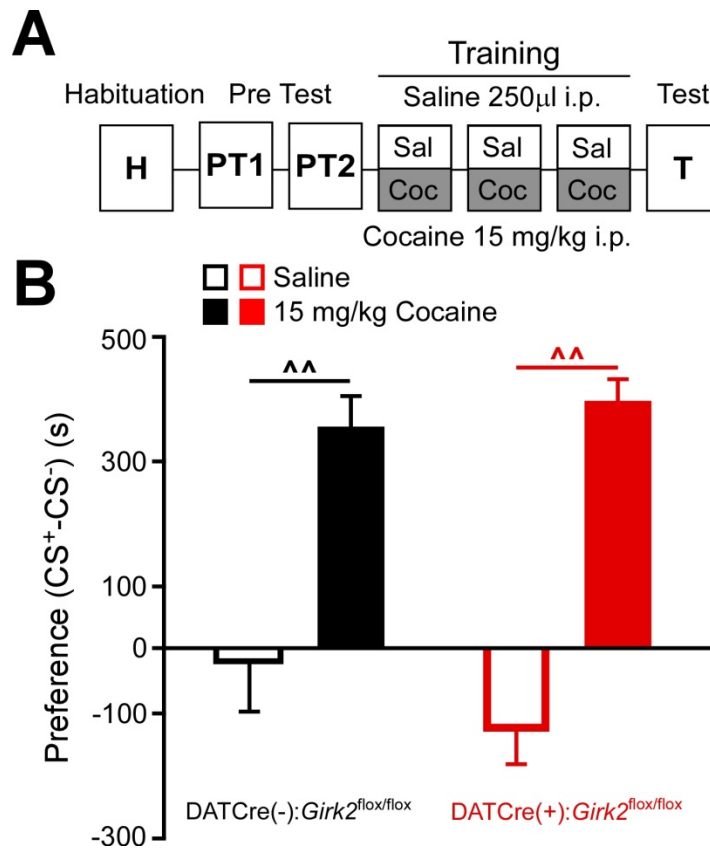


Figure 3.4. DA neuron-specific ablation of GIRK channels does not impact cocaine reward. (A) Schematic of CPP testing protocol. (B) Difference in time spent by DATCre(-):*Girk2*^{flox/flox} and DATCre(+):*Girk2*^{flox/flox} mice between the drug-paired (CS⁺) and saline-paired (CS⁻) sides of a CPP chamber (preference (CS⁺-CS⁻)), measured after three conditioning days with cocaine (15mg/kg i.p.). Both cocaine-paired groups showed a significant preference to saline, but there was no difference between genotype. Symbols (^^p<0.01) n=10-14 mice per treatment/genotype.

Discussion

Cocaine exposure triggers an increase in extracellular DA levels within the VTA and in downstream targets of the VTA, such as the NAc [256, 310]. The increase in extracellular DA within the VTA triggers a key autoinhibitory mechanism designed to reduce VTA DA neurotransmission [298, 318, 319]. The extracellular DA binds to D₂R autoreceptors, which triggers the activation of G_{i/o} G proteins and subsequently GIRK channels [150]. GIRK channel activation results in cellular hyperpolarization and suppresses VTA DA neuron activity [164, 165]. Dysregulation of this autoinhibitory mechanism of VTA DA neurons may contribute to the addictive properties of drugs of abuse [150, 233, 236, 326].

The present studies sought to evaluate the role of autoinhibitory feedback on inhibitory signaling in VTA DA neurons and its relevance to addiction-related behaviors linked to acute and repeated cocaine exposure. We found that VTA DA neurons in DATCre(+):*Girk2*^{fllox/fllox} mice do not show any difference in excitability at baseline (RMP, spontaneous activity, and rheobase), but they do show a significant reduction in inhibitory D₂R-GIRK signaling [321], suggesting that the DATCre(+):*Girk2*^{fllox/fllox} line represents a loss-of-function model for the autoinhibitory feedback of VTA DA neurons. Interestingly, mice lacking inhibitory feedback to VTA DA neurons, exhibited enhanced cocaine-induced motor stimulation following both acute and repeated cocaine exposure. What is missing from these studies is confirmation that DATCre(+):*Girk2*^{fllox/fllox} mice do, in fact, show enhanced DA neurotransmission. Fast-scan cyclic voltammetry (FSCV) can be used to measure DA levels in the VTA and downstream targets, including the NAc. Previous studies with mice lacking autoinhibitory D₂R in DA neurons revealed

increased DA release in the striatum compared to wild-type controls [235, 236]. Based on these findings, and our initial results characterizing D₂R-GIRK signaling in VTA DA neurons of our conditional knockout line, we would expect to see elevated extracellular DA levels following VTA stimulation in DATCre(+):*Girk2*^{lox/lox} mice .

The loss of *Girk2* in DA neurons alters the locomotor response to cocaine, with DATCre(+):*Girk2*^{lox/lox} mice showing “pre-sensitization”, or an enhanced initial response to cocaine. However, to further examine the influence of GIRK-dependent signaling on cocaine reward, we need to test this mouse model using established reward behavioral paradigms, such as CPP or self-administration. Our initial CPP results show that cocaine was effective at establishing a CPP in both DATCre(+):*Girk2*^{lox/lox} and DATCre(-):*Girk2*^{lox/lox} mice, but there was no difference in the magnitude of CPP as we predicted. Previous studies characterizing cocaine CPP in C57BL/6J mice have concluded, however, that there is no dose-response relationship to CPP magnitude for cocaine in the 4-12 mg/kg range. Nevertheless, a significant effect of the number of cocaine-pairing sessions on CPP magnitude can be observed; indeed, CPP magnitude following four cocaine training sessions is greater than that observed following 1 or 2 sessions [327-330]. In order to better test the influence of GIRK-dependent signaling on cocaine reward, we have designed a low dose cocaine CPP protocol to assess the impact of the number of training sessions on CPP magnitude. This protocol consists of two pretest preference days, 4 days of training (2 saline/2 cocaine) followed by a test day, and another 4 days of training (2 saline/ 2 cocaine) followed by test day. If the loss of *Girk2* in DA neurons enhances the sensitivity of the subject to cocaine, we would expect DATCre(+):*Girk2*^{lox/lox} mice to show enhanced CPP magnitude between 4 cocaine

training sessions than that observed following 2 sessions, or a difference in CPP preference between genotypes on the first test day after only 2 drug paired sessions.

The GIRK channel found in VTA DA neurons, GIRK2/GIRK3 heteromer, is unique and may be a key mediator of behavioral sensitivity to drugs of abuse [180, 188]. Previous studies involving the global loss of *Girk2* have established its importance in the motor-stimulatory effects of drugs of abuse, such as systemic morphine and cocaine [228, 321]. The results of this study confirm that DA neuron specific loss of *Girk2* results in a similar enhanced motor-stimulatory profile to drugs of abuse. Interestingly, constitutive *Girk3*^{-/-} exhibit decreased sensitivity to the motor-stimulatory effect of morphine, ethanol, and cocaine [228, 321, 331, 332], which can be restored by viral re-repression of *Girk3* in the VTA. These results, and work done by Dr. Christian Lüscher's lab, suggest that GIRK channel subunit composition in VTA DA neurons can influence the sensitivity to drugs of abuse. Thus, the loss of *Girk2* (*i.e.* no functional GIRK channels) removes all GPCR-GIRK inhibitory control, while the loss of *Girk3* (*i.e.* GIRK2 homomeric channels) results in enhanced sensitivity to GPCR-dependent activation. Collectively, these findings support the emerging hypothesis that the unique GIRK channel found in VTA DA neurons, the GIRK2/GIRK3 channel, mediates the behavioral sensitivity to various drugs of abuse, and might prove to be a useful target for treating some aspects of drug addiction.

CHAPTER 4

Discussion

General Summary

Although drugs of abuse exert their effects through a variety of molecular and cellular targets, they also produce overlapping behavioral effects, suggesting a common neuronal target that is critical to addiction-related behaviors [197, 198]. GIRK channel activity has been shown to be a key regulator of excitability in the CNS. Disruption or dysregulation of GIRK-dependent signaling is associated with many disorders, including Down syndrome, Parkinson's disease, psychiatric disorders (depression, anxiety, ADHD, epilepsy, schizophrenia), and drug addiction [165, 186, 187, 190-193]. GIRK-dependent signaling has also been shown to play a role in alterations to cellular activity and behavior following exposure to various drugs of abuse [188, 195, 199, 200]. Current pharmacotherapies for addiction are targeted toward relevant GPCRs, but are not very effective in treating addiction and often have undesirable off target effects [135]. More direct targeting of GIRK channels may provide more effective treatment of addiction and other CNS disorders.

Using electrophysiological, genetic, and pharmacologic approaches, we have shown the nuances of subunit composition, expression, function, and drug-induced adaptations of GPCR-GIRK signaling in the mesocorticolimbic DA system. We demonstrated in Chapter 2 that GIRK-dependent signaling in VTA DA neurons, but not midbrain GABA neurons, is required for opioid-induced motor-stimulation. Further, the GIRK2/GIRK3 heteromer found in VTA DA neurons regulates sensitivity to the motor-stimulatory effect of opioids [321]. In addition to its importance to opioid reward, the GIRK2/GIRK3 heteromer found in VTA DA neurons may also play an important role in regulating cocaine sensitivity. This is supported by work described in Chapter 3 showing

the loss of GIRK-dependent signaling in VTA DA neurons blunts D₂R autoinhibitory feedback and enhances reward-related behavioral responses to cocaine. Thus, the unique GIRK channel expressed in VTA DA neurons, GIRK2/GIRK3 heteromer, may be a therapeutic target to regulate the sensitivity of the mesocorticolimbic DA system to various drugs of abuse.

The relevance of GIRK channels to the cellular and reward-related behavioral effects of opioids

The mesocorticolimbic DA system, comprised of the VTA and reciprocal connections to downstream targets, mediates the reward-related behavioral effects of drugs of abuse, including opioids [74]. Since the 1980s, it was believed that GIRK channels were responsible for mediating the direct inhibitory effect of opioids on VTA GABA neurons, thus disinhibiting the VTA DA neurons, resulting in enhanced DA neurotransmission [83, 214]. The direct inhibitory effect of opioids was thought to be mediated by the MOR activation of GIRK channels.

Accordingly, we predicted that a loss of GIRK signaling in VTA GABA neurons would reduce the opioid-induced disinhibition of VTA DA neurons, resulting in reduced motor-stimulation. We found that VTA GABA neurons do indeed express GIRK channels comprised of GIRK1 and GIRK2 subunits; however, genetic ablation of either GIRK subunit failed to reduce the motor-stimulatory effect of opioids. Additionally, direct stimulation of GIRK1-containing channels on VTA GABA neurons with the novel small molecule activator, ML297, was not sufficient to induce locomotor activation. Surprisingly, our results indicate that manipulations to GIRK-dependent signaling in VTA DA neurons were capable of altering opioid-induced motor-stimulation. These

genetic manipulations to enhance or suppress GIRK channel function correlated with decreased and increased sensitivity to opioid-induced motor-stimulation. Collectively, this work suggests that the unique GIRK channel found in VTA DA neurons, a GIRK2/GIRK3 heteromer, regulates the behavioral sensitivity to opioids.

While many cellular and behavioral effects of opioids have been linked to GIRK-dependent signaling, opioids can also exert their effects through presynaptic and non-GIRK somatodendritic mechanisms. The site(s) of action of MOR agonists that are most relevant to opioid reward-related signaling and behavior remain unclear. MOR signaling results in the activation of a number of downstream targets including the inhibition of adenylyl cyclase, the activation of other K^+ channels, and the inhibition of voltage-gated Ca^{2+} channels. MORs are found at neuron terminals (presynaptic) and somatodendritic sites in VTA GABA neurons [208]. MORs found at presynaptic sites are commonly-associated with the inhibition of voltage-gated Ca^{2+} channels, leading to reduced Ca^{2+} entry and neurotransmitter release [333]. MORs found at somatodendritic locations are commonly-associated with K^+ channel activation [208]. Although our studies argue against the involvement of GIRK channels, there may be other K^+ channels present that can be activated by opioids, such as TREK channels [334]. One explanation for our results showing the lack of GIRK involvement in VTA GABA neurons to opioid-induced motor stimulation is that MOR may couple preferentially to a non-GIRK effector in VTA GABA neurons. The relevant contributions of non-GIRK mechanisms can be assessed using genetic and pharmacologic approaches to modulate different K^+ and/or voltage-gated Ca^{2+} channel activity, and/or measure the opioid-induced disinhibition of VTA DA neurons, as well as opioid-induced changes in firing rate (Hz). At this point, it is still

unclear through which mechanism opioids work to exert their direct inhibitory effects on midbrain GABA neurons, and how this results in disinhibition of VTA DA neurons.

The understanding of neuron subtypes within the VTA has significantly evolved since our initial investigations into opioid signaling in the VTA. Recent studies have found that VTA DA neurons are actually a heterogeneous population of DA neurons that vary in projection targets and electrophysiological properties. VTA DA neurons exhibit distinct functional properties that can be directly linked to their projection target [297, 335, 336]. As a result, a random evaluation of VTA DA neurons might give variable results and make outcomes difficult to interpret. An example of VTA DA diversity can be seen in Figure 2. 4D/E, which showed that only 1/3 of the VTA DA neurons sampled, showed a direct inhibitory response to DAMGO. Given the relevance of DA neurotransmission in the NAc and mPFC to the acute rewarding effect of opioids, a retrograde labeling approach could be used to label specific VTA DA neuron subpopulations based on their projection sites, as was previously done for neurons from the mPFC [187]. These experiments would be done in Pitx3-eGFP(+) mice to allow for easy identification of eGFP(+) cells (*i.e.* DA neurons) that also express fluorescent retrobeads. eGFP(+)/retrobead(+) neurons would be targeted for electrophysiological evaluation to probe the impact of projection targets on direct MOR actions on VTA DA neurons.

While evidence has shown that GIRK-dependent signaling mediates the spinal analgesic effects of opioids and sensitivity to the motor-stimulatory effect of opioids [270, 271, 321, 337], it is unclear if alterations to GIRK channels activity would alter opioid reward. For the studies discussed in Chapter 2, we focused on opioid-induced

motor activation because it is an unconditioned behavioral response to opioids that is dependent on VTA-NAc DA neurotransmission [212, 260]. However, to directly connect GIRK-dependent signaling in VTA DA neurons to opioid reward, we would need to use alternative behavioral paradigms, such as CPP and/or self-administration (SA). We would begin by looking at morphine CPP in our GADCre:*Girk2*^{fllox/fllox} and DATCre:*Girk2*^{fllox/fllox} mice. Given that the loss of *Girk2* in GABA neurons had no apparent effect on morphine-induced motor stimulation, we would not expect to see any effect on CPP responses to morphine in GADCre(+/-):*Girk2*^{fllox/fllox} mice. However, we would expect that the DATCre(+):*Girk2*^{fllox/fllox} mice would exhibit altered CPP to morphine compared to DATCre(-):*Girk2*^{fllox/fllox} controls.

In addition to the improvements made in our understanding of VTA DA neuron heterogeneity, recent studies have highlighted the importance of another GABAergic structure to the activity of VTA DA neurons, the rostromedial tegmental nucleus (RMTg). While it has been shown that RMTg GABA neurons can regulate the activity of VTA DA neurons and are sensitive to opioids [266, 267, 287, 288, 290-294], our initial studies suggest that opioid-induced suppression of GIRK-dependent signaling in the RMTg is not responsible for the disinhibition of VTA DA neurons. However, there is still much to be learned regarding the GIRK subunit composition and the importance of MOR-GIRK signaling to the activity of RMTg GABA neurons. We chose to use *Girk1*^{-/-} mice for our RMTg studies based on the evidence that the predominant GIRK channel within the CNS is the GIRK1/GIRK2 heteromer. Although this was a logical first step, RMTg GABA neurons may not express GIRK1 and instead express a more unique GIRK channel subtype, as is the case for VTA DA neurons. The lack of GIRK1-containing

channels could explain why we did not see a difference in DAMGO-induced locomotor activity between *Girk1*^{-/-} and WT mice. Direct activation of GIRK1-containing channels with ML297 in the RMTg also failed to stimulate motor activity. More experiments are needed to examine the GIRK subunit composition of RMTg GABA neurons. This could be accomplished using single cell RT-PCR, similar to the approach used for VTA DA and GABA neurons [187, 188], to detect the presence of GIRK1, GIRK2, GIRK3, and GIRK4 mRNAs. Constitutive *Girk*^{-/-} mice could also be used to electrophysiologically characterize GIRK subunit contribution to GIRK-dependent signaling in RMTg GABA neurons. It is also possible that independent of GIRK channel subtype, GIRK-dependent signaling is not the primary mechanism through which opioids inhibit RMTg GABA neurons. If this is the case, there are other postsynaptic mechanisms through which opioids could act that could be more relevant to the disinhibition of VTA DA neurons, and subsequent reward-related behavioral responses.

The studies covered in Chapter 2 have challenged long-held assumptions about opioid signaling in the VTA. We have shown that VTA DA neuron GIRK channel subunit composition can influence the behavioral sensitivity to opioids. Further insight into the relevance of GIRK-dependent signaling in VTA DA neurons to morphine reward is required to determine whether it is possible to dissect the clinically beneficial outcomes (analgesia) from the undesirable side effects (addiction) of opioids.

The relevance of VTA DA neuron GIRK-dependent signaling to the cellular and reward-related behavioral effects of cocaine

Cocaine exposure has been shown to produce acute alterations and persistent synaptic adaptations to both excitatory and inhibitory signaling within the mesocorticolimbic DA system, with inhibitory changes linked to alterations in GPCR-dependent signaling [91, 123, 150, 298, 299, 312-316]. Cocaine exposure results in increased extracellular DA levels, both in downstream targets and within the VTA. Extracellular DA in the VTA binds to inhibitory metabotropic receptors, D₂Rs. D₂R activation represents a key autoinhibitory mechanism that tempers VTA DA neurotransmission, and it is mediated in large part by the activation of GIRK channels [299, 318, 319]. DA neurons express a unique GIRK channel, a heteromer containing GIRK2 and GIRK3 subunits [188]. To evaluate the behavioral impact of the loss of GIRK-dependent signaling in DA neurons, we created a novel conditional *Girk2*^{-/-} mouse line, referred to as DATCre:*Girk2*^{fllox/fllox}.

Our previous work using the DATCre:*Girk2*^{fllox/fllox} model showed that the selective loss of *Girk2* in DA neurons attenuates baclofen- and opioid-induced currents in VTA DA neurons, while enhancing systemic opioid-induced locomotor stimulation [321]. Selective loss of *Girk2* in DA neurons significantly blunted currents evoked by the D₂R agonist quinpirole. In addition to a reduction in inhibitory signaling, the DA neuron-selective loss of *Girk2* enhances both the acute and repeated locomotor-stimulatory response to cocaine; however, the importance of VTA DA GIRK-dependent signaling to cocaine reward (CPP) requires further evaluation. The impact *Girk2* expression has on cocaine-induced locomotor activity suggests that the unique GIRK channel expressed in

VTA DA neurons may be a therapeutic target to regulate the sensitivity of the mesolimbic DA system to various drugs of abuse.

Cocaine triggers enhanced excitatory neurotransmission within the mesocorticolimbic DA system [128, 338-342]. We have reported that the global loss of *Girk2*^{-/-} results in enhanced excitatory neurotransmission in VTA DA neurons and the medium spiny neurons (MSNs) of the NAc [228]. Cocaine exposure also triggers a transient suppression of GIRK-dependent signaling and a reduction in GIRK membrane expression in the VTA and mPFC [187, 199]. The reported locomotor responses to acute and repeated cocaine in Chapter 3 are interesting given that the loss of GIRK-dependent signaling seems to pre-sensitize mice to the behavioral effects of cocaine. Collectively, the results of these studies beg the question: what is the relationship between the cocaine-induced suppression of GIRK channel activity and the cocaine-induced adaptation in excitatory signaling? One possibility is that the cocaine-induced suppression of GIRK channel activity removes a threshold that allows for the enhancement of excitatory neurotransmission in the mesocorticolimbic DA system. To test this hypothesis, we could measure excitatory neurotransmission following the selective loss of *Girk2* in DA neurons. These studies would look at miniature excitatory postsynaptic current (mEPSCs) amplitude and frequency in VTA DA and NAc MSNs of DATCre(+):*Girk2*^{lox/lox} and control mice.

One concern regarding the behavioral studies in Chapter 3 is the selectivity of the manipulations to VTA DA signaling in our DATCre(+):*Girk2*^{lox/lox} mice. The use of a transgenic Cre-driven ablation of a “floxed” gene, in this case *Girk2*, should remove the gene from all neurons expressing the DAT promoter. DAT expression, while primarily

restricted to DA neurons, is not limited to the VTA. Thorough characterization of this DATCre line shows that Cre expression is strong in other DAergic populations, including the substantia nigra [280, 343]. To support our assertion that the Cre-induced reduction in *Girk2* expression is restricted to DA neurons, we have shown that changes to GIRK-dependent signaling in DATCre(+):*Girk2*^{lox/lox} mice are seen in VTA DA neurons but not VTA GABA neurons. This genetic approach to *Girk2* ablation also means that *Girk2* is not present in DA neurons during development, allowing for compensatory adaptations to occur. Since this is a more targeted loss of *Girk2*, we would not expect to see the same level of compensatory adaptations, notably enhanced mesocorticolimbic excitatory transmission, as we see with the global loss of *Girk2*; a possibility that still confounds clean interpretation of data.

These initial findings in Chapter 3 regarding the role of GIRK-dependent signaling in the cellular and behavioral effects of cocaine were done with the DATCre(+/-):*Girk2*^{lox/lox} mice line, meaning GIRK2-dependent signaling has been lost in all DA neurons. To more specifically link GIRK-dependent signaling in VTA DA neurons to the cellular and behavioral effects of cocaine, we need to restrict our manipulations to DA neurons within the VTA. One way to achieve this is through viral suppression of *Girk2*. We have developed a Cre-dependent shRNA viral construct (LV-CMV-eGFP-DIO-U6-shRNA) that targets *Girk2*, as well as a scrambled control. Viral infusion targeting the VTA of DATCre(+) mice should restrict GIRK2 knockdown to DA neurons within the VTA [187, 321]. A Cre-dependent shRNA viral strategy offers many advantages to constitutive genetic manipulations, such as anatomic specificity and allowing *Girk2* to be present throughout development until viral treatment in adulthood.

Based on our initial findings, it is expected that a viral knockdown of *Girk2* would result in diminished baclofen- and quinpirole-induced currents. If GIRK signaling in VTA DA neurons is critical to the reward-related behavioral effects of cocaine, we would also expect that mice experiencing a knockdown of *Girk2* would exhibit enhanced motor-stimulation and altered CPP compared to scrambled viral controls.

There are many unanswered questions pertaining to the studies discussed in Chapter 3 that will require further examination. Although we have seen that loss of GIRK2 in DA neurons alters the motor-stimulatory response to cocaine, with DATCre(+):*Girk2*^{lox/lox} mice showing pre-sensitization to the initial exposure of cocaine, to answer our questions about the influence of GIRK-dependent signaling on cocaine reward, we need to test this mouse model using established reward-related behavioral paradigms, such as CPP and SA.

Although we expect to see an enhanced magnitude of CPP from DATCre(+):*Girk2*^{lox/lox} mice in our low-dose session dependent model, the CPP paradigm is not the best behavioral model of human addiction. In the CPP behavioral paradigm, the drug is experimenter-administered and not contingent on the subject behavior. Cocaine SA is a response-contingent paradigm where the subject must work to receive cocaine, and this is a more accurate model of human drug intake and willingness to work for the drug. These studies would be done in collaboration with Dr. Michael Beckstead to look at what effect the loss of GIRK-dependent signaling in VTA DA neurons has on acquisition of cocaine SA [344]. We would also want to establish a dose-response relationship for cocaine SA based on the differences we have seen in acute cocaine motor-stimulation. My hypothesis is that the DATCre(+):*Girk2*^{lox/lox} mice

would show enhanced SA and have more robust responses at lower doses of cocaine compared to DATCre(-):*Girk2*^{fllox/fllox} controls, if in fact the loss of *Girk2* in DA neurons enhances the subject's sensitivity to cocaine. Further insight into the relevance of GIRK-dependent signaling in VTA DA neurons to cocaine reward could be useful to our understanding and the treatment of cocaine addiction.

Future Directions

VTA DA GIRK2/GIRK3 channel: a key regulator of the behavioral sensitivity to drugs of abuse

The work discussed in this dissertation and additional studies from other labs, has implicated the unique GIRK channel subtype found in VTA DA neurons, the GIRK2/GIRK3 heteromer, in the sensitivity of the mesocorticolimbic DA system to circuit-level and behavioral effects of drugs of abuse [180, 188, 321]. Previous studies involving VTA DA neurons from *Girk3*^{-/-} mice, which express GIRK2 homomeric channels in VTA DA neurons, are more sensitive to GPCR stimulation than wild-type counterparts [180]. The VTA DA GIRK channel has also been shown to play a role in the motor-stimulatory effect evoked by drugs of abuse. Constitutive *Girk2*^{-/-} mice have been shown to be more sensitive to the motor-stimulatory effect of cocaine and morphine [228], whereas, constitutive *Girk3*^{-/-} mice appear to be less sensitive to the motor-stimulatory effects of opioids and ethanol [321, 331, 332]. The work presented in Chapters 2 and 3 has shown that weakening GIRK channel activity (*i.e.* *Girk2* ablation, no functional GIRK channels) in VTA DA neurons correlates with enhanced behavioral sensitivity to drugs of abuse, presumably reflecting a manifestation of enhanced DA

neurotransmission in downstream targets of the VTA in the mesocorticolimbic DA system. Alternatively, enhancing GIRK channel activity (*i.e.* loss of *Girk3*, GIRK2 homomeric channels) in VTA DA neurons correlates with decreased behavioral sensitivity to morphine, presumably reflecting reduced DA neurotransmission. These results would suggest that this unique GIRK channel subtype plays a vital role in controlling VTA DA activity, and may serve as a barrier to the rewarding effects of various drugs of abuse, such as morphine and cocaine. Based on these findings therapeutic approaches, genetic or pharmacologic, targeting the activity of the GIRK2/GIRK3 channel might prove useful for treating some aspects of drug addiction.

DATCre:Girk3^{lox/lox} mouse line

To complement the studies we have done with the conditional *Girk2^{-/-}* line looking at the relevance of GIRK-dependent signaling in VTA DA neurons to the rewarding effects of drugs of abuse, we have acquired a conditional *Girk3^{-/-}* line from the Medical Research Council Harwell (strain: Kcnj9^{tm1a(EUCOMM)Hmgu}). These mice will be crossed with the DATCre line (strain: #006660, Jackson Labs) to generate a DA neuron specific loss of *Girk3*, DATCre:*Girk3^{lox/lox}* mice. To characterize this novel line, we would begin by crossing the DATCre:*Girk3^{lox/lox}* line with our Pitx3-eGFP mouse line. The Pitx3-eGFP line allows for easier electrophysiological identification of DA neurons within the VTA, as they express eGFP under the control of a DA neuron-specific promoter, the Pitx3 gene. Immunohistochemistry could be done on brain slices containing the VTA from DATCre(+):*Girk3^{lox/lox}* and DATCre(-):*Girk3^{lox/lox}* mice, staining for *Girk3* (Cat# APC-038, Alomone labs) expression.

Functional validation of this line could also be done using whole-cell patch-clamp slice electrophysiology. Previous work suggests that the DA neuron-selective loss of *Girk3* should have no impact in our saturating concentration baclofen-current response assay, since constitutive ablation of *Girk3* has no impact [180, 228]. To answer this question we would use a baclofen concentration-response protocol, and based on our hypothesis that the GIRK2/GIRK3 channel in VTA DA neurons can regulate the sensitivity to various drugs of abuse, we would expect to see that VTA DA neurons from DATCre(+):*Girk3*^{flox/flox} mice were more sensitive to baclofen than VTA DA neurons from DATCre(-):*Girk3*^{flox/flox} controls. In relation to the work done in Chapter 3, DATCre:*Girk3*^{flox/flox} mice could then be used to test the impact of *Girk3* ablation on D₂R-dependent signaling and DA neurotransmission in VTA DA neurons. Assuming successful functional validation of DATCre:*Girk3*^{flox/flox} line, we would assess the reward-related behavioral effects of various drugs of abuse, such as morphine and cocaine, through similar efforts described in Chapters 2 and 3. Based on our hypothesis that the strength of GIRK-dependent signaling in VTA DA neurons is a key regulatory of the behavioral sensitivity to drugs of abuse, I would expect that DATCre(+):*Girk3*^{flox/flox} mice will exhibit reduced reward-related behavioral responses to morphine and cocaine, as compared to DATCre(-):*Girk3*^{flox/flox} controls; this phenotype should be revealed by a downward shift in drug dose/reward-related behavior activity relationship.

Selective GIRK2/GIRK3 pharmacology

The importance of GIRK channel function to many physiological processes makes them a logical candidate for various therapeutic indications. However, due to the fact that GIRK channels are vital to proper cardiac function [345, 346], we must be able

to achieve regional and/or subunit-selective pharmacology to tap into the therapeutic potential of GIRK channels. Until recently the compounds available for modulation of GIRK channel activity have lacked potency and selectivity, but a novel family of small molecule modulators of GIRK channels was discovered by Dr. C. David Weaver at Vanderbilt University, using high-throughput screen technology [279]. The compound ML297 has been shown to activate GIRK1-containing channels selectively, in a G protein-independent manner, through binding structures unique to the GIRK1 subunit [347]. ML297 was also shown to have therapeutic potential as an antiepileptic and anxiolytic compound, and it lacks significant reward liability as assessed by CPP [279, 347]. The ability of ML297 to alter GIRK-dependent signaling in a subunit-dependent manner, with low abuse liability, represents a promising tool to further study the therapeutic potential of GIRK channel manipulations in other clinical disorders.

Aside from analgesia, GIRK channel activity has also been linked to the development of addiction. The work discussed in Chapters 2 and 3 has highlighted the importance of the unique VTA DA GIRK channel, the GIRK2/GIRK3 heteromer, to the sensitivity of various drugs of abuse. As shown in Chapters 2 and 3, removal of GIRK-dependent signaling in VTA DA neurons, as shown in the DATCre(+):*Girk2*^{flox/flox} mice, resulted in decrease inhibitory GPCR GIRK-dependent signaling and enhanced reward-related behavioral responses. These findings suggest that the discovery of a GIRK2/GIRK3 channel selective compound, such as a GIRK2/GIRK3 selective agonist, could be useful as a therapeutic for the treatment and/or prevention of addiction to various drugs of abuse.

Concluding Thoughts

Drug abuse is a critical global problem, with opioid and cocaine abuse being among the top offenders [3, 4]. With the high social and economic costs associated with illicit drug use, it is vital that we gain a better understanding of the cellular and molecular targets of drugs of abuse, and how modulation of these mechanisms can ultimately lead to the development of addiction. Much of the recent work in the field has focused on drug-induced adaptations to excitatory signaling within the mesocorticolimbic DA system; however, the adaptations and importance of inhibitory signaling requires further investigation. The goal of this dissertation was to enhance our understanding of the role of GIRK-dependent inhibitory signaling throughout the mesocorticolimbic DA system, and in particular, how it mediates the cellular and behavioral effects of drugs of abuse.

The work discussed here has challenged the opioid signaling “dogma” and shown that GIRK-dependent signaling in midbrain GABA neurons is not required for disinhibition of VTA DA neurons and subsequent opioid-induced motor-stimulation. Rather, it appears the unique GIRK2/GIRK3 channel found in VTA DA neurons can regulate the behavioral sensitivity to morphine. Interestingly, we found that this unique GIRK channel subtype can also modulate the behavioral sensitivity to cocaine, and possibly other drugs of abuse. This work supports the feasibility of dissecting the clinically-desired analgesic effects from undesirable effects of opioids, by exploiting subunit differences in GIRK channel composition.

Further, the discovery of ML297 and validation of its selectivity for GIRK1-containing channels gives us hope for GIRK subunit-selective compounds as useful therapeutics. The work covered here would suggest that while important to the analgesic

effect of opioids in the spinal cord, GIRK1-containing channels do not seem to be vital to the reward-related behavioral effects of opioids. These results, and the fact that ML297 lacks significant reward liability as assessed by CPP, support further studies to examine the analgesic ability of ML297. ML297 may be able to provide what current opioid therapies cannot, analgesia without the risk of tolerance and abuse. The specificity and effectiveness of ML97 also gives hope for the discovery of a GIRK2/GIRK3 channel-selective agonist that could be useful to reduce cravings or the propensity for relapse in addicts. Collectively, the findings of these studies demonstrate that inhibitory signaling through GIRK channels in neurons of the VTA is critical to the molecular and behavioral effects of drugs of abuse, and suggest that novel compounds that modulate GIRK channel function in a subunit-dependent manner could help us prevent and/or treat addiction.

REFERENCES

1. Association, A.P., *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*. 2013, Arlington, VA: American Psychiatric Association.
2. Services, S.A.a.M.H. and Administration, *Results from the 2012 National Survey on Drug Use and Health: Summary of National Findings*, S.A.a.M.H. Services and Administration, Editors. 2013: Rockville, MD.
3. Nicholls, L., L. Bragaw, and C. Ruetsch, *Opioid dependence treatment and guidelines*. *Journal of managed care pharmacy : JMCP*, 2010. 16(1 Suppl B): p. S14-21.
4. *World Drug Report 2008*, U. Nations, Editor. 2008, United Nations Publication.
5. CASAColumbia, *Addiction medicine: Closing the gap between science and practice*, T.N.C.o.A.a.S.A.C. University, Editor. 2012: New York, NY.
6. *World Drug Report 2012* UN, Editor. 2012, United Nations publication.
7. Center, N.D.I., *The Economic Impact of Illicit Drug Use on American Society*, U.S.D.o. Justice, Editor. 2011, United States Department of Justice: Washington D.C.
8. CASAColumbia, *The Cost of Substance Abuse to America's Health Care System* T.N.C.o.A.a.S.A.a.C. University, Editor. 1993, CASA: New York, NY.
9. Everitt, B.J., *Neural and psychological mechanisms underlying compulsive drug seeking habits and drug memories--indications for novel treatments of addiction*. *Eur J Neurosci*, 2014. 40(1): p. 2163-82.
10. McLellan, A.T., et al., *Drug dependence, a chronic medical illness: implications for treatment, insurance, and outcomes evaluation*. *JAMA*, 2000. 284(13): p. 1689-95.
11. Wise, R.A., *Neurobiology of addiction*. *Curr Opin Neurobiol*, 1996. 6(2): p. 243-51.
12. Adinoff, B., *Neurobiologic processes in drug reward and addiction*. *Harv Rev Psychiatry*, 2004. 12(6): p. 305-20.
13. Wise, R.A. and M.A. Bozarth, *Brain substrates for reinforcement and drug self-administration*. *Progress in neuro-psychopharmacology*, 1981. 5(5-6): p. 467-74.
14. Devine, D.P. and R.A. Wise, *Self-administration of morphine, DAMGO, and DPDPE into the ventral tegmental area of rats*. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 1994. 14(4): p. 1978-84.
15. Pierce, R.C. and V. Kumaresan, *The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse?* *Neurosci Biobehav Rev*, 2006. 30(2): p. 215-38.
16. Ritz, M.C., E.J. Cone, and M.J. Kuhar, *Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters: a structure-activity study*. *Life Sci*, 1990. 46(9): p. 635-45.
17. Treatment, C.f.S.A., *Treatment Improvement Protocol (TIP)* D.S.A.a.M.H. Administration, Editor. 2004, DHHS Publication NO. (SMA) 04-3939.
18. O'Brien, C.P. and A.T. McLellan, *Myths about the treatment of addiction*. *Lancet*, 1996. 347(8996): p. 237-40.
19. NIH, *Treatment Approaches for Drug Addiction*, NIDA, Editor. 2009.

20. Smyth, B.P., et al., *Lapse and relapse following inpatient treatment of opiate dependence*. Ir Med J, 2010. 103(6): p. 176-9.
21. Simpson, D.D., et al., *A national evaluation of treatment outcomes for cocaine dependence*. Arch Gen Psychiatry, 1999. 56(6): p. 507-14.
22. Bevacqua, B.K., *Book Review*. New England Journal of Medicine, 2004. 350(20): p. 2109-2111.
23. Brownstein, M.J., *A brief history of opiates, opioid peptides, and opioid receptors*. Proc Natl Acad Sci U S A, 1993. 90(12): p. 5391-3.
24. Rosenblum, A., et al., *Opioids and the treatment of chronic pain: controversies, current status, and future directions*. Exp Clin Psychopharmacol, 2008. 16(5): p. 405-16.
25. Trescot, A.M., et al., *Opioid pharmacology*. Pain physician, 2008. 11(2 Suppl): p. S133-53.
26. Booth, M., *Opium: A History*. 1996, New York NY: St Martin's Griffin.
27. Helen Askitopoulou, I.A.R., Eleni Konsolaki, *Archaeological evidence on the use of opium in the Minoan world*. International Congress Series 2002. 1242: p. 23-29.
28. Frank Dikötter, L.L., Zhou Xun, *Narcotic Culture: A History of Drugs in China*. 2004, London: C. Hurst & Co. Ltd.
29. Breasted, J.H., *Ancient Records of Egypt*. University of Chicago Oriental Institute Publications. Vol. III. June 2001: University of Chicago Press.
30. Li Hui Chen, H.H., Margaret Warner, *Drug-poisoning Deaths Involving Opioid Analgesics: United States, 1999-2011*, N.C.f.H. Statistics, Editor. 2014, NCHS data brief: Hyattsville, MD.
31. in *Approaching Death: Improving Care at the End of Life*, M.J. Field and C.K. Cassel, Editors. 1997: Washington (DC).
32. Schnoll, S.H. and M.F. Weaver, *Addiction and pain*. Am J Addict, 2003. 12 Suppl 2: p. S27-35.
33. Breitbart, W., et al., *Patient-related barriers to pain management in ambulatory AIDS patients*. Pain, 1998. 76(1-2): p. 9-16.
34. Schmitz, R., *Friedrich Wilhelm Serturmer and the discovery of morphine*. Pharm Hist, 1985. 27(2): p. 61-74.
35. Pattinson, K.T., *Opioids and the control of respiration*. Br J Anaesth, 2008. 100(6): p. 747-58.
36. Ganesh, A. and L.G. Maxwell, *Pathophysiology and management of opioid-induced pruritus*. Drugs, 2007. 67(16): p. 2323-33.
37. Porreca, F. and M.H. Ossipov, *Nausea and vomiting side effects with opioid analgesics during treatment of chronic pain: mechanisms, implications, and management options*. Pain Med, 2009. 10(4): p. 654-62.
38. Byas-Smith, M.G., et al., *The effect of opioids on driving and psychomotor performance in patients with chronic pain*. Clin J Pain, 2005. 21(4): p. 345-52.
39. Swegle, J.M. and C. Logemann, *Management of common opioid-induced adverse effects*. Am Fam Physician, 2006. 74(8): p. 1347-54.
40. Benyamin, R., et al., *Opioid complications and side effects*. Pain Physician, 2008. 11(2 Suppl): p. S105-20.

41. Pud, D., et al., *Opioids and abnormal pain perception: New evidence from a study of chronic opioid addicts and healthy subjects*. Drug Alcohol Depend, 2006. 82(3): p. 218-23.
42. Pacifici, R., et al., *Effect of morphine and methadone acute treatment on immunological activity in mice: pharmacokinetic and pharmacodynamic correlates*. J Pharmacol Exp Ther, 1994. 269(3): p. 1112-6.
43. Roy, S., et al., *Opioid drug abuse and modulation of immune function: consequences in the susceptibility to opportunistic infections*. J Neuroimmune Pharmacol, 2011. 6(4): p. 442-65.
44. Daniell, H.W., *Hypogonadism in men consuming sustained-action oral opioids*. J Pain, 2002. 3(5): p. 377-84.
45. Daniell, H.W., *DHEAS deficiency during consumption of sustained-action prescribed opioids: evidence for opioid-induced inhibition of adrenal androgen production*. J Pain, 2006. 7(12): p. 901-7.
46. Daniell, H.W., *Opioid endocrinopathy in women consuming prescribed sustained-action opioids for control of nonmalignant pain*. J Pain, 2008. 9(1): p. 28-36.
47. Collett, B.J., *Opioid tolerance: the clinical perspective*. Br J Anaesth, 1998. 81(1): p. 58-68.
48. Vowles, K.E., et al., *Rates of opioid misuse, abuse, and addiction in chronic pain: a systematic review and data synthesis*. Pain, 2015. 156(4): p. 569-76.
49. Fiellin, D.A. and P.G. O'Connor, *Clinical practice. Office-based treatment of opioid-dependent patients*. N Engl J Med, 2002. 347(11): p. 817-23.
50. Treatment, C.f.S.A., *Detoxification and substance abuse treatment training manual-based on treatment improvement protocol (TIP) Series 45*, D.S.A.a.m.H.S. Administration, Editor. 2008, DHHS Publication NO. (SAS) 08-4331.
51. Helm, S., et al., *Opioid antagonists, partial agonists, and agonists/antagonists: the role of office-based detoxification*. Pain Physician, 2008. 11(2): p. 225-35.
52. WHO, *World Health Organization: Guidelines for the Psychosocially Assisted Pharmacological Treatment of Opioid Dependence*. 2009: WHO Press.
53. Fraud, C.A.I., *Prescription for peril: how insurance fraud finances theft and abuse of addictive prescription drugs*. 2007, Coalition Against Insurance Fraud: Washington DC.
54. Subcommittee, P.D.A., *Addressing Prescription Drug Abuse in the United States: Current Activities and Future Opportunities*, U.S.D.o.H.a.H. Services, Editor. 2013: Washington, DC.
55. Siegel, R.K., *Cocaine smoking*. J Psychoactive Drugs, 1982. 14(4): p. 271-359.
56. Naranjo, P., *Social function of coca in pre-Columbian American*. J Ethnopharmacol, 1981. 3(2-3): p. 161-72.
57. George F. Koob, M.L.M., *Neurobiology of Addiction*. 2006, Elsevier Inc. .
58. Karch, S.B., *Cocaine: history, use, abuse*. J R Soc Med, 1999. 92(8): p. 393-7.
59. Orr, K. and D. Taylor, *Psychostimulants in the treatment of depression : a review of the evidence*. CNS Drugs, 2007. 21(3): p. 239-57.
60. Wood, S., et al., *Psychostimulants and cognition: a continuum of behavioral and cognitive activation*. Pharmacol Rev, 2014. 66(1): p. 193-221.

61. Das, G., *Cocaine abuse in North America: a milestone in history*. J Clin Pharmacol, 1993. 33(4): p. 296-310.
62. Phillips, K.A., D.H. Epstein, and K.L. Preston, *Psychostimulant addiction treatment*. Neuropharmacology, 2014. 87: p. 150-60.
63. Winger G, W.J.H., Hofmann F.G., *A Handbook on Drug and Alcohol Abuse: The Biomedical Aspects*. 4th ed ed. 2004, New York, NY: Oxford University Press.
64. Romanelli, F. and K.M. Smith, *Clinical effects and management of methamphetamine abuse*. Pharmacotherapy, 2006. 26(8): p. 1148-56.
65. Ellinwood, E.H., Jr. and T.H. Lee, *Dose- and time-dependent effects of stimulants*. NIDA Res Monogr, 1989. 94: p. 323-40.
66. Fasano, A., et al., *Cocaine addiction: from habits to stereotypical-repetitive behaviors and punning*. Drug Alcohol Depend, 2008. 96(1-2): p. 178-82.
67. Gay, G.R., *Clinical management of acute and chronic cocaine poisoning*. Ann Emerg Med, 1982. 11(10): p. 562-72.
68. Karch, S.B., *Cocaine cardiovascular toxicity*. South Med J, 2005. 98(8): p. 794-9.
69. Mooney, M.E., et al., *Effects of oral methamphetamine on cocaine use: a randomized, double-blind, placebo-controlled trial*. Drug Alcohol Depend, 2009. 101(1-2): p. 34-41.
70. Grabowski, J., et al., *Dextroamphetamine for cocaine-dependence treatment: a double-blind randomized clinical trial*. J Clin Psychopharmacol, 2001. 21(5): p. 522-6.
71. George, T.P., et al., *Disulfiram versus placebo for cocaine dependence in buprenorphine-maintained subjects: a preliminary trial*. Biol Psychiatry, 2000. 47(12): p. 1080-6.
72. Carroll, K.M., et al., *Efficacy of disulfiram and cognitive behavior therapy in cocaine-dependent outpatients: a randomized placebo-controlled trial*. Arch Gen Psychiatry, 2004. 61(3): p. 264-72.
73. Jayaram-Lindstrom, N., et al., *Naltrexone attenuates the subjective effects of amphetamine in patients with amphetamine dependence*. Neuropsychopharmacology, 2008. 33(8): p. 1856-63.
74. Koob, G.F. and E.J. Nestler, *The neurobiology of drug addiction*. J Neuropsychiatry Clin Neurosci, 1997. 9(3): p. 482-97.
75. Koob, G.F. and N.D. Volkow, *Neurocircuitry of addiction*. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2010. 35(1): p. 217-38.
76. Pfaus, J.G., *Neurobiology of sexual behavior*. Curr Opin Neurobiol, 1999. 9(6): p. 751-8.
77. Kelley, A.E., *Memory and addiction: shared neural circuitry and molecular mechanisms*. Neuron, 2004. 44(1): p. 161-79.
78. Dichter, G.S., C.A. Damiano, and J.A. Allen, *Reward circuitry dysfunction in psychiatric and neurodevelopmental disorders and genetic syndromes: animal models and clinical findings*. J Neurodev Disord, 2012. 4(1): p. 19.
79. Koob, G.F. and N.D. Volkow, *Neurocircuitry of addiction*. Neuropsychopharmacology, 2010. 35(1): p. 217-38.
80. Luscher, C. and R.C. Malenka, *Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling*. Neuron, 2011. 69(4): p. 650-63.

81. Ikemoto, S., *Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex*. Brain Res Rev, 2007. 56(1): p. 27-78.
82. Carr, D.B. and S.R. Sesack, *GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex*. Synapse, 2000. 38(2): p. 114-23.
83. Johnson, S.W. and R.A. North, *Two types of neurone in the rat ventral tegmental area and their synaptic inputs*. The Journal of physiology, 1992. 450: p. 455-68.
84. Yamaguchi, T., W. Sheen, and M. Morales, *Glutamatergic neurons are present in the rat ventral tegmental area*. The European journal of neuroscience, 2007. 25(1): p. 106-18.
85. Morales, M. and D.H. Root, *Glutamate neurons within the midbrain dopamine regions*. Neuroscience, 2014. 282C: p. 60-68.
86. Dobi, A., et al., *Glutamatergic and nonglutamatergic neurons of the ventral tegmental area establish local synaptic contacts with dopaminergic and nondopaminergic neurons*. J Neurosci, 2010. 30(1): p. 218-29.
87. Van Bockstaele, E.J. and V.M. Pickel, *GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain*. Brain Res, 1995. 682(1-2): p. 215-21.
88. Kalivas, P.W., et al., *Enkephalin action on the mesolimbic system: a dopamine-dependent and a dopamine-independent increase in locomotor activity*. The Journal of pharmacology and experimental therapeutics, 1983. 227(1): p. 229-37.
89. Baumeister, A.A., et al., *The antinociceptive and motivational effects of intranigral injection of opioid agonists*. Neuropharmacology, 1993. 32(12): p. 1299-303.
90. Nestler, E.J., *The neurobiology of cocaine addiction*. Sci Pract Perspect, 2005. 3(1): p. 4-10.
91. Brebner, K., R. Phelan, and D.C. Roberts, *Intra-VTA baclofen attenuates cocaine self-administration on a progressive ratio schedule of reinforcement*. Pharmacol Biochem Behav, 2000. 66(4): p. 857-62.
92. Ranaldi, R. and R.A. Wise, *Blockade of D1 dopamine receptors in the ventral tegmental area decreases cocaine reward: possible role for dendritically released dopamine*. J Neurosci, 2001. 21(15): p. 5841-6.
93. Bozarth, M.A. and R.A. Wise, *Localization of the reward-relevant opiate receptors*. NIDA research monograph, 1982. 41: p. 158-64.
94. Vezina, P., *Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs*. Neurosci Biobehav Rev, 2004. 27(8): p. 827-39.
95. Roberts, D.C. and G.F. Koob, *Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats*. Pharmacol Biochem Behav, 1982. 17(5): p. 901-4.
96. Bozarth, M.A., *Neural basis of psychomotor stimulant and opiate reward: evidence suggesting the involvement of a common dopaminergic system*. Behavioural brain research, 1986. 22(2): p. 107-16.
97. Siddiqui, S.V., et al., *Neuropsychology of prefrontal cortex*. Indian J Psychiatry, 2008. 50(3): p. 202-8.

98. Masterman, D.L. and J.L. Cummings, *Frontal-subcortical circuits: the anatomic basis of executive, social and motivated behaviors*. J Psychopharmacol, 1997. 11(2): p. 107-14.
99. Anderson, S.W., et al., *Impairments of emotion and real-world complex behavior following childhood- or adult-onset damage to ventromedial prefrontal cortex*. J Int Neuropsychol Soc, 2006. 12(2): p. 224-35.
100. Berlin, H.A., E.T. Rolls, and U. Kischka, *Impulsivity, time perception, emotion and reinforcement sensitivity in patients with orbitofrontal cortex lesions*. Brain, 2004. 127(Pt 5): p. 1108-26.
101. Floden, D., et al., *Impulsivity and risk-taking behavior in focal frontal lobe lesions*. Neuropsychologia, 2008. 46(1): p. 213-23.
102. Bechara, A., et al., *Insensitivity to future consequences following damage to human prefrontal cortex*. Cognition, 1994. 50(1-3): p. 7-15.
103. Conde, F., et al., *Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents*. J Comp Neurol, 1995. 352(4): p. 567-93.
104. Gabbott, P.L., et al., *Local-circuit neurones in the medial prefrontal cortex (areas 25, 32 and 24b) in the rat: morphology and quantitative distribution*. J Comp Neurol, 1997. 377(4): p. 465-99.
105. Sesack, S.R., et al., *Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin*. J Comp Neurol, 1989. 290(2): p. 213-42.
106. Carr, D.B. and S.R. Sesack, *Terminals from the rat prefrontal cortex synapse on mesoaccumbens VTA neurons*. Ann N Y Acad Sci, 1999. 877: p. 676-8.
107. Sesack, S.R. and V.M. Pickel, *Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area*. J Comp Neurol, 1992. 320(2): p. 145-60.
108. Kelley, A.E., *Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning*. Neurosci Biobehav Rev, 2004. 27(8): p. 765-76.
109. Kalivas, P.W. and N.D. Volkow, *The neural basis of addiction: a pathology of motivation and choice*. Am J Psychiatry, 2005. 162(8): p. 1403-13.
110. Goldstein, R.Z. and N.D. Volkow, *Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex*. Am J Psychiatry, 2002. 159(10): p. 1642-52.
111. Volkow, N.D., et al., *Relationship between psychostimulant-induced "high" and dopamine transporter occupancy*. Proc Natl Acad Sci U S A, 1996. 93(19): p. 10388-92.
112. Maas, L.C., et al., *Functional magnetic resonance imaging of human brain activation during cue-induced cocaine craving*. Am J Psychiatry, 1998. 155(1): p. 124-6.
113. Childress, A.R., et al., *Limbic activation during cue-induced cocaine craving*. Am J Psychiatry, 1999. 156(1): p. 11-8.
114. Steketee, J.D. and P.W. Kalivas, *Drug wanting: behavioral sensitization and relapse to drug-seeking behavior*. Pharmacol Rev, 2011. 63(2): p. 348-65.

115. Luscher, C., *Drug-evoked synaptic plasticity causing addictive behavior*. J Neurosci, 2013. 33(45): p. 17641-6.
116. McFarland, K. and P.W. Kalivas, *The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior*. J Neurosci, 2001. 21(21): p. 8655-63.
117. Baker, D.A., et al., *Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse*. Nat Neurosci, 2003. 6(7): p. 743-9.
118. van Huijstee, A.N. and H.D. Mansvelder, *Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction*. Front Cell Neurosci, 2014. 8: p. 466.
119. McFarland, K., C.C. Lapish, and P.W. Kalivas, *Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior*. J Neurosci, 2003. 23(8): p. 3531-7.
120. See, R.E., *Dopamine D1 receptor antagonism in the prelimbic cortex blocks the reinstatement of heroin-seeking in an animal model of relapse*. Int J Neuropsychopharmacol, 2009. 12(3): p. 431-6.
121. Sun, W. and G.V. Rebec, *The role of prefrontal cortex D1-like and D2-like receptors in cocaine-seeking behavior in rats*. Psychopharmacology (Berl), 2005. 177(3): p. 315-23.
122. Everitt, B.J. and M.E. Wolf, *Psychomotor stimulant addiction: a neural systems perspective*. J Neurosci, 2002. 22(9): p. 3312-20.
123. Steketee, J.D., *Neurotransmitter systems of the medial prefrontal cortex: potential role in sensitization to psychostimulants*. Brain Res Brain Res Rev, 2003. 41(2-3): p. 203-28.
124. Pierce, R.C., et al., *Repeated intra-ventral tegmental area administration of SKF-38393 induces behavioral and neurochemical sensitization to a subsequent cocaine challenge*. J Pharmacol Exp Ther, 1996. 278(1): p. 384-92.
125. Kalivas, P.W. and P. Duffy, *Repeated cocaine administration alters extracellular glutamate in the ventral tegmental area*. J Neurochem, 1998. 70(4): p. 1497-502.
126. Wolf, M.E., *Addiction: making the connection between behavioral changes and neuronal plasticity in specific pathways*. Mol Interv, 2002. 2(3): p. 146-57.
127. Thomas, M.J., P.W. Kalivas, and Y. Shaham, *Neuroplasticity in the mesolimbic dopamine system and cocaine addiction*. Br J Pharmacol, 2008. 154(2): p. 327-42.
128. Ungless, M.A., et al., *Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons*. Nature, 2001. 411(6837): p. 583-7.
129. Saal, D., et al., *Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons*. Neuron, 2003. 37(4): p. 577-82.
130. Argilli, E., et al., *Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area*. J Neurosci, 2008. 28(37): p. 9092-100.
131. Kauer, J.A. and R.C. Malenka, *Synaptic plasticity and addiction*. Nat Rev Neurosci, 2007. 8(11): p. 844-58.
132. Pignatelli, M. and A. Bonci, *Role of Dopamine Neurons in Reward and Aversion: A Synaptic Plasticity Perspective*. Neuron, 2015. 86(5): p. 1145-57.
133. Robinson, T.E. and K.C. Berridge, *Addiction*. Annu Rev Psychol, 2003. 54: p. 25-53.
134. Wettschureck, N. and S. Offermanns, *Mammalian G proteins and their cell type specific functions*. Physiol Rev, 2005. 85(4): p. 1159-204.

135. Schlyer, S. and R. Horuk, *I want a new drug: G-protein-coupled receptors in drug development*. Drug Discov Today, 2006. 11(11-12): p. 481-93.
136. Drews, J., *Drug discovery: a historical perspective*. Science, 2000. 287(5460): p. 1960-4.
137. Kobilka, B.K., *G protein coupled receptor structure and activation*. Biochim Biophys Acta, 2007. 1768(4): p. 794-807.
138. Fredriksson, R., et al., *The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints*. Mol Pharmacol, 2003. 63(6): p. 1256-72.
139. Bjarnadottir, T.K., et al., *Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse*. Genomics, 2006. 88(3): p. 263-73.
140. George, S.R., B.F. O'Dowd, and S.P. Lee, *G-protein-coupled receptor oligomerization and its potential for drug discovery*. Nat Rev Drug Discov, 2002. 1(10): p. 808-20.
141. Milligan, G., *G protein-coupled receptor dimerization: function and ligand pharmacology*. Mol Pharmacol, 2004. 66(1): p. 1-7.
142. Kristiansen, K., *Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function*. Pharmacol Ther, 2004. 103(1): p. 21-80.
143. Oldham, W.M. and H.E. Hamm, *Heterotrimeric G protein activation by G-protein-coupled receptors*. Nat Rev Mol Cell Biol, 2008. 9(1): p. 60-71.
144. Tuteja, N., *Signaling through G protein coupled receptors*. Plant Signal Behav, 2009. 4(10): p. 942-7.
145. Downes, G.B. and N. Gautam, *The G protein subunit gene families*. Genomics, 1999. 62(3): p. 544-52.
146. Carter, B.D. and F. Medzihradsky, *Go mediates the coupling of the mu opioid receptor to adenylyl cyclase in cloned neural cells and brain*. Proc Natl Acad Sci U S A, 1993. 90(9): p. 4062-6.
147. Wittpoth, C., et al., *Regions on adenylyl cyclase that are necessary for inhibition of activity by beta gamma and G(ialpha) subunits of heterotrimeric G proteins*. Proc Natl Acad Sci U S A, 1999. 96(17): p. 9551-6.
148. Johnson, D.A., et al., *Dynamics of cAMP-dependent protein kinase*. Chem Rev, 2001. 101(8): p. 2243-70.
149. Wickman, K. and D.E. Clapham, *Ion channel regulation by G proteins*. Physiol Rev, 1995. 75(4): p. 865-85.
150. Hearing, M.C., A.N. Zink, and K. Wickman, *Cocaine-induced adaptations in metabotropic inhibitory signaling in the mesocorticolimbic system*. Rev Neurosci, 2012. 23(4): p. 325-51.
151. Jeong, S.W. and S.R. Ikeda, *Effect of G protein heterotrimer composition on coupling of neurotransmitter receptors to N-type Ca(2+) channel modulation in sympathetic neurons*. Proc Natl Acad Sci U S A, 2000. 97(2): p. 907-12.
152. Herlitze, S., et al., *Modulation of Ca²⁺ channels by G-protein beta gamma subunits*. Nature, 1996. 380(6571): p. 258-62.

153. Wickman, K.D., et al., *Recombinant G-protein beta gamma-subunits activate the muscarinic-gated atrial potassium channel*. *Nature*, 1994. 368(6468): p. 255-7.
154. Krapivinsky, G., et al., *G beta gamma binds directly to the G protein-gated K⁺ channel, IKACH*. *J Biol Chem*, 1995. 270(49): p. 29059-62.
155. Inanobe, A., et al., *Characterization of G-protein-gated K⁺ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra*. *J Neurosci*, 1999. 19(3): p. 1006-17.
156. Reuveny, E., et al., *Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits*. *Nature*, 1994. 370(6485): p. 143-6.
157. Karschin, C., et al., *IRK(1-3) and GIRK(1-4) inwardly rectifying K⁺ channel mRNAs are differentially expressed in the adult rat brain*. *J Neurosci*, 1996. 16(11): p. 3559-70.
158. Padgett, C.L. and P.A. Slesinger, *GABAB receptor coupling to G-proteins and ion channels*. *Adv Pharmacol*, 2010. 58: p. 123-47.
159. Saenz del Burgo, L., et al., *Distribution and neurochemical characterization of neurons expressing GIRK channels in the rat brain*. *J Comp Neurol*, 2008. 510(6): p. 581-606.
160. Fernandez-Alacid, L., et al., *Developmental regulation of G protein-gated inwardly-rectifying K⁺ (GIRK/Kir3) channel subunits in the brain*. *Eur J Neurosci*, 2011. 34(11): p. 1724-36.
161. Ciruela, F., et al., *Evidence for oligomerization between GABAB receptors and GIRK channels containing the GIRK1 and GIRK3 subunits*. *Eur J Neurosci*, 2010. 32(8): p. 1265-77.
162. Ulens, C., P. Daenens, and J. Tytgat, *The dual modulation of GIRK1/GIRK2 channels by opioid receptor ligands*. *Eur J Pharmacol*, 1999. 385(2-3): p. 239-45.
163. Kim, K.M., Y. Nakajima, and S. Nakajima, *G protein-coupled inward rectifier modulated by dopamine agonists in cultured substantia nigra neurons*. *Neuroscience*, 1995. 69(4): p. 1145-58.
164. Lujan, R., et al., *New insights into the therapeutic potential of Girk channels*. *Trends Neurosci*, 2014. 37(1): p. 20-9.
165. Luscher, C. and P.A. Slesinger, *Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease*. *Nature reviews. Neuroscience*, 2010. 11(5): p. 301-15.
166. Yamada, M., A. Inanobe, and Y. Kurachi, *G protein regulation of potassium ion channels*. *Pharmacol Rev*, 1998. 50(4): p. 723-60.
167. Lujan, R. and C. Aguado, *Localization and Targeting of GIRK Channels in Mammalian Central Neurons*. *Int Rev Neurobiol*, 2015. 123: p. 161-200.
168. Ma, D., et al., *Diverse trafficking patterns due to multiple traffic motifs in G protein-activated inwardly rectifying potassium channels from brain and heart*. *Neuron*, 2002. 33(5): p. 715-29.
169. Lesage, F., et al., *Molecular properties of neuronal G-protein-activated inwardly rectifying K⁺ channels*. *J Biol Chem*, 1995. 270(48): p. 28660-7.
170. Slesinger, P.A., et al., *Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K⁺ channels*. *Neuron*, 1996. 16(2): p. 321-31.

171. Jelacic, T.M., et al., *Functional and biochemical evidence for G-protein-gated inwardly rectifying K⁺ (GIRK) channels composed of GIRK2 and GIRK3*. J Biol Chem, 2000. 275(46): p. 36211-6.
172. Jelacic, T.M., S.M. Sims, and D.E. Clapham, *Functional expression and characterization of G-protein-gated inwardly rectifying K⁺ channels containing GIRK3*. J Membr Biol, 1999. 169(2): p. 123-9.
173. Kofuji, P., N. Davidson, and H.A. Lester, *Evidence that neuronal G-protein-gated inwardly rectifying K⁺ channels are activated by G beta gamma subunits and function as heteromultimers*. Proc Natl Acad Sci U S A, 1995. 92(14): p. 6542-6.
174. Kennedy, M.E., et al., *GIRK4 confers appropriate processing and cell surface localization to G-protein-gated potassium channels*. J Biol Chem, 1999. 274(4): p. 2571-82.
175. Lunn, M.L., et al., *A unique sorting nexin regulates trafficking of potassium channels via a PDZ domain interaction*. Nat Neurosci, 2007. 10(10): p. 1249-59.
176. Balana, B., et al., *Mechanism underlying selective regulation of G protein-gated inwardly rectifying potassium channels by the psychostimulant-sensitive sorting nexin 27*. Proc Natl Acad Sci U S A, 2011. 108(14): p. 5831-6.
177. Kulik, A., et al., *Subcellular localization of metabotropic GABA(B) receptor subunits GABA(B1a/b) and GABA(B2) in the rat hippocampus*. J Neurosci, 2003. 23(35): p. 11026-35.
178. Luscher, C., et al., *G protein-coupled inwardly rectifying K⁺ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons*. Neuron, 1997. 19(3): p. 687-95.
179. Lujan, R., J. Maylie, and J.P. Adelman, *New sites of action for GIRK and SK channels*. Nat Rev Neurosci, 2009. 10(7): p. 475-80.
180. Labouebe, G., et al., *RGS2 modulates coupling between GABAB receptors and GIRK channels in dopamine neurons of the ventral tegmental area*. Nature neuroscience, 2007. 10(12): p. 1559-68.
181. Koyrakh, L., et al., *Molecular and cellular diversity of neuronal G-protein-gated potassium channels*. J Neurosci, 2005. 25(49): p. 11468-78.
182. Aguado, C., et al., *Cell type-specific subunit composition of G protein-gated potassium channels in the cerebellum*. J Neurochem, 2008. 105(2): p. 497-511.
183. Ladera, C., et al., *Pre-synaptic GABA receptors inhibit glutamate release through GIRK channels in rat cerebral cortex*. J Neurochem, 2008. 107(6): p. 1506-17.
184. Liao, Y.J., Y.N. Jan, and L.Y. Jan, *Heteromultimerization of G-protein-gated inwardly rectifying K⁺ channel proteins GIRK1 and GIRK2 and their altered expression in weaver brain*. J Neurosci, 1996. 16(22): p. 7137-50.
185. Koyrakh, L., et al., *Molecular and cellular diversity of neuronal G-protein-gated potassium channels*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2005. 25(49): p. 11468-78.
186. Cruz, H.G., et al., *Absence and rescue of morphine withdrawal in GIRK/Kir3 knock-out mice*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2008. 28(15): p. 4069-77.
187. Hearing, M., et al., *Repeated cocaine weakens GABA(B)-Girk signaling in layer 5/6 pyramidal neurons in the prelimbic cortex*. Neuron, 2013. 80(1): p. 159-70.

188. Cruz, H.G., et al., *Bi-directional effects of GABA(B) receptor agonists on the mesolimbic dopamine system*. Nature neuroscience, 2004. 7(2): p. 153-9.
189. Wydeven, N., et al., *Structural elements in the Girk1 subunit that potentiate G protein-gated potassium channel activity*. Proc Natl Acad Sci U S A, 2012. 109(52): p. 21492-7.
190. Signorini, S., et al., *Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K⁺ channel GIRK2*. Proceedings of the National Academy of Sciences of the United States of America, 1997. 94(3): p. 923-7.
191. Mitrovic, I., et al., *Contribution of GIRK2-mediated postsynaptic signaling to opiate and alpha 2-adrenergic analgesia and analgesic sex differences*. Proceedings of the National Academy of Sciences of the United States of America, 2003. 100(1): p. 271-6.
192. Morgan, A.D., et al., *Decreased cocaine self-administration in Kir3 potassium channel subunit knockout mice*. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2003. 28(5): p. 932-8.
193. Harkins, A.B. and A.P. Fox, *Cell death in weaver mouse cerebellum*. Cerebellum, 2002. 1(3): p. 201-6.
194. Sago, H., et al., *Ts1Cje, a partial trisomy 16 mouse model for Down syndrome, exhibits learning and behavioral abnormalities*. Proc Natl Acad Sci U S A, 1998. 95(11): p. 6256-61.
195. Tipps, M.E. and K.J. Buck, *GIRK Channels: A Potential Link Between Learning and Addiction*. Int Rev Neurobiol, 2015. 123: p. 239-77.
196. Luscher, C. and M.A. Ungless, *The mechanistic classification of addictive drugs*. PLoS medicine, 2006. 3(11): p. e437.
197. Nestler, E.J., *Is there a common molecular pathway for addiction?* Nature neuroscience, 2005. 8(11): p. 1445-9.
198. Nestler, E.J., *Cellular basis of memory for addiction*. Dialogues Clin Neurosci, 2013. 15(4): p. 431-43.
199. Arora, D., et al., *Acute cocaine exposure weakens GABA(B) receptor-dependent G-protein-gated inwardly rectifying K⁺ signaling in dopamine neurons of the ventral tegmental area*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2011. 31(34): p. 12251-7.
200. Marron Fernandez de Velasco, E., N. McCall, and K. Wickman, *GIRK Channel Plasticity and Implications for Drug Addiction*. Int Rev Neurobiol, 2015. 123: p. 201-38.
201. Dhawan, B.N., et al., *International Union of Pharmacology. XII. Classification of opioid receptors*. Pharmacol Rev, 1996. 48(4): p. 567-92.
202. Feng, Y., et al., *Current research on opioid receptor function*. Curr Drug Targets, 2012. 13(2): p. 230-46.
203. Ahlbeck, K., *Opioids: a two-faced Janus*. Curr Med Res Opin, 2011. 27(2): p. 439-48.
204. Inturrisi, C.E., *Clinical pharmacology of opioids for pain*. Clin J Pain, 2002. 18(4 Suppl): p. S3-13.
205. Law, P.Y., Y.H. Wong, and H.H. Loh, *Molecular mechanisms and regulation of opioid receptor signaling*. Annu Rev Pharmacol Toxicol, 2000. 40: p. 389-430.

206. Sesack, S.R. and V.M. Pickel, *Ultrastructural relationships between terminals immunoreactive for enkephalin, GABA, or both transmitters in the rat ventral tegmental area*. Brain Res, 1995. 672(1-2): p. 261-75.
207. Capogna, M., B.H. Gähwiler, and S.M. Thompson, *Mechanism of mu-opioid receptor-mediated presynaptic inhibition in the rat hippocampus in vitro*. J Physiol, 1993. 470: p. 539-58.
208. Williams, J.T., M.J. Christie, and O. Manzoni, *Cellular and synaptic adaptations mediating opioid dependence*. Physiological reviews, 2001. 81(1): p. 299-343.
209. Torrecilla, M., et al., *G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. 22(11): p. 4328-34.
210. Al-Hasani, R. and M.R. Bruchas, *Molecular mechanisms of opioid receptor-dependent signaling and behavior*. Anesthesiology, 2011. 115(6): p. 1363-81.
211. Devine, D.P. and R.A. Wise, *Self-Administration of Morphine, Damgo, and Dpdpe into the Ventral Tegmental Area of Rats*. Journal of Neuroscience, 1994. 14(4): p. 1978-1984.
212. Kalivas, P.W., et al., *Behavioral and neurochemical effects of neurotensin microinjection into the ventral tegmental area of the rat*. Neuroscience, 1983. 8(3): p. 495-505.
213. Fields, H.L. and E.B. Margolis, *Understanding opioid reward*. Trends Neurosci, 2015. 38(4): p. 217-25.
214. Johnson, S.W. and R.A. North, *Opioids excite dopamine neurons by hyperpolarization of local interneurons*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 1992. 12(2): p. 483-8.
215. Matthews, R.T. and D.C. German, *Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine*. Neuroscience, 1984. 11(3): p. 617-25.
216. Gysling, K. and R.Y. Wang, *Morphine-induced activation of A10 dopamine neurons in the rat*. Brain research, 1983. 277(1): p. 119-27.
217. Di Chiara, G. and A. Imperato, *Preferential stimulation of dopamine release in the nucleus accumbens by opiates, alcohol, and barbiturates: studies with transcerebral dialysis in freely moving rats*. Ann N Y Acad Sci, 1986. 473: p. 367-81.
218. Chefer, V.I., B.L. Kieffer, and T.S. Shippenberg, *Basal and morphine-evoked dopaminergic neurotransmission in the nucleus accumbens of MOR- and DOR-knockout mice*. Eur J Neurosci, 2003. 18(7): p. 1915-22.
219. Howell, L.L. and H.L. Kimmel, *Monoamine transporters and psychostimulant addiction*. Biochem Pharmacol, 2008. 75(1): p. 196-217.
220. Volkow, N.D., et al., *Imaging dopamine's role in drug abuse and addiction*. Neuropharmacology, 2009. 56 Suppl 1: p. 3-8.
221. Ciccarone, D., *Stimulant abuse: pharmacology, cocaine, methamphetamine, treatment, attempts at pharmacotherapy*. Prim Care, 2011. 38(1): p. 41-58, v-vi.
222. Gainetdinov, R.R., T.D. Sotnikova, and M.G. Caron, *Monoamine transporter pharmacology and mutant mice*. Trends Pharmacol Sci, 2002. 23(8): p. 367-73.

223. Torres, G.E., R.R. Gainetdinov, and M.G. Caron, *Plasma membrane monoamine transporters: structure, regulation and function*. Nat Rev Neurosci, 2003. 4(1): p. 13-25.
224. Chen, R., et al., *Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter*. Proc Natl Acad Sci U S A, 2006. 103(24): p. 9333-8.
225. Di Chiara, G., *A motivational learning hypothesis of the role of mesolimbic dopamine in compulsive drug use*. Journal of psychopharmacology, 1998. 12(1): p. 54-67.
226. Bradberry, C.W. and R.H. Roth, *Cocaine increases extracellular dopamine in rat nucleus accumbens and ventral tegmental area as shown by in vivo microdialysis*. Neurosci Lett, 1989. 103(1): p. 97-102.
227. Thomas, M.J. and R.C. Malenka, *Synaptic plasticity in the mesolimbic dopamine system*. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 2003. 358(1432): p. 815-9.
228. Arora, D., et al., *Altered neurotransmission in the mesolimbic reward system of Girk mice*. Journal of neurochemistry, 2010. 114(5): p. 1487-97.
229. Padgett, C.L., et al., *Methamphetamine-evoked depression of GABA(B) receptor signaling in GABA neurons of the VTA*. Neuron, 2012. 73(5): p. 978-89.
230. Steketee, J.D. and C.E. Beyer, *Injections of baclofen into the ventral medial prefrontal cortex block the initiation, but not the expression, of cocaine sensitization in rats*. Psychopharmacology (Berl), 2005. 180(2): p. 352-8.
231. Jayaram, P. and J.D. Steketee, *Effects of repeated cocaine on medial prefrontal cortical GABAB receptor modulation of neurotransmission in the mesocorticolimbic dopamine system*. J Neurochem, 2004. 90(4): p. 839-47.
232. Jayaram, P. and J.D. Steketee, *Effects of cocaine-induced behavioural sensitization on GABA transmission within rat medial prefrontal cortex*. Eur J Neurosci, 2005. 21(7): p. 2035-9.
233. Adell, A. and F. Artigas, *The somatodendritic release of dopamine in the ventral tegmental area and its regulation by afferent transmitter systems*. Neurosci Biobehav Rev, 2004. 28(4): p. 415-31.
234. Lalive, A.L., et al., *Firing modes of dopamine neurons drive bidirectional GIRK channel plasticity*. J Neurosci, 2014. 34(15): p. 5107-14.
235. Bello, E.P., et al., *Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors*. Nat Neurosci, 2011. 14(8): p. 1033-8.
236. Rouge-Pont, F., et al., *Changes in extracellular dopamine induced by morphine and cocaine: crucial control by D2 receptors*. J Neurosci, 2002. 22(8): p. 3293-301.
237. Muscat, R., M.M. Iravani, and Z.L. Kruk, *The role of ventral tegmental dopamine neurons in locomotor sensitization following quinpirole or (+)-amphetamine: ex vivo voltammetric evidence*. Neuroscience, 1996. 75(4): p. 1175-84.
238. Sesack, S.R., C. Aoki, and V.M. Pickel, *Ultrastructural localization of D2 receptor-like immunoreactivity in midbrain dopamine neurons and their striatal targets*. J Neurosci, 1994. 14(1): p. 88-106.

239. Beckstead, M.J., et al., *Vesicular dopamine release elicits an inhibitory postsynaptic current in midbrain dopamine neurons*. *Neuron*, 2004. 42(6): p. 939-46.
240. Marinelli, M., et al., *Impulse activity of midbrain dopamine neurons modulates drug-seeking behavior*. *Psychopharmacology (Berl)*, 2003. 168(1-2): p. 84-98.
241. Ackerman, J.M. and F.J. White, *A10 somatodendritic dopamine autoreceptor sensitivity following withdrawal from repeated cocaine treatment*. *Neurosci Lett*, 1990. 117(1-2): p. 181-7.
242. Gao, W.Y., et al., *Alterations in baseline activity and quinpirole sensitivity in putative dopamine neurons in the substantia nigra and ventral tegmental area after withdrawal from cocaine pretreatment*. *Neuropsychopharmacology*, 1998. 18(3): p. 222-32.
243. Nimitvilai, S. and M.S. Brodie, *Reversal of prolonged dopamine inhibition of dopaminergic neurons of the ventral tegmental area*. *J Pharmacol Exp Ther*, 2010. 333(2): p. 555-63.
244. Volkow, N.D., et al., *Effects of chronic cocaine abuse on postsynaptic dopamine receptors*. *Am J Psychiatry*, 1990. 147(6): p. 719-24.
245. Jin, W. and Z. Lu, *A novel high-affinity inhibitor for inward-rectifier K⁺ channels*. *Biochemistry*, 1998. 37(38): p. 13291-9.
246. Ransom, C.B. and H. Sontheimer, *Biophysical and pharmacological characterization of inwardly rectifying K⁺ currents in rat spinal cord astrocytes*. *J Neurophysiol*, 1995. 73(1): p. 333-46.
247. Shirasaka, T., H. Kannan, and M. Takasaki, *Activation of a G protein-coupled inwardly rectifying K⁺ current and suppression of I_h contribute to dexmedetomidine-induced inhibition of rat hypothalamic paraventricular nucleus neurons*. *Anesthesiology*, 2007. 107(4): p. 605-15.
248. Jin, W. and Z. Lu, *Synthesis of a stable form of tertiapin: a high-affinity inhibitor for inward-rectifier K⁺ channels*. *Biochemistry*, 1999. 38(43): p. 14286-93.
249. Kanjhan, R., et al., *Tertiapin-Q blocks recombinant and native large conductance K⁺ channels in a use-dependent manner*. *J Pharmacol Exp Ther*, 2005. 314(3): p. 1353-61.
250. Bettahi, I., et al., *Contribution of the Kir3.1 subunit to the muscarinic-gated atrial potassium channel IK_{ACh}*. *J Biol Chem*, 2002. 277(50): p. 48282-8.
251. Cohen-Tannoudji, M. and C. Babinet, *Beyond 'knock-out' mice: new perspectives for the programmed modification of the mammalian genome*. *Mol Hum Reprod*, 1998. 4(10): p. 929-38.
252. Berridge, K.C., *From prediction error to incentive salience: mesolimbic computation of reward motivation*. *Eur J Neurosci*, 2012. 35(7): p. 1124-43.
253. Robinson, T.E. and K.C. Berridge, *The neural basis of drug craving: an incentive-sensitization theory of addiction*. *Brain Res Brain Res Rev*, 1993. 18(3): p. 247-91.
254. Grimm, J.W., et al., *Neuroadaptation. Incubation of cocaine craving after withdrawal*. *Nature*, 2001. 412(6843): p. 141-2.
255. Salamone, J.D. and M. Correa, *The mysterious motivational functions of mesolimbic dopamine*. *Neuron*, 2012. 76(3): p. 470-85.

256. Di Chiara, G. and A. Imperato, *Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats*. Proceedings of the National Academy of Sciences of the United States of America, 1988. 85(14): p. 5274-8.
257. Bontempi, B. and F.R. Sharp, *Systemic morphine-induced Fos protein in the rat striatum and nucleus accumbens is regulated by mu opioid receptors in the substantia nigra and ventral tegmental area*. J Neurosci, 1997. 17(21): p. 8596-612.
258. Garcia, M.M., H.E. Brown, and R.E. Harlan, *Alterations in immediate-early gene proteins in the rat forebrain induced by acute morphine injection*. Brain Res, 1995. 692(1-2): p. 23-40.
259. Kalivas, P.W., P. Duffy, and H. Eberhardt, *Modulation of A10 dopamine neurons by gamma-aminobutyric acid agonists*. J Pharmacol Exp Ther, 1990. 253(2): p. 858-66.
260. Stinus, L., et al., *Locomotor activation induced by infusion of endorphins into the ventral tegmental area: evidence for opiate-dopamine interactions*. Proc Natl Acad Sci U S A, 1980. 77(4): p. 2323-7.
261. Longoni, R., L. Spina, and G. Di Chiara, *Dopaminergic D-1 receptors: essential role in morphine-induced hypermotility*. Psychopharmacology (Berl), 1987. 93(3): p. 401-2.
262. Hnasko, T.S., B.N. Sotak, and R.D. Palmiter, *Morphine reward in dopamine-deficient mice*. Nature, 2005. 438(7069): p. 854-7.
263. Tian, M., et al., *Altered hematopoiesis, behavior, and sexual function in mu opioid receptor-deficient mice*. J Exp Med, 1997. 185(8): p. 1517-22.
264. Contarino, A., et al., *Lack of reward and locomotor stimulation induced by heroin in mu-opioid receptor-deficient mice*. Eur J Pharmacol, 2002. 446(1-3): p. 103-9.
265. Yoo, J.H., et al., *Differential effects of morphine and cocaine on locomotor activity and sensitization in mu-opioid receptor knockout mice*. Neurosci Lett, 2003. 344(1): p. 37-40.
266. Matsui, A. and J.T. Williams, *Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons*. J Neurosci, 2011. 31(48): p. 17729-35.
267. Matsui, A., et al., *Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal*. Neuron, 2014. 82(6): p. 1346-56.
268. Bergevin, A., et al., *Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons*. Neuropharmacology, 2002. 42(8): p. 1065-78.
269. Marker, C.L., et al., *Distinct populations of spinal cord lamina II interneurons expressing G-protein-gated potassium channels*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2006. 26(47): p. 12251-9.
270. Marker, C.L., M. Stoffel, and K. Wickman, *Spinal G-protein-gated K⁺ channels formed by GIRK1 and GIRK2 subunits modulate thermal nociception and contribute to morphine analgesia*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2004. 24(11): p. 2806-12.

271. Marker, C.L., et al., *Spinal G-protein-gated potassium channels contribute in a dose-dependent manner to the analgesic effect of mu- and delta- but not kappa-opioids*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2005. 25(14): p. 3551-9.
272. Tamamaki, N., et al., *Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse*. The Journal of comparative neurology, 2003. 467(1): p. 60-79.
273. Zhao, S., et al., *Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons*. The European journal of neuroscience, 2004. 19(5): p. 1133-40.
274. Wei, J., et al., *Characterization of murine Girk2 transcript isoforms: structure and differential expression*. Genomics, 1998. 51(3): p. 379-90.
275. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method*. Methods, 2001. 25(4): p. 402-8.
276. Pravetoni, M. and K. Wickman, *Behavioral characterization of mice lacking GIRK/Kir3 channel subunits*. Genes, brain, and behavior, 2008. 7(5): p. 523-31.
277. Costa, A.C., et al., *G-protein-gated potassium (GIRK) channels containing the GIRK2 subunit are control hubs for pharmacologically induced hypothermic responses*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2005. 25(34): p. 7801-4.
278. Taniguchi, H., et al., *A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex*. Neuron, 2011. 71(6): p. 995-1013.
279. Kaufmann, K., et al., *ML297 (VU0456810), the first potent and selective activator of the GIRK potassium channel, displays antiepileptic properties in mice*. ACS Chem Neurosci, 2013. 4(9): p. 1278-86.
280. Backman, C.M., et al., *Characterization of a mouse strain expressing Cre recombinase from the 3' untranslated region of the dopamine transporter locus*. Genesis, 2006. 44(8): p. 383-90.
281. Cameron, D.L., M.W. Wessendorf, and J.T. Williams, *A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids*. Neuroscience, 1997. 77(1): p. 155-66.
282. Ford, C.P., G.P. Mark, and J.T. Williams, *Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2006. 26(10): p. 2788-97.
283. Margolis, E.B., et al., *Direct bidirectional mu-opioid control of midbrain dopamine neurons*. J Neurosci, 2014. 34(44): p. 14707-16.
284. Margolis, E.B., et al., *Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons*. J Neurosci, 2003. 23(31): p. 9981-6.
285. Garzon, M. and V.M. Pickel, *Plasmalemmal mu-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area*. Synapse, 2001. 41(4): p. 311-28.
286. Bocklisch, C., et al., *Cocaine disinhibits dopamine neurons by potentiation of GABA transmission in the ventral tegmental area*. Science, 2013. 341(6153): p. 1521-5.

287. Omelchenko, N. and S.R. Sesack, *Ultrastructural analysis of local collaterals of rat ventral tegmental area neurons: GABA phenotype and synapses onto dopamine and GABA cells*. Synapse, 2009. 63(10): p. 895-906.
288. Chieng, B., et al., *Distinct cellular properties of identified dopaminergic and GABAergic neurons in the mouse ventral tegmental area*. The Journal of physiology, 2011. 589(Pt 15): p. 3775-87.
289. Steffensen, S.C., et al., *Contingent and non-contingent effects of heroin on mu-opioid receptor-containing ventral tegmental area GABA neurons*. Exp Neurol, 2006. 202(1): p. 139-51.
290. Barrot, M., et al., *Braking dopamine systems: a new GABA master structure for mesolimbic and nigrostriatal functions*. J Neurosci, 2012. 32(41): p. 14094-101.
291. Bourdy, R. and M. Barrot, *A new control center for dopaminergic systems: pulling the VTA by the tail*. Trends Neurosci, 2012. 35(11): p. 681-90.
292. Hong, S., et al., *Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates*. J Neurosci, 2011. 31(32): p. 11457-71.
293. Jalabert, M., et al., *Neuronal circuits underlying acute morphine action on dopamine neurons*. Proceedings of the National Academy of Sciences of the United States of America, 2011. 108(39): p. 16446-50.
294. Lecca, S., et al., *Effects of drugs of abuse on putative rostromedial tegmental neurons, inhibitory afferents to midbrain dopamine cells*. Neuropsychopharmacology, 2011. 36(3): p. 589-602.
295. Watabe-Uchida, M., et al., *Whole-brain mapping of direct inputs to midbrain dopamine neurons*. Neuron, 2012. 74(5): p. 858-73.
296. Kalivas, P.W., L. Churchill, and M.A. Klitenick, *GABA and enkephalin projection from the nucleus accumbens and ventral pallidum to the ventral tegmental area*. Neuroscience, 1993. 57(4): p. 1047-60.
297. Margolis, E.B., et al., *Identification of rat ventral tegmental area GABAergic neurons*. PLoS One, 2012. 7(7): p. e42365.
298. Sugita, S., S.W. Johnson, and R.A. North, *Synaptic inputs to GABAA and GABAB receptors originate from discrete afferent neurons*. Neurosci Lett, 1992. 134(2): p. 207-11.
299. Einhorn, L.C., P.A. Johansen, and F.J. White, *Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: studies in the ventral tegmental area*. J Neurosci, 1988. 8(1): p. 100-12.
300. Vezina, P. and J. Stewart, *Amphetamine administered to the ventral tegmental area but not to the nucleus accumbens sensitizes rats to systemic morphine: lack of conditioned effects*. Brain Res, 1990. 516(1): p. 99-106.
301. Vezina, P., et al., *Environment-specific cross-sensitization between the locomotor activating effects of morphine and amphetamine*. Pharmacol Biochem Behav, 1989. 32(2): p. 581-4.
302. DuMars, L.A., L.D. Rodger, and P.W. Kalivas, *Behavioral cross-sensitization between cocaine and enkephalin in the A10 dopamine region*. Behav Brain Res, 1988. 27(1): p. 87-91.

303. Cador, M., Y. Bijiou, and L. Stinus, *Evidence of a complete independence of the neurobiological substrates for the induction and expression of behavioral sensitization to amphetamine*. *Neuroscience*, 1995. 65(2): p. 385-95.
304. McDaid, J., et al., *Cross-sensitization to morphine in cocaine-sensitized rats: behavioral assessments correlate with enhanced responding of ventral pallidal neurons to morphine and glutamate, with diminished effects of GABA*. *J Pharmacol Exp Ther*, 2005. 313(3): p. 1182-93.
305. Lett, B.T., *Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine*. *Psychopharmacology (Berl)*, 1989. 98(3): p. 357-62.
306. Nair-Roberts, R.G., et al., *Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat*. *Neuroscience*, 2008. 152(4): p. 1024-31.
307. Yamaguchi, T., et al., *Mesocorticolimbic glutamatergic pathway*. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 2011. 31(23): p. 8476-90.
308. Maxwell, J.C., *The prescription drug epidemic in the United States: a perfect storm*. *Drug Alcohol Rev*, 2011. 30(3): p. 264-70.
309. Munoz, M.B. and P.A. Slesinger, *Sorting nexin 27 regulation of G protein-gated inwardly rectifying K(+) channels attenuates in vivo cocaine response*. *Neuron*, 2014. 82(3): p. 659-69.
310. Hyman, S.E., R.C. Malenka, and E.J. Nestler, *Neural mechanisms of addiction: the role of reward-related learning and memory*. *Annu Rev Neurosci*, 2006. 29: p. 565-98.
311. Van den Oever, M.C., et al., *Prefrontal cortex plasticity mechanisms in drug seeking and relapse*. *Neurosci Biobehav Rev*, 2010. 35(2): p. 276-84.
312. Beyer, C.E. and J.D. Steketee, *Intra-medial prefrontal cortex injection of quinpirole, but not SKF 38393, blocks the acute motor-stimulant response to cocaine in the rat*. *Psychopharmacology (Berl)*, 2000. 151(2-3): p. 211-8.
313. Beyer, C.E. and J.D. Steketee, *Characterization of the role of medial prefrontal cortex dopamine receptors in cocaine-induced locomotor activity*. *Behav Neurosci*, 2001. 115(5): p. 1093-100.
314. Brebner, K., R. Phelan, and D.C. Roberts, *Effect of baclofen on cocaine self-administration in rats reinforced under fixed-ratio 1 and progressive-ratio schedules*. *Psychopharmacology (Berl)*, 2000. 148(3): p. 314-21.
315. Tzschentke, T.M., *Pharmacology and behavioral pharmacology of the mesocortical dopamine system*. *Prog Neurobiol*, 2001. 63(3): p. 241-320.
316. Backes, E.N. and S.E. Hemby, *Contribution of ventral tegmental GABA receptors to cocaine self-administration in rats*. *Neurochem Res*, 2008. 33(3): p. 459-67.
317. Niehaus, J.L., M. Murali, and J.A. Kauer, *Drugs of abuse and stress impair LTP at inhibitory synapses in the ventral tegmental area*. *Eur J Neurosci*, 2010. 32(1): p. 108-17.
318. Rahman, S. and W.J. McBride, *Feedback control of mesolimbic somatodendritic dopamine release in rat brain*. *J Neurochem*, 2000. 74(2): p. 684-92.

319. Rahman, S. and W.J. McBride, *D1-D2 dopamine receptor interaction within the nucleus accumbens mediates long-loop negative feedback to the ventral tegmental area (VTA)*. J Neurochem, 2001. 77(5): p. 1248-55.
320. Blednov, Y.A., et al., *Hyperactivity and dopamine D1 receptor activation in mice lacking girk2 channels*. Psychopharmacology (Berl), 2002. 159(4): p. 370-8.
321. Kotecki, L., et al., *GIRK Channels Modulate Opioid-Induced Motor Activity in a Cell Type- and Subunit-Dependent Manner*. J Neurosci, 2015. 35(18): p. 7131-42.
322. Morgan, A.D., et al., *Decreased cocaine self-administration in Kir3 potassium channel subunit knockout mice*. Neuropsychopharmacology, 2003. 28(5): p. 932-8.
323. Mirkovic, K., et al., *Behavioral characterization of mice lacking Trek channels*. Front Behav Neurosci, 2012. 6: p. 60.
324. Nyberg, F., *Structural plasticity of the brain to psychostimulant use*. Neuropharmacology, 2014. 87: p. 115-24.
325. Schmidt, H.D. and R.C. Pierce, *Cocaine-induced neuroadaptations in glutamate transmission: potential therapeutic targets for craving and addiction*. Ann N Y Acad Sci, 2010. 1187: p. 35-75.
326. Steketee, J.D., et al., *Possible role for G-proteins in behavioral sensitization to cocaine*. Brain Res, 1991. 545(1-2): p. 287-91.
327. Cunningham, C.L., et al., *Genetic differences in cocaine-induced conditioned place preference in mice depend on conditioning trial duration*. Psychopharmacology (Berl), 1999. 146(1): p. 73-80.
328. Zhang, Y., et al., *Conditioned place preference after single doses or "binge" cocaine in C57BL/6J and 129/J mice*. Pharmacol Biochem Behav, 2002. 73(3): p. 655-62.
329. Brabant, C., E. Quertemont, and E. Tirelli, *Influence of the dose and the number of drug-context pairings on the magnitude and the long-lasting retention of cocaine-induced conditioned place preference in C57BL/6J mice*. Psychopharmacology (Berl), 2005. 180(1): p. 33-40.
330. Spyraki, C., H.C. Fibiger, and A.G. Phillips, *Dopaminergic substrates of amphetamine-induced place preference conditioning*. Brain Res, 1982. 253(1-2): p. 185-93.
331. Herman, M.A., et al., *GIRK3 gates activation of the mesolimbic dopaminergic pathway by ethanol*. Proc Natl Acad Sci U S A, 2015. 112(22): p. 7091-6.
332. Kozell, L.B., et al., *Mapping a barbiturate withdrawal locus to a 0.44 Mb interval and analysis of a novel null mutant identify a role for Kcnj9 (GIRK3) in withdrawal from pentobarbital, zolpidem, and ethanol*. J Neurosci, 2009. 29(37): p. 11662-73.
333. Vaughan, C.W. and M.J. Christie, *Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey in vitro*. The Journal of physiology, 1997. 498 (Pt 2): p. 463-72.
334. Honore, E., *The neuronal background K2P channels: focus on TREK1*. Nature reviews. Neuroscience, 2007. 8(4): p. 251-61.
335. Matsumoto, M. and O. Hikosaka, *Two types of dopamine neuron distinctly convey positive and negative motivational signals*. Nature, 2009. 459(7248): p. 837-41.

336. Zhao-Shea, R., et al., *Nicotine-mediated activation of dopaminergic neurons in distinct regions of the ventral tegmental area*. *Neuropsychopharmacology* : official publication of the American College of Neuropsychopharmacology, 2011. 36(5): p. 1021-32.
337. Marker, C.L., et al., *Hyperalgesia and blunted morphine analgesia in G protein-gated potassium channel subunit knockout mice*. *Neuroreport*, 2002. 13(18): p. 2509-13.
338. Engblom, D., et al., *Glutamate receptors on dopamine neurons control the persistence of cocaine seeking*. *Neuron*, 2008. 59(3): p. 497-508.
339. Kourrich, S., et al., *Cocaine experience controls bidirectional synaptic plasticity in the nucleus accumbens*. *J Neurosci*, 2007. 27(30): p. 7921-8.
340. Kourrich, S. and M.J. Thomas, *Similar neurons, opposite adaptations: psychostimulant experience differentially alters firing properties in accumbens core versus shell*. *J Neurosci*, 2009. 29(39): p. 12275-83.
341. Zweifel, L.S., et al., *Role of NMDA receptors in dopamine neurons for plasticity and addictive behaviors*. *Neuron*, 2008. 59(3): p. 486-96.
342. Jedynek, J., et al., *Cocaine and Amphetamine Induce Overlapping but Distinct Patterns of AMPAR Plasticity in Nucleus Accumbens Medium Spiny Neurons*. *Neuropsychopharmacology*, 2015.
343. Lammel, S., et al., *Diversity of transgenic mouse models for selective targeting of midbrain dopamine neurons*. *Neuron*, 2015. 85(2): p. 429-38.
344. Sharpe, A.L., et al., *Methamphetamine self-administration in mice decreases GIRK channel-mediated currents in midbrain dopamine neurons*. *Int J Neuropsychopharmacol*, 2015. 18(5).
345. Krapivinsky, G., et al., *The G-protein-gated atrial K⁺ channel IKACH is a heteromultimer of two inwardly rectifying K⁽⁺⁾-channel proteins*. *Nature*, 1995. 374(6518): p. 135-41.
346. Wickman, K., et al., *Abnormal heart rate regulation in GIRK4 knockout mice*. *Neuron*, 1998. 20(1): p. 103-14.
347. Wydeven, N., et al., *Mechanisms underlying the activation of G-protein-gated inwardly rectifying K⁺ (GIRK) channels by the novel anxiolytic drug, ML297*. *Proc Natl Acad Sci U S A*, 2014. 111(29): p. 10755-60.