A comprehensive analysis of sexual dimorphism in the midbrain dopamine system

Amanda Shinae Zila

A dissertation submitted
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2016

Reading Committee:
Larry S. Zweifel (Chair)
Richard D. Palmiter
Paul E. M. Phillips

Program Authorized to Offer Degree:

Pharmacology

University of Washington

Abstract

A comprehensive analysis of sexual dimorphism in the midbrain dopamine system

Amanda Shinae Zila

Chair of the Supervisory Committee:
Assistant Professor Larry S. Zweifel
Department of Pharmacology

The dopamine system is widely thought to play a role in many crucial behaviors, including reward association, motivation, and addiction. Additionally, dopamine is also linked to multiple diseases, such as depression, post-traumatic stress disorder, schizophrenia, and autism. What is striking about these diseases is they present with sex differences in multiple aspects including susceptibility, progression, and response to treatment. However, we know very little about sex differences in the dopamine system, especially at a baseline state. In the current study, I provided a comprehensive analysis of the dopamine system in males and females, including circuitry, physiology, gene expression, and behavior.

Employing retrograde viral tools, we characterized the inputs to the entire ventral tegmental area (VTA), to VTA dopamine neurons specifically, and compared the number of GABAergic, glutamatergic, and serotonergic inputs to the VTA; we also mapped VTA dopaminergic outputs through use of excitatory DREADDs. However, a comparison of the number of inputs in each brain area between males and females revealed no differences. An interesting discovery was the high amount of GABAergic inputs to the VTA, relative to

glutamatergic and serotonergic. Further interrogation of this observation uncovered the presence of a strong inhibitory input onto VTA dopamine neurons, which was confirmed through slice electrophysiology and selective expression of neurotransmitter receptor mRNA transcripts.

Isolation of ribosome-associated mRNA transcripts in dopamine neurons and subsequent microarray analysis yielded only two mRNAs with significant sex-dependent expression. Interestingly, these mRNAs encode for genes that are sex chromosome linked. Finally, a comparison of male and female mice in a serious of appetitive dopamine-dependent tasks revealed no consequential differences resulting from sex or hormone state. However, examination of locomotor response to cocaine sensitization indicated a strong effect of hormones in both males and females. Altogether, these data suggest that VTA dopamine circuitry, gene expression, and behavior are largely the same between male and female mice at a baseline state. This implies the need to look at upstream structures that may impart sex-specific qualities which may explain the sex differences we see in dopamine-related diseases.

Table of Contents

Title 1
Abstract 3
Table of Contents 5
List of Figures 6
Glossary 7
Acknowledgments 10

Chapter 1 – Introduction 11
The Dopamine System 11
Dopamine Function 12
Dopamine-dependent Behaviors 15
Dopamine Circuitry 20
Dopamine and Disease 26
Sex Differences 30
Dopamine Function 30
Dopamine-Dependent Behaviors 33
Dopamine and Disease 35

Chapter 2 – VTA Circuitry 40
VTA Afferents 40
GABA 46
Glutamate 47
Comparison of Inhibitory and Excitatory Input 49
Serotonin 51
VTA Efferents 52

Chapter 3 – Dopamine Translatome 54

Chapter 4 – Dopamine-dependent Behavior 60

Chapter 5 – Discussion, Conclusions, and Future Directions 65

Experimental Methods 72

References 79

List of Figures

Chapter 2:

1. Viral approach for mapping VTA afferents
2. Mapping of inputs to VTA neurons
3. Viral approach for mapping afferents of VTA dopamine neurons
4. Quantification of viral efficiency and specificity showed no sex differences
5. Mapping of inputs to VTA dopamine neurons
6. Viral approach for mapping GABAergic afferents of VTA neurons
7. Mapping of GABAergic inputs to VTA neurons
8. Mapping of glutamatergic inputs to VTA neurons
9.
10. Dopamine neurons displayed elevated mIPSC amplitude and frequency
11. Baseline electrophysiology properties of dopamine neurons showed no sex difference in slice
12. Mapping of serotonergic inputs to VTA neurons
13. Mapping of VTA dopamine output areas

Chapter 3:

14. Viral approach for isolation of VTA dopamine neuron mRNA
15. RNA of immunoprecipitated VTA tissue showed enrichment for dopamine markers
16. Genes enriched in dopamine neurons
17. Two genes expressed in dopamine neurons demonstrated sex difference
18. Gene expression profile of neurotransmitter receptor subtypes in IP

Chapter 4:

19. Pavlovian appetitive conditioned task revealed no sex differences
20. Instrumental conditioning and progressive ratio paradigms
21. Locomotor sensitization to cocaine showed no sex difference, but revealed effect of hormones

Glossary

Abbreviation:	Term:
AAV	Adeno-associated viral vector
ACh	Acetylcholine
ACo	Anterior cortical amygdaloid nucleus
AHA	Anterior hypothalamic area, anterior
AI	Agranular insular cortex
Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc	Arcuate hypothalamic nucleus
BLA	Basolateral amygdaloid nucleus
BNST	Bed nucleus of the stria terminalis
CAST	Castration
CAV	Canine adenovirus
CCK	Cholecystokinin
CeA	Central amygdaloid nucleus
Cg	Cingulate cortex
Cl	Claustrum
CNO	Clozapine N-oxide
COMT	Catechol-O-methyltransferase
CPu	Caudate putamen
Crh	Corticotropin-releasing hormone
CS	Conditioned stimulus
DAT	Dopamine transporter
DBH	Dopamine beta-hydroxylase
DIO	Double-floxed inverted open reading frame
DM	Dorsomedial hypothalamic nucleus
DOPAC	3,4-Dihydroxyphenylacetic acid
DP	Dorsal peduncular nucleus
DRN	Dorsal raphe nucleus
DREADD	Designer receptors exclusively activated by designer drugs
DStr	Dorsal striatum
DTT	Dorsal tenia tecta
ER	Estrogen receptor
FrA	Frontal association cortex
GABA	Gamma-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
Glu	Glutamate
Gly	Glycine
GP	Globus pallidus
GPCR	G-protein coupled receptors

HA	Hemagglutinin
HDB	Nucleus of the horizontal limb of the diagonal band
HRP	Horseradish peroxidase
InG	Intermediate geniculate nucleus
InWh	Intermediate white layer of the superior colliculus
LA	Lateral amygdaloid nucleus
LC	Locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine, levodopa
LDTg	Laterodorsal tegmental nucleus
LH	Lateral hypothalamic area
LHb	Lateral Habenular nucleus
LO	Lateral orbital cortex
LOT	Nucleus of the lateral olfactory tract
LPB	Lateral parabrachial nucleus
LPO	Lateral preoptic area
LS	Lateral septal nucleus
LSI	Lateral septal nucleus, intermediate part
LSV	Lateral septal nucleus, ventral part
LTD	Lateral septal nucleus, dorsal part
LTP	Long-term potentiation
M1	Primary motor cortex
M2	Secondary motor cortex
mAChR	Muscarinic acetylcholine receptors
mEPSC	Miniature excitatory postsynaptic currents
mHb	Medial habenular nucleus
mIPSC	Miniature inhibitory postsynaptic currents
MO	Medial orbital cortex
MPA	Medial preoptic area
MPB	Medial parabrachial nucleus
mPFC	Medial prefrontal cortex
MPO	Medial preoptic nucleus
MS	Medial septal nucleus
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptors
NE	Norepinephrine
NK-B	Neurokinin B
nM	Nanomolar
NMDA	N-methyl-D-aspartate
Nts	Nucleus of the solitary tract
OVX	Ovariectomy
PAG	Periaqueductal gray
PBN	Parabrachial nucleus
PD	Parkinson’s disease
PFC	Prefrontal cortex

PH	Posterior hypothalamic area
PM	Premammillary nucleus
PP2A	Protein phosphatase 2
PPTg	Pedunculopontine tegmental nucleus
PtA	Parietal association cortex
PTSD	Post-traumatic stress disorder
Pv	Periventricular fiber system
PVH	Paraventricular thalamic nucleus
RMTg	Rostromedial tegmental nucleus
RRF	Retrorubral field
S1	Primary somatosensory cortex
SI	Substantia innominata
SK3	Small conductance calcium-activated potassium channel 3
SNc	Substantia nigra
SoN	Supraoptic nucleus
SSRI	Selective serotonin reuptake inhibitor
TBS	1x tris buffered solution
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
US	Unconditioned stimulus
V2MM	Secondary visual cortex, mediomedial area
VDB	Nucleus of the vertical limb of the diagonal band
vGluT	Vesicular glutamate transporter
VMH	Ventromedial hypothalamic nucleus
VO	Ventral orbital cortex
VP	Ventral pallidum
VTA	Ventral tegmental area
YFP	Yellow fluorescent protein
ZI	Zona incerta

Acknowledgments

First and foremost, I would like to thank Dr. Larry S. Zweifel for his mentorship and support throughout my graduate career. I greatly appreciate your patience and the positive environment you fostered in the lab. Without your guidance I certainly would not have been challenged to develop into the scientist I am today. I am very proud to say that I was your first graduate student. I would also like to thank my committee, Drs. Paul Phillips, Stan McKnight, Richard Palmiter, and Jeansok Kim for their advice and direction. Special thanks goes to Dr. Palmiter and his lab for their thoughtful input during lab meeting, as well as their willingness to allow me to use their equipment.

To my fellow Zweifelites, thank you for your optimism, inspiration, and encouragement. Without you all and the atmosphere you provided on a daily basis, I surely would not have been able to make it through graduate school. Special thanks to Dr. Marta Soden for her constant mentorship and input from the beginning. Big props to Jesse Resnick, Samara Miller, and Cerise Knakal for their help in completing experiments and for being such amazing friends. I am incredibly glad to have gotten to know you as people and scientists. Thank you to all of my classmates for being a fantastic support system. I am grateful for your friendship and support throughout graduate school.

Thank you to my parents who, despite not really understanding what I was doing on a daily basis, have supported me throughout graduate school. Without the strong foundation you provided I doubt I would have accomplished all that I have. Special thanks goes to my friend, Traci Brooks, who always provided a sympathetic ear for my frustrations. You are one of the best people I know and I treasure our friendship. Last, thank you to my husband John. I don’t think you really knew what you were getting into when we first met and I said I was a graduate student, but you were there for every breakdown, success, and challenge. This absolutely would not have been possible without your unconditional support and love. I look forward to the adventures our future holds.

CHAPTER 1

INTRODUCTION

Dopamine is one of the primary catecholamine neurotransmitters in the central nervous system. Although it has been studied in detail for decades due to its role in many critical behaviors, several gaps in our knowledge remain. In particular, where different neurotransmitters systems that influence dopamine neurons arise, the degree of selectivity in gene expression in these neurons, and the extent of sex differences.

The purpose of this dissertation is to describe in detail a thorough exploration of the dopamine system in the brain, gene expression profiles, and sex differences using a mouse model system. To accomplish this, I have performed descriptive experiments illustrating the pattern of inputs to the dopamine neurons from cells expressing the primary neurotransmitters known to project to the VTA. Additionally, I have mapped the areas throughout the brain to which VTA dopamine neurons send signals. To complement this, I have characterized the expression of mRNA transcripts in dopamine neurons. These data together provide a picture of the input and output patterns of the VTA dopamine neurons, as well as how these patterns may be reflected in gene expression. These findings are completed with functional experiments, including slice electrophysiology and behavior in male and female mice.

The presence of sex differences in the dopamine system at a baseline state, as well as how these may affect dopamine-dependent behaviors, provides vital insight into the prevalence of sex differences in multiple aspects of dopamine-related diseases.

Dopamine Neurons

Dopamine is synthesized in the brain from the amino acid tyrosine. Tyrosine is converted to dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH), which is the rate-limiting step in the synthesis of dopamine and often serves as a marker of neurons that produce dopamine. From here, the enzyme dopa decarboxylase can convert L-DOPA to dopamine[1,2]. Following its synthesis, dopamine is transported to vesicles primarily in the terminals, where it can be released following an action potential. After release from neuron terminals, dopamine can either bind to one of its receptors, or get taken back up into the dopamine terminals through transporters, or be degraded by monoamine oxidase and catechol-O-methyltransferase (COMT), which ultimately break down dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid.

Dopamine is located primarily in the ventral tegmental area (VTA) and zona compacta of the substantia nigra (SNc) neurons of the midbrain. The dopaminergic neurons located in the VTA are known as the mesolimbic and mesocortical dopamine systems due to their projection patterns to the nucleus accumbens and prefrontal cortex , respectively, while the neurons of the SNc are referred to as the nigrostriatal system[3].

Extracellular recordings *in vivo* of dopamine neurons has shown that these cells typically fire at a rate between 4 and 5 Hz[4]. Their activity can be classified into two patterns: tonic and phasic[1,4–9]. Tonic firing is typically characterized as slow, baseline activity exhibiting single spike action potentials and results in an extracellular concentration of 5-10 nM[10]. This firing pattern is regulated primarily by a slow depolarization followed by a hyperpolarization that is mediated by SK3 channels[4,11]. Release of dopamine resulting from tonic firing is responsible for maintaining a low baseline level of dopamine[12]. Phasic or burst firing is classified by 3-10 spikes with an interval of no more than 160 ms[5]. The product of this firing pattern is an increase of dopamine release into the synapse reaching concentrations of 150-400 nM, which plays an important role in many behaviors[8,10].

Importantly, dysregulation of tonic and phasic dopamine firing has been implicated in psychiatric diseases, including schizophrenia and depression[11,12].

Dopamine Receptors

Dopamine receptors are classified into two categories: D1-type and D2-type. This classification was established based on differences in pharmacology, physiology, and signaling. There are five different dopamine receptors, which fall within one of the two categories. The D1 and D5 receptors are D1-type, while the D2, D3, and D4 receptors are D2-type. The D1-type receptors are seven transmembrane G-coupled protein receptors (GPCRs) that activate $G\alpha_s$ proteins, which stimulate adenylyl cyclase and lead to an increase in cAMP production. D2-type receptors are also GPCRs, but instead couple to $G\alpha_i$ proteins, resulting in an inhibition of adenylyl cyclase and an overall decrease in cAMP. After prolonged activation, the receptors can also form a complex with β-arrestin, PP2A, and Akt. This change in proximity results in an inactivation of Akt by the nearby PP2A, as well as internalization of the receptor[13,14].

In addition to the interaction with adenylyl cyclase and its consequent signaling cascade, dopamine receptors can also increase intracellular calcium levels through stimulation of phospholipase C, which then increases phosphatidylinositol hydrolysis[15]. Another noncanonical D2 receptor signaling cascade includes the Gβγ subunits that have separated from Gα following agonist binding. These subunits can inhibit L/N-type calcium channels, as well as activate G protein-coupled inwardly-rectifying potassium channels, resulting in hyperpolarization of the neuron[15]. To make matters even more complex, dopamine receptors can also interact with glutamatergic and GABAergic receptors[13].

The localization of the D1- and D2-type receptors is similar in terms of brain structure expression but unique in sub-cellular positioning. Dopamine receptors are highly expressed in the olfactory bulb, substantia nigra, amygdala, and other subdivisions of the basal ganglia[16,17]. More specifically, D1 receptors are distributed primarily in the substantia

nigra, prefrontal cortex, amygdala, striatum, and nucleus accumbens. D2 receptors are primarily distributed in the striatum, olfactory bulb, and nucleus accumbens core[13,16]. They are also located in the ventral tegmental area dopamine neurons, where they act as inhibitory autoreceptors. On the other hand, D1 receptors are found mostly in the postsynaptic dendrites of dopamine target areas. This distinction is important, as the D2 autoreceptors located on dopamine neurons, in response to high levels of dopamine in the synapse, will decrease activity of dopamine neurons by hyperpolarizing the cell, resulting in inhibition of action potentials and a lower level of neurotransmitter release[18].

This leads us to the differential activation of the two dopamine receptor types, which is instrumental in their classification. Early studies looking at the affinity of D1 and D2 receptors used quantitative autoradiography to look at the affinity of each receptor for dopamine. D1 receptors were found to have low affinity for dopamine, while D2 receptors had high affinity[17,19]. Based on these findings, the universally held belief is that D2 receptors mediate signaling for tonic dopamine release, while D1 receptors mediate phasic dopamine release[5].

Dopamine Neuron Gene Expression

Studies that identify gene expression in dopamine neurons primarily examine differences between VTA and SNc populations[20–23]. Canonical dopaminergic markers that contribute to the function of dopamine in the cell include TH, D2 receptors, the dopamine active transporter *Slc6a3* (DAT), DOPA decarboxylase (DDC), and the vesicular monoamine transporter *Slc18a2* (VMAT2)[24]. Other genes that have been identified in dopamine neurons including those that encode calbindin, cholecystokinin, cadherin, neuropilin, voltage-gated potassium channels (*Kcna5, Kcnab1*), sodium channels (*Scn3a*, *Scn6a*), ion channel receptors (*Gabra4, Grin2c, Gria1*), gastrin releasing peptide, roundabout guidance receptor 2, aldehyde dehydrogenase (*Aldh1a1*), forkhead box protein A1 (*Foxa1*), nuclear receptor (*Nr4a2*), and many others[21–24].

Beyond this, the dopaminergic neurons of the VTA can be further subdivided into populations based on differential gene expression. Genes that facilitate this categorization include *Aldh1a1*, *Snca* (synuclein), *Slc32a1* (Vgat), *Lpl* (lipoprotein lipase), and Calb1 (calbindin)[24]. These subdivisions of dopaminergic neurons may prove to be important, as it has become clear that within populations of neurons once thought to be homogeneous, there exists clear heterogeneity that can be shown through disease, electrophysiology, and projection targets. However, one aspect of dopaminergic neuron gene expression that has not been explored is whether expression of mRNA is sex-dependent.

Dopamine-Dependent Behaviors

Reward

Traditionally, dopamine neurons are associated with rewarding behaviors. This assumption is based on early work that found an increase in phasic dopamine neuron firing when a rewarding unconditioned stimulus (US), such as sucrose, was presented to a rat. With training the rat learned to associate the reward with an unconditioned stimulus (CS) such as a light. As the rat made this connection between the US and CS, eventually the dopamine neurons increased in firing frequency to the predictive CS, instead of the US. This type of behavioral training is called Pavlovian conditioning[25]. In addition to this, if the expected reward does not occur, a decrease in phasic firing is seen; this is called a reward prediction error[10]. If one inhibits the dopamine system with manipulations such as dopamine receptor antagonists or D1 receptor knockout mice, subjects fail to make the association between US and CS, further supporting dopamine's role in positive reinforcement and reward learning[26]. Conversely, optogenetic stimulation of dopaminergic neurons in the VTA increases reward learning when a rewarding stimulus is present[27]. Additional evidence for this is illustrated using fast scan cyclic voltammetry *in vivo*, which uses electrodes to measure phasic dopamine in VTA projection areas during behavior. Rats, when trained on a Pavlovian conditioning paradigm, separate into two groups. The first are goal trackers;

these rats engage primarily with the area where the reward will appear. The second are sign trackers; these rats engage with the cue that predicts the upcoming reward. Similar to data showing dopamine neuron firing, there is an increase in dopamine release to the US in the beginning of the subjects' training. However, over time this signal shifts to show an increase in response to the CS presentation. Importantly, this is only demonstrated in rats that are sign trackers[28]. Together, these findings demonstrate that dopamine is important for reinforcement of reward-cue associations.

On a molecular level, an increase in phasic firing, which underlies the association between cues and rewarding stimuli, is dependent on the D1 receptor (which we now know mediates phasic dopamine signals), and excitatory inputs. Stimulation of glutamatergic inputs, as well as application of glutamate has been shown to increase burst firing. Additionally, manipulation of the primary glutamate receptor, NMDA receptor, has been shown to affect phasic firing[6]. More specifically, genetic manipulation resulting in inactivation of the NR1 subunit of the NMDA receptor in dopamine neurons has been shown to decrease burst firing and disrupt drug-seeking, fear, and some cue-dependent learning behaviors[29,30].

One of the origins of this glutamatergic input lies in the dorsal raphe (DRN) of the hindbrain. The DRN is primarily known as the main nucleus containing serotonin (5-HT) neurons. Early studies showed that stimulation of the DRN itself proved rewarding[31,32]. Optogenetic stimulation of 5-HT neurons in the DRN also has a reinforcing effect in reward behaviors such as sucrose preference, open field, and operant conditioning. These findings were further supported by electrophysiology *in vivo* that revealed and increase in 5-HT neuron firing leading up to and during a reward-predictive cue. However, it seems that 5-HT is not the sole neurotransmitter responsible for this effect. Glutamate was also found to co-localize with 5-HT in the DRN, as well as contribute to reward signaling[33]. It wasn't until recently that a reward link between the DRN and VTA was established. Activation of VGluT3–expressing neurons in the DRN results in increased dopamine release in the nucleus

accumbens, a well-known target of VTA dopamine neurons. Additionally, activation of these neurons also reinforces conditioned place preference, which is reversed by an AMPA receptor antagonist, while a 5-HT antagonist has no effect[34]. Another study, however, showed that activation of 5-HT neurons in the DRN not only has no reward effect, but stimulation of non-5-HT neurons are the key to reinforcing this behavior[35]. Based on these data, it is likely that indeed it is glutamate neurons synapsing onto VTA dopamine neurons that drive reward learning.

Another VTA dopamine-projecting structure implicated in reward-seeking behaviors is the basolateral amygdala (BLA). Infusion of GABA antagonists into the BLA resulted in decreased dopamine release in the NAc and an inhibition of lever-approach behavior, although there was no change in the number of times animals would press the lever to receive the reward[36]. An additional VTA-projecting area, the laterodorsal tegmentum (LDTg), has been linked to reward. Optical stimulation of glutamatergic LDTg neurons projecting to the VTA increased conditioned place preference, and these neurons were shown to synapse primarily onto dopamine neurons[37].

The outputs of VTA dopamine neurons and their role in reward behavior are also important. The prefrontal cortex (PFC), caudate putamen (CPu), amygdala, and NAc have all been shown to mediate this behavior[38–44]. In the case of the NAc, this area can be further subdivided in its influence on various reward-related behaviors. Specifically, dopamine in the core of the NAc is responsible for Pavlovian conditioned approach and cocaine sensitization/preference, while the shell of the NAc plays no role. Both the shell and core mediate dopamine-dependent instrumental conditioning[43–45].

Drug Seeking

The effects of drugs are often rewarding, contributing to their addictive nature. The signals from VTA dopamine neurons to the NAc are primarily responsible for this effect, although dopamine in the olfactory tubercle has also been implicated[44,46–50]. While many drugs may act in the VTA, cocaine is the most widely studied due to its direct blockade of

the DAT. When animals are allowed to perform a task in order to self-administer cocaine, performance of this task (such as lever pressing) can be used to determine drug-seeking behavior. When dopamine receptor antagonists are infused into the NAc, there is an attenuation of this behavior[51–53]. Studies have shown that it is the neurons of the VTA that project to the NAc shell that undergo a synaptic strengthening, demonstrated by an increase in AMPAR/NMDAR ratio. Projections from the SNc to dorsal striatum or VTA to PFC, however, show no change in this ratio[47]. It is also evident that glutamate signaling to dopamine neurons is critical for this drug-seeking behavior to occur. Repeated self-administration of cocaine increases glutamate in the VTA, and perfusion of glutamate antagonists into the VTA decreases behavior. However, glutamate is also seen if animals received an unexpected saline infusion instead of the usual cocaine, suggesting that glutamate in the VTA is critical in reinforcement of drug-seeking and drug-related cues[54]. In addition to glutamate, acetylcholine (ACh) inputs onto VTA dopamine neurons may also influence cocaine behaviors. An increase of ACh in the VTA is seen in response to the cue predicting cocaine, as well as to cocaine itself. ACh, which increases DA release, may therefore influence both reward-association and reward effect[55].

The rewarding effects of many other drugs of abuse have been found to be mediated by the VTA and increase dopamine release. Self-administration of nicotine, alcohol, cannabinoids, opiates, and amphetamine is a robust behavior in murine models[56–60]. Much like cocaine, the primary dopamine release target area is the nucleus accumbens, and dopamine receptor antagonists reduce this behavior[44,60–62]. Together, these studies suggest the possibility of a common circuit underlying drug reward and drug-mediated associations[63].

Aversion

The processing of fear and fear learning in response to aversive stimuli is performed primarily in the amygdala. Put simply, the basolateral nucleus of the amygdala (BLA) receives information about an aversive event from the prefrontal cortex, sensory cortex,

parabrachial nucleus, and thalamus[64]. The BLA projects to the central nucleus of the amygdala (CeA), which then relays response information to autonomic areas to formulate a reaction to the stimulus[65,66]. The lateral amygdala demonstrates an increase in firing when mice are subjected to a mild footshock[67]. An additional input to the BLA that is instrumental to fear learning is dopaminergic, originating from the VTA. Early studies demonstrated that dopamine neurons themselves respond to aversive stimuli, such as a tail pinch, with a decrease in burst firing[30,68]. On the other hand, there is also data showing that a small population of dopamine neurons increase activity to aversive stimuli[30,69,70]. Additional evidence for this circuit revealed that application of dopamine to BLA neurons affected their firing differentially; BLA neurons with low baseline firing rates decrease activity in response to dopamine, while high firing neurons increase their activity[65]. Also, stimulation of BLA neurons through optogenetics increases anxiety behavior[71]. Administration of a D1 receptor agonist enhances fear responses, while a D2 receptor antagonist in the VTA inhibited fear expression[72,73]. Additionally, genetic knock-out models of the D1 receptor demonstrated that this receptor is necessary for fear learning[74]. In a dopamine-deficient mouse model, mice were unable to exhibit fear-potentiated startle, which was rescued with L-Dopa administration[75].

The specific circuits responsible for processing aversive stimuli and fear learning have been further explored thanks to recent technological advances. Inputs exerting influence on the VTA dopamine neuron fear circuit primarily arise from glutamatergic cell bodies in the lateral habenula (LHb). Optogenetic activation of neurons in this area that project to the VTA result in conditioned place aversion, implicating this pathway in the expression of fear behavior[37,76]. In the VTA itself, dopamine neurons undergo plasticity in response to Pavlovian fear conditioning. Knock-out of the NR1 subunit of the NMDA receptor in dopamine neurons impairs the response to cued fear[30]. Furthermore, calcium imaging of dopamine neurons using a genetically encoded calcium indicator, GCaMP3, shows an increase in activity of these cells to a cue after multiple pairings with an aversive shock

stimulus[77]. This plasticity proves to be crucial to fear processing in the lateral amygdala (LA). Lack of NR1 specifically in dopamine neurons reduces activation of the LA to an unconditioned aversive stimulus and a conditioned stimulus, which results in an abnormal behavioral output reflecting this lack of plasticity[67]. So, similar to reward learning, dopamine neurons in the VTA undergo plasticity to regulate association learning of stimuli, and this plasticity is vital to the ability of the amygdala to regulate fear coding.

While the amygdala is a key structure in the processing of fear, projections from VTA dopamine neurons to other areas have also been studied. Exposure to stress reveals an increase of dopamine release in the mPFC, as well as the role of dopamine receptors here in the expression of fear[78–80]. Additional evidence the role of the mPFC is shown by the discovery that neurons from the LHb that project to the VTA to mediate aversion do so preferentially onto dopamine neurons that synapse onto the mPFC. Furthermore, local infusion of a D1 receptor antagonist into the mPFC prior to stimulation of these LHb neurons prevents conditioned aversion[37]. One more VTA efferent area worth mentioning in relation to aversive stimuli is the NAc. The role of the NAc in fear behavior is unclear, due to its divergent subdivisions[79]. For example, infusion of a D1 receptor antagonist into the NAc shell impaired fear learning, while having no effect in the NAc core[81]. An increase of dopamine in the NAc has also been observed in response to a footshock, as well as a shock-paired cue[82]. However, dopamine is higher in the NAc shell during a shock-paired tone, while this is only seen in the NAc core during a shock-paired context[83]. While the exact role of VTA dopaminergic innervation of the NAc in fear conditioning remains unclear, it does appear that dopamine is contributing to fear learning and expression.

Dopaminergic Circuitry

Inputs

Studies describing VTA circuitry have been published going back some 60-odd years. As science technology advanced, so has our knowledge of this circuit. Beginning in the

1970s, scientists used a technique called autoradiography to examine projections. This method involves injecting a radioactive amino acid into an area in which you want to know its projections; brain sections are then imaged using x-ray. One of the first studies to do this injected radioactive leucine into the dorsal raphe (DRN) of cats and found a strong projection to the VTA[84]. Soon after this same method was used to look at projections of the bed nucleus of the stria terminalis (BNST) and horizontal limb of the diagonal band of Broca (HDB) in the rat, which also revealed fibers in the VTA. However, the septum did not show this same expression[85]. The first thorough characterization of projections to the VTA was made possible through the use of horseradish peroxidase (HRP) in female rats. HRP travels retrograde from the site of injection, enabling the identification of areas sending afferents to the VTA. This technique revealed that the PFC, NAc, amygdala, substantia innominata (SI), preoptic areas, lateral hypothalamus (LH), zona incerta (ZI), habenula, and parabrachial nucleus (PBN) provide strong inputs to the VTA. Additionally, the previous findings of the BNST, HDB and DRN were confirmed[86]. A comprehensive study combining both HRP and autoradiography in male rats was later published, which confirmed projections to the VTA from the DRN and habenula and noted the addition of the locus ceruleus (LC) to the list of VTA afferents[87].

A confounding factor in the use of these tools is the possibility of uptake by fibers of passage in addition to synaptic terminals. To avoid this problem, the retrograde tracer fluorogold can be used instead. In addition to the areas described previously, this method revealed additional areas in male rats, including the dorsal peduncle (DP), ventral pallidum (VP), lateral septum (LS), periaqueductal gray (PAG), pedunculopontine nucleus (PPTg), and laterodorsal tegmental nucleus (LDTg). Most notably, it was observed that the VTA inputs were loosely organized into an "elongated formation" or continuous band descending from the forebrain down through the lateral hypothalamus[88].

It was not until many years later that a new technology became available allowing for cell-type specific circuit mapping, specifically the use of a modified rabies virus. This

technique involves the use of two viruses: one is a Cre-conditional rabies virus envelope glycoprotein. This protein is required for the rabies virus to be able to spread retrograde. The virus is injected into the VTA of a mouse expressing Cre in dopamine neurons (DAT-Cre) where it will express in a cell-type specific manner. After a period of time, a second injection is performed into the VTA of a modified rabies virus with the essential glycoprotein replaced with a fluorescent marker. This viral approach allows for visualization of neurons projecting specifically to VTA dopamine neurons. Use of this strategy in male mice confirmed that many of the afferent areas previously identified did indeed synapse onto dopamine neurons. Areas exhibiting a high density of inputs include the DRN, NAc, VP, BNST, LH, and dorsal striatum. The septum and medial portion of the habenula (mHb) were not labeled as strongly as previous studies, implying that these areas project to non-dopaminergic neurons[89]. An additional study replicating this technique also in mice found similar results[90]. Of note is the complete lack of data demonstrating whether there are any differences in the strength of these inputs to the VTA between males and females.

As technology progressed, it became possible to explore what neurotransmitters were being released from these afferents and regulating dopamine output. The VTA receives input of primarily three types: glutamatergic, GABAergic, and cholinergic[91,92]. As mentioned previously, functioning NMDA receptors and the plasticity they mediate are extremely important for the ability of the VTA dopamine neurons to function correctly and regulate aversive and reward behaviors. One of the primary sources of glutamatergic input to VTA dopamine neurons arises from the PFC; stimulation of the PFC results in a burst-like response from dopamine neurons and glutamate infusion into the PFC increases release of dopamine[91,93,94]. Through retrograde tracers and in situ labeling of afferents, these neurons are found to express primarily the vesicular glutamate transporter VGluT1[95]. Another source of glutamatergic input are the LDTg and PPTg, the majority of which are VGluT2-positive[95–98]. Additional excitatory areas projecting to the VTA (although it is unknown whether these

are dopamine specific) include the claustrum (Cl), medial septum, VP, medial preoptic, LH, LHb, DRN, and BNST[95].

Inhibitory GABAergic input to the VTA likely arises from the dorsal striatum, NAc, RMTg, and globus pallidus (GP)[91]. However, the evidence for this is weak; while these afferents do project to the VTA dopamine neurons and contain populations of GABA neurons, there is little direct evidence showing that this neurotransmitter is acting on dopamine neurons. One study demonstrated through retrograde Fluorogold labeling and in situ hybridization, the presence of GABAergic neurons in the NAc and diagonal band of Broca that project to the VTA, although not in a dopamine-specific manner[99]. The lateral habenula has been studied in more detail, as stimulation of the LHb strongly inhibits spontaneous firing of dopamine neurons; bicuculline, a GABA receptor antagonist, decreases this effect[100]. Also, lesions of the LHb result in increased dopamine levels[101,102]. The VP has also been shown to produce increased dopamine release[98]. One other source of inhibition that should be noted is the VTA itself, where GABAergic interneurons synapse locally onto dopamine neurons[103].

While GABA and glutamate are the most common neurotransmitters in the brain, there are others of note that influence midbrain dopamine neurons. The dorsal raphe holds the primary population of 5-HT neurons in the brain. These neurons synapse onto dopaminergic VTA neurons and stimulation of these cells results in inhibition and less often excitation[91,104–106]. As mentioned previously, DRN neurons that express vGluT3 and 5-HT mediate a glutamate-driven reward signal to VTA dopamine neurons[33,35]. Neurons in the DR projecting specifically to dopamine neurons also express GAD 1/2 and vGluT 2/3, which may account for why DRN stimulation produces varying responses[90].

Another important neurotransmitter in the VTA is acetylcholine (ACh), which binds to nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors. Cholinergic input originates primarily from the PPTg and LDTg, and stimulation of these neurons shows an increase in dopamine burst firing and release in the NAc, while inhibition of the LDTg

decreases firing and burst rate[98,107–110]. Likewise, receptor activation of all cholinergic receptors increases dopamine firing rate, while application of nicotine causes a depolarization and muscarine shortens the afterhyperpolarization, which leads to increased burst firing[111–115]. Conversely, muscarinic receptor antagonism decreases NAc dopamine levels[116]. The mechanisms of receptor action on dopamine neurons exhibit unique timing properties driven by the fast ionotropic nicotinic receptors and the slower metabotropic muscarinic receptors[116,117]. Behavioral effects of cholinergic input include modulation of reward learning and drug-seeking. Mice will self-administer nicotine into the VTA, and this behavior is blocked by a D1 receptor or nAChR antagonist[62]. Cocaine increases ACh in the VTA, while blockade of mAChRs in the VTA decreased self-administration of cocaine and food pellets[118,119]. Interestingly, lesioning of the PPTg alone has no effect on cocaine-seeking behaviors; on the other hand, inhibition of the LDTg decreases CPP for cocaine, as does inhibition of mAChRs and nAChRs[120,121].

Other types of afferents worth noting include noradrenergic inhibitory inputs from the LC and pons[122,123]. Also, excitatory neurotensin projections from the preoptic area, DRN, LS, PPTg, LDTg, BNST, NAc shell and lateral hypothalamus synapse in the VTA where neurotensin can decrease D2 receptor affinity in addition to opening nonselective cation channels[7,124–128]. There is also some evidence for cholecystokinin innervation of VTA dopamine neurons, as injection of CCK into the VTA increases dopamine release in the NAc and amygdala[129].

Put together, it is commonly thought that excitatory afferents onto the dopaminergic neurons drive burst firing in response to a stimulus. Under baseline conditions the neurons are under inhibitory influence that keeps them firing at a low tonic state[91,130,131]. However, there has been no direct comparison of the input density of the various neurotransmitters to VTA dopamine neurons. Furthermore, there has been no examination of the possibility of sex differences.

Outputs

Many of the early studies examining dopaminergic VTA efferents used the same techniques described above to look at VTA afferents. The use of autoradiography in the VTA revealed VTA projections in the PFC, LS, BNST, NAc, amygdala, Cl, LHb, and DR[87,132]. Subsequent studies began to parse out a topographic map of these outputs, revealing that they group into distinct regions. Generally, dopamine neurons located medially tend to project to more medial forebrain areas (such as the olfactory tubercle and LHb), while lateral dopamine neurons project more laterally to structures like the amygdala[91]. There is also a strong dorsal-ventral pattern, where ventral neurons project to dorsal areas such as the LS, PFC, BNST, hippocampus, NAc and olfactory tubercle, while the dorsal dopamine neurons synapse onto the thalamus and LHb[133,134]. The NAc targets can be further subdivided, with the medial shell receiving dopamine fibers from the posteromedial VTA and the core and lateral shell receiving them from the lateral VTA[44]. A third region synapses in the hindbrain regions, including the LC and PAG[135]. Interestingly, these subdivisions also express different proteins; the dorsal VTA neurons express calbindin and lower levels of DAT, while the ventral neurons lack calbindin, instead expressing high DAT and GIRK[136]. An additional level to this is the discovery of a group of fast-firing dopamine neurons projecting to the PFC, NAc core, medial NAc shell, and BLA that express low levels of DAT. Morphologically, these neurons are also smaller and have shorter dendrites. The more canonical dopamine cells project to the lateral NAc shell and dorsal striatum and express high DAT[137]. More recent technological advances have allowed for single-cell axon tracing of projections from the VTA. This method allows for even more finite projection pattern analysis of how dopamine neuron location may correspond to its target area. The neurons in the lateral-rostral area of the VTA project strongly to the NAc shell, dorsal striatum, lateral hypothalamus, and olfactory bulb. Medial-rostral neurons project to the NAc shell, motor cortex, and olfactory tubercle. Altogether, a profile of VTA projection neurons was created. The mesocorticolimbic neurons project to the substantia innominata, amygdala, and cortical

structures. The mesocortical cells project to the substantia innominata and somatosensory cortex. Mesolimbic cells synapse in the dorsal striatum, septum, NAc, VP, amygdala, and BNST. Mesostriatal neurons send projections only to the dorsal striatum, and the last subset send projections to the hypothalamus, thalamus, BNST, septum, and hindbrain[138]. These data illustrate that a single dopaminergic neuron can send axons to multiple targets, which could provide insight into the functionality of these cell subsets in behavior.

Dopamine-Dependent Diseases

Dysregulation of the dopamine system has been implicated in many diseases including Parkinson's disease (PD), as well as psychiatric disorders such as autism, schizophrenia, depression, and addiction[139–150]. Parkinson's disease is perhaps the most well-characterized of these due to our knowledge of its mechanistic pathology. In PD, there is a loss of dopaminergic neurons resulting in an abnormal formation of Lewy bodies and aggregation of α-synuclein primarily in the SNc. This decrease in dopaminergic innervation resulting from cell death is manifested in several physiological symptoms in patients, including general loss of motor control (including tremors and slower movement), depression, anxiety, and sleep problems. Generally, the loss of dopaminergic innervation of the striatum is considered the cause of most motor symptoms, due to the ability of dopamine to affect movement through the direct and indirect pathways[151]. This decrease in input results in impaired LTP and LTD, causing dysregulation of synaptic plasticity and resulting in many of the symptoms the PD patients experience[152]. However, as the VTA has largely not been implicated in this disease, I will not go into further specifics here; refer to [148,151,153,154] for more details.

Schizophrenia is a psychological disorder for which the cause is not well understood. While there are multiple genetic factors that are involved, environmental factors also play a role. The symptoms of schizophrenia are generally grouped into two categories: positive and negative. Positive symptoms include hallucinations, delusions, and abnormal behavior;

negative symptoms include apathy/affective flattening, anhedonia, and inability to concentrate[155]. D2 receptor blockade using atypical antipsychotics is a common treatment for schizophrenic patients; manipulations to increase dopamine, such as L-Dopa, amphetamine and methylphenidate exacerbate the symptoms of schizophrenia[156]. Dysfunctional dopaminergic signaling has been implicated in the positive symptoms of schizophrenia as well; an abnormally high level of dopamine in the forebrain is thought to underlie hallucinations[139,146,157,158]. Accordingly, a decrease in PFC activity, which would in turn lead to loss of inhibition onto dopamine neurons, could underlie this mechanism[157,159]. A more recent hypothesis postulates that it is actually a deficit in dopamine in schizophrenic patients that underlies symptoms; this deficit leads to an uncontrolled increase of dopamine release in an attempt to compensate[160]. However, an examination of a spontaneous mutation in a calcium-activated small conductance potassium channel, SK3, found in a schizophrenic patient, reveals that inhibition of SK3 channel function in mice allows for increased dopamine neuron burst firing, increased dopamine release in the NAc, and a behavioral phenotype exhibiting dysfunctional attention gating[11]. This impairment in attention is exhibited by some schizophrenic patients.

The link between dopamine deficiency and depression is currently controversial. Many of the anti-depressant medications prescribed to patients with this disorder affect the dopamine system. Selective serotonin reuptake inhibitors (SSRIs), which are often used to treat depression, have been shown to decrease dopamine release in the NAc and striatum[161]. However, another study demonstrated a SSRI-stimulated increase in dopamine in the PFC in a 5-HT independent manner[162]. The link between dopamine and serotonin is, as alluded to previously, also complicated. While it may be tempting to believe that the increase in 5-HT caused by SSRIs may lead to an increase in dopamine release, there is contradicting evidence in this regard. Activation of 5-HT1 receptors increase dopamine release, while 5-HT2 agonists inhibit it; additionally, fluoxetine was shown to inhibit dopaminergic activity[163,164]. A lack of motivation is a common symptom in depression

patients, and is thought to be caused by a misregulation of reward circuitry involving the VTA-NAc dopaminergic circuit[165]. Human studies showed an increased response to a rewarding substance in depressed patients[166]. Previous studies show that in a forced swim test, which is a measure of depression and motivation in murine models, a D2 receptor antagonist decreased the amount of time spent immobile, indicating increased motivation presumably resulting from increased dopamine release[167]. Overall, while it seems that dopamine may play a role in depression and depression-related mental states, there is little direct evidence that abnormal dopamine release or signaling is directly responsible for the disease phenotype.

Autism spectrum disorder is a wide-ranging, genetically linked mental disorder. Autistic patients can exhibit numerous symptoms including social difficulties/abnormal social interactions and repetitive motor movements. The role of dopamine in autism is demonstrated through multiple factors. Medications that increase dopamine levels, such as a dopamine reuptake inhibitor and a D2 receptor antagonist, relieve some of the symptoms of autism[140,168]. However, a study examining levels of dopamine transporter binding showed higher levels in the frontal cortex of autistic patients, suggesting that there is an overactive dopamine system in these subjects[169]. A search for genes related to dopamine pathway in autistic patients revealed several mutations in genes encoding for proteins related to dopamine synthesis, as well as in the D2 receptor[140,170]. Additionally, a rat model of autism exhibited increased fear generalization and fear conditioning, behaviors which are dopamine-dependent[171].

Post-traumatic stress disorder (PTSD) is a disease that often manifests after an individual experiences an extremely fearful event. PTSD may develop due to repeated exposure to trauma, after a particularly threatening event, or because an individual is not able to recover from exposure to such an experience. Symptoms may include generalization of fear to stimuli remotely related to the events, hypervigilance, anxiety, and repeated recall[172]. Treatment for PTSD primarily involves cognitive and exposure therapy, no drug

interventions have been effective. While the link between dopamine and PTSD has not been thoroughly explored, the role of dopamine in fear-association learning makes it a crucial factor for study of this disease. A study of plasma dopamine levels in PTSD patients found increased levels compared to controls; a complementary finding from the same lab revealed higher DBH levels in patients[144,173]. A follow-up to these experiments showed an increased prevalence of a D2 receptor polymorphism in PTSD individuals that corresponds with a decrease in dopamine binding to the receptor[174,175]. Accordingly, another human study demonstrated an increase in binding of the dopamine transporter in the striatum in patients, which could either be due to an overall increase in dopamine levels, or due to abnormal increased activity of DAT[145]. Examination of polymorphisms in the *Slc6a3* revealed a higher occurrence of repeat alleles in PTSD patients, which may indicate a dysregulation of dopamine[149]. More functional studies showed the amygdala, which as described previously is involved in fear learning, is significantly more active when exposed to fearful stimuli[176,177]. Altogether, these data suggest that abnormal dopamine signaling may be involved in PTSD.

Dopamine and its association with reward learning make it a logical factor in the development of drug addiction. In addition, many drugs such as amphetamine, nicotine and cocaine, act directly on the dopamine system and increase dopamine release in the NAc[63,178]. More specifically, drugs of abuse seem to preferentially increase dopamine and Fos levels in the NAc shell[178–180]. Phasic dopamine signaling, while not required for the motor effects of drugs, is necessary for drug-cue association and behavioral sensitization[181]. As the relation of dopamine, drug seeking, and reward learning has already been discussed, further detail about the nature of dopamine and addiction will not be covered further here. Dopamine is not only involved in the direct effects produced by drugs of abuse, but is also instrumental in forming memories of stimuli related to drug association[150,182]. This plasticity is a critical aspect of addiction.

Sex Differences in the Dopamine System

The study of sex differences is one that is vital to our understanding of the brain and human body as a whole. However, current research is lacking and existing data are convoluted, not well-controlled, and contradictory. In 2014 the NIH announced its goal to make sex differences research a priority through increased funding and the implementation of policies in grant reviews. The hope is that encouragement of sex differences research will result in a better understanding of the effect of sex and the possible translational implications in disease treatment[183]. In April 2016 a PubMed search for dopamine results in 144,686 articles; a search for sex differences gave 197,132 results; the terms sex differences AND dopamine produced only 1,424 articles. This demonstrates a need for sex differences research in the dopamine system, particularly in characterizing any distinctions at a baseline state without manipulation.

Dopamine Neuron Function

In cell culture, the examination of dopamine on hormone receptor gene transcription reveals that dopamine can increase transcription of progesterone and estrogen receptors, which is mediated through dopamine receptors[184]. Conversely, application of sex steroids on dopamine neurons in culture decrease DOPA synthesis, although another lab found that while this same effect was replicated in DAT levels with estrogen, the opposite effect was shown in TH protein levels[185–188]. Exploration of these effects *in vivo* revealed that administration of estrogen in ovariectomized (OVX) rats and intact male rats increased activity of one subset of SNc dopamine neurons, while decreasing the firing rate of a second group[189]. This biphasic response is further supported through counts of TH+ neurons in the SNc and VTA of OVX rats and found a reduction of the cells of the SNc and medial VTA but not the lateral, suggesting that estrogen may differentially affect individual populations of dopamine neurons[190]. Additionally, injection of estrogen into castrated (CAST) or OVX rats had variable effects; acutely, females showed a decrease in D2 receptor affinity, which was also seen after 4 hours in males[191]. These data indicate that hormones can act on dopamine

neurons, implying the presence of hormone receptors in dopamine neurons. Indeed, estrogen receptors (ERs) were found in the VTA (but absent from the SNc) of both intact male and female rats. In males, over 80% of ER+ neurons were also TH+, while around 50% of TH neurons were also ER+. However, in females only about 10% of TH neurons contained ERs[192]. Examination of androgen receptors (ARs) in TH+ neurons projecting to the PFC revealed that less than 5% of these were AR+ in females, while about 25% were also AR+ in males[193]. Together, this evidence indicates that sex steroids, particularly estrogen, may have an effect on the dopamine neurons of the VTA, although it is unclear whether it is mediated through hormone receptors or dopamine receptors.

There are many more studies looking at the effect of hormones on dopaminergic innervation and release in target areas. The presence of TH+ fibers in the cingulate and insular cortex showed sensitivity to the presence of hormones in male rats. CAST resulted in a large decrease in TH innervation, which interestingly rebounded to above-baseline levels after 28 days; CAST rats supplemented with testosterone do not demonstrate these changes[194,195]. This finding was further characterized in the PFC with similar results[196]. However, gonadectomy followed by hormone replacement in male rats shows an increase and decrease, respectively, in PFC dopamine levels while estrogen produced no effect[197]. Stimulation of dopamine release using amphetamine seems to vary across sexes and be dependent on hormone levels. For example, dopamine levels in the striatum in OVX females are greatly decreased over intact females and are restored to baseline levels with estrogen+progesterone treatment. Intact male rats have similar dopamine levels to diestrus/estrus females (when estrogen, leutenizing hormone, and follicle stimulating hormone levels are lower) and gonadectomy has no effect, although a separate study demonstrated that estrogen+progesterone treatment in CAST males increases amphetamine-stimulated dopamine release[198,199]. These findings suggest that ovarian hormones can affect dopamine release, while testicular hormones have no effect. Follow-up studies showed that OVX rats with lower baseline striatal dopamine compared to CAST rats.

Estrogen treatment increased stimulated dopamine release in OVX but not CAST subjects[200]. Additional findings from the same lab showed that, again, striatal dopamine was unaffected by hormone levels in male rats, but OVX/diestrus dopamine was significantly lower than proestrus/estrus levels[201]. However, a comparison of dorsal and ventral striatum dopamine release in intact male rats showed that both areas demonstrated a rise in dopamine after testosterone administration[202].

The prevailing mechanistic model behind these data was that estrogen downregulates D2 receptors, increasing dopaminergic release; it may act directly on post-synaptic GABAergic neurons in the striatum, inhibiting their activity and thereby allowing for increased dopamine neuron activity[203]. An examination of dopamine receptor mRNA and ligand binding in the striatum and NAc of male rats showed a decrease in *Drd2* mRNA after estrogen administration using in situ hybridization; however, this did not correspond to a decrease in binding[204]. At odds with this finding are other results indicating an increase in density of striatal dopamine receptors after application of estrogen[205]. However, the possibility of post-translational mechanisms to explain these conflicting reports has not been sussed out.

The first study to use fast-scan cyclic voltammetry to interrogate sex differences in striatal dopamine release showed much higher stimulated release in female rats, which is at odds with previous findings using microdialysis; however, the methods of stimulation were not the same, which could account for this discrepancy[206]. A human study looking at amphetamine-stimulated dopamine release revealed a significantly larger dopamine release in males in the striatum, as well as the putamen and caudate nucleus[207]. Another human study reporting a higher uptake of striatal fluorodopa (a radiolabeled dopamine precursor) in women than men[208]. Dopamine release in areas other than the striatum has also been explored. For example, dopamine levels in the BLA at baseline are much higher in intact male versus female rats, although restraint stress evokes a much higher dopaminergic response in females[209]. Nonetheless, it is evident that there are a great deal of contradictory

findings surrounding dopamine innervation and release from the VTA to the forebrain, and no mechanism that explains any sex differences has been rigorously tested.

Data from examination of sex differences in dopamine neuronal firing properties is extremely scarce. In fact, only one such study exists in rats and none exist in mice. Zhang et al found in anesthetized intact female rats that the firing and burst rate of VTA dopamine neurons were significantly increased during estrus and diestrus as compared to proestrus[210]. This is contradictory to studies looking at the hormonal effect of dopamine release, where estrogen administration increases dopamine levels in the OVX striatum, but in line with measurements of dopamine in the PFC during the estrous cycle, implying that some compensatory mechanisms may occur after OVX[198–200,211]. Indeed, when dopaminergic firing properties of OVX rats were measured, the replacement of estradiol or progesterone had a positive effect on firing and burst rate[210]. Additionally, cocaine had no effect on the firing rate or locomotor activity in OVX rats, but hormone replacement rescued this[210,212,213].

Dopamine-Dependent Behaviors

Aversion

As might be expected in light of the conflicting reports of sex differences in dopamine neuron function and release, sex differences in behavioral tests also produced mixed results. In humans, males and females often exhibit different preferences for learning strategies[214]. Hormones may play a role in modulating the ability of each sex to perform various tasks. Examination of intact female and male rats in fear conditioning shows that males exhibit more freezing to a shock-paired context, while there is no sex difference in cued fear conditioning[215]. In fear conditioning where there are two cues, one that predicts a shock (CS+) and a second that indicates safety (CS-), estrogen seems to disrupt the ability of female rats to learn the difference between these cues. Gonadectomized male and female rats, along with estrogen-administered CAST rats are able to recognize a safety cue, while estrogen-administered OVX rats show more generalized fear. However, in a fear

conditioning paradigm with a single shock-paired cue and no CS- these rats were able to form this association, implying that estrogen inhibits the ability of female rats to decrease their fear when the safe CS- was presented[216]. However, in another study testing single-cue fear conditioning, estrogen-treated OVX mice demonstrated increased freezing over controls during the CS[217]. In addition, estrogen treatment of intact female mice also increases both contextual and cued fear learning[218].

Extinction of fear is an important part of learning. When a previously fear-predicting cue is encountered multiple times without the previously paired aversive stimulus, expression of fear to the cue should decrease. Fear recall tests the ability of a subject to re-learn the fear-cue association following extinction. Extinction during the proestrus phase of the estrous cycle when hormone levels are high, was facilitated, indicating that hormones increase extinction learning. To support this observation, female mice in metestrus, when hormone levels are low, were injected with estrogen and progesterone, resulting in facilitated extinction[219]. Another study demonstrated increased extinction in intact females as compared to intact males and OVX females; estrogen treatment also facilitated extinction in OVX females as compared to vehicle injected OVX females[220]. While data on fear conditioning seems conflicting, the results are relatively consistent in regards to fear extinction. Estrogen levels modulate the ability of murine models to extinguish fear learning, although the ability of estrogen to do this in males remains unexplored.

Reward

A common paradigm used to measure reward learning is operant conditioning, where subjects have to perform a task, such as press a lever, to receive a reward. Often this is in the form of an appetitive food pellet. Although females are more active in general, males show a tendency to contact the lever more often. As a result, males perform better in instrumental conditioning tasks[221,222]. After gonadectomization, female performance decreases dramatically, but male performance is largely unaffected[223]. Studies conflict in their results of hormone manipulations, as others find no difference with OVX or hormone

replacement and others still find no sex difference at all[224,225]. However, in a version of this task where subjects are required to press a lever an exponentially increasing number of times to receive a reward, females exceed males, implying greater motivation to work for the reward; additionally, male performance is worsened by CAST[214,226]. Again, results are conflicted, as another study reported no sex differences in the progressive-ratio task, and no effect of gonadectomy[227]. Data for this particular behavior are difficult to compare, as the required lever presses increases at a rate that varies across studies. In a Pavlovian conditioning task, where learning is measured by the number of times the subject puts their head in the food receptacle during a CS+ that predicts food delivery, females learn this cue-reward association faster, although this sex difference disappears by day 5 of training[228,229]. Furthermore, estrous cycle does not affect this behavior[229]. Overall, the presence of sex differences and the effect of sex hormones in dopamine-dependent behaviors are unclear, as many studies report conflicting data.

Dopamine-Dependent Diseases

There is a large disparity in the prevalence of Parkinson's disease between men and women, with a ratio of 1.6:1, respectively[230]. Besides the overt prevalence of PD, there are also sex differences in the symptoms presented in patients. For example, the age of onset is later in women and they have an overall milder presentation of the disease, indicating that the disease progression is slower for females[231]. However, some symptoms are more common in women, including tremor, dyskinesia, and depression[232,233]. The factors contributing to this cover both physiological and environmental aspects.

The role of hormones in PD has garnered a great deal of interest. A correlation study examining the possibility of a link between female hormones and PD development suggested that estrogen may contribute to the delayed/milder PD presentation in women[233]. In the MPTP mouse model of Parkinson's, which itself displays a more toxic effect in males, male mice show a greater dopaminergic loss, as well as an increased response to methamphetamine than females. This was confirmed in lower levels of DOPAC, HVA, and

DAT binding as well. β-estradiol treatment at doses similar to physiological levels proves to have neuroprotective effects in both male and female MPTP mice, but cannot reverse the damage already done. Additionally, administration of testosterone does not produce the same protective effect[234]. In humans, preliminary evidence demonstrates this hormone influence as well. Estrogen hormone replacement has shown some relief of PD symptoms, as well as a decreased risk of disease development[232]. The mechanism by which this interaction occurs is theorized to be mediated through ERα, as agonists of this receptor, but not ERβ are protective against dopaminergic loss. Furthermore, ERα knockout mice exhibit a worsening of dopamine loss, while ERβ knockout mice do not[234,235]. Additional proposed mechanisms involve signaling pathways that affect cell survival, including activation of the PI3K/Akt pathway leading to inhibition of pro-apoptotic GSK3β, as well as expression of the Y-linked gene *SRY*[232,234,236].

Schizophrenia is another dopamine-dependent disease exhibiting sex differences with the prevalence in men ranging from 40-50% more than women[237,238]. Additionally, men experience an earlier age of onset, a decreased responsivity to treatment, and more severe symptom presentation, in particular negative symptoms[239–243]. Interestingly, this disparity is sustained over time[244]. Contributing factors to these differences include increased likelihood of brain structure abnormalities in men, where men have significantly larger ventricles than women in addition to other brain volume abnormalities in areas including the temporal lobe[242,245]. A natural result of these reported sex differences is the exploration of the role of hormones. The use of estrogen as a therapeutic agent in treating schizophrenia produces mixed results, although comparison across the estrous cycle, as well as pre- versus post-menopausal suggests that estrogen may be protective in females with schizophrenia[237,239]. In a mouse model of schizophrenia, induced by administration of haloperidol and apomorphine, β-estradiol injection in female rats decreases symptoms, implying that estrogen inhibits dopamine activity, as the current mechanistic theory of schizophrenia asserts that it is caused by excess dopamine[246]. However, other studies in mice

demonstrated that estrogen increases stimulated dopamine firing and release, which is at odds with these findings. It is clear that much needs to be clarified in the cause of sex differences in schizophrenia; it is likely that it can be attributed to multiple factors, including but not limited to sex hormones.

Unlike schizophrenia and PD, depression is far more prevalent in women[247]. The cause for this has been theorized to be explained by genetic predispositions, environmental factors or biological differences primarily explained by changes in hormone levels. Evidence for the latter is the existence of postpartum depression, an increase in depression rates at mid-puberty, as well as premenstrual depression[248]. During these periods, ovarian hormones are low, leading some to believe that these decreased levels of estrogen and progesterone may be linked to the incidence of depression[249]. If one takes into consideration the influence of these hormones on dopamine, and the fact that there is believed to be a low level of dopamine in depression patients, this association may be real. However, the mechanisms behind this theory have yet to be explored.

The gap between men and women in the incidence of autism is one of the most extreme; men are four times more likely to be diagnosed[250]. It is notable that despite this large sex difference, dopamine's proposed role in autism and the link between hormone levels and dopamine, little to no research has been done into whether hormone manipulations might relieve autistic symptoms. Finally, PTSD is another dopamine-dependent disease that shows higher incidence in women. This can be at least partially explained by the differences in the types of trauma that each sex is exposed to; women are more likely to experience emotional or sexual trauma, while men are more likely to experience combat and disaster situations. However, even within groups of mixed sex experiencing the same type of trauma, women were more likely to develop PTSD. Therefore, other factors may be involved[251]. As detailed previously, fear learning and extinction are heavily influenced by dopamine signaling. While the effects of sex hormones have not been well-explored in PTSD patients, there is some evidence that women taking

oral contraceptives, and therefore maintaining a low level of estrogen and progesterone, show an elevated response to the CS+ in the extinction of a fear conditioning paradigm, indicating that hormones facilitate extinction[252]. Animal models of fear have examined this and show similar effects; compared to males, females have a slightly elevated increase in extinction, and administration of estrogen, progesterone, or both, increase the ability of female rats to extinguish fear, although the effect of hormones in males was not explored[219]. Still, these data are contrary to the larger prevalence of PTSD in women. More research must be done to determine how the experiences, processing, and neurobiology of women may underlie these differences.

Overall, this review of current literature demonstrates that dopamine is an important neurotransmitter in many behaviors, including aversion and reward. Dopamine receives input via multiple neurotransmitters, including glutamate, GABA, neurotensin, and serotonin; however, comparison of the density of these inputs has never been done. These inputs project to the midbrain from many areas that facilitate the ability of the brain to process environmental stimuli and promote learning. Dopaminergic neurons then transmit outputs to multiple areas that can then formulate an appropriate response. Additionally, dopamine is involved in multiple diseases, many of which exhibit strong sex differences in their prevalence. The findings reported surrounding sex differences are highly variable and often contradictory.

Our goal was to thoroughly characterize the VTA dopaminergic system at a baseline state. To accomplish this, we mapped the inputs of the major neurotransmitters synapsing onto the VTA; the outputs of the VTA dopamine neurons were also explored. In addition, we examined the expression of mRNA transcripts in VTA dopamine neurons, analyzing how the expression levels of many receptors mirror our input findings. Basic electrophysiology properties were also tested, both in vivo and in slice. Finally, we tested mice in multiple dopamine-dependent behaviors. To add to these data, all experiments were performed in male and female and analyzed for sex differences. This comprehensive description of the

dopamine system and the examination of sex will facilitate our understanding of diseases involving dopamine, as well as the presentation of sex differences in these diseases. Our hope is that these data will further our understanding of the dopamine system as a whole and perhaps lead to treatments that might be tailored based on sex; without this understanding we cannot hope to comprehend the underlying baseline state of the brain or the cause of sex differences in a disease state.

CHAPTER 2

VTA CIRCUITRY

VTA Afferents

The VTA is composed primarily of either dopaminergic or GABAergic neurons. Dopamine neurons comprise somewhere from 55-70% of the VTA, while the rest of the population is primarily local inhibitory interneurons, with a small percentage also expressing glutamate[253,254]. A population of dopamine neurons co-release glutamate or GABA, while non-dopaminergic neurons in the medial VTA release exclusively glutamate[255–257]. Therefore, we sought to examine the inputs to the VTA by using a viral tracing approach. Although many studies have detailed the areas projecting to the VTA using techniques such as horseradish peroxidase and fluorogold, we utilized the retrograde virus CAV2-Cre in conjunction with a mouse reporter line, Rosa26-fs-TdTomato. Following injection into the VTA, this vector is taken up by cell bodies and terminals in the injection site and travels retrograde to neuronal bodies. There, it turns on the TdTomato reporter, allowing for fluorescent expression in the cell bodies of VTA afferents (Fig 1A-D).

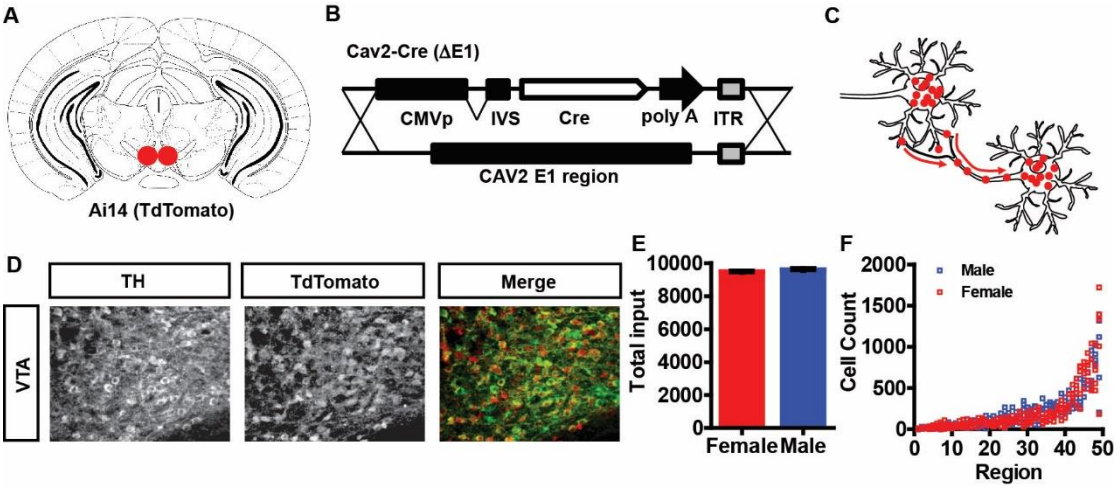


Figure 1. Viral approach for mapping VTA afferents. A) Schematic of VTA targeting by CAV2-Cre in Rosa26-fs-TdTomato mouse line. B) Schematic of CAV2-Cre viral vector. C) Representation of CAV2-Cre retrograde movement. D) Immunohistochemistry of coronal VTA slices from Rosa26-fs-TdTomato mice injected with CAV2-Cre. Left: anti-TH, a marker of dopamine neurons. Right: anti-dsRed. E) Total average number of cells counted per brain shows no sex difference. Unpaired t-test, n=5 males, 5 females. F) Positive correlation between number of cells counted and each brain region from rostral to caudal. $p<0.0001$. All values are shown as mean ± SEM.

Existing literature primarily uses male subjects, disregarding the possibility of sex differences. I used both male and female cohorts with the purpose of uncovering any sex differences in VTA afferents. Whole brains were sectioned and approximately every third section was imaged. Fluorescent cells were counted in all areas, summed for each animal, and averaged across sexes. The total number of fluorescent cells in each brain was not different between males and females (Fig 1E). A correlation between the number of cells counted in each area and their position in the rostral-caudal axis revealed a significantly positive relationship in males and females, indicating a higher density of inputs to the VTA in the posterior area of the brain (Fig 1F). Several areas exhibited fluorescent expression; areas of high projection strength include the prefrontal cortex (PFC), dorsal striatum (Dstr), cingulate cortex (Cg), lateral hypothalamus (LH), medial habenula (MHb), and dorsal raphe (DRN). These afferents match closely with previously reported results[86–88]. An examination of the number of input neurons in each area reveals no sex differences in the total afferents

per area (Fig 2). These novel results show that there are no sex differences in the number of neurons per afferent area projecting to the VTA.

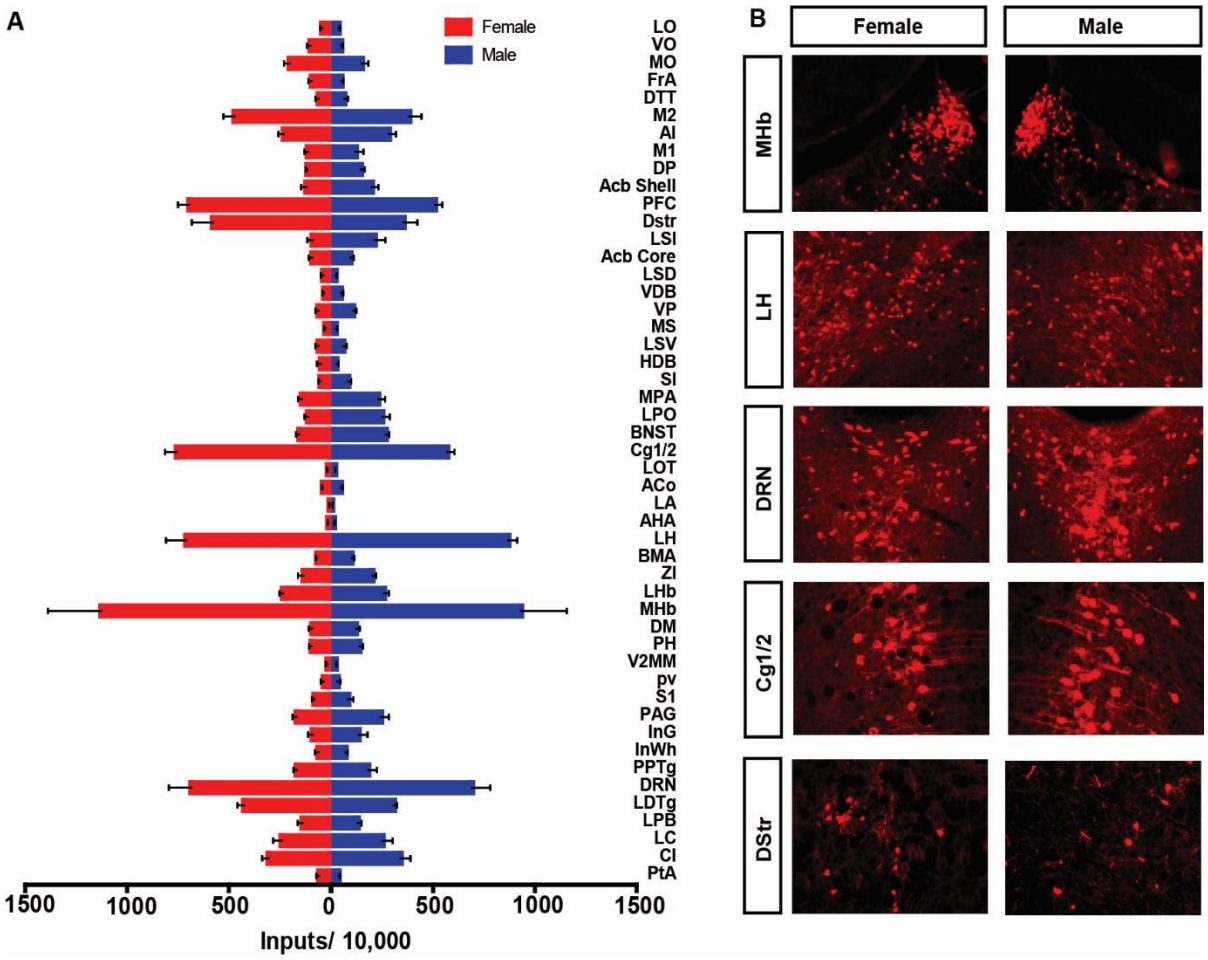


Figure 2. Mapping of inputs to VTA neurons. A) The number of afferent neurons in each area, normalized to total number of input neurons/10,000, reveals no sex differences. Two-way ANOVA with Bonferroni post-hoc multiple comparisons. B) Representative images of input neurons in male and female mice in the medial habenula (MHb), lateral hypothalamus (LH), dorsal raphe (DRN), cingulate cortex (Cg1/2), and dorsal striatum (DStr) from top to bottom. All values are shown as mean ± SEM.

Next, I investigated the inputs to the VTA in a cell-type specific manner; in particular, the afferents of the VTA dopaminergic neurons, which comprise the majority of cells in the VTA. To accomplish this I utilized a modified rabies virus approach. First, male and female *Slc6a3*$^{Cre/+}$ mice were injected into the VTA with a Cre-dependent virus expressing the rabies virus glycoprotein in addition to the fluorescent tag GFP (AAV1-EF1α-

FLEX-GFP). This resulted in expression of the rabies glycoprotein and GFP exclusively in dopaminergic neurons of the VTA. After two weeks a modified rabies virus, (EnvA)-SADΔG-mCherry, was also injected into the VTA. Here, the glycoprotein required for transynaptic spread was removed and the fluorescent marker mCherry was added. Therefore, the rabies virus will infect all neurons, but only express in and spread in a retrograde manner transsynaptically from dopamine neurons containing the glycoprotein virus (Fig 3A-D). mCherry-positive neurons can then be quantified across the entire brain in males and females the same way as described in the previous CAV-Cre experiment. Here, the positive correlation between number of cells and position on the rostral-caudal axis was also highly significant in both males and females (Fig 3E).

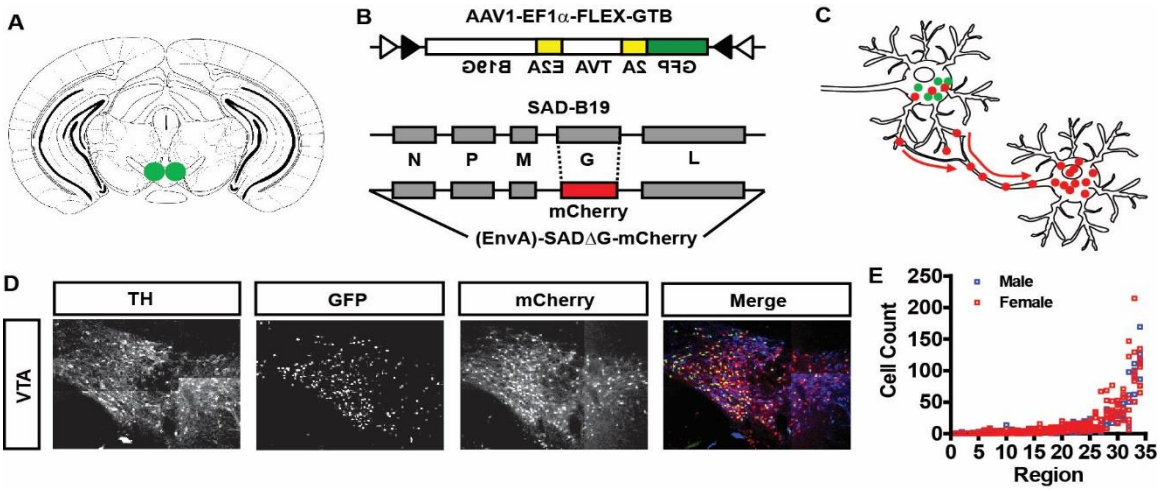


Figure 3. Viral approach for mapping afferents of VTA dopamine neurons. A) Schematic of VTA viral targeting by a Cre-conditional glycoprotein-GFP followed by modified rabies-mCherry in *Slc6a3*$^{Cre/+}$ mice. B) Schematic of AAV1-EF1α-FLEX-GFP and (EnvA)-SADΔG-mCherry viral vectors. C) Representation of dopaminergic glycoprotein expression and retrograde rabies virus transynaptic spread. D) Immunohistochemistry of coronal VTA slices from *Slc6a3*$^{Cre/+}$ mice injected with glycoprotein and modified rabies viruses. Left: anti-TH, a marker of dopamine neurons. Middle: anti-GFP. Right: anti-dsRed. E) Positive correlation between number of cells counted and each brain region from rostral to caudal. $p<0.0001$, n=5 males, 7 females.

To control for any possible sex differences in viral injection or efficiency, I quantified the number of GFP cells across the entire VTA (Fig 4A). I also counted the percent of TH+ neurons that showed overlap with mCherry+ or GFP+ to demonstrate viral efficiency, in addition to the percent of GFP cells that were co-labeled for TH as a measure of viral specificity (Fig 4B). Importantly, viral expression exhibited sex differences. A quantification of inputs to VTA dopamine neurons also revealed similar results to previously published literature[89,90]. Areas with high projection strength included the NAc, BNST, LH, PPTg, and DRN. An analysis of sex differences showed that, again, the number of dopamine-projecting cells in each area was not different between males and females (Fig 5). Mapping of the afferents of the VTA in a nonspecific manner showed high congruency with previously published results. Mapping of inputs to dopamine neurons of the VTA also mirrored existing literature. However, the possibility of sex differences in these inputs had not been examined. I show new data indicating that there is no difference in VTA afferent projection strength between male and female mice, both in a nonspecific and dopamine-specific manner.

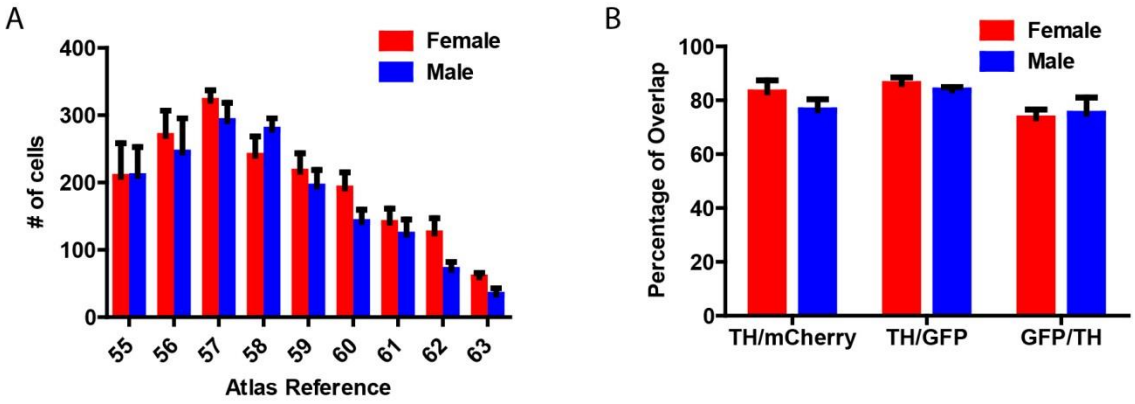


Figure 4. Quantification of viral efficiency and specificity showed no sex sex differences. A) Number of GFP+ neurons in across the entire VTA based on the Paxinos and Franklin Atlas. Two-way ANOVA. B) Percent of TH+ neurons that also expressed mCherry or GFP, in addition to the percent of GFP+ neurons that are dopaminergic. Unpaired T-test. All values are shown as mean ± SEM.

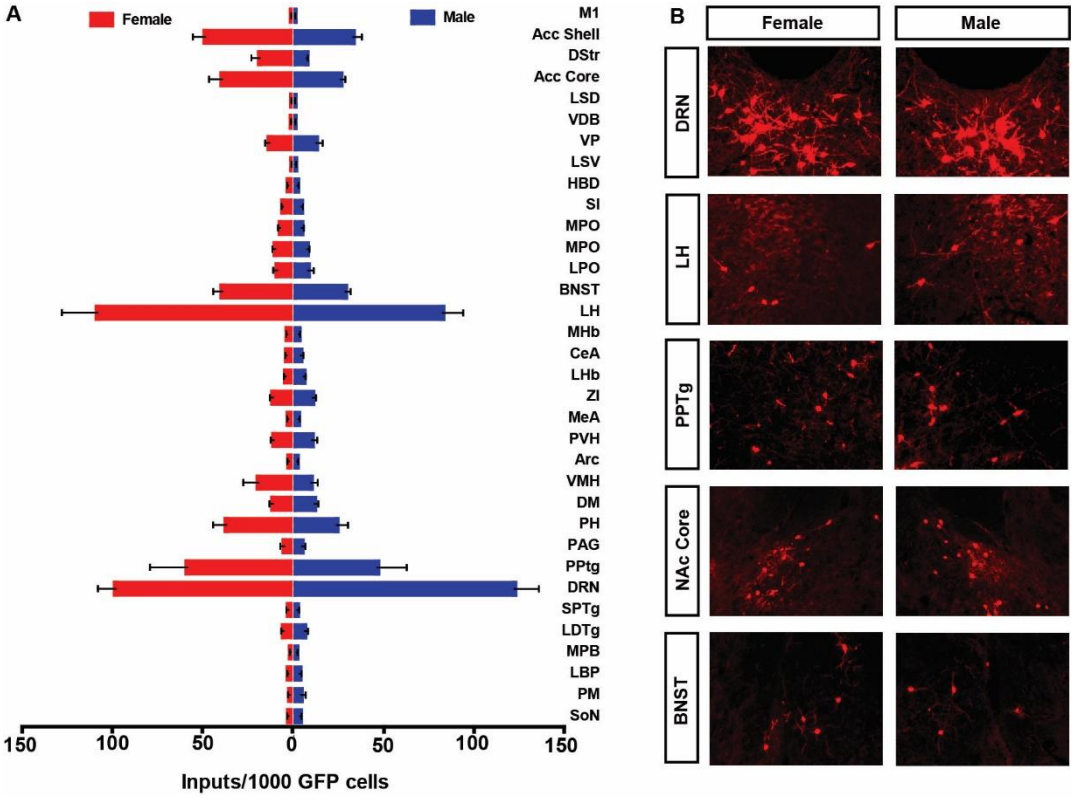


Figure 5. Mapping of inputs to VTA dopamine neurons. A) The number of afferent neurons in each area, normalized to total number of GFP+ neurons/1,000, reveals no sex differences. Two-way ANOVA . B) Representative images of input neurons in male and female mice in the DRN, LH, pedunculopontine tegmental nucleus (PPTg), nucleus accumbens core (NAc), and bed nucleus of the stria terminalis (BNST) from top to bottom. Data are shown as mean ± SEM.

Mapping Neurotransmitter-specific VTA Inputs

While several of the inputs to the VTA have been well-characterized individually in terms of neurotransmitter release and consequent effect on VTA-driven behavior, the strength of these afferents has never been compared across areas expressing various signaling molecules. I quantified the inputs to the VTA in areas expressing markers for GABA, glutamate, serotonin, and acetylcholine. Each of these neurotransmitters have been reported to synapse onto VTA neurons. To accomplish this goal, I utilized a conditional retrograde virus, CAV-FLEX-ZsGreen. Similar to CAV2-Cre, this virus is taken up by terminals in the injection area and travels in a retrograde manner. I used this approach in combination with a variety of genetic mouse lines expressing Cre in neuronal subtypes

expressing the aforementioned neurotransmitters. Here, loxP sites undergo recombination with Cre and allow for expression of ZsGreen.

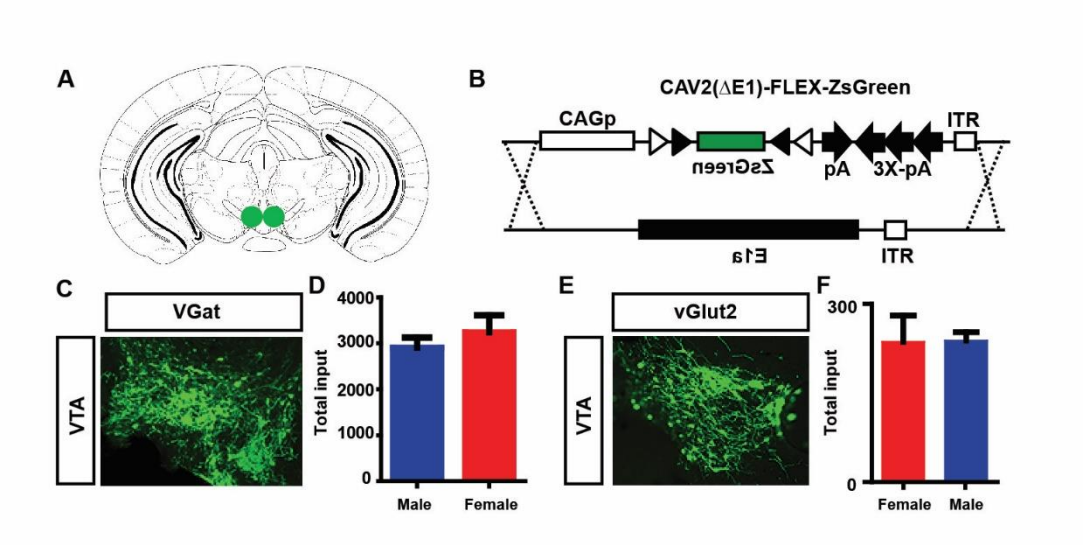


Figure 6. Viral approach for mapping GABAergic afferents of VTA neurons. A) Schematic of VTA viral targeting by CAV2-FLEX-zsGreen in *Slc32a1*$^{Cre/+}$ mice. B) Schematic of AAV1-EF1α-FLEX-ZsGreen viral vector. C) Representative image of ZsGreen expression in the VTA of a *Slc32a1*$^{Cre/+}$ mouse at the injection site. D) Total average number of cells counted per brain shows no sex difference. Unpaired t-test. E) Representative image of ZsGreen expression in the VTA of a *Slc17a6*$^{Cre/+}$ mouse at the injection site. . F) Total average number of cells counted per brain shows no sex difference. Unpaired t-test. Data are shown as mean ± SEM.

GABA

First, I injected CAV2-FLEX-ZsGreen into the VTA of *Slc32a1*$^{Cre/+}$ mice. This resulted in fluorescent expression in GABAergic neurons projecting to the VTA (Fig 6A-C). An examination of afferents providing inhibitory input to the VTA revealed multiple areas overlapping with structures seen using CAV2-Cre. Notably, areas of high expression included the DStr, NAc core, VP, BNST, medial preoptic area (MPA), lateral preoptic area (LPO), LH, retrorubral field (RRF), DRN, and LDTg. Regions where I saw no fluorescent expression in, but have been previously reported as those releasing GABA in the VTA include the RMTg, GP, and Broca's diagonal band. I provide novel areas of GABAergic input

including the medial amygdala and RRF. An analysis of sex differences revealed no difference in total number of input neurons, as well as similar projection strength in all areas between males and females (Fig 6D,7).

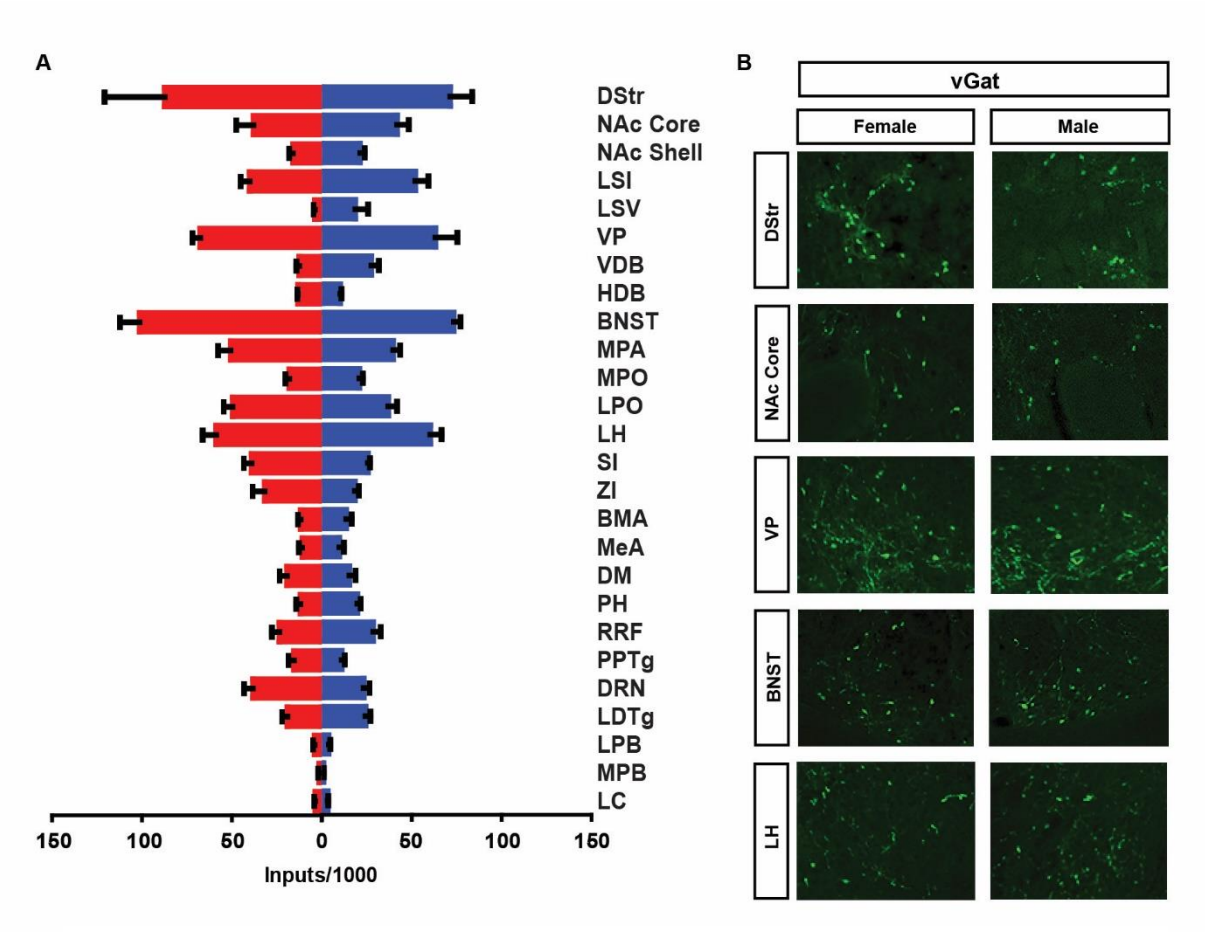


Figure 7. Mapping of GABAergic inputs to VTA neurons. A) The number of afferent neurons in each area, normalized to total number of input neurons/1,000, reveals no sex differences. Two-way ANOVA, n=5 males, 6 females. B) Representative images of input neurons in male and female mice in the dorsal striatum, NAc core, ventral pallidum, bed nucleus of the stria terminalis, and lateral hypothalamus, from top to bottom. Data are shown as mean ± SEM.

Glutamate

Use of the same viral strategy in *Slc17a6*$^{Cre/+}$ mice allowed for visualization of glutamatergic inputs to the VTA (Fig 6E). Areas that displayed ZsGreen expression included the NAc, LH, DRN, PPTg, LDTg, and LC (Fig 8). Again, we saw no sex difference in the number of cells per afferent area. Surprisingly, when I compared total glutamatergic and

GABAergic cell counts, I found there were significantly more GABAergic VTA afferents (Fig 9). This finding indicates that similar to the SNc, the VTA remains largely under the influence of inhibitory tone[130]. Previous studies have detailed the extensive GABAergic input to the SNc. Between 50-70% of the synapses in the SNc express GABA, primarily originating in the globus pallidus, striatum, and the SNc itself[258]. The influence of this input on SNc dopamine activity and behavior has been detailed in multiple studies[130,258–261]. However, whether or not the VTA also operates under a similar inhibitory influence has not been explored, making this a novel finding.

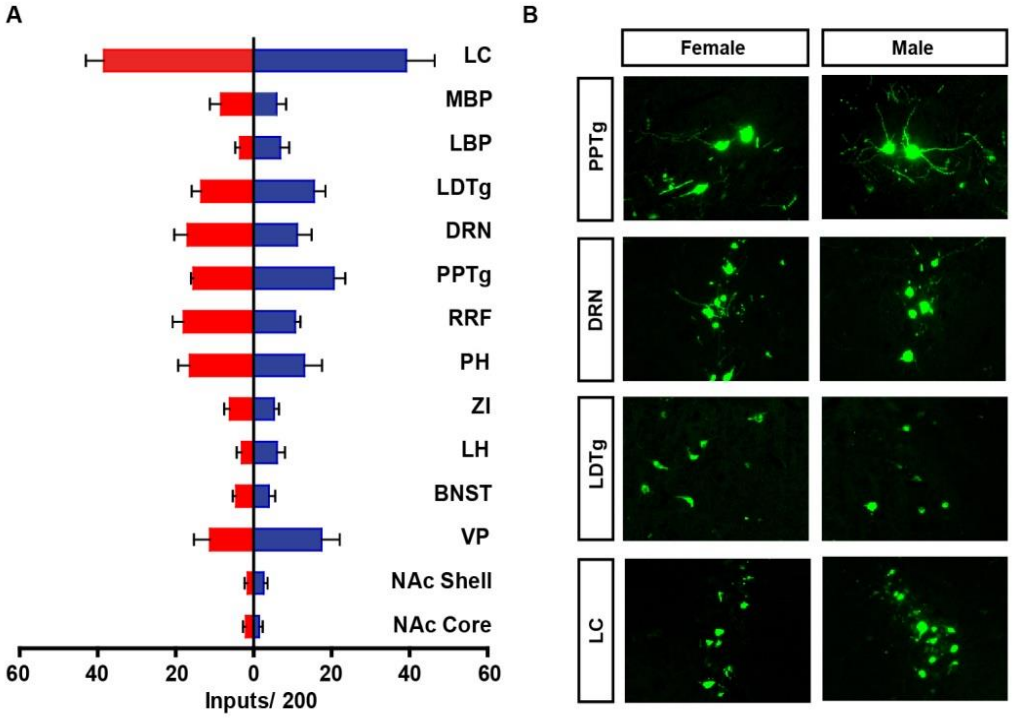


Figure 8. Mapping of glutamatergic inputs to VTA neurons. A) The number of afferent neurons in each area, normalized to total number of input neurons/200, revealed no sex differences. Two-way ANOVA, n=4M, 6F B) Representative images of input neurons in male and female mice in the pedunculopontine tegmental nucleus, dorsal raphe nucleus, laterodorsal tegmental nucleus, and locus ceruleus, from top to bottom. Data are shown as mean ± SEM.

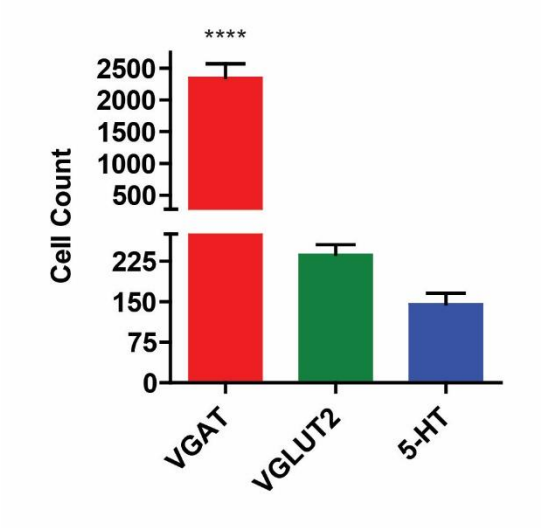


Figure 9. Comparison of total neurotransmitter-specific inputs to the VTA. The total number of GABAergic neurons projecting to the VTA was significantly higher when compared to glutamatergic and serotonergic inputs. One-way ANOVA, $F_{(2,31)}$=103.4. ****p<0.0001, Bonferroni's multiple comparison test. Data are shown as mean ± SEM.

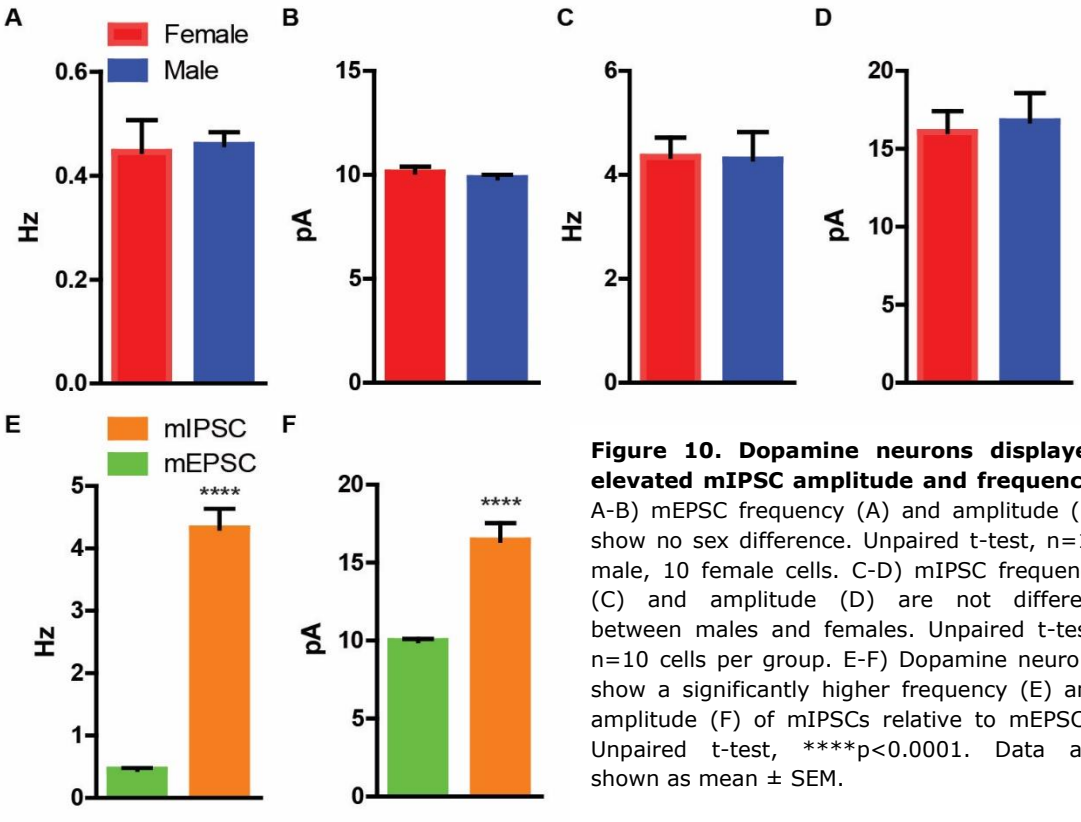


Figure 10. Dopamine neurons displayed elevated mIPSC amplitude and frequency. A-B) mEPSC frequency (A) and amplitude (B) show no sex difference. Unpaired t-test, n=12 male, 10 female cells. C-D) mIPSC frequency (C) and amplitude (D) are not different between males and females. Unpaired t-test, n=10 cells per group. E-F) Dopamine neurons show a significantly higher frequency (E) and amplitude (F) of mIPSCs relative to mEPSCs. Unpaired t-test, ****p<0.0001. Data are shown as mean ± SEM.

Our mapping studies demonstrate no sex difference in excitatory or inhibitory inputs. To confirm these observations, we recorded mIPSCs and mEPSCs from VTA dopamine neurons. Consistent with our mapping results, an examination of spontaneous mIPSCs and mEPSCs revealed no sex differences in the amplitude or frequency, indicating the pre- and postsynaptic activity is not sex-dependent (Fig 10A-D). Interestingly, we observed an approximately 10-fold higher amplitude and frequency in mIPSCs, indicating increased inhibitory synaptic transmission in dopamine neurons, as compared to excitatory synaptic activity (Fig 10E-F). This difference is proportional to the difference we observed in our mapping study.

However, a lack of differences in mIPSCs and mEPSCs does not exclude potential differences in neuronal excitability between males and females. To address this, we next examined the electrophysiological properties of dopamine neurons in slice. Dopamine neurons were identified by fluorescent expression either genetically in a *Slc6a3*$^{Cre/+}$*; Rosa26-fs-TdTomato* line or through injection of AAV1-DIO-GCaMP into the VTA of *Slc6a3*$^{Cre/+}$ mice. Dopamine neurons are often identified in slice through the presence of an I_h current, which is mediated by HCN channels[262,263]. We found no difference in the I_h current of dopamine neurons (Fig 11A). Although dopamine neurons do not burst fire in slice, we recorded spontaneous firing activity and observed no difference between males and females (Fig 11B). We also found no difference in the membrane capacitance, indicating the size of dopamine neurons are unaffected by sex (Fig 11C). SK3 channels are calcium-activated small conductance potassium channels that mediate the afterhyperpolarization and regulate the burst properties of dopamine neurons[11]. Here, we also observed no sex differences (Fig 11D). Membrane resistance was also the same in males and females (Fig 11E).

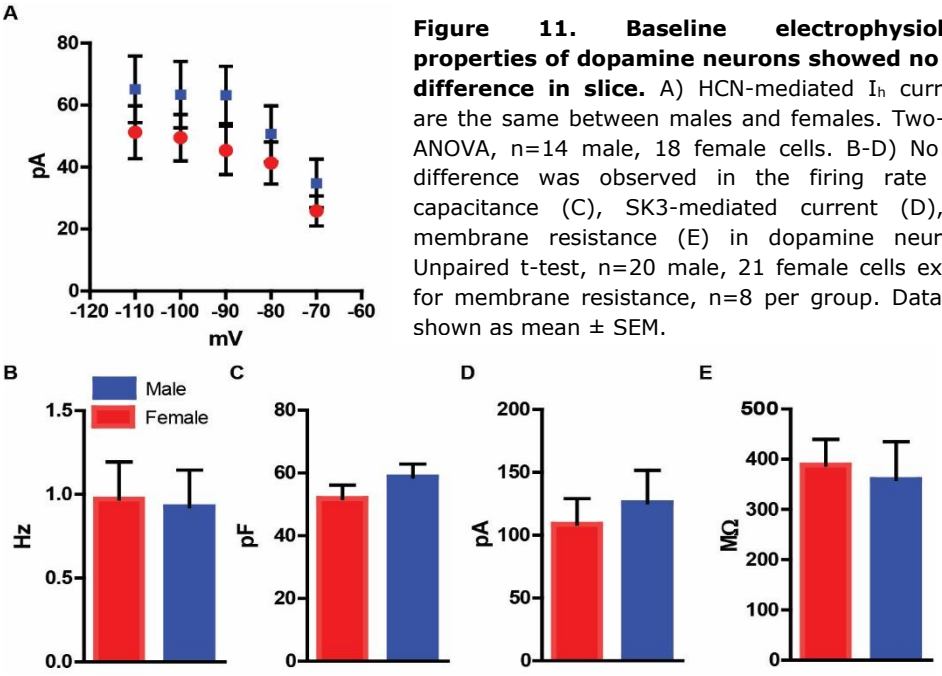


Figure 11. Baseline electrophysiology properties of dopamine neurons showed no sex difference in slice. A) HCN-mediated I_h currents are the same between males and females. Two-way ANOVA, n=14 male, 18 female cells. B-D) No sex difference was observed in the firing rate (B), capacitance (C), SK3-mediated current (D), or membrane resistance (E) in dopamine neurons. Unpaired t-test, n=20 male, 21 female cells except for membrane resistance, n=8 per group. Data are shown as mean ± SEM.

Serotonin

We again employed CAV2-DIO-ZsGreen into the VTA, this time to examine the serotonergic afferents of the VTA in *ePet1*$^{Cre/+}$ mice. Confirmation of the specificity of the virus was performed using immunohistochemistry for ZsGreen and tryptophan hydroxylase (TPH2), the rate-limiting enzyme involved in the synthesis of serotonin(Fig 12C)[264]. The primary serotonergic input to the VTA, the dorsal raphe nucleus, indicated no difference between males and females in the strength of these inputs (Fig 12A-B)[105,265,266].

My results describe a comprehensive analysis of the afferents to VTA dopamine neurons. In addition, a thorough investigation of the areas of major types of neuromodulatory VTA inputs was done, including quantification of GABA, glutamate, and serotonin neurons. I saw a striking difference in the number of GABAergic neurons projecting to the VTA in comparison to all other neurotransmitters, suggesting a powerful inhibitory tone in the VTA. This was further confirmed using slice electrophysiology, which demonstrated an elevated frequency and amplitude of mIPSCs in dopamine neurons, implying elevated inhibitory activity at these synapses. Importantly, I saw no signs of

sexual dimorphism in any VTA afferents, indicating that any sex differences in the dopamine system are likely not due to a disparity in afferent projection strength. Further evidence for this notion is shown in a lack of sex differences in multiple electrophysiological properties of dopamine neurons. Consequently, I examined the possibility of a sex difference in the strength of dopaminergic VTA outputs.

VTA Efferents

Injection of AAV1-hM3Dq-FLEX-eYFP into the VTA of *Slc6a3*$^{Cre/+}$mice allowed for visualization of dopamine projections following injection of CNO[267]. Inspection of Fos, a marker of neuronal activity, in areas known to receive dopaminergic input revealed high activation in the Cg, NAc, DStr, LSD, and LH, among other areas[268]. Here, an analysis of sex differences revealed no sex difference in the total Fos or the amount of Fos in each efferent area, indicating VTA dopamine neurons do not send differential projections to any areas in males or females (Fig 13).

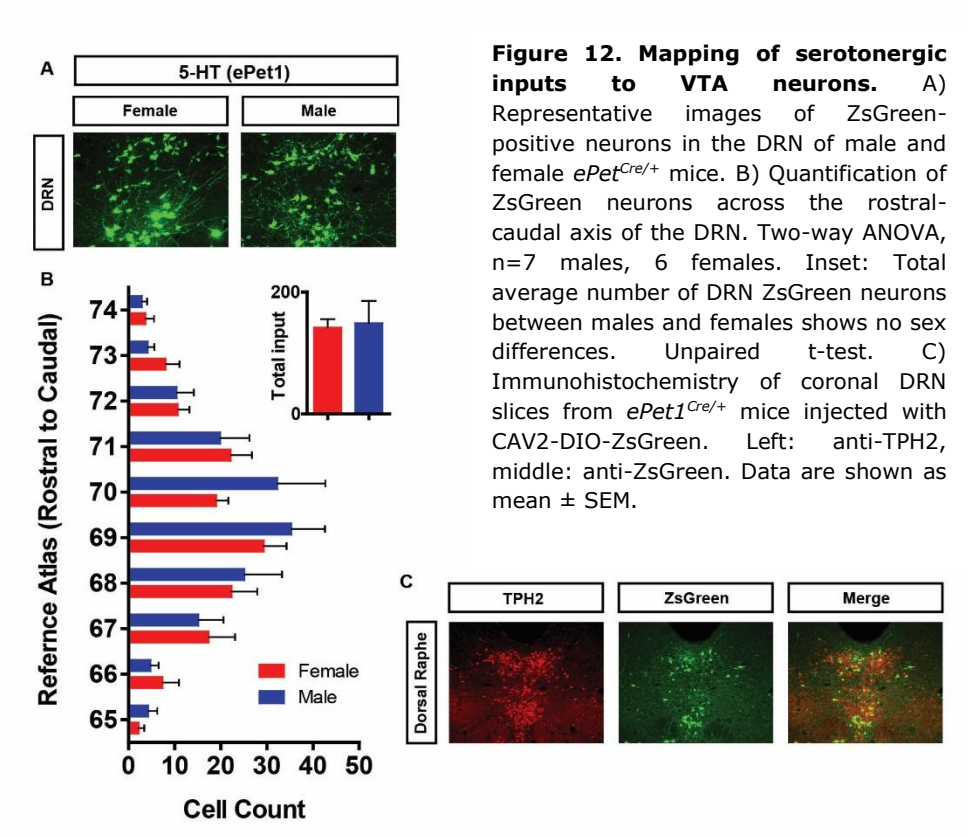


Figure 12. Mapping of serotonergic inputs to VTA neurons. A) Representative images of ZsGreen-positive neurons in the DRN of male and female *ePet*$^{Cre/+}$ mice. B) Quantification of ZsGreen neurons across the rostral-caudal axis of the DRN. Two-way ANOVA, n=7 males, 6 females. Inset: Total average number of DRN ZsGreen neurons between males and females shows no sex differences. Unpaired t-test. C) Immunohistochemistry of coronal DRN slices from *ePet1*$^{Cre/+}$ mice injected with CAV2-DIO-ZsGreen. Left: anti-TPH2, middle: anti-ZsGreen. Data are shown as mean ± SEM.

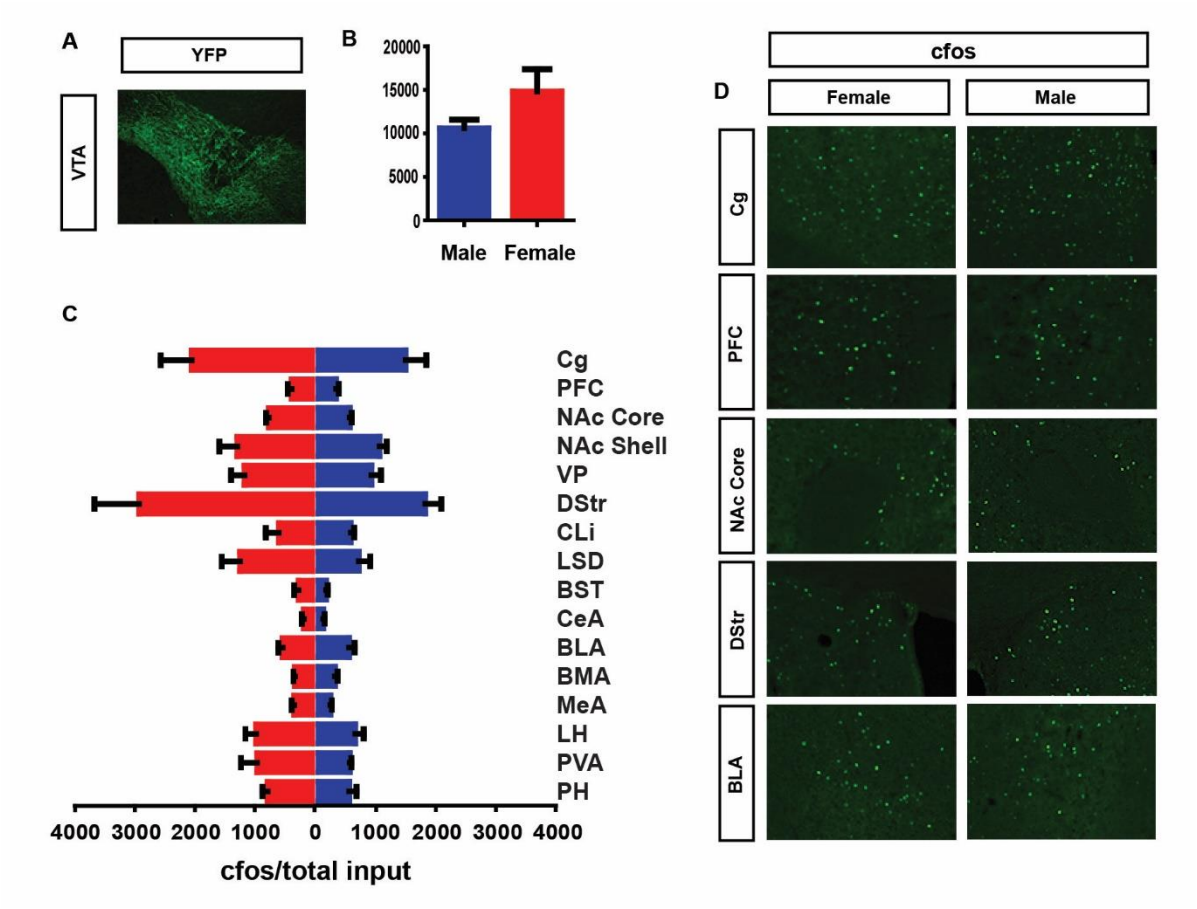


Figure 13. Mapping of VTA dopamine output areas. A) Representative image of YFP expression in the VTA of a *Slc6a3*$^{Cre/+}$mouse. B) Average total amount of cfos was not different between males and females. Unpaired t-test, n=5 males, 4 females. C) Quantification of cfos in VTA efferent areas. Two-way ANOVA. Data are shown as mean ± SEM.

CHAPTER 3

DOPAMINE TRANSLATOME

While examination of gene expression in dopamine neurons has been performed using both *in situ* hybridization and microarray analysis, these studies investigated differences in the molecular characterization between various dopaminergic populations and none assessed enriched genes of dopamine neurons as a whole compared to non-dopamine neurons[20–24]. Here, I examined the gene expression profiles of dopamine neurons in male and female mice and compare the VTA dopaminergic population to non-dopamine neurons in the VTA. In addition, I draw attention to the possible relationship between the expression of neurotransmitter receptor transcripts in dopamine neurons and the VTA afferent input of neurons expressing these neurotransmitters.

In order to isolate the ribosome-associated mRNA of dopamine neurons, I utilized a viral RiboTag strategy[269]. Injection of a Cre-dependent AAV1-FLEX-Rpl22HA into the VTA of a *Slc6a3*$^{Cre/+}$ mouse allows for replacement of WT exon 4 of the ribosomal protein Rpl22 with an exon 4 that contains a HA tag, specifically in dopamine neurons (Fig 14). I then

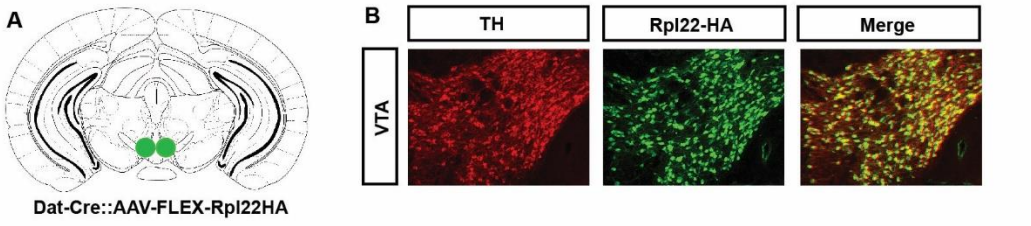


Figure 14. Viral approach for isolation of VTA dopamine neuron mRNA. A) Schematic of VTA viral targeting by a Cre-conditional HA-tagged ribosomal protein, Rpl22, in *Slc6a3*$^{Cre/+}$ mice. B) Immunohistochemistry of coronal VTA slices from *Slc6a3*$^{Cre/+}$ mice injected with AAV1-FLEX-Rpl22HA. Left: anti-TH, middle: anti-HA,

isolated these HA-tagged polyribosomes through immunoprecipitation (IP) of VTA polysomes. The RNA obtained was then amplified, purified, analyzed using microarray, and confirmed with qRT-PCR. To confirm the isolation and identity of dopamine neuron-associated mRNA in the IP, I first verified the enrichment of dopaminergic mRNAs. These included *Th*, *Ddc*, *Slc6a3* (Dat), vesicular monoamine transporter 2 *Slc18a2* (Vmat2), and Drd2. Both in the samples used for the microarray and those used for qRT-PCR, I saw high enrichment for all of these transcripts (Fig 15). qRT-PCR revealed no effect of sex or hormone on the degree of enrichment (Fig 15B).

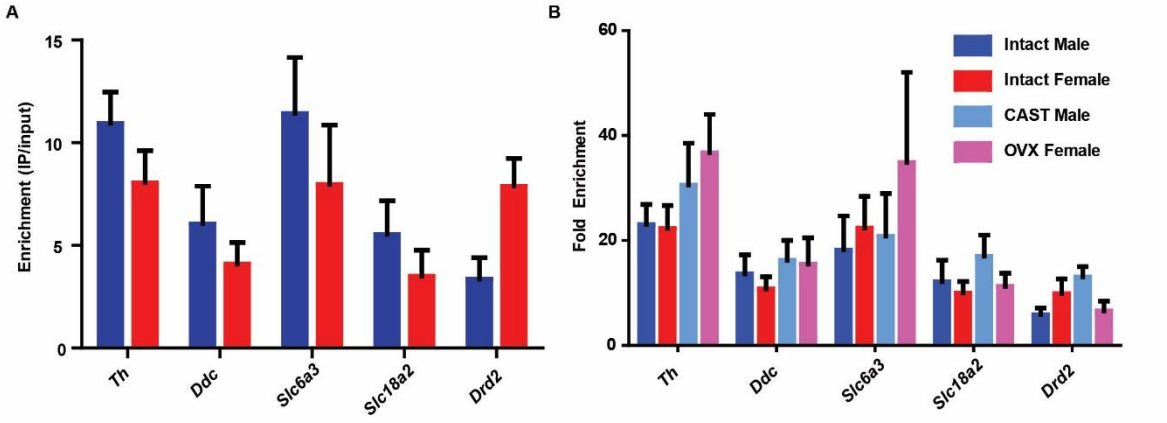


Figure 15. RNA of immunoprecipitated VTA tissue showed enrichment for dopamine markers. A) Microarray of male and female samples was enriched for *Th, Ddc, Dat, Vmat2,* and *Drd2*. There were no sex differences in the level of enrichment. Two-way ANOVA, n=4 males, 3 females. B) qRT-PCR of intact and gonadectomized tissue showed enrichment for dopamine markers. There was no effect of sex or hormone in the level of enrichment. Two-way ANOVA, n=4 intact males, 5 intact females, 3 CAST males, 4 OVX females. Data are shown as mean ± SEM.

To establish a baseline for looking at enriched genes, I chose the minimum enrichment value for dopaminergic markers. Therefore, I filtered the data to show the genes that had a signal at least four times more in IP than total mRNA input, eliminating genes with weak expression (Fig 16A). The results further validate our isolation of dopamine neurons with enriched mRNAs including cholinergic receptors (*Chrna6, Chrna4, Chrnb3.1*), cholecystokinin (*Cck*), and transcription factor engrailed homeobox protein 1 (*En1*)[24]. I chose several of these mRNAs to be confirmed with qRT-PCR, which affirmed their

enrichment in dopamine neurons (Fig 16B). *Gapdh* was chosen as a housekeeping gene and showed no enrichment. I saw no sex difference or effect of hormone on the enrichment level of these genes.

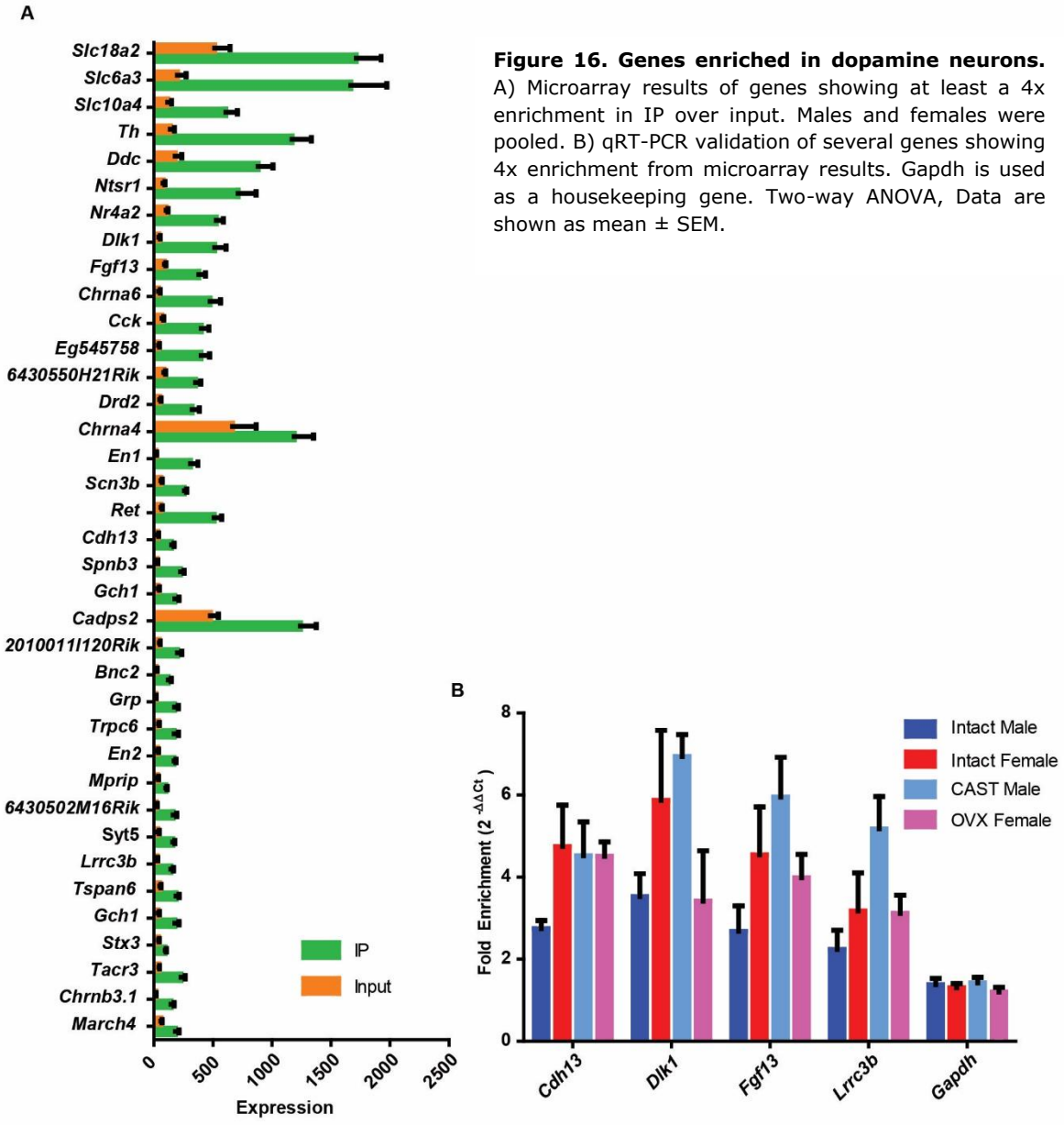


Figure 16. Genes enriched in dopamine neurons. A) Microarray results of genes showing at least a 4x enrichment in IP over input. Males and females were pooled. B) qRT-PCR validation of several genes showing 4x enrichment from microarray results. Gapdh is used as a housekeeping gene. Two-way ANOVA, Data are shown as mean ± SEM.

Surprisingly, analysis of sex differences in IP samples revealed only two genes, both of which are chromosomally linked. *Xist*, which is X-linked and expressed only in females, is responsible for a gene dosing effect. In females, which express two X chromosomes, *Xist*

acts on the inactive X chromosome by coating it and effectively silencing it[270]. I found significantly higher expression of *Xist* in female IP samples (Fig 17B). The second gene, Eif2s3y, is expressed on the Y chromosome and is essential for spermatogenesis. Accordingly, I find enrichment of *Eif2s3y* in the IP of males versus females (Fig 17A). However, this gene is not present in humans. It should be noted that neither *Xist* nor *Eif2s3y* were expressed at a higher level in IP than input, and the sex difference seen was maintained in the input samples. Therefore, I found no genes that were enriched in IP over total mRNA and also exhibited a sex difference. Additionally, *Xist* and *Eif2s3y* showed no change in enrichment after gonadectomy, demonstrating that the expression of these transcripts is not hormone-dependent (Fig 17C-D).

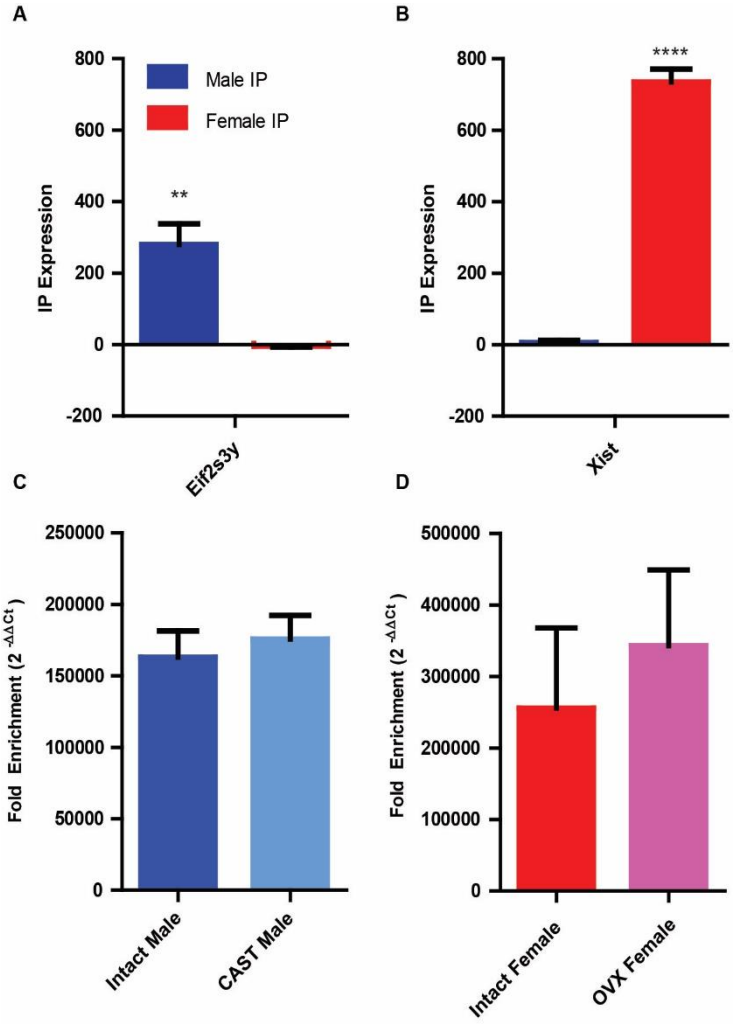


Figure 17. Two genes expressed in dopamine neurons showed a sex difference. A) Microarray analysis of transcripts in IP samples showed higher expression of *Eif2s3y* in males. Unpaired t-test, **p<0.01. B) Microarray results showed higher levels of *Xist* in female IP samples. Unpaired t-test. ****p<0.0001. C) qRT-PCR confirmed enrichment of *Eif2s3y* in both intact and CAST males as compared to females. Unpaired t-test. D) Confirmation of Xist enrichment in intact and OVX females compared to males. Unpaired t-test. Data are shown as mean ± SEM.

Our investigation of dopaminergic circuitry and physiology led to our finding of a strong afferent input from GABAergic neurons. I also observed inputs from areas expressing glutamate and serotonin. Therefore, I examined the expression of receptors associated with these neurotransmitters and others known to be expressed in the VTA. Microarray analysis revealed gene expression of several classes of receptors in the dopaminergic IP including GABA, glycine, acetylcholine, norepinephrine, serotonin, neurotensin, cholecystokinin, neurokinin B, ghrelin, glutamate, corticotropin releasing hormone, and opioid receptors (Fig 18A). Enrichment of several of these receptor transcripts in the IP relative to input is also seen, including GABA, cholinergic, glycinergic, ghrelin, and neurokinin B. There is also reduced enrichment of the mRNAs encoding for the adrenergic receptor Adra2a, as well as the neurotensin receptor Ntsr2. Interestingly, the metabotropic glutamate receptor mGluR3 (encoded by the gene *Grm3*), is also highly de-enriched (Fig 18B). Further analysis averaging enrichment across each neurotransmitter receptor type demonstrates the possible selectivity of those neurotransmitters for dopamine neurons over other neurons in the VTA (Fig 18C). Receptors binding norepinephrine are selective for non-dopamine neurons, whereas glutamate receptors are non-selective. In contrast, receptors mediating acetylcholine, GABA, glycine, serotonin, neurotensin, neurokinin B, and ghrelin signaling are highly selectively expressed in dopamine neurons. This finding mirrors our observation of dopaminergic circuitry, which shows relatively robust projections from serotonergic and GABAergic afferents, but fewer glutamatergic inputs. These data, along with physiological evidence of stronger presynaptic inhibitory influence, support an overall larger inhibitory tone onto VTA dopamine neurons.

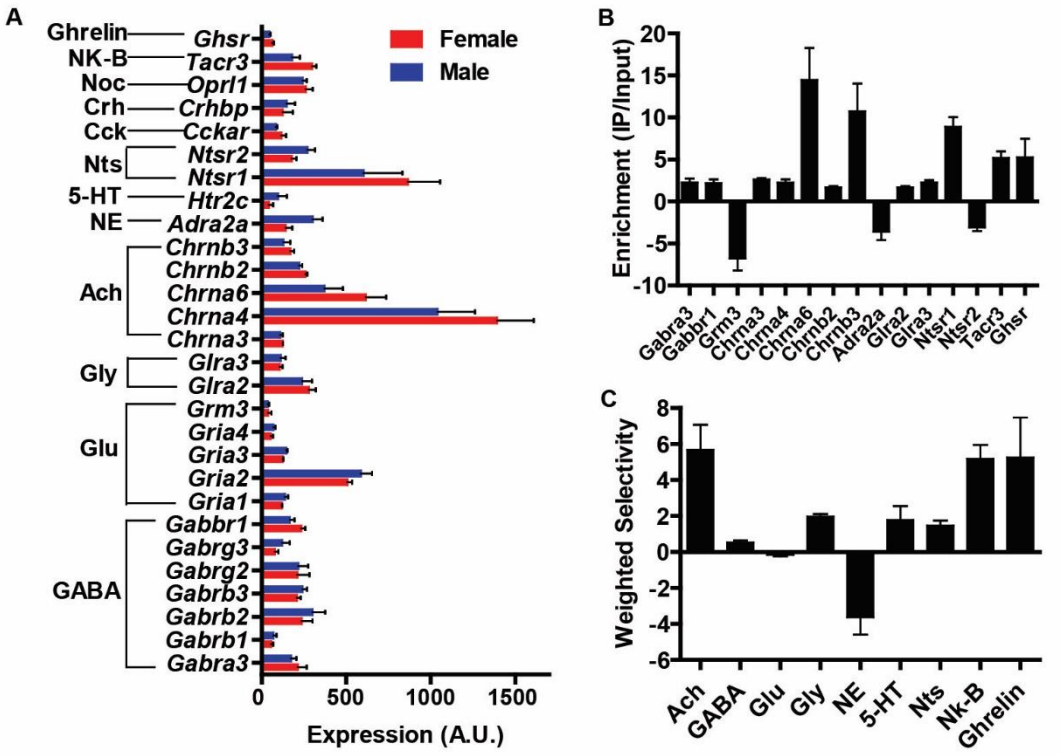


Figure 18. Gene expression profile of neurotransmitter receptor subtypes in IP. A) Microarray analysis showed expression of several receptor types in dopamine neurons (IP), including GABA, glutamate, glycine, acetylcholine, norepinephrine, serotonin, neurotensin, cholecystokinin, corticotropin-releasing hormone, nociception, neurokinin B, and ghrelin. B) Enrichment and de-enrichment of several receptors in dopamine neurons relative to total mRNA. C) Selectivity of receptor subtypes is shown by average IP expression of all receptors within a classification relative to average expression of those receptors in input. Data are shown as mean ± SEM.

CHAPTER 4

DOPAMINE-DEPENDENT BEHAVIOR

Thus far, I have determined that there are few differences between males and females in terms of gene expression and circuitry of the VTA dopamine neurons. Dopamine is important in behaviors including stimulus-response reward learning and motivation[10,29,32,271]. As existing literature on sex differences in these behaviors are largely done in rats and produce conflicting results, I tested both intact and gonadectomized mice in a variety of appetitive- reward tasks. Mice were food restricted and trained in a Pavlovian appetitive conditioning task where a 10-second cue presentation of levers preceded delivery of a food pellet. Learning was measured by an increase in the number of head entries performed during the cue over time versus during the intertrial interval. I observed no overt difference of sex-specific learning during this task, although a two-way ANOVA revealed a small but significant interaction (Fig 19A-B). Likewise, gonadectomized female and male mice learned this association to a similar degree (Fig 19C-D). Further examination of activity patterns during the cue showed that intact mice are, as previously reported, largely goal trackers, and males and females exhibit similar behavior while the lever is presented[43,272,273] (Fig 19E-F).

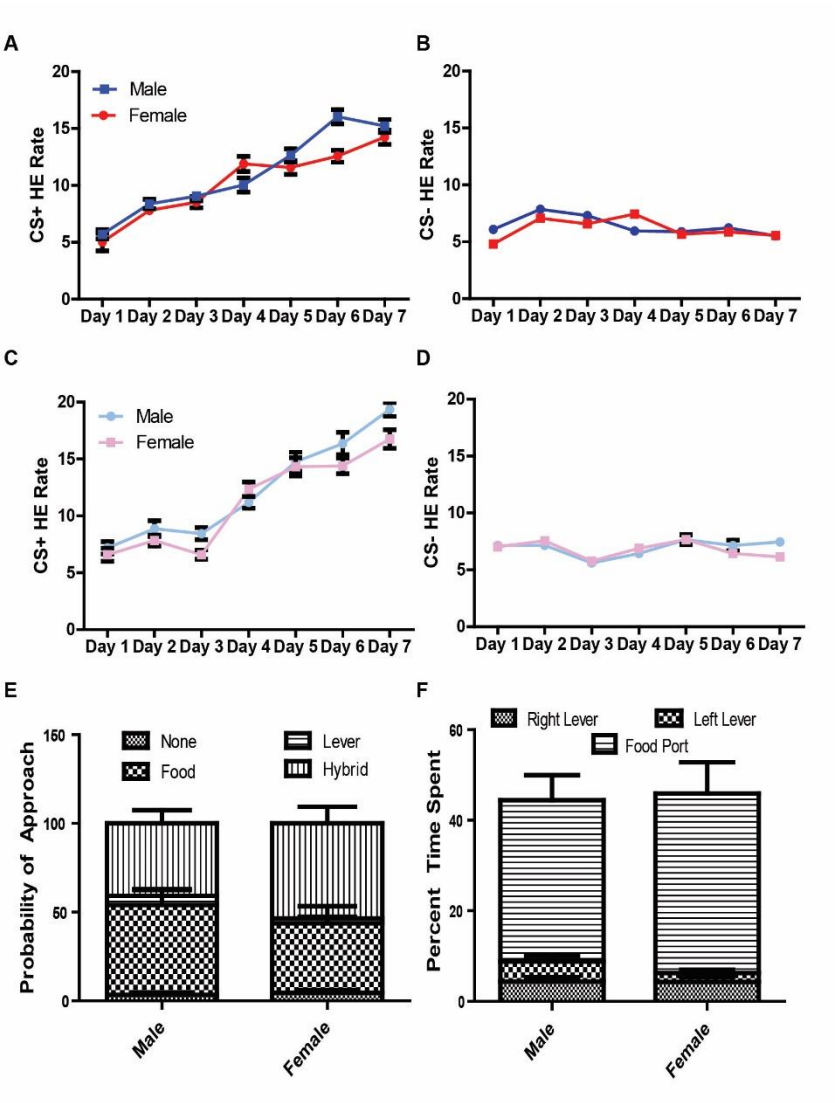


Figure 19. Pavlovian appetitive conditioning task revealed no sex differences. A) Intact males and females show a similar increase in head entries during the CS+ over training days. Two-way ANOVA, n=22 intact males, 22 intact females sex x day, $F_{(1,6)}=3.85$, **p<0.01. B) Intact males and females maintain a similarly low rate of head entries during the CS- over training days. Two-way ANOVA, $F_{(1,6)}=6.13$, ****p<0.0001. C) Gonadectomized males and females show a similar increase in head entries during the CS+ over training days. Two-way ANOVA, n=12 intact males, 12 intact females. D) Gonadectomized males and females maintain a low level of head entries during the CS- over training days. E) Manual analysis of behavior during the cue revealed no sex difference in intact animals. Two-way ANOVA. F) Ethovision analysis of behavior during the cue also showed no sex difference. Two-way ANOVA. Data are shown as mean ± SEM.

Following Pavlovian conditioning, mice were trained on a FR1 instrumental conditioning task where one lever press resulted in delivery of a food pellet. There was a slight effect of hormone in females, with OVX females pressing the levers at a significantly higher rate than intact females on the first day of training. However, this disparity was no longer significant by the fourth day of training (Fig 20A-B). I observed no effect of sex or male hormone level. Assessment of motivation through a progressive ratio task, where the number of times mice are required to press the lever increases exponentially, showed no difference between intact or gonadectomized males and females (Fig 20C). These results

suggest there is no sex difference in the performance of male and female mice in reward learning or motivation.

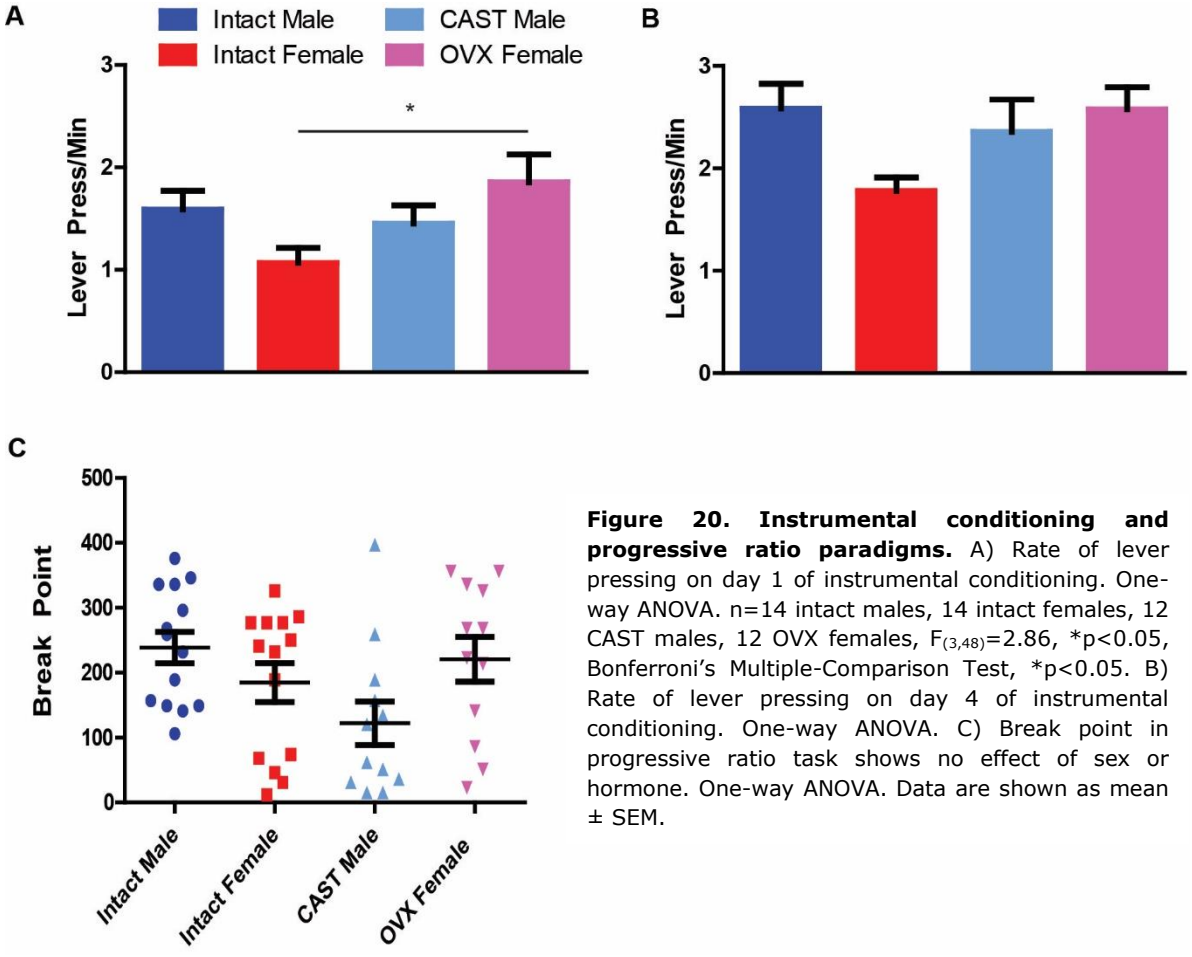


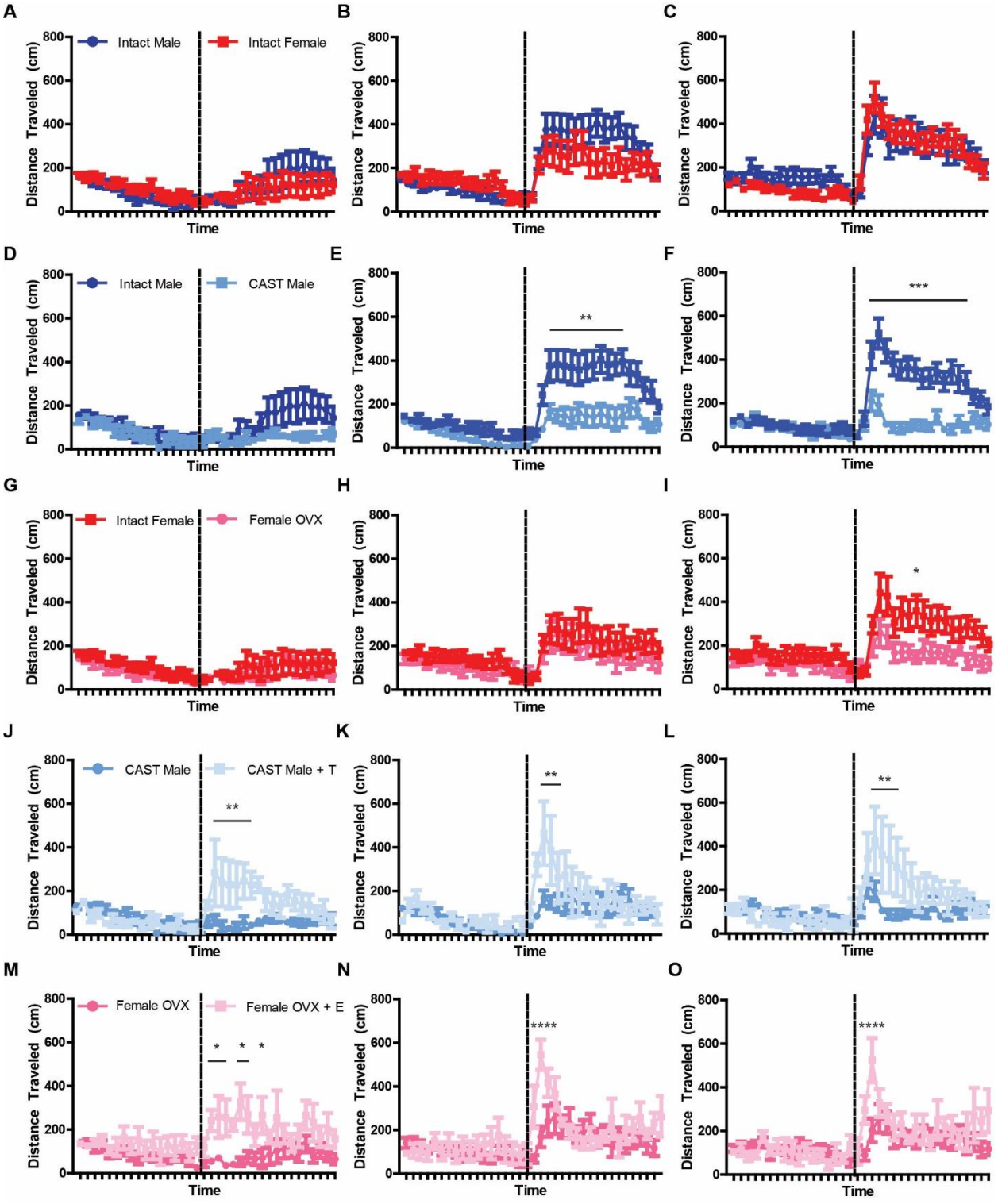
Figure 20. Instrumental conditioning and progressive ratio paradigms. A) Rate of lever pressing on day 1 of instrumental conditioning. One-way ANOVA. n=14 intact males, 14 intact females, 12 CAST males, 12 OVX females, $F_{(3,48)}=2.86$, *p<0.05, Bonferroni's Multiple-Comparison Test, *p<0.05. B) Rate of lever pressing on day 4 of instrumental conditioning. One-way ANOVA. C) Break point in progressive ratio task shows no effect of sex or hormone. One-way ANOVA. Data are shown as mean ± SEM.

Cocaine acts at the dopamine transporter by blocking it and thereby inhibiting the reuptake of dopamine into the presynaptic terminal. This results in increased dopamine in the synapse and an inhibition of dopamine neuron firing. Repeated administration of cocaine typically results in behavioral sensitization, which can be measured as an increase in locomotor activity over days. Following 5 days of cocaine injections, both intact males and females sensitized as expected, with no sex difference (Fig 21A-C). However, neither OVX females nor CAST males exhibited a robust locomotor response when compared to their intact counterparts (Fig 21D-I). This effect was more pronounced in males. Hormone

replacement with either estrogen or testosterone in females and males, respectively, rescued locomotor sensitization (Fig 21J-O).

Overall, these data indicate that in dopamine-dependent behaviors, mice showed no obvious sex or hormone effect in appetitive-reward paradigms, including instrumental appetitive conditioning, Pavlovian conditioning, and progressive ratio. However, both males and females display sensitivity to hormone levels as measured by locomotor cocaine sensitization.

Figure 21. Locomotor sensitization to cocaine showed no sex difference, but revealed effect of hormones. A-C) Locomotor response to cocaine injection on day 1 (A), day 3 (B), and day 5 (C) revealed no sex difference between intact males and females. Two-way RM ANOVA, n=14 intact males, 14 intact females. D) Locomotor response to cocaine injection on day 1 between intact and CAST males revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=1.76, **p<0.01, n=14 intact males, 11 CAST males. E) Locomotor response to cocaine injection on day 3 between intact and CAST males revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=4.70, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. F) Locomotor response to cocaine injection on day 5 between intact and CAST males revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=9.77, ****p<0.0001. Bonferroni's multiple-comparison test, ***p<0.001. G-H) Locomotor response to cocaine injection on day 1 and day 3 between intact and OVX females revealed no effect of hormone. Two-way RM ANOVA, n=14 intact females, 12 OVX females. F) Locomotor response to cocaine injection on day 5 between intact and OVX females revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=2.37, ****p<0.0001. Bonferroni's multiple-comparison test, *p<0.05. J) Locomotor response to cocaine injection on day 1 between CAST males and CAST males with testosterone replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=5.76, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. K) Locomotor response to cocaine injection on day 3 between CAST males and CAST males with testosterone replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=2.79, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. L) Locomotor response to cocaine injection on day 5 between CAST males and CAST males with testosterone replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=3.37, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. M) Locomotor response to cocaine injection on day 1 between OVX females and OVX females with estrogen replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=2.61, ****p<0.0001. Bonferroni's multiple-comparison test, *p<0.05. N) Locomotor response to cocaine injection on day 3 between OVX females and OVX females with estrogen replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=2.94, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. O) Locomotor response to cocaine injection on day 5 between OVX females and OVX females with estrogen replacement revealed an effect of hormone. Two-way RM ANOVA, condition x time, $F_{(35,35)}$=3.24, ****p<0.0001. Bonferroni's multiple-comparison test, **p<0.01. Data are shown as mean ± SEM.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Intact Male
Intact Female
CAST Male
CAST Male + T
Female OVX
Female OVX + E
Distance Traveled (cm)
Time
0
200
400
600
800
*
**
***
****

CHAPTER 5

DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

This study presents a comprehensive collection of findings analyzing the midbrain dopamine system by investigating circuitry, gene expression, and behavior. Through extensive circuit mapping of the midbrain, I show that while there is no sexual dimorphism in the VTA afferents, there is a prominent inhibitory input that is mirrored in the electrophysiological synaptic properties of dopamine neurons in slice preparation. Examination of the VTA dopaminergic outputs revealed an enhanced projection in females to the dorsal striatum. Microarray analysis of dopamine neurons resulted in only two sexually dimorphic genes, both of which were sex chromosome-linked. However, further analysis also revealed an enhanced expression and selectivity of certain neurotransmitter receptor subtypes, again supporting our circuit-mapping results. Finally, while appetitive dopamine-dependent behaviors and locomotor cocaine sensitization showed minimal sex differences, I observed a striking effect of hormone on the locomotor response to repeated cocaine administration.

Dopamine Circuitry

Previous studies detailed midbrain dopamine circuitry using a variety of techniques, including fluorogold, horseradish peroxidase, and modified rabies virus[86–90]. However, none of these studies explored the possibility of sex differences. Using CAV2-Cre and a modified

rabies virus approach, I was able to look at the inputs to the VTA in a dopamine independent and dependent manner. Our findings matched with what has been previously reported, with the highest density of VTA projections originating in the DRN, BNST, NAc, PPTg, and LH. A large number of neurons from the cingulate cortex, motor cortex, and MHb, were also found to project to the VTA, although these were not seen in projections specifically to dopamine neurons. Comparison of male and female subjects revealed no sex differences in the number of cells in VTA afferents.

Following these findings, I explored the possibility of sexual dimorphism within the major neurotransmitter VTA afferents. Employing CAV-DIO-ZsGreen with several Cre driver mouse lines allowed us to visualize VTA-projecting neurons expressing GABA, glutamate, and serotonin. While I did not observe any differences between males and females, I did note a relatively low number of glutamatergic inputs to the VTA. However, GABAergic afferents displayed a much higher projection strength. In order to see if this striking difference translated to a synaptic effect, we examined the properties of VTA dopamine neurons in slice preparation. Here, we also saw no sex differences in spontaneous firing rate, Ih current, SK3 current, capacitance, membrane resistance, or mIPSC and mEPSC amplitude and frequency. However, analysis of mIPSC and mEPSC amplitude and frequency revealed a highly significant difference. Both amplitude and frequency of mIPSCs was substantially higher in dopamine neurons, indicating higher inhibitory activity both at the pre- and post-synaptic level. Increased mIPSC frequency is associated with higher vesicular release from the presynapse, while increased mIPSC amplitude implies greater activation of postsynaptic receptors. While a strong GABAergic presence has been previously characterized in the SNc, I am the first to provide substantial circuitry and physiological evidence that the VTA is largely innervated by GABAergic inputs outside of local interneurons, as compared to glutamatergic input[7,130,258–261,274]. Most of the GABAergic inputs to the SNc originate in the globus pallidus, striatum, and from within the SNc itself[130]. A comprehensive map of GABA afferents to the VTA, surprisingly, has never been

performed, and we show that these same areas also provide strong inhibitory input to the VTA, along with the lateral septum, bed nucleus, lateral hypothalamus, and preoptic areas, among others.

By expressing DREADDs in dopamine neurons of the VTA, I was able to visualize dopamine projection strength by looking for cfos, a marker of neuronal activity. I observed high levels of cfos in areas known to receive dopaminergic input, including the cingulate cortex, NAc, DStr, LH, and septum. However, no difference between males and females was seen in efferent VTA areas.

Dopamine Translatome

To examine gene expression in isolated dopamine neurons, I used a viral RiboTag technique. Immunoprecipitation and microarray analysis of HA-tagged polyribosomes allowed for examination of transcripts in dopamine neurons. Consequently, I was able to characterize the dopamine transcriptome. While comparison of gene expression between various dopaminergic populations had been performed, our study was the first to look for sex differences[20–24]. I found no sex differences in genes that were preferentially upregulated in dopamine neurons. However, our analysis revealed two sexually dimorphic genes: *Xist* and *Eif2s3y*. *Xist* is a X-linked gene whose purpose is to silence the second inactive X chromosome in females, allowing for gene dosage in an effort to minimize differences in levels of X chromosome gene expression between males and females[270]. Accordingly, I observed significantly higher expression of *Xist* in female dopamine neurons as compared to males. However, this sex difference was also seen in the total mRNA input, and therefore is not unique to dopamine neurons. *Eif2s3y* is a Y-linked gene that is vital for spermatogenesis[275,276]. As would be expected, I found significantly higher levels of this transcript in male dopamine neurons. Also like *Xist* this sex difference was not limited to only dopamine neurons. Expression of neither *Xist* nor *Eif2s3y* was affected by gonadectomy, demonstrating that these genes are not regulated by hormone levels. I also

saw no difference in gene expression of any dopaminergic transcripts with gonadectomy, including those encoding for Th, Dat, Vmat, D2 receptor, or Ddc.

After discovering a novel predominant inhibitory tone in VTA dopamine neurons, I was curious if this was also reflected in the gene expression of neurotransmitter receptors. Analysis of microarray results showed that dopamine neurons express transcripts for a variety of neurotransmitter receptors, including GABAergic, glutamatergic, cholinergic, serotonergic, and adrenergic receptors. There was little to no enrichment of ionotropic or metabotropic glutamatergic receptors in dopamine neurons compared to total input. Other de-enriched receptor transcripts include the neurotensin receptor *Ntsr2* and the adrenergic receptor *Adra2a*. On the other hand, several receptors were enriched, including cholinergic, glycinergic, ghrelin, and neurotensin receptors. Importantly, I see an enrichment in dopamine neurons for GABAergic receptors. This enrichment, along with a de-enrichment of glutamatergic receptors, further supports our findings of strong inhibitory tone onto dopamine neurons of the VTA.

Additional analysis explored the selective expression of receptor types in dopamine neurons. Here, the average enrichment for all receptors of a subtype (e.g. glutamate, GABA, etc.) was averaged, to illustrate the specificity of that neurotransmitter for dopamine neurons over all other neurons of the VTA. This reveals a high selectivity of acetylcholine, glycine, neurokinin B, and ghrelin for dopamine neurons. GABA shows a slight selectivity for dopamine neurons, while glutamate shows none, again contributing to our evidence of strong inhibitory input to VTA dopamine neurons, as compared to excitatory.

Dopamine-Dependent Behavior

It is well established that dopamine is required for reward learning. Dopamine in the striatum, in particular, is necessary for motivated reward learning[75]. While I found few sex differences in VTA connectivity and gene expression, I could not rule out the possibility of other sexually dimorphic factors that could result in an observable difference in behavioral

output. Therefore, I tested mice in a variety of appetitive learning tasks, including Pavlovian conditioning, instrumental conditioning, and progressive ratio. In all three paradigms, I saw few differences between males and females. Pavlovian conditioning revealed no sex difference, and gonadectomized mice performed equally well in this task. I also observed no difference in their conditioned approach behavior, as defined by goal or sign tracking. Instrumental conditioning also showed no sex difference or effect of hormone, except on day 1 of training where OVX females exhibit a small but significantly higher lever press rate. This effect is gone by the last day of training. As dopamine in the striatum is necessary for food motivation and learning, and I observed higher female dopaminergic input in this area, I tested animals in a progressive ratio task[75]. However, I also saw no effect of sex or hormone in this paradigm.

Finally, I examined the locomotor sensitization of mice to repeated injections of cocaine. Current literature reporting sex differences in cocaine sensitization is extremely convoluted, contradictory, and primarily performed in rats. Most studies agree that female rats have a greater locomotor response to chronic cocaine than males[277–279]. Some studies observe that CAST and testosterone decrease and restore sensitization, respectively, while others see no change with CAST[280–282]. OVX females reported show a decreased locomotor response to cocaine, while estrogen seems to restore this[210,277,282]. Chronic administration of cocaine over 5 days to intact male and female mice revealed an equal locomotor sensitization. Gonadectomy greatly reduced this behavioral response and hormone replacement restored it. While our results largely mirror what is seen with hormone manipulation, I see no sex difference; this may be due to our use of a mouse model instead of rats.

Conclusions

This study demonstrates that overall, the midbrain dopaminergic system of male and female mice is largely very similar. Examination of inputs to the VTA both in a nonspecific

and dopamine-specific manner revealed no sexual dimorphism in the strength of inputs from multiple afferent areas. Additionally, the neurotransmitter makeup of these afferents showed no sex difference. The basic electrophysiological properties of the dopamine neuron are also the same between males and females. Dopamine-dependent appetitive behaviors showed similar performance between sexes as well. Some small differences were seen, however. The dorsal striatum receives more input from dopamine neurons in females. Also, microarray analysis of ribosome-associated transcripts showed a sex difference in two genes, *Xist* and *Eif2s3y*, both of which are X- and Y-linked, respectively. However, neither of these genes is known to affect the dopamine system. The most striking effect I observed was not one of sex, but rather of hormone on locomotor cocaine sensitization. In both male and female mice, gonadectomy and hormone replacement decreased and increased, respectively, this behavioral response.

What is the most intriguing result of this study is the finding that dopaminergic neurons are under a largely inhibitory input. This is shown not only by the relatively large number of GABAergic neurons projecting to the VTA in comparison to other major neurotransmitters, but also in the synaptic properties. Recording mEPSCs and mIPSCs from dopamine neurons in slice demonstrates a significantly higher amplitude and frequency of mIPSCs. This evidence suggests that both at the pre- and postsynapse of dopamine neurons, there is greater inhibitory activity, which is a novel and exciting finding. This, along with our comprehensive mapping of VTA afferent areas expressing GABA, provides a plethora of inhibitory sources that could be influencing behavior. How these areas and their inhibitory input contribute to behaviors could reveal important information about VTA function.

Future Directions

Characterization of afferent VTA circuitry from neurons expressing neurotransmitters other than those I detailed would provide an exhaustive picture of midbrain inputs. Other

studies suggest that neurons expressing neurotensin, cholecystokinin, dynorphin, glycine, substance P, ghrelin, and enkephalin may synapse and influence VTA dopamine neurons[7,124,125,128]. The use of our CAV-DIO-zsGreen virus along with various Cre mouse lines would provide a means to complete this characterization.

Our microarray results also show that some of these neurotransmitters may selectively synapse onto dopamine neurons versus other neuron types in the VTA, implying a functional role. Identification of the influence of each of these neurotransmitters in regulating dopamine neuron physiological properties and dopamine-dependent behavior would also add to our knowledge of how the dopamine system is regulated. This could be accomplished through the simultaneous injection of Cre-conditional tetanus toxin into afferent areas with strong ZsGreen expression and CAV-Cre into the VTA of various Cre lines. The result would be neuronal inactivation of VTA-projecting cells expressing a specific neurotransmitter, after which behavioral effects could be tested. Electrophysiology effects could be explored through expression of a Cre-conditional channelrhodopsin in the afferents of these same mouse lines, after which slice recordings of dopamine neurons could be performed to observe the influence of terminal photostimulation.

As cocaine sensitization demonstrated an effect of hormone in males and females, exploration of this mechanism would be interesting. Others have speculated on the role of hormones in regulating D2 receptor binding, but whether this is the mechanism responsible for the effect I see in cocaine locomotor response is unclear[191,210,213].

METHODS

Chapter 2

Animals

All methods and experiments were approved by the University of Washington Institutional Animal Care and Use Committee. *Ai14:426(Sor)tdTom* mice aged 10 weeks were used for all CAV-Cre experiments. *Slc6a3*$^{Cre/+}$mice aged 10 weeks were used for all rabies virus experiments. Slc32a1Cre/+, Slc17a6Cre/+, and ePet1Cre/+ mice aged 10 weeks were used for all CAV-DIO-zsGreen experiments. Both male and female mice were used in these experiments.

Surgery

All mice were anesthetized using isoflurane and stereotaxically injected bilaterally with viral vector (0.5 uL/side) into the VTA. Stereotaxic injection coordinates from bregma in mm, A-P: -3.25*x, M-L: ±0.5, D-V: -4.5 (x=lambda:bregma distance/4.21) for the VTA. Stereotaxic injection coordinates from bregma in mm, A-P: -4.7*x, M-L: ±0.5, D-V: -3.25 (x=lambda:bregma distance/4.21) for the DRN. Mice were allowed to recover for two weeks before sacrifice.

Histology

Mice were anesthetized with 50 mg/kg of Beuthenasia and perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. Whole brains were dissected and fixed overnight in paraformaldehyde, followed by immersion in a 30% sucrose solution for at least 48 hours. Brains were frozen in OCT at -20 degrees Celsius and immediately sectioned

coronally on a cryostat in 30um sections. Sections were then stored in PBS and 0.1% sodium azide until immunostaining and/or mounting onto slides for imaging.

Immunostaining

cfos: Every other section from the entire brain was washed in 1x tris buffered solution (TBS) + 0.3% TritonX 100 (TBST) with 3% donkey serum for 30 minutes. Sections were then incubated overnight at 4 degrees Celsius or for 4 hours at room temperature in primary antibody (rabbit anti cfos, 1:2000, CalBiochem). This was followed by a 3x wash in TBS for 10 minutes and a 1 hour incubation at room temperature in secondary antibody conjugated to Cy3 or AF-488 at a 1:200 dilution. Finally, sections were washed in 1x TBS 3 more times before mounting onto slides.

TPH: Sections from the dorsal raphe were selected and processed in the same was as described above. Primary antibody used was rabbit anti TPH2 at a 1:500 dilution (Millipore).

Image Analysis

Whole sections were imaged based on corresponding atlas reference figures (Mouse Brain Atlas, Franklin and Paxinos). Cells were then counted manually with the exception of cfos, which was counted using ImageJ software.

Statistical Analysis

All statistical analysis was done using Prism (GraphPad).

Chapter 3

Animals

All methods and experiments were approved by the University of Washington Institutional Animal Care and Use Committee. *Slc6a3*$^{Cre/+}$mice aged 10 weeks were used for all RiboTag experiments. Both male and female mice were used in these experiments.

Surgery

All mice were anesthetized using isoflurane and stereotaxically injected bilaterally with AAV1-DIO-Rpl22-HA (0.5 uL/side) into the VTA. Stereotaxic injection coordinates from bregma in mm, A-P: -3.25*x, M-L: ±0.5, D-V: -4.5 (x=lambda:bregma distance/4.21). Mice were allowed to recover for four weeks before sacrifice.

For gonadectomy (CAST or OVX), the gonads were removed from mice anesthetized using isoflurane. Vasculature to the gonad was sutured and sutures were used to seal the incision.

RiboTag

Brain tissue from the VTA area was collected using a tissue punch and homogenized, as previously described[269]. Tissue was then incubated with 5 ul of anti-HA primary antibody (Covance) for four hours at 4 degrees Celsius, followed by overnight incubation with 200 ul of magnetic beads (Pierce). Next, RNA-conjugated beads were washed using a high salt buffer and the RNA was extracted from the magnetic beads. RNA was then purified using a RNeasy Plus Micro kit (Qiagen).

For samples intended for qRT-PCR analysis, RNA was quantified using a Ribogreen RNA kit (Invitrogen) and converted to cDNA using Superscript IV and oligo dT primers (Invitrogen). TaqMan primers (Applied Biosystems) for chosen genes of interest were used to measure gene expression. Expression was quantified using the Ct values normalized to *Actb* (ΔCt). Fold enrichment of IP over input was calculated for figures 15 and 16 using $2^{-\Delta\Delta Ct}$.

Fold enrichment for figure 17 was calculated as $2^{-\Delta\Delta Ct}$ with ΔΔCt = (Male IP ΔCt – Avg Female ΔCt) for each gene, in order to show sex differences in enrichment.

For samples intended for microarray analysis, following purification using the RNeasy kit, confirmation of enrichment for dopamine markers was performed using TaqMan primers. After this, RNA was amplified (Ovation PicoSL WTA RNA Amplification System), purified (Qiagen MinElute Reaction Cleanup Kit), and the quantity was measured again using a nanodrop machine. Confirmation of dopamine marker enrichment was again performed using TaqMan primers, after which biotinylation (Encore BiotinIL) and purification (Qiagen) was done. Samples were then checked for quality (Agilent RNA 6000 Nano) and hybridized to the microarray chip (Illumina Mouse 8 Channel V2). Finally, the chips were read and analyzed (Illumina iScan, GenomeStudio).

Slice Electrophysiology

Whole-cell recordings were made using an Axopatch 700B amplifier (Molecular Devices) with filtering at 1 KHz using 4-6 MΩ electrodes filled with an internal solution containing (in mM): 130 K-gluconate, 10 HEPES, 5 NaCl, 1 EGTA, 5 Mg-ATP, 0.5 Na-GTP, pH 7.3, 280 mOsm.. Horizontal brain slices (200 μm) were prepared from 8 week old mice in an ice slush solution containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2, 10 MgSO4, pH 7.3-7[283]. Slices recovered for ~12 min in the same solution at 32 degrees Celsius and then were transferred to a room temperature solution including (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiouria, 5 Na-ascorbate, 3 Na-pyruvate, 2 CaCl2, 2 MgSO4. Slices recovered for an additional 60 min. All solutions were continually bubbled with O2/CO2, and all recordings were made in ACSF at 32 degrees Celsius continually perfused over slices at a rate of ~2 ml/min. Ih currents were induced by 2-s hyperpolarizing voltage steps from -70 mV to -120 mV. SK currents were induced by

depolarizing voltage steps from -70 to 0 mV. Capacitance measurements were calculated by software using 5 mV hyperpolarizing steps (Clampex).

For recording miniature excitatory postsynaptic currents, electrodes were filled with an internal solution containing (in mM): 130 K-gluconate, 10 HEPES, 5 NaCl, 1 EGTA, 5 Mg-ATP, 0.5 Na-GTP, pH 7.3, 280 mOsm and 200mM picrotoxin was bath applied through the ACSF to block inhibit GABAA receptor-mediated events. For recording spontaneous excitatory postsynaptic currents, electrodes were filled with an internal solution containing (in mM): 135 KCl, 12 NaCl, 0.05 EGTA, 100 HEPES, 0.2 Mg-ATP, 0.02, Na-GTP 2mM kynurenic acid was bath applied through the ACSF to block inhibit AMPA and NMDA events. For all miniature current recordings, cells were clamped at a holding potential of -60 mV for a minimum of 5 minutes and were recorded in the presence of 1mM tetrodoxin (TTX) to block action potentials. Access resistance was monitored throughout all experiments.

Histology

Histology was performed as described in the previous chapter in order to check for viral specificity and spread.

Immunohistochemistry

Coronal brain sections were processed as described in the previous chapter using primary antibodies of mouse anti-HA (1:1000, ABM) and rabbit anti-TH (1:1000, Millipore).

Statistical Analysis

All statistical analysis was done using Prism (GraphPad).

Chapter 4

Animals

All methods and experiments were approved by the University of Washington Institutional Animal Care and Use Committee. WT mice aged 10 weeks were used for all RiboTag experiments. Both male and female mice were used in these experiments.

Surgery

Gonadectomy was performed as described in chapter 3.

Pavlovian Conditioning

After mice were food restricted to 85% of their normal body weight, they were placed in a standard operant chamber containing two levers and a food port (MedAssociates). Mice were trained to obtain food pellets immediately following a 10 second lever presentation (45mg, Bio-Serv). Trials were separated by a 90 second variable intertrial interval and repeated 25 times over 7 days.

Instrumental Conditioning

Following Pavlovian conditioning, mice were placed in the same operant chamber and trained to press either lever based on a FR1 schedule. Specifically, one lever press performed resulted in one food pellet. Levers stayed retracted until the mice made a head entry into the food port. This was repeated until the mice performed 50 lever presses or 2 hours had elapsed. Each subject repeated this over 4 days.

Progressive Ratio

After completing 4 days of instrumental conditioning, mice were then trained on a progressive ratio task, where the number of lever presses required to receive one pellet increased nonarithmatically with each trial (1, 1, 4, 7, 13, 19, 25, 34, 43, 52, 61, 73…).

Break point was calculated as the number of lever presses completed to successfully receive the last pellet after 4 hours had elapsed, or after 3 minutes had elapsed between lever presses.

Cocaine Sensitization

Mice were placed into cages where locomotion could be measured (Opto-M3; Columbus Instruments). For the first 2 days mice were injected with 0.2 ul of 0.9% saline in order to obtain baseline measurements over 90 minutes pre and post injection. Following this, mice were injected with 20 mg/kg of cocaine (Sigma) subcutaneously and locomotion was measured for 90 minutes prior to and after injection. Gonadectomized male and female mice were then injected subcutaneously with testosterone (50 ug/kg, Sigma) or B-Estradiol (10 ug/100 ul, Sigma) dissolved in sesame oil once a day over 7 days. The cocaine locomotion regime was then repeated as described previously, followed by daily injections of hormone after each trial.

Statistical Analysis

All statistical analysis was done using Prism (GraphPad).

REFERENCES

1. Elsworth, J. D. & Roth, R. H. Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Exp. Neurol.* **144,** 4–9 (1997).
2. Icard-Liepkalns, C. *et al.* Tyrosine hydroxylase regulation in neurotransmission and neuroplasticity. *J. Physiol. - Paris* **87,** 153–157 (1993).
3. Wise, R. A. Dopamine, learning and motivation. *Nat. Rev. Neurosci.* **5,** 483–494 (2004).
4. Grace, A. & Bunney, B. The control of firing pattern in nigral dopamine neurons: single spike firing. *J. Neurosci.* **4,** 2866–2876 (1984).
5. Dreyer, J. K., Herrik, K. F., Berg, R. W. & Hounsgaard, J. D. Influence of phasic and tonic dopamine release on receptor activation. *J. Neurosci.* **30,** 14273–83 (2010).
6. Grace, A. a, Floresco, S. B., Goto, Y. & Lodge, D. J. Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.* **30,** 220–7 (2007).
7. Kalivas, P. W. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. *Brain Res. Rev.* **18,** 75–113 (1993).
8. Grace, a a. The tonic/phasic model of dopamine system regulation and its implications for understanding alcohol and psychostimulant craving. *Addiction* **95 Suppl 2,** S119–28 (2000).
9. Grace, A. & Bunney, B. The control of firing pattern in nigral dopamine neurons: burst firing. *J. Neurosci.* **4,** 2877–2890 (1984).
10. Schultz, W. Predictive reward signal of dopamine neurons. *J. Neurophysiol.* **80,** 1–27 (1998).
11. Soden, M. E. *et al.* Disruption of dopamine neuron activity pattern regulation through selective expression of a human KCNN3 mutation. *Neuron* **80,** 997–1009 (2013).
12. Goto, Y., Otani, S. & Grace, A. a. The Yin and Yang of dopamine release: a new perspective. *Neuropharmacology* **53,** 583–7 (2007).
13. Beaulieu, J. & Gainetdinov, R. R. The Physiology , Signaling , and Pharmacology of Dopamine Receptors. *Pharmacol. Rev.* **63,** 182–217 (2011).
14. Beaulieu, J. M. *et al.* An Akt/β-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* **122,** 261–273 (2005).
15. Missale, C., Nash, S. R. S., Robinson, S. W., Jaber, M. & Caron, M. G. Dopamine receptors: from structure to function. *Physiol. …* **78,** 189–225 (1998).
16. Levey, A. I. *et al.* Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8861–8865 (1993).
17. Richfield, E. K., Penney, J. B. & Young, a B. Anatomical and affinity state comparisons between dopamine D1 and D2 receptors in the rat central nervous system. *Neuroscience* **30,** 767–77 (1989).
18. Lacey, M. G., Mercuri, N. B. & North, R. a. Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J. Physiol.* **392,** 397–416 (1987).
19. Marcellino, D., Kehr, J., Agnati, L. F. & Fuxe, K. Increased affinity of dopamine for D(2) -like versus D(1) -like receptors. Relevance for volume transmission in interpreting PET findings. *Synapse* **66,**

196–203 (2012).
20. Blanchard, V. *et al.* Differential expression of tyrosine hydroxylase and membrane dopamine transporter genes in subpopulations of dopaminergic neurons of the rat mesencephalon. *Mol. Brain Res.* **22,** 29–38 (1994).
21. Grimm, J., Mueller, A., Hefti, F. & Rosenthal, A. Molecular basis for catecholaminergic neuron diversity. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 13891–6 (2004).
22. Greene, J. G., Dingledine, R. & Greenamyre, J. T. Gene expression profiling of rat midbrain dopamine neurons: Implications for selective vulnerability in parkinsonism. *Neurobiol. Dis.* **18,** 19–31 (2005).
23. Chung, C. Y. *et al.* Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. *Hum. Mol. Genet.* **14,** 1709–1725 (2005).
24. Poulin, J. F. *et al.* Defining midbrain dopaminergic neuron diversity by single-cell gene expression profiling. *Cell Rep.* **9,** 930–943 (2014).
25. Schultz, W., Dayan, P. & Montague, P. R. A neural substrate of prediction and reward. *Science (80-.).* **275,** 1593–1599 (1997).
26. Fields, H. L., Hjelmstad, G. O., Margolis, E. B. & Nicola, S. M. Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.* **30,** 289–316 (2007).
27. Adamantidis, A. R. *et al.* Optogenetic Interrogation of Dopaminergic Modulation of the Multiple Phases of Reward-Seeking Behavior. *J. Neurosci.* **31,** 10829–10835 (2011).
28. Flagel, S. B. *et al.* A selective role for dopamine in stimulus-reward learning. *Nature* **469,** 53–57 (2011).
29. Zweifel, L. S. *et al.* Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 7281–8 (2009).
30. Zweifel, L. S. *et al.* Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nat. Neurosci.* **14,** 620–6 (2011).
31. Corbett, D. & Wise, R. A. Intracranial self-stimulation in relation to the ascending noradrenergic fiber systems of the pontine tegmentum and caudal midbrain: a moveable electrode mapping study. *Brain Res.* **177,** 423–436 (1979).
32. Wise, R. Brain Dopamine And Reward. *Annu. Rev. Psychol.* **40,** 191–225 (1989).
33. Liu, Z. *et al.* Dorsal raphe neurons signal reward through 5-HT and glutamate. *Neuron* **81,** 1360–74 (2014).
34. Qi, J. *et al.* A glutamatergic reward input from the dorsal raphe to ventral tegmental area dopamine neurons. *Nat. Commun.* **5,** 5390 (2014).
35. McDevitt, R. A. *et al.* Serotonergic versus nonserotonergic dorsal raphe projection neurons: Differential participation in reward circuitry. *Cell Rep.* **8,** 1857–1869 (2014).
36. Jones, J. L. *et al.* Basolateral Amygdala Modulates Terminal Dopamine Release in the Nucleus Accumbens and Conditioned Responding. *Biol. Psychiatry* **67,** 737–744 (2010).
37. Lammel, S. *et al.* Input-specific control of reward and aversion in the ventral tegmental area. *Nature* **491,** 212–217 (2012).
38. Baldwin, A. E., Sadeghian, K. & Kelley, A. E. Appetitive instrumental learning requires coincident activation of NMDA and dopamine D1 receptors within the medial prefrontal cortex. *J. Neurosci.* **22,** 1063–1071 (2002).
39. Lovinger, D. M. Neurotransmitter roles in synaptic modulation, plasticity and learning in the dorsal striatum. *Neuropharmacology* **58,** 951–961 (2010).
40. Tye, K. M., Cone, J. J., Schairer, W. W. & Janak, P. H. Amygdala neural encoding of the absence of reward during extinction. *J. Neurosci.* **30,** 116–25 (2010).

41. Smith-Roe, S. L. & Kelley, A. E. Coincident activation of NMDA and dopamine D1 receptors within the nucleus accumbens core is required for appetitive instrumental learning. *J. Neurosci.* **20,** 7737–7742 (2000).
42. Berglind, W. J., Case, J. M., Parker, M. P., Fuchs, R. A. & See, R. E. Dopamine D1 or D2 receptor antagonism within the basolateral amygdala differentially alters the acquisition of cocaine-cue associations necessary for cue-induced reinstatement of cocaine-seeking. *Neuroscience* **137,** 699–706 (2006).
43. Gore, B. B. & Zweifel, L. S. Genetic reconstruction of dopamine D1 receptor signaling in the nucleus accumbens facilitates natural and drug reward responses. *J. Neurosci.* **33,** 8640–9 (2013).
44. Ikemoto, S. Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* **56,** 27–78 (2007).
45. Miller, C. A. & Marshall, J. F. Molecular substrates for retrieval and reconsolidation of cocaine-associated contextual memory. *Neuron* **47,** 873–884 (2005).
46. Ikemoto, S. Involvement of the olfactory tubercle in cocaine reward: intracranial self-administration studies. *J. Neurosci.* **23,** 9305–9311 (2003).
47. Lammel, S., Ion, D. I., Roeper, J. & Malenka, R. C. Projection-Specific Modulation of Dopamine Neuron Synapses by Aversive and Rewarding Stimuli. *Neuron* **70,** 855–862 (2011).
48. Addy, N. a, Daberkow, D. P., Ford, J. N., Garris, P. a & Wightman, R. M. Sensitization of rapid dopamine signaling in the nucleus accumbens core and shell after repeated cocaine in rats. *J. Neurophysiol.* **104,** 922–931 (2010).
49. Roberts, D. C. S., Corcoran, M. E. & Fibiger, H. C. On the role of ascending catecholaminergic systems in intravenous self-administration of cocaine. *Pharmacol. Biochem. Behav.* **6,** 615–620 (1977).
50. Roberts, D. C. S., Koob, G. F., Klonoff, P. & Fibiger, H. C. Extinction and recovery of cocaine self-administration following 6-hydroxydopamine lesions of the nucleus accumbens. *Pharmacol. Biochem. Behav.* **12,** 781–787 (1980).
51. Anderson, S. M., Bari, A. A. & Pierce, R. C. Administration of the D1-like dopamine receptor antagonist SCH-23390 into the medial nucleus accumbens shell attenuates cocaine priming-induced reinstatement of drug-seeking behavior in rats. *Psychopharmacology (Berl).* **168,** 132–138 (2003).
52. Wise, N. S. and R. A. Satiating effects of cocaine are controlled by dopamine actions in the nucleus accumbens core. *J. Neurosci.* **31,** 17917–17922 (2011).
53. Cervo, L., Carnovali, F., Stark, J. A. & Mennini, T. Cocaine-seeking behavior in response to drug-associated stimuli in rats: involvement of D3 and D2 dopamine receptors. *Neuropsychopharmacology* **28,** 1150–1159 (2003).
54. You, Z.-B., Wang, B., Zitzman, D., Azari, S. & Wise, R. A. A Role for Conditioned Ventral Tegmental Glutamate Release in Cocaine Seeking. *J. Neurosci.* **27,** 10546–10555 (2007).
55. Zhi-Bing You, Bin Wang, Dawnya Zitzman, and R. A. W. Acetylcholine release in the mesocorticolimbic dopamine system during cocaine-seeking: Conditioned and unconditioned contributions to reward and motivation. *J. Neurosci.* **72,** 181–204 (2011).
56. Bozarth, M. A. & Wise, R. A. Intracranial self-administration of morphine into the ventral tegmental area in rats. *Life Sci.* **28,** 551–555 (1981).
57. Zangen, A., Solinas, M., Ikemoto, S., Goldberg, S. R. & Wise, R. A. Two Brain Sites for Cannabinoid Reward. *J. Neurosci.* **26,** 4901–4907 (2006).
58. Gatto, G. J., McBride, W. J., Murphy, J. M., Lumeng, L. & Li, T. K. Ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats. *Alcohol* **11,** 557–564 (1994).
59. Corrigall, W. a. Nicotine self-administration in animals as a dependence model. *Nicotine Tob. Res.* **1,** 11–20 (1999).

60. Sellings, L. H. L., Baharnouri, G., McQuade, L. E. & Clarke, P. B. S. Rewarding and aversive effects of nicotine are segregated within the nucleus accumbens. *Eur. J. Neurosci.* **28,** 342–352 (2008).
61. Ikemoto, S. Primary Reinforcing Effects of Nicotine Are Triggered from Multiple Regions Both Inside and Outside the Ventral Tegmental Area. *J. Neurosci.* **26,** 723–730 (2006).
62. David, V., Besson, M., Changeux, J. P., Granon, S. & Cazala, P. Reinforcing effects of nicotine microinjections into the ventral tegmental area of mice: Dependence on cholinergic nicotinic and dopaminergic D1 receptors. *Neuropharmacology* **50,** 1030–1040 (2006).
63. Wise, R. a. Addictive drugs and brain stimulation reward. *Annu. Rev. Neurosci.* **19,** 319–340 (1996).
64. Han, S., Soleiman, M., Soden, M., Zweifel, L. & Palmiter, R. D. Elucidating an Affective Pain Circuit that Creates a Threat Memory. *Cell* **162,** 363–374 (2015).
65. Rosenkranz, J. a & Grace, a a. Modulation of basolateral amygdala neuronal firing and afferent drive by dopamine receptor activation in vivo. *J. Neurosci.* **19,** 11027–11039 (1999).
66. Fanselow, M. S. & LeDoux, J. E. Why we think plasticity underlying pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron* **23,** 229–232 (1999).
67. Jones, G. L. *et al.* A genetic link between discriminative fear coding by the lateral amygdala, dopamine, and fear generalization. *Elife* **4,** 1–18 (2015).
68. Ungless, M. a, Magill, P. J. & Bolam, J. P. Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. *Science (80-.).* **303,** 2040–2042 (2004).
69. Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B. & Uchida, N. Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature* **482,** 85–88 (2012).
70. Schultz, W. Updating dopamine reward signals. *Curr. Opin. Neurobiol.* **23,** 229–38 (2013).
71. Tye, K. M. *et al.* Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature* **471,** 358–362 (2011).
72. Borowski, T. B. & Kokkinidis, L. The effects of cocaine, amphetamine, and the dopamine D1 receptor agonist SKF 38393 on fear extinction as measured with potentiated startle: implications for psychomotor stimulant psychosis. *Behav. Neurosci.* **112,** 952–65 (1998).
73. Munro, L. J. & Kokkinidis, L. Infusion of quinpirole and muscimol into the ventral tegmental area inhibits fear-potentiated startle: Implications for the role of dopamine in fear expression. *Brain Res.* **746,** 231–238 (1997).
74. Fadok, J. P., Dickerson, T. M. K. & Palmiter, R. D. Dopamine is necessary for cue-dependent fear conditioning. *J. Neurosci.* **29,** 11089–97 (2009).
75. Palmiter, R. D. Dopamine signaling in the dorsal striatum is essential for motivated behaviors: Lessons from dopamine-deficient mice. *Ann. N. Y. Acad. Sci.* **1129,** 35–46 (2008).
76. Lammel, S., Lim, B. K. & Malenka, R. C. Reward and aversion in a heterogeneous midbrain dopamine system. *Neuropharmacology* **76,** 351–359 (2014).
77. Gore, B. B., Soden, M. E. & Zweifel, L. S. Visualization of plasticity in fear-evoked calcium signals in midbrain dopamine neurons. *Learn. Mem.* **21,** 575–9 (2014).
78. Dazzi, L. *et al.* Inhibition of stress- or anxiogenic-drug-induced increases in dopamine release in the rat prefrontal cortex by long-term treatment with antidepressant drugs. *J. Neurochem.* **76,** 1212–1220 (2001).
79. Pezze, M. A. & Feldon, J. Mesolimbic dopaminergic pathways in fear conditioning. *Prog. Neurobiol.* **74,** 301–320 (2004).
80. Pezze, M. a, Bast, T. & Feldon, J. Significance of dopamine transmission in the rat medial prefrontal cortex for conditioned fear. *Cereb. Cortex* **13,** 371–380 (2003).
81. Fenu, S., Bassareo, V. & Di Chiara, G. A role for dopamine D1 receptors of the nucleus accumbens shell in conditioned taste aversion learning. *J. Neurosci.* **21,** 6897–6904 (2001).
82. Young, a M., Joseph, M. H. & Gray, J. a. Latent inhibition of conditioned dopamine release in rat

nucleus accumbens. *Neuroscience* **54,** 5–9 (1993).
83. Pezze, M. A., Heidbreder, C. A., Feldon, J. & Murphy, C. A. Selective responding of nucleus accumbens core and shell dopamine to aversively conditioned contextual and discrete stimuli. *Neuroscience* **108,** 91–102 (2001).
84. Bobillier, P., Petitjean, F., Salvert, D., Ligier, M. & Seguin, S. Differential Projections of the Nucleus Raphe Dorsalis and Nucleus Raphe Centralis as Revealed by Autoradiography. *Brain Res.* **85,** 205–210 (1975).
85. Meibach, R. C. & Siegel, A. Efferent connections of the septal area in the rat: An analysis utilizing retrograde and anterograde transport methods. *Brain Res.* **119,** 1–20 (1977).
86. Phillipson, O. Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. *J. Comp. Neurol.* **187,** 117–143 (1979).
87. Simon, H., Le Moal, M. & Calas, A. Efferents and afferents of the ventral tegmental-A10 region studied after local injection of [3H]leucine and horseradish peroxidase. *Brain Res.* **178,** 17–40 (1979).
88. Geisler, S. & Zahm, D. S. Afferents of the ventral tegmental area in the rat-anatomical substratum for integrative functions. *J. Comp. Neurol.* **490,** 270–94 (2005).
89. Watabe-Uchida, M., Zhu, L., Ogawa, S. K., Vamanrao, A. & Uchida, N. Whole-Brain Mapping of Direct Inputs to Midbrain Dopamine Neurons. *Neuron* **74,** 858–873 (2012).
90. Beier, K. T. *et al.* Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic Input-Output Mapping. *Cell* **162,** 622–634 (2015).
91. Sesack, S. R. & Grace, A. a. Cortico-Basal Ganglia reward network: microcircuitry. *Neuropsychopharmacology* **35,** 27–47 (2010).
92. Bonci, A. & Malenka, R. C. Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. *J. Neurosci.* **19,** 3723–30 (1999).
93. Tong, Z.-Y., Overton, P. G. & Clark, D. Chronic administration of (+)-amphetamine alters the reactivity of midbrain dopaminergic neurons to prefrontal cortex stimulation in the rat. *Brain Res.* **674,** 63–74 (1995).
94. Tong, Z. Y., Overton, P. G. & Clark, D. Stimulation of the prefrontal cortex in the rat induces patterns of activity in midbrain dopaminergic neurons which resemble natural burst events. *Synapse* **22,** 195–208 (1996).
95. Geisler, S., Derst, C., Veh, R. W. & Zahm, D. S. Glutamatergic afferents of the ventral tegmental area in the rat. *J. Neurosci.* **27,** 5730–43 (2007).
96. Omelchenko, N. & Sesack, S. R. Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. *J. Comp. Neurol.* **483,** 217–35 (2005).
97. Charara, a, Smith, Y. & Parent, a. Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. *J. Comp. Neurol.* **364,** 254–66 (1996).
98. Floresco, S. B., West, A. R., Ash, B., Moore, H. & Grace, A. A. Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat. Neurosci.* **6,** 968–73 (2003).
99. Kalivas, P. W., Churchill, L. & Klitenick, M. A. GABA and enkephalin projection from the nucleus accumbens and ventral pallidum to the ventral tegmental area. *Neuroscience* **57,** 1047–1060 (1993).
100. Ji, H. & Shepard, P. D. Lateral Habenula Stimulation Inhibits Rat Midbrain Dopamine Neurons through a GABAA Receptor-Mediated Mechanism. *J. Neurosci.* **27,** 6923–6930 (2007).
101. Nishikawa, T., Fage, D. & Scatton, B. Evidence for, and nature of, the tonic inhibitory influence of habenulointerpeduncular pathways upon cerebral dopaminergic transmission in the rat. *Brain*

Res. **373,** 324–336 (1986).
102. Lisoprawski, A., Herve, D., Blanc, G., Glowinski, J. & Tassin, J. P. Selective activation of the mesocortico-frontal dopaminergic neurons induced by lesion of the habenula in the rat. *Brain Res.* **183,** 229–234 (1980).
103. Omelchenko, N. & Sesack, S. R. Ultrastructural analysis of local collaterals of rat ventral tegmental area neurons: GABA phenotype and synapses onto dopamine and GABA cells. *Synapse* **63,** 895–906 (2009).
104. Gervais, J., Rouillard, C. & Gervais, J. Dorsal raphe stimulation differentially modulates dopaminergic neurons in the ventral tegmental area and substantia nigra. *Synapse* **291,** 1–11 (2000).
105. Hervé, D., Pickel, V. M., Joh, T. H. & Beaudet, A. Serotonin axon terminals in the ventral tegmental area of the rat: fine structure and synaptic input to dopaminergic neurons. *Brain Res.* **435,** 71–83 (1987).
106. Van Bockstaele, E. J., Cestari, D. M. & Pickel, V. M. Synaptic structure and connectivity of serotonin terminals in the ventral tegmental area: potential sites for modulation of mesolimbic dopamine neurons. *Brain Res.* **647,** 307–322 (1994).
107. Blaha, C. D. *et al.* Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. *J. Neurosci.* **16,** 714–722 (1996).
108. Lodge, D. J. & Grace, a a. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 5167–5172 (2006).
109. Lester, D. B., Miller, A. D. & Blaha, C. D. Muscarinic receptor blockade in the ventral tegmental area attenuates cocaine enhancement of laterodorsal tegmentum stimulation-evoked accumbens dopamine efflux in the mouse. *Synapse* **64,** 216–223 (2010).
110. Dautan, D. *et al.* A Major External Source of Cholinergic Innervation of the Striatum and Nucleus Accumbens Originates in the Brainstem. *J. Neurosci.* **34,** 4509–4518 (2014).
111. Lacey, M. G., Calabresi, P. & North, R. Muscarine Depolarizes and Ventral Tegmental Rat Substantia Nigra Zona Compacta Neurons in Vitro Through M1-Like it mediation receptors. *J. Pharmacol. Exp. Ther.* **253,** 395–400 (1990).
112. Scroggs, R. S., Cardenas, C. G., Whittaker, J. a & Kitai, S. T. Muscarine reduces calcium-dependent electrical activity in substantia nigra dopaminergic neurons. *J. Neurophysiol.* **86,** 2966–72 (2001).
113. Kitai, S. T., Shepard, P. D., Callaway, J. C. & Scroggs, R. Afferent modulation of dopamine neuron firing patterns. *Curr. Opin. Neurobiol.* **9,** 690–697 (1999).
114. Calabresi, P., Lacey, M. G. & North, R. A. Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br. J. Pharmacol.* **98,** 135–40 (1989).
115. Gronier, B. & Rasmussen, K. Activation of midbrain presumed dopaminergic neurones by muscarinic cholinergic receptors: an in vivo electrophysiological study in the rat. *Br. J. Pharmacol.* **124,** 455–464 (1998).
116. Miller, A. D. & Blaha, C. D. Midbrain muscarinic receptor mechanisms underlying regulation of mesoaccumbens and nigrostriatal dopaminergic transmission in the rat. *Eur. J. Neurosci.* **21,** 1837–1846 (2005).
117. Mena-Segovia, J., Winn, P. & Bolam, J. P. Cholinergic modulation of midbrain dopaminergic systems. *Brain Res. Rev.* **58,** 265–271 (2008).
118. Mark, G. P., Shabani, S., Dobbs, L. K. & Hansen, S. T. Cholinergic modulation of mesolimbic dopamine function and reward. *Physiol. Behav.* **104,** 76–81 (2011).
119. You, Z. B., Wang, B., Zitzman, D. & Wise, R. A. Acetylcholine release in the mesocorticolimbic dopamine system during cocaine seeking: conditioned and unconditioned contributions to reward and motivation. *J. Neurosci.* **28,** 9021–9029 (2008).

120. Steidl, S., Wang, H. & Wise, R. A. Lesions of cholinergic pedunculopontine tegmental nucleus neurons fail to affect cocaine or heroin self-administration or conditioned place preference in rats. *PLoS One* **9,** (2014).
121. Shinohara, F., Kihara, Y., Ide, S., Minami, M. & Kaneda, K. Critical role of cholinergic transmission from the laterodorsal tegmental nucleus to the ventral tegmental area in cocaine-induced place preference. *Neuropharmacology* **79,** 573–579 (2014).
122. Mejias-Aponte, C. A., Drouin, C. & Aston-Jones, G. Adrenergic and Noradrenergic Innervation of the Midbrain Ventral Tegmental Area and Retrorubral Field: Prominent Inputs from Medullary Homeostatic Centers. *J. Neurosci.* **29,** 3613–3626 (2009).
123. Paladini, C. A. Noradrenergic Inhibition of Midbrain Dopamine Neurons. *J. Neurosci.* **24,** 4568–4575 (2004).
124. Geisler, S. & Zahm, D. S. Neurotensin afferents of the ventral tegmental area in the rat: [1] Re-examination of their origins and [2] responses to acute psychostimulant and antipsychotic drug administration. *Eur. J. Neurosci.* **24,** 116–134 (2006).
125. Binder, E. B., Kinkead, B., Owens, M. J. & Nemeroff, C. B. Neurotensin and dopamine interactions. *Pharmacol. Rev.* **53,** 453–486 (2001).
126. Kortleven, C., Bruneau, L. C. & Trudeau, L. E. Neurotensin inhibits glutamate-mediated synaptic inputs onto ventral tegmental area dopamine neurons through the release of the endocannabinoid 2-AG. *Neuropharmacology* **63,** 1161–1171 (2012).
127. Jiang, Z. G., Pessia, M. & North, R. a. Neurotensin excitation of rat ventral tegmental neurones. *J. Physiol.* **474,** 119–29 (1994).
128. Kalivas, P. W. Interactions between neuropeptides and dopamine neurons in the ventromedial mesencephalon. *Neurosci. Biobehav. Rev.* **9,** 573–587 (1985).
129. Hamilton, M. E. & Freeman, A. S. Effects of administration of cholecystokinin into the VTA on DA overflow in nucleus accumbens and amygdala of freely moving rats. *Brain Res.* **688,** 134–142 (1995).
130. Tepper, J. M. & Lee, C. R. GABAergic control of substantia nigra dopaminergic neurons. *Prog. Brain Res.* **160,** 189–208 (2007).
131. Paladini, C. A. & Tepper, J. M. GABA(A) and GABA(B) antagonists differentially affect the firing pattern of substantia nigra dopaminergic neurons in vivo. *Synapse* **32,** 165–176 (1999).
132. Fallon, J. H. & Moore, R. Y. Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. *J. Comp. Neurol.* **180,** 545–580 (1978).
133. Fallon, J. H. Topographic organization of ascending dopaminergic projections. *Ann. N. Y. Acad. Sci.* **537,** 1–9 (1988).
134. Del-Fava, F., Hasue, R. H., Ferreira, J. G. P. & Shammah-Lagnado, S. J. Efferent connections of the rostral linear nucleus of the ventral tegmental area in the rat. *Neuroscience* **145,** 1059–1076 (2007).
135. Swanson, L. W. The projections of the ventral tegmental area and adjacent regions: A combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res. Bull.* **9,** 321–353 (1982).
136. Bjorklund, A. & Dunnett, S. B. Dopamine neuron systems in the brain: an update. *Trends Neurosci.* **30,** 194–202 (2007).
137. Lammel, S. *et al.* Unique Properties of Mesoprefrontal Neurons within a Dual Mesocorticolimbic Dopamine System. *Neuron* **57,** 760–773 (2008).
138. Aransay, A., Rodríguez-López, C., García-Amado, M., Clascá, F. & Prensa, L. Long-range projection neurons of the mouse ventral tegmental area: a single-cell axon tracing analysis. *Front. Neuroanat.* **9,** 59 (2015).

139. Abi-Dargham, A. *et al.* Increased striatal dopamine transmission in schizophrenia: Confirmation in a second cohort. *Am. J. Psychiatry* **155,** 761–767 (1998).
140. Anderson, B. M. *et al.* Examination of association to autism of common genetic variationin genes related to dopamine. *Autism Res.* **1,** 364–369 (2008).
141. Davis, K., Kahn, R., Ko, G. & Davidson, M. Dopamine in schizophrenia: a review and reconceptualization. *Am. J. Psychiatry* **148,** 1474–1486 (1991).
142. Cross, A. J. *et al.* Reduced dopamine-beta-hydroxylase activity in Alzheimer's disease. *Br. Med. J.* **282,** 93–4 (1981).
143. Dunlop BW & Nemeroff CB. The role of dopamine in the pathophysiology of depression. *Arch. Gen. Psychiatry* **64,** 327–337 (2007).
144. Hamner, M. B. & Diamond, B. I. Elevated plasma dopamine in posttraumatic stress disorder: A preliminary report. *Biol. Psychiatry* **33,** 304–306 (1993).
145. Hoexter, M. Q. *et al.* Higher striatal dopamine transporter density in PTSD: An in vivo SPECT study with [99mTc]TRODAT-1. *Psychopharmacology (Berl).* **224,** 337–345 (2012).
146. Howes, O. D. & Kapur, S. The dopamine hypothesis of schizophrenia: Version III - The final common pathway. *Schizophr. Bull.* **35,** 549–562 (2009).
147. Lake, C., Ziegler, M. G. & Murphy, D. L. Increased norepinephrine levels and decreased dopamine-β-hydroxylase activity in primary autism. *Arch. Gen. Psychiatry* **34,** 553–556 (1977).
148. Ouchi, Y. *et al.* Microglial activation and dopamine terminal loss in early Parkinson's disease. *Ann. Neurol.* **57,** 168–175 (2005).
149. Segman, R. H. *et al.* Association between the dopamine transporter gene and posttraumatic stress disorder. *Mol. Psychiatry* **7,** 903–907 (2002).
150. Volkow, N. D. *et al.* Cocaine Cues and Dopamine in Dorsal Striatum: Mechanism of Craving in Cocaine Addiction. *J. Neurosci.* **26,** 6583–6588 (2006).
151. Lotharius, J. & Brundin, P. Pathogenesis of Parkinson's disease: Dopamine, vesicles and alpha-synuclein. *Nat. Rev. Neurosci.* **3,** 932–942 (2002).
152. Calabresi, P., Centonze, D. & Bernardi, G. Electrophysiology of dopamine in normal and denervated striatal neurons. *Trends Neurosci.* **23,** S57–S63 (2000).
153. Chase, T. N. & Oh, J. D. Striatal dopamine- and glutamate-mediated dysregulation in experimental parkinsonism. *Trends Neurosci.* **23,** S86–S91 (2000).
154. Sulzer, D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends Neurosci.* **30,** 244–250 (2007).
155. Addington, J. & Addington, D. Positive and negative symptoms of schizophrenia: Their course and relationship over time. *Schizophr. Res.* **5,** 51–59 (1991).
156. Snyder, S., Banerjee, S., Yamamura, H. & Greenberg, D. Drugs, Neurotransmitters, and Schizophrenia. *Science (80-.).* **184,** 1243–1253 (1974).
157. Sesack, S. R. & Carr, D. B. Selective prefrontal cortex inputs to dopamine cells: Implications for schizophrenia. *Physiol. Behav.* **77,** 513–517 (2002).
158. Laruelle, M., Abi-Dargham, A., Gil, R., Kegeles, L. & Innis, R. Increased dopamine transmission in schizophrenia: Relatioship to illness phase. *Biol. Psychiatry* **46,** 56–72 (1999).
159. Carlsson, A., Waters, N., Waters, S. & Carlsson, M. L. Network interactions in schizophrenia - Therapeutic implications. *Brain Res. Rev.* **31,** 342–349 (2000).
160. Carlsson, A. & Carlsson, M. L. A dopaminergic deficit hypothesis of schizophrenia: The path to discovery. *Dialogues Clin. Neurosci.* **8,** 137–142 (2006).
161. Ichikawa, J. & Meltzer, H. Y. Effect of antidepressants on striatal and accumbens extracellular dopamine levels. *Eur. J. Pharmacol.* **281,** 255–261 (1995).
162. Pozzi, L., Invernizzi, R., Garavaglia, C. & Samanin, R. Fluoxetine increases extracellular dopamine in the prefrontal cortex by a mechanism not dependent on serotonin: A comparison with

citalopram. *J. Neurochem.* **73,** 1051–1057 (1999).
163. Dailly, E., Chenu, F., Renard, C. E. & Bourin, M. Dopamine, depression and antidepressants. *Fundam. Clin. Pharmacol.* **18,** 601–607 (2004).
164. Prisco, S. & Esposito, E. Differential effects of acute and chronic fluoxetine administration on the spontaneous activity of dopaminergic neurones in the ventral tegmental area. *Br. J. Pharmacol.* **116,** 1923–1931 (1995).
165. Nestler, E. J. & Carlezon, W. A. The Mesolimbic Dopamine Reward Circuit in Depression. *Biol. Psychiatry* **59,** 1151–1159 (2006).
166. Tremblay, L. K. *et al.* Functional neuroanatomical substrates of altered reward processing in major depressive disorder revealed by a dopaminergic probe. *Arch. Gen. Psychiatry* **62,** 1228–36 (2005).
167. Cervo, L., Grignaschi, G. & Samanin, R. The role of the mesolimbic dopaminergic system in the despramine effect in the forced swimming test. *Eur. J. Pharmacol.* **178,** 129–133 (1990).
168. R.P., M., S.S., G., M.A., D. & S.B., H. Advances in drug treatments for children and adolescents with autism and other pervasive developmental disorders. *CNS Drugs* **19,** 923–934 (2005).
169. Nakamura, K. *et al.* Brain Serotonin and Dopamine Transporter Bindings in Adults With High-Functioning Autism. *Arch. Gen. Psychiatry* **67,** 59–68 (2010).
170. Comings, D. E. *et al.* The dopamine D2 receptor locus as a modifying gene in neuropsychiatric disorders. *JAMA* **266,** 1793–1800 (1991).
171. Markram, K., Rinaldi, T., La Mendola, D., Sandi, C. & Markram, H. Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology* **33,** 901–912 (2008).
172. Bisson, J., Cosgrove, S., Lewis, C. & Roberts, N. Post-traumatic stress disorder. *BMJ* **6161,** 1–7 (2015).
173. Hamner, M. B. & Gold, P. B. Plasma Dopamine Beta-Hydroxylase Activity in Psychotic and Non-Psychotic Post-Traumatic Stress Disorder. *Psychiatry Res.* **77,** 175–181 (1998).
174. Comings, D. E., Muhleman, D. & Gysin, R. Dopamine D2 receptor (DRD2) gene and susceptibility to posttraumatic stress disorder: A study and replication. *Biol. Psychiatry* **40,** 368–372 (1996).
175. Thompson, T. L. & Moss, R. L. Modulation of mesolimbic dopaminergic activity over the rat estrous cycle. *Neurosci. Lett.* **229,** 145–148 (1997).
176. Liberzon, I. *et al.* Brain activation in PTSD in response to trauma-related stimuli. *Biol. Psychiatry* **45,** 817–826 (1999).
177. Bremner, J. D. *et al.* Positron emission tomographic imaging of neural correlates of a fear acquisition and extinction paradigm in women with childhood sexual-abuse-related post-traumatic stress disorder. *Psychol. Med.* **35,** 791–806 (2005).
178. Di Chiara, G. Drug addiction as dopamine-dependent associative learning disorder. *Eur. J. Pharmacol.* **375,** 13–30 (1999).
179. Barrot, M. Functional heterogeneity in dopamine release and in the expression of Fos-like proteins within the rat striatal complex. *Eur. J. Neurosci.* **11,** 1155–1166 (1999).
180. Cadoni, C. & Di Chiara, G. Reciprocal changes in dopamine responsiveness in the nucleus accumbens shell and core and in the dorsal caudate-putamen in rats sensitized to morphine. *Neuroscience* **90,** 447–455 (1999).
181. Zweifel, L. S., Argilli, E., Bonci, A. & Palmiter, R. D. Role of NMDA receptors in dopamine neurons for plasticity and addictive behaviors. *Neuron* **59,** 486–96 (2008).
182. Kelley, A. E. Memory and addiction: Shared neural circuitry and molecular mechanisms. *Neuron* **44,** 161–179 (2004).
183. Clayton, J. a & Collins, F. S. Policy: NIH to balance sex in cell and animal studies. *Nature* **509,** 282–3 (2014).

184. Power, R. F., Mani, S. K., Codina, J., Conneely, O. M. & O'Malley, B. W. Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science* **254,** 1636–1639 (1991).
185. Ivanova, T. & Beyer, C. Estrogen regulates tyrosine hydroxylase expression in the neonate mouse midbrain. *J. Neurobiol.* **54,** 638–47 (2003).
186. Kipp, M. *et al.* Estrogen and the development and protection of nigrostriatal dopaminergic neurons: Concerted action of a multitude of signals, protective molecules, and growth factors. *Front. Neuroendocrinol.* **27,** 376–390 (2006).
187. Beyer, C., Eusterschulte, B., Pilgrim, C. & Reisert, I. Sex steroids do not alter sex differences in tyrosine hydroxylase activity of dopaminergic neurons in vitro. *Cell Tissue Res.* **270,** 547–552 (1992).
188. Pasqualini, C., Olivier, V., Guibert, B., Frain, O. & Leviel, V. Acute Stimulatory Effect of Estradiol on Striatal Dopamine Synthesis. *J. Neurochem.* **65,** 1651–1657 (1995).
189. chiodo, L.A., Caggiula, A. R. Substantia nigra dopamine neurons: alterations in basal discharge rates and autoreceptor sensitivity induced by estrogen. *Neuropharmacology* **22,** 593–599 (1983).
190. Zsarnovszky, A., Scalise, T. J., Horvath, T. L. & Naftolin, F. Estrogen effects on tyrosine hydroxylase-immunoreactive cells in the ventral mesencephalon of the female rat: Further evidence for the two cell hypothesis of dopamine function. *Brain Res.* **868,** 363–366 (2000).
191. Bazzett, T. J. & Becker, J. B. Sex differences in the rapid and acute effects of estrogen on striatal D2 dopamine receptor binding. *Brain Res.* **637,** 163–72 (1994).
192. Creutz, L. M. & Kritzer, M. F. Estrogen Receptor- Immunoreactivity in the Midbrain of Adult Rats : Regional , Subregional , and Cellular Localization in the A10 , A9 , and A8 Dopamine. *J. Comp. Neurol.* **300,** 288–300 (2002).
193. Kritzer, M. F. & Creutz, L. M. Region and sex differences in constituent dopamine neurons and immunoreactivity for intracellular estrogen and androgen receptors in mesocortical projections in rats. *J. Neurosci.* **28,** 9525–35 (2008).
194. Kritzer, M. F., Adler, a, Marotta, J. & Smirlis, T. Regionally selective effects of gonadectomy on cortical catecholamine innervation in adult male rats are most disruptive to afferents in prefrontal cortex. *Cereb. Cortex* **9,** 507–18 (1999).
195. Kritzer, M. F. Effects of acute and chronic gonadectomy on the catecholamine innervation of the cerebral cortex in adult male rats: insensitivity of axons immunoreactive for dopamine-beta-hydroxylase to gonadal steroids, and differential sensitivity of axons immunoreact. *J. Comp. Neurol.* **427,** 617–33 (2000).
196. Kritzer, M. F. Long-term gonadectomy affects the density of tyrosine hydroxylase- but not dopamine-beta-hydroxylase-, choline acetyltransferase- or serotonin-immunoreactive axons in the medial prefrontal cortices of adult male rats. *Cereb. Cortex* **13,** 282–96 (2003).
197. Aubele, T. & Kritzer, M. F. Gonadectomy and hormone replacement affects in vivo basal extracellular dopamine levels in the prefrontal cortex but not motor cortex of adult male rats. *Cereb. Cortex* **21,** 222–32 (2011).
198. Becker, J. B. & Ramirez, V. D. Sex differences in the amphetamine stimulated release of catecholamines from rat striatal tissue in vitro. *Brain Res.* **204,** 361–372 (1981).
199. Dluzen, D.E., Ramirez, V. D. In vitro progesterone modulates amphetamine-stimulated dopamine release from the corpus striatum of castrated male rats treated with estrogen. *Neuroendocrinology* **52,** 517–520 (1990).
200. Castner, S. a, Xiao, L. & Becker, J. B. Sex differences in striatal dopamine: in vivo microdialysis and behavioral studies. *Brain Res.* **610,** 127–34 (1993).
201. Xiao, Li and Becker, J. Quantitative microdialysis determination of extracellular striatal dopamine concentration in male and female rats: effects of estrous cycle and gonadectomy. *Neurosci. Lett.*

180, 155–158 (1994).
202. de Souza Silva, M. a, Mattern, C., Topic, B., Buddenberg, T. E. & Huston, J. P. Dopaminergic and serotonergic activity in neostriatum and nucleus accumbens enhanced by intranasal administration of testosterone. *Eur. Neuropsychopharmacol.* **19,** 53–63 (2009).
203. Becker, J. B. Gender differences in dopaminergic function in striatum and nucleus accumbens. *Pharmacol. Biochem. Behav.* **64,** 803–12 (1999).
204. Lammers, C. H. *et al.* Regulation of striatal dopamine receptors by estrogen. *Synapse* **34,** 222–227 (1999).
205. Hruska, R. E., Ludmer, L. M., Pitman, K. T., De Ryck, M. & Silbergeld, E. K. Effects of Estrogen on Striatal Dopamine receptor function in male and female rats. *Pharmacol. Biochem. Behav.* **16,** 285–91 (1982).
206. Walker, Q. D., Rooney, M. B., Wightman, R. M. & Kuhn, C. M. Dopamine release and uptake are greater in female than male rat striatum as measured by fast cyclic voltammetry. *Neuroscience* **95,** 1061–1070 (2000).
207. Munro, C. a *et al.* Sex differences in striatal dopamine release in healthy adults. *Biol. Psychiatry* **59,** 966–74 (2006).
208. Laakso, A. *et al.* Sex differences in striatal presynaptic dopamine synthesis capacity in healthy subjects. *Biol. Psychiatry* **52,** 759–763 (2002).
209. Mitsushima, D., Yamada, K., Takase, K., Funabashi, T. & Kimura, F. Sex differences in the basolateral amygdala: the extracellular levels of serotonin and dopamine, and their responses to restraint stress in rats. *Eur. J. Neurosci.* **24,** 3245–54 (2006).
210. Zhang, D., Yang, S., Yang, C., Jin, G. & Zhen, X. Estrogen regulates responses of dopamine neurons in the ventral tegmental area to cocaine. *Psychopharmacology (Berl).* **199,** 625–35 (2008).
211. Dazzi, L. *et al.* Estrous cycle-dependent changes in basal and ethanol-induced activity of cortical dopaminergic neurons in the rat. *Neuropsychopharmacology* **32,** 892–901 (2007).
212. Sell, S. L., Scalzitti, J. M., Thomas, M. L. & Cunningham, K. a. Influence of ovarian hormones and estrous cycle on the behavioral response to cocaine in female rats. *J. Pharmacol. Exp. Ther.* **293,** 879–86 (2000).
213. Becker, J. B., Molenda, H. & Hummer, D. L. Gender differences in the behavioral responses to cocaine and amphetamine. Implications for mechanisms mediating gender differences in drug abuse. *Ann. N. Y. Acad. Sci.* **937,** 172–187 (2001).
214. Dalla, C. & Shors, T. J. Sex differences in learning processes of classical and operant conditioning. *Physiol. Behav.* **97,** 229–38 (2009).
215. Maren, S., De Oca, B. & Fanselow, M. S. Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. *Brain Res.* **661,** 25–34 (1994).
216. Toufexis, D. J., Myers, K. M., Bowser, M. E. & Davis, M. Estrogen disrupts the inhibition of fear in female rats, possibly through the antagonistic effects of estrogen receptor alpha (ERalpha) and ERbeta. *J. Neurosci.* **27,** 9729–35 (2007).
217. Morgan, M. a & Pfaff, D. W. Effects of estrogen on activity and fear-related behaviors in mice. *Horm. Behav.* **40,** 472–482 (2001).
218. Jasnow, A. M., Schulkin, J. & Pfaff, D. W. Estrogen facilitates fear conditioning and increases corticotropin-releasing hormone mRNA expression in the central amygdala in female mice. *Horm. Behav.* **49,** 197–205 (2006).
219. Milad, M. R., Igoe, S. a, Lebron-Milad, K. & Novales, J. E. Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience* **164,** 887–95 (2009).
220. Gupta, R. R., Sen, S., Diepenhorst, L. L., Rudick, C. N. & Maren, S. Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats(1).

Brain Res. **888,** 356–365 (2001).
221. van Haaren, F., van Hest, a & Heinsbroek, R. P. Behavioral differences between male and female rats: effects of gonadal hormones on learning and memory. *Neurosci. Biobehav. Rev.* **14,** 23–33 (1990).
222. Mishima, N., Higashitani, F., Teraoka, K. & Yoshioka, R. Sex differences in appetitive learning of mice. *Physiol. Behav.* **37,** 263–8 (1986).
223. Beatty, W. W. Effects of gonadectomy on sex differences in DRL behavior. *Physiol. Behav.* **10,** 177–178 (1973).
224. Lentz, F., Pool, G. & Milner, J. Effects of ovariectomy and hormone replacement on DRL behavior in the rat. *Physiol. Behav.* **20,** 477–480 (1978).
225. Marx, M. H. A test of sex differences in instrumental appetitive learning by rats. *Behav. Res. Methods Instrum.* **I,** 208–210 (1969).
226. Kritzer, M. F., Brewer, a, Montalmant, F., Davenport, M. & Robinson, J. K. Effects of gonadectomy on performance in operant tasks measuring prefrontal cortical function in adult male rats. *Horm. Behav.* **51,** 183–94 (2007).
227. van Hest, A., van Haaren, F. & van de Poll, N. E. The behavior of male and female Wistar rats pressing a lever for food is not affected by sex differences in food motivation. *Behav. Brain Res.* **27,** 215–221 (1988).
228. Hammerslag, L. R. & Gulley, J. M. Age and sex differences in reward behavior in adolescent and adult rats. *Dev. Psychobiol.* **56,** 611–21 (2014).
229. Pitchers, K. K. *et al.* Individual variation in the propensity to attribute incentive salience to a food cue: Influence of sex. *Behav. Brain Res.* **278C,** 462–469 (2014).
230. Shulman, L. M. & Bhat, V. Gender disparities in Parkinson's disease. *Expert Rev Neurother* **6,** 407–416 (2006).
231. Lubomski, M., Louise Rushworth, R., Lee, W., Bertram, K. L. & Williams, D. R. Sex differences in Parkinson's disease. *J. Clin. Neurosci.* **21,** 1503–1506 (2014).
232. Gillies, G. E., Pienaar, I. S., Vohra, S. & Qamhawi, Z. Sex differences in Parkinson's disease. *Front. Neuroendocrinol.* **35,** 370–384 (2014).
233. Haaxma, C. A. *et al.* Gender differences in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **78,** 819–24 (2007).
234. Bourque, M., Dluzen, D. E. & Di Paolo, T. Neuroprotective actions of sex steroids in Parkinson's disease. *Front. Neuroendocrinol.* **30,** 142–157 (2009).
235. Al-Sweidi, S., Morissette, M., Bourque, M. & Di Paolo, T. Estrogen receptors and gonadal steroids in vulnerability and protection of dopamine neurons in a mouse model of Parkinson's disease. *Neuropharmacology* **61,** 583–591 (2011).
236. Smith, K. M. & Dahodwala, N. Sex differences in Parkinson's disease and other movement disorders. *Exp. Neurol.* **259,** 44–56 (2014).
237. Abel, K. M., Drake, R. & Goldstein, J. M. Sex differences in schizophrenia. *Int. Rev. Psychiatry* **22,** 417–28 (2010).
238. Aleman, a., Kahn, R. S. & Selten, J. Sex Differences in the Risk of Schizophrenia. *Arch. Gen. Psychiatry* **60,** 565–571 (2003).
239. Seeman, M. V & Lang, M. The role of estrogens in schizophrenia gender differences. Special Issue: : The role of estrogens in schizophrenia gender differences. *Schizophr. Bull.* **16,** 185–194 (1990).
240. Castle, D. J. & Murray, R. M. The neurodevelopmental basis of sex differences in schizophrenia. *Psychol. Med.* **21,** 565–575 (1991).
241. Hafner, H., Maurer, K., Loffler, W. & Riecher-Rossler, A. The influence of age and sex on the onset and early course of schizophrenia. *Br.J Psychiatry* **162,** 80–86 (1993).

242. Leung, A. & Chue, P. Sex differences in schizophrenia, a review of the literature. *Acta Psychiatr. Scand. Suppl.* **401,** 3–38 (2000).
243. Grossman, L. S., Harrow, M., Rosen, C. & Faull, R. Sex differences in outcome and recovery for schizophrenia and other psychotic and nonpsychotic disorders. *Psychiatr. Serv.* **57,** 844–850 (2006).
244. Grossman, L. S., Harrow, M., Rosen, C., Faull, R. & Strauss, G. P. Sex differences in schizophrenia and other psychotic disorders: a 20-year longitudinal study of psychosis and recovery. *Compr. Psychiatry* **49,** 523–529 (2008).
245. Nopoulos, P., Flaum, M. & Andreasen, N. C. Sex differences in brain morphology in schizophrenia. *Am J Psychiatry* **154,** 1648–1654 (1997).
246. Häfner, H., Behrens, S., De Vry, J. & Gattaz, W. F. An animal model for the effects of estradiol on dopamine-mediated behavior: implications for sex differences in schizophrenia. *Psychiatry Res.* **38,** 125–34 (1991).
247. Weissman, M. M. *et al.* Sex differences in rates of depression: cross-national perspectives. *J. Affect. Disord.* **29,** 77–84 (1993).
248. Piccinelli, M. & Wilkinson, G. Gender differences in depression. Critical review. *Br. J. Psychiatry* **177,** 486–92 (2000).
249. Nolen-Hoeksema, S. Sex differences in unipolar depression: Evidence and theory. *Psychol. Bull.* **101,** 259–282 (1987).
250. Giarelli, E. *et al.* Sex differences in the evaluation and diagnosis of autism spectrum disorders among children. *Disabil. Health J.* **3,** 107–116 (2010).
251. Tolin, D. F. & Foa, E. B. Sex differences in trauma and posttraumatic stress disorder: a quantitative review of 25 years of research. *Psychol. Bull.* **132,** 959–992 (2006).
252. Merz, C. J. *et al.* Neuronal correlates of extinction learning are modulated by sex hormones. *Soc. Cogn. Affect. Neurosci.* **7,** 819–830 (2012).
253. Johnson, S. W. & North, R. a. Two types of neurone in the rat ventral tegmental area and their synaptic inputs. *J. Physiol.* **450,** 455–468 (1992).
254. Margolis, E. B., Lock, H., Hjelmstad, G. O. & Fields, H. L. The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? *J. Physiol.* **577,** 907–924 (2006).
255. Hnasko, T. S., Hjelmstad, G. O., Fields, H. L. & Edwards, R. H. Ventral Tegmental Area Glutamate Neurons: Electrophysiological Properties and Projections. *J. Neurosci.* **32,** 15076–15085 (2012).
256. Hnasko, T. S. & Edwards, R. H. Neurotransmitter corelease: mechanism and physiological role. *Annu. Rev. Physiol.* **74,** 225–43 (2012).
257. Tritsch, N. X., Ding, J. B. & Sabatini, B. L. Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. *Nature* **490,** 262–6 (2012).
258. Bolam, J. P. & Smith, Y. The GABA and substance P input to dopaminergic neurones in the substantia nigra of the rat. *Brain Res.* **529,** 57–78 (1990).
259. Celada, P., Paladini, C. A. & Tepper, J. M. GABAergic control of rat substantia nigra dopaminergic neurons: Role of globus pallidus and substantia nigra pars reticulata. *Neuroscience* **89,** 813–825 (1999).
260. Cheramy, A., Nieoullon, A. & Glowinski, J. GABAergic processes involved in the control of dopamine release from nigrostriatal dopaminergic neurons in the cat. *Eur. J. Pharmacol.* **48,** 281–295 (1978).
261. Santiago, M. & Westerink, B. H. C. The role of GABA receptors in the control of nigrostriatal dopaminergic neurons : dual-probe microdialysis study in awake rats. *Eur. J. Pharmacol.* **219,** 175–181 (1992).
262. Chu, H. & Zhen, X. Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels in the regulation of midbrain dopamine systems. *Acta Pharmacol. Sin.* **31,** 1036–1043 (2010).

263. Mercuri, N. B., Bonci, A., Calabresi, P., Stefani, A. & Bernardi, G. Properties of the hyperpolarization-activated cation current I(h) in rat midbrain dopaminergic neurons. *Eur. J. Neurosci.* **7,** 462–469 (1995).
264. Walther, D. J. *et al.* Synthesis of Serotonin by a Second Tryptophan Hydroxylase Isoform. *Science (80-.).* **299,** 76 (2003).
265. Domínguez, R., Cruz-Morales, S. E., Carvalho, M. C., Xavier, M. & Brandao, M. L. Sex differences in serotonergic activity in dorsal and median raphe nucleus. *Physiol. Behav.* **80,** 203–210 (2003).
266. Di Giovanni, G., Esposito, E. & Di Matteo, V. Role of serotonin in central dopamine dysfunction. *CNS Neurosci. Ther.* **16,** 179–194 (2010).
267. Urban, D. J. & Roth, B. L. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annu. Rev. Pharmacol. Toxicol.* **55,** 399–417 (2015).
268. Dragunow, M. & Faull, R. The use of c-fos as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Methods* **29,** 261–265 (1989).
269. Sanz, E. *et al.* Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13939–44 (2009).
270. Gendrel, A.-V. & Heard, E. Noncoding RNAs and Epigenetic Mechanisms During X-Chromosome Inactivation. *Annu. Rev. Cell Dev. Biol.* **30,** 561–580 (2014).
271. Berridge, K. C. & Robinson, T. E. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Res. Brain Res. Rev.* **28,** 309–69 (1998).
272. Parker, J. G. *et al.* Absence of NMDA receptors in dopamine neurons attenuates dopamine release but not conditioned approach during Pavlovian conditioning. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 13491–6 (2010).
273. Darvas, M., Wunsch, A. M., Gibbs, J. T. & Palmiter, R. D. Dopamine dependency for acquisition and performance of Pavlovian conditioned response. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 2764–9 (2014).
274. Smith, Y. & Bolam, J. P. The output neurones and the dopaminergic neurones of the substantia nigra receive a GABA-containing input from the globus pallidus in the rat. *J. Comp. Neurol.* **296,** 47–64 (1990).
275. Ehrmann, I. E. *et al.* Characterization of genes encoding translation initiation factor eIF-2gamma in mouse and human: sex chromosome localization, escape from X-inactivation and evolution. *Hum. Mol. Genet.* **7,** 1725–1737 (1998).
276. Mazeyrat, S. *et al.* A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. *Nat. Genet.* **29,** 49–53 (2001).
277. Festa, E. D. & Quinones-Jenab, V. Gonadal hormones provide the biological basis for sex differences in behavioral responses to cocaine. *Horm. Behav.* **46,** 509–519 (2004).
278. van Haaren, F. & Meyer, M. E. Sex differences in locomotor activity after acute and chronic cocaine administration. *Pharmacol. Biochem. Behav.* **39,** 923–7 (1991).
279. Sershen, H., Hashim, A. & Lajtha, A. Gender differences in kappa-opioid modulation of cocaine-induced behavior and NMDA-evoked dopamine release. *Brain Res.* **801,** 67–71 (1998).
280. Walker, Q. D. *et al.* Sex differences in cocaine-stimulated motor behavior: Disparate effects of gonadectomy. *Neuropsychopharmacology* **25,** 118–130 (2001).
281. Menendez-Delmestre, R. & Segarra, A. C. Testosterone is essential for cocaine sensitization in male rats. *Physiol. Behav.* **102,** 96–104 (2011).
282. Hu, M. & Becker, J. B. Effects of sex and estrogen on behavioral sensitization to cocaine in rats. *J. Neurosci.* **23,** 693–9 (2003).
283. Ting, J. T., Daigle, T. L., Chen, Q. & Feng, G. Patch-Clamp Methods and Protocols. *Methods Mol. Biol.* **1183,** 221–242 (2014).