THE ROLE OF MESOINTERPEDUNCULAR CIRCUITRY IN ANXIETY

A Dissertation Presented

By

Steven Randall DeGroot

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 14, 2019

PROGRAM OF NEUROSCIENCE
THE ROLE OF MESOINTERPEDUNCULAR CIRCUITRY IN

ANXIETY

A Dissertation Presented

By

Steven Randall DeGroot

This work was undertaken in the Graduate School of Biomedical Sciences

Department of Neurobiology

Under the mentorship of

Andrew R. Tapper, Thesis Advisor

Kensuke Futai, Member of Committee

Gilles Martin, Member of Committee

William Kobertz, Member of Committee

Joseph Bergan, External Member of Committee

Ann Rittenhouse, Chair of Committee

Marry Ellan Lane, Ph.D., Dean of the Graduate School of Biomedical Sciences

May, 14th 2019
DEDICATION

This dissertation is dedicated to all my family in California patiently waiting for my return. Thank you for all your understanding and calm words whenever I am frustrated with the scientific process.

To my fiancé who is waiting with great anticipation for my return. There’s nowhere else I’d rather be.
ACKNOWLEDGEMENTS

I would like to thank my parents for getting me here and Ruth for the abundant care.

Thank you to all the lab members starting out at the BNRI building and ending in the LRB. Rubing Zhao-Shea, Liwang Liu, Jennifer Ngolab, Susanna Casacuberta, Paul Klenowski, In-Jee Yu, Leeyup Chung, Xueyan Pang, Melissa Guilford, Alison Casserly, Ciearra Smith, Anthony Sacino, Lindsey Soll, Linzy Hendrickson, Reina Improgo, Eric Hogan.

Thank you to my mentors. Especially my thesis advisor Andrew. Thank you for holding the lab together. I’ve learned a lot about e-phys, science, and zen. Sorry I still can’t bring myself to drink coffee.

Thank you to Paul Gardner for the “two on ones” and finding CRFR1 in the Allen Brain Atlas. You always read my sweaters.

Thank you to Liwang for all the e-phys advice and the proper daily practices for consistent data.

Thank you to Rubing for being exasperated about the IPN with me and much collaboration.

Thank you to Susanna for the advice. I wish you great luck furthering your career.

Thank you to Lindsey for teaching me perfusion.

Thank you to In-Jee for always thinking of us when you return from trips.
Thank you to Anthony for making all our lives easier.

Thank you to Paul Klenowski for the help with biocytin. Don’t lose another passport.

Thank you to Jen for the enthusiasm and all the viruses.

Thank you to Ann Rittenhouse for the calm and collected advice and notice on important seminars.

Thank you to Haley. Your enthusiasm is infectious.

Thank you to my qualifying committee members:

   Ann Rittenhouse
   Haley Melikian
   José Lemos
   Larry Hayward
   Alonzo Ross
   David Weaver

Thank you to my TRAC members: The advice helped a lot.

   Ann Rittenhouse
   Kenny Futai
   Gilles Martin
José Lemos

Thank you to my Dissertation Committee members: Thank you for your time.

Ann Rittenhouse

Kenny Futai

Gilles Martin

William Kobertz

And my outside member: Thank you for your carefully reasoned advice and edits.

Joseph Bergan
ABSTRACT

Anxiety is an affective state defined by heightened arousal and unease in the absence of a clear and present fear-inducing stimulus. Chronic and inappropriate anxiety leads to anxiety disorders, the most common class of human mental disorder. Recent work suggests projections to the ventral tegmental area (VTA), are critical for anxiety behavior expression. However, the relationship between efferent VTA projections and anxiety is unclear. This thesis resolves anxiety circuitry connecting the dopaminergic (DAergic) VTA to the interpeduncular nucleus (IPN), coined the mesointerpeduncular circuit. I hypothesize the mesointerpeduncular circuit affects anxiety through the release of anxiogenic corticotropin releasing factor (CRF) during nicotine withdrawal and anxiolytic dopamine (DA) during drug naïve behavior. Electrophysiological and pharmacological data suggest CRF release from the DAergic VTA during nicotine withdrawal activates CRF receptor 1 (CRFR1) potentiating the glutamatergic activation of “Type 2” neurons and anxiety-like behavior in mice. However, in nicotine naïve conditions CRF production is negligible. Instead, in vivo DA release is anticorrelated with anxiety-like behaviors. Optogenetic stimulation and inhibition drives decreased and increased anxiety-like behaviors, respectively. Electrophysiological experiments reveal a complex interpeduncular microcircuit where D1-like DA receptor expressing “Type C” neurons in the caudal IPN (cIPN) regulate glutamatergic release in the ventral IPN (vIPN) through presynaptic GABA receptors. The result is propagation of the signal to excite “Type A” and inhibit
“Type B” vlPN neurons. Finally, pharmacological activation or inhibition of interpeduncular D1-like DA receptors is sufficient to decrease and increase anxiety-like behaviors respectively. Thus, this circuit is important for modulating anxiety-like behavior.
# Table of Contents

TITLE PAGE .......................................................................................................... i

REVIEWER PAGE ............................................................................................... ii

DEDICATION ....................................................................................................... iii

ACKNOWLEDGEMENTS ..................................................................................... iv

ABSTRACT ......................................................................................................... vii

LIST OF TABLES ............................................................................................... xiii

LIST OF FIGURES ............................................................................................. xiv

LIST OF ABBREVIATIONS ............................................................................... xvii

LIST OF COPYRIGHTED MATERIALS ............................................................ xxv

CHAPTER I: INTRODUCTION ............................................................................. 1

  Anxiety Disorders .............................................................................................. 1

  The Impact of Anxiety Disorders ...................................................................... 1

  Current Treatments for Anxiety Disorders ......................................................... 3

  Anxiety Circuitry of the Extended Amygdala ..................................................... 6

  Anxiety Circuitry of the VTA .............................................................................. 9

  Anxiety in the Dorsal Diencephalic Conduction System .................................. 11

  The IPN ........................................................................................................... 14

  The IPN’s Connection to the Anxiety Circuitry of the Extended Amygdala ..... 16
# Table of Contents

Data analysis ................................................................. 59
Acknowledgements ....................................................... 59

**CHAPTER III** .......................................................... 60
Midbrain dopamine controls anxiety-like behaviors by engaging unique interpeduncular nucleus microcircuitry .................................................. 60

Abstract .............................................................................. 61
Introduction .......................................................................... 62
Results .................................................................................. 64
Figures .................................................................................. 76
Discussion ............................................................................. 109

Acknowledgments ............................................................. 112
Methodology ................................................................. 114
Animals ................................................................................. 114
Viral Preparation .............................................................. 114
Stereotaxic injections .......................................................... 115
Implantation of cannulas and optic fibers ................................ 116
Post-surgery procedures ...................................................... 117
Intra-cerebral Infusion ............................................................ 117
Fiber Photometry ............................................................... 118
LIST OF TABLES

Table II.1 — Raw EPSC frequency data from IPI CRF pharmacology 46

Table II.2 — IPI specific genes 47

Table III.1 — General morphology parameters of Type A and B neurons 105

Table III.2 — Branch order characteristics of Type A and B neurons 106

Table III.3 — Sholl analysis differences for Type A and B neurons 108

Table IV.1 — Significance between groups in Figure IV.1 138
LIST OF FIGURES

Figure I.1 Anxiety circuitry of the extended amygdala in rodent CNS. 25

Figure I.2 Afferent and efferent connections of the MHb-IPN axis in rodent CNS 27

Figure I.3 Hypothetical circuitry combining the MHb-IPN axis and the anxiety circuitry of the extended amygdala in rodent CNS. 29

Figure II.1 Characterization of IPI neurons. 39

Figure II.2 CRF modulates glutamatergic activity in a Type 1 but not Type 2 IPI neurons. 41

Figure II.3 Type 2, but not Type 1, neurons exhibit increased activation during nicotine withdrawal. 43

Figure II.4 Model of neuroadaptations in response to nicotine in the mesointerpeduncular circuit. 46

Figure III.1 VTA→IPN DA input controls anxiolytic behavior. 76

Figure III.2 Optogenetic stimulation and inhibition of VTA afferents in the IPN during the OFT. 79

Figure III.3 DA modulates neuronal activity in two vlPN neuron sub-populations. 81
Figure III.4 VLPN neurons respond to DA through presynaptic D1-like but not D2-like DA receptors.

Figure III.5 D2-like DA receptor agonist does not significantly affect VLPN spAPs.

Figure III.6 VLPN neurons spAPs are blocked by exposure to glutamate receptor antagonists CNQX and AP-5.

Figure III.7 Optogenetic stimulation of VTA→IPN DAergic terminals modulate cLPN neurons via D1 receptors.

Figure III.8 Characterization of cLPN neurons and their responses to VTA terminal stimulation.

Figure III.9 VTA DAergic predominantly innervate the cLPN.

Figure III.10 D1 DA receptor expression in the IPN.

Figure III.11 cLPN Type C putative D1 receptor-expressing neurons project to the VLPN and modulate Type A and Type B neuronal activity via GABA.

Figure III.12 Type A neurons receive stronger innervation from MHb cholinergic/glutamatergic neurons than Type B neurons.

Figure III.13 Manipulation of D1 receptors in the IPN controls anxiety-like behaviors.
Figure III.14  Effects of VTA D1 drug infusion on anxiety-like behavior.  102

Figure III.15  Circuit model for DA signal amplification in the IPN.  104

Figure IV.1  Comparison of physiological properties across proposed neuronal types of the IPN.  137

Figure IV.2  Expanded schematic diagram of IPN anxiety circuitry  139
LIST OF ABBREVIATIONS

µm – micrometer

5-HT — 5-hydroxytryptamine, Serotonin

AAV — adeno associated virus

ACh — acetylcholine

ACSF — artificial cerebrospinal fluid

AMPA — α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA — analysis of variance

AP-5 — NMDA receptor antagonist

BAC — bed nucleus of the anterior commissure

BDNF — brain-derived neurotrophic factor

BLA — basolateral amygdala

BNST — bed nucleus of the stria terminalis

Ca²⁺ — calcium

CeA — central nucleus of the amygdala

CeL — central lateral nucleus of the amygdala

CeM — central medial nucleus of the amygdala

ChAT — choline acetyltransferase

ChR2 — channelrhodopsin 2

cIPN — caudal interpeduncular nucleus

cm — centimeter

CNQX — 6-cyano-7-nitroquinoxaline-2,3-dione (AMPA receptor antagonist)
CNS — central nervous system
Cre — Cre recombinase
CRF — corticotropin releasing factor
CRFR1 — corticotropin releasing factor receptor 1
D1 — dopamine receptor 1
D2 — dopamine receptor 2
D3 — dopamine receptor 3
D4 — dopamine receptor 4
D5 — dopamine receptor 5
DA — dopamine
DAergic — dopaminergic
DAT — dopamine transporter
DIO — double-inverted open reading frame
DMSO — dimethyl sulfoxide
DR — dorsal raphe
DRD1 — dopamine receptor 1 gene
DREADD — designer receptors exclusively activated by designer drugs
DTg — dorsal tegmental nucleus
DTR — dorsal tegmental region
Ef1a — Elongation Factor 1 Alpha
EPM — elevated plus maze
EPSC — excitatory postsynaptic current
eYFP — enhanced yellow fluorescent protein
fMRI — functional magnetic resonance imaging
fr — fasciculus retroflexus
GABA — γ-Aminobutyric Acid
GABA\textsubscript{A}R GABA(A) receptor
GABA\textsubscript{B}R GABA(B) receptor
Gad1 — Glutamic Acid Decarboxylase 1
Gad2 — Glutamic Acid Decarboxylase 2
GFP — Green Fluorescent Protein
Glu — glutamate
GPCR — G-Protein Coupled Receptor
GRAB\textsubscript{DA} — G protein-coupled receptor [GPCR]-activation-based DA sensor
h — hour
HPC — hippocampus
HRP — horseradish peroxidase
Hz — hertz
I/V — current/voltage
IC — inferior colliculus
ip — intraperitoneal
IPA — apical subnucleus of the IPN
IPC — central subnucleus of the IPN
IPDL — dorsolateral subnucleus of the IPN
IPDM — dorsomedial subnucleus of the IPN
IPI — intermediate subnucleus of the IPN
IPL — lateral subnucleus of the IPN
IPN — interpeduncular nucleus
IPR — rostral subnucleus of the IPN
IPSC — inhibitory postsynaptic current
kHz — kilohertz
KO — knock out
L — liter
LC — locus coeruleus
LCM — laser capture microdissection
LDTg — laterodorsal tegmentum
LH — lateral hypothalamus
LHb — lateral habenula
LS — lateral septum
LTD — long-term depression
LTP — long-term potentiation
MAOI — monoamine oxidase inhibitors
MAPK — Mitogen Activated Protein Kinase
MBT — marble burying test
MD — medial dorsal nucleus of the thalamus
Mec — mecamylamine
mEPSC — miniature excitatory postsynaptic current
mg/kg — milligrams per kilogram
mGluR — metabotropic glutamate receptor
MHb — medial habenula
min — minute
miRNA — micro ribonucleic acid
ml — milliliter
mm — millimeter
mM — millimolar
MnR — medial raphe
mPFC — medial prefrontal cortex
mRNA — messenger ribonucleic acid
MS — medial septum
ms — millisecond
mV — millivolt
MΩ — megaohm
n.s. — non-significant
NA — numerical aperture
Na+ — sodium
NAc — nucleus accumbens

nAChR — nicotinic acetylcholine receptor

NDB — nucleus of the diagonal band

NI — nucleus incertus

Nic — nicotine

nm — nanometer

NMDA — N-methyl-D-aspartate

NMDAR — NMDA receptor

NpHR — halorhodopsin

OFT — open field test

pA — picoamps

PAG — periaqueductal gray/central gray

PBG — parabigeminal nucleus

PFA — paraformaldehyde

PFC — prefrontal cortex

PTSD — post-traumatic stress disorder

qRT-PCR — quantitative reverse transcription- polymerase chain reaction

RMTg — rostromedial tegmentum

RN — raphe nuclei

s — second

s.c. — subcutaneouse
SACSF — sucrose based artificial cerebrospinal fluid
SC — superior colliculus
SCH39166 — ecopipam (D1 antagonist)
sec — second
SEM — standard error of the mean
SFi — septofimbrial nucleus
SI — substantia innominate
SKF82958 — (D1 agonist)
SNr — substantia nigra
SRNI — selective serotonin norepinephrine inhibitor
SP — substance P
spAP — spontaneous action potentials
spEPSC — spontaneous excitatory post synaptic current
SSRI — selective serotonin re-uptake inhibitor
SUM — supramammillary nucleus
TH — tyrosine hydroxylase
TS — triangular septal nucleus
TTX — tetrodotoxin
VIP — vasoactive intestinal peptide
vIPN — ventral interpeduncular nucleus
VTA — ventral tegmental area
VTg — ventral tegmental nuclei of Gudden
WT — wild type

μM — micromolar
LIST OF COPYRIGHTED MATERIALS THIS AUTHOR PRODUCED

CHAPTER I

Part of the writing in this chapter was modified from a separate publication:


CHAPTER II

Figures 2 and 3 have appeared in a separate publication:


CHAPTER III

This chapter is currently under revision for a separate publication:

CHAPTER I: INTRODUCTION

Anxiety Disorders

Everyone experiences anxiety. It has a profound impact on our behavior. While it can help us avoid dangerous or damaging situations, chronic, inappropriate anxiety impairs perceived quality of life and is known as an anxiety disorder. Anxiety disorders are often defined as near daily bouts of anxiety lasting longer than 6 months however, they can be less frequent but more debilitating as in the case of panic attack (American Psychiatric Association, 2013). These disorders comprise the most common class of human mental disorders (Chisholm et al., 2016; Kessler et al., 2005). This class of disorders includes several separable disorders including generalized anxiety disorder (GAD), panic disorder, phobia-related disorders, agoraphobia, separation anxiety disorder, and selective mutism (American Psychiatric Association, 2013; Guze, 1995; Lieb, 2005; Ruscio et al., 2017). The many types of anxiety disorders reveal the complexity of the anxious state. An important question remains— How do people become susceptible to repeated, sustained bouts of anxiety?

The Impact of Anxiety Disorders

An estimated 18.1% of the United States English speaking adult population has an anxiety disorder lasting at least 12 months (Kessler et al., 2005). This is the
majority of the 26.2% of the population estimated to have any psychiatric disorder. 22.8% of these anxiety disorders were considered “serious” because the disorder led to a suicide attempt, missing 30+ days of work in a year, or substantial limitations in ability at work. Anxiety disorders are more prominent among those with disadvantaged social status, those who are female, those who are unmarried, those that live in more developed societies, and those who have low socioeconomic status (Chisholm et al., 2016; Kessler et al., 2005; Lieb, 2005; Ruscio et al., 2017).

The estimated annual cost of anxiety disorders in the United States in 1990 was approximately $42.3 billion and €74.4 billion in Europe (2010) (Greenberg et al., 1999; Olesen et al., 2012). These totals come from a sum of direct medical care cost (37% of total), direct nonmedical expenses (23%), and the estimated cost of lost productivity (40%). The loss of productivity is a cost associated with ineffective treatment which illustrates the need for the development of novel, effective treatments. A 2016 study estimated expanding effective anxiety treatments to every person with an anxiety disorder in the 36 most populated countries, which make up a combined 80% of the world’s population, would result in an increase of 169 billion dollars to the global GDP (Chisholm et al., 2016). This total was derived after subtracting the estimated increased cost of expanding similar cost treatments to the whole population. Taken together, there is a need for more effective diagnosis and treatment of anxiety disorders, which can be aided by a better
understanding of anxiety. It also suggests a large financial incentive for studying and treating anxiety disorders.

The financial studies highlighted above also miss other potential benefits of anxiety treatment due to the co-morbidity of anxiety and other disorders. Anxiety disorders are highly co-morbid with other psychiatric conditions, particularly mood disorders like depression and substance use disorders (Kessler et al., 2005). The comorbidity between the effects of anxiety and the co-expressed illness often have a multiplicative effect on behavioral dysfunction and discomfort (Kessler et al., 2005; Noyes, 2001). Therefore, potent anxiety disorder treatments may also be an important supplementary treatment in other psychiatric disorders.

**Current Treatments for Anxiety Disorders**

Available treatments for anxiety and their effectiveness inform us about the nature of anxiety and suggest how treatments could be improved. The neurotransmitter and receptor systems targeted by the pharmacological treatments provide evidence for the features common in regions of the central nervous system (CNS) that process anxiety information.

Current treatments for anxiety utilize psychotherapy, anti-depressants, or, in rare cases, brain stimulation (Bandelow et al., 2017; Farb and Ratner, 2014). The fact that the molecules effective for anxiety are also anti-depressants illustrates the close ties between anxiety disorders and depression.
Psychotherapy has a significant effect compared to placebos on all forms of anxiety disorders (Barlow and Lehman, 1996). This suggests that areas of cognitive function must be part of or have a degree of control over anxiety circuitry. However, psychotherapy alone is often not enough for those with anxiety disorders. Instead psychotherapy is used in conjunction with anti-depressant medications (Bandelow et al., 2017). This suggests anxiety disorders may create neuroadaptations in circuits beyond those under direct executive control.

The recommended first anti-depressants for patients with anxiety disorders are selective serotonin re-uptake inhibitors (SSRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs) (Ballenger et al., 1998a, 1998b, 2001; Bandelow et al., 2002). These medications increase the concentration of extracellular serotonin, and norepinephrine in the case of SNRIs, throughout the brain. This implies that serotonin and norepinephrine should modulate anxiety circuitry. Different SSRIs and SNRIs will be effective for different people in a currently unpredictable fashion (Bystritsky, 2006). The lack of predictive factors prevents pre-tailoring medications for patients, so there is often a need to switch between drugs to find the most effective ones and minimize debilitating side effects (Bandelow et al., 2017; Bystritsky, 2006). Future anxiety research should strive to find predictive factors to avoid improper prescription of medication. Additionally, the treatment is often only effective after 2-8 weeks, and causes symptoms to worsen before relieving them (Bandelow et al., 2002, 2017). This class of medication is effective in 60-85% of cases (Bystritsky, 2006). SSRIs and SNRIs need to be taken for several months
while patients endure long-term side effects like sexual dysfunction only to face a remission rate of 50-60% (Ballenger, 2004). The lag period before effectiveness and wide variability in response to these drugs may be a result of the complexity of the serotonin receptor subunits and their widespread presence in the CNS. In the end SSRIs and SNRIs are far from a perfect solution.

The second class of anti-depressants prescribed are benzodiazepines which have a significant effect in 80% of cases (Bystritsky, 2006). Benzodiazepines enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at GABA\textsubscript{A} receptors (Costa et al., 1975). These receptors are found throughout the CNS and thus have a broad effect over multiple brain regions (Benke et al., 1994). Despite guidelines stating benzodiazepines should be avoided in favor of SSRIs and SNRIs, benzodiazepines are actually the most prescribed treatment for anxiety disorders partly due to the practice of prescribing multiple medications to a single patient (Stahl, 2002). However, benzodiazepines are criticized because of the tendency for patients to develop tolerance and dependence, their depressive effects on cognition and motor activity, and their ineffectiveness against the common comorbid conditions, i.e. depression (Klein, 2002).

The last line of defense for anxiety disorder medications are monoamine oxidase inhibitors (MAOIs) (Bandelow et al., 2017). MAOIs increase the concentration of the monoamines serotonin, dopamine, norepinephrine, and melatonin by inhibiting the enzymes responsible for their metabolism (Davison, 1957). However, the patient must avoid several potentially fatal, seizure inducing drug interactions and
change their diet to avoid certain foods (Grady and Stahl, 2012). Consuming MAOIs may also lead to dependence and withdrawal symptoms (Selikoff et al., 1953).

From the details above, it is obvious that broad action over much of the CNS is a common theme among current anxiety disorder treatments. As a result, treatments are often not potent enough or include a plethora of side effects. To develop a potent, specialized treatment for anxiety disorders with less side effects, a better understanding of the specific anxiety circuitry is necessary.

**Anxiety Circuitry of the Extended Amygdala**

This section contains materials modified from (Molas et al., 2017a)

A complex behavior like anxiety is bound to have complex circuitry. This complexity is hinted at by the multiple forms of anxiety disorders, the mixed responses to treatments, and the myriad of situations in which anxiety appears. Because anxiety is vital for survival, it would not be surprising to find multiple redundant circuits that function together to minimize errors in judgement. So which circuits encode anxiety?

Historically, anxiety circuitry has been difficult to differentiate from fear circuitry because of their shared structural and behavioral features. Indeed, numerous studies have confirmed that the prefrontal cortex, hippocampus, and the extended
amygdala play vital roles in both fear and anxiety in rodents and humans (Fanselow and Gale, 2003; Farb and Ratner, 2014; Kim et al., 2011; Shackman and Fox, 2016). For reference the extended amygdala is a macrostructure that includes the various subdivisions of the amygdala, the substantia innominata, the nucleus accumbens (NAc) shell, and the bed nucleus of the stria terminalis (BNST). Although there are many similarities between fear and anxiety and in many cases the terms are interchangeable, the two can be distinct in terms of temporal differences present in both behavior and circuit activation. Anxiety, unlike fear, is long lasting and can exist independently from a fear inducing cue (American Psychiatric Association, 2013; Lieb, 2005). Specifically, fear is a reaction to an external threat that abates with the removal of the cue while anxiety can appear before and after a fear inducing cue. (American Psychiatric Association, 2013; Davis et al., 1989; Lieb, 2005). Additionally, anxiolytics do not occlude defensive fear behaviors (Blanchard et al., 1993). Although it is important to note the similarities and differences between fear and anxiety, this thesis focuses on anxiety.

Anxiety can be measured via verbal or written communication i.e. surveys of perceived anxiety in humans, but measurement of anxiety in mice requires observable behaviors associated with anxiety known as anxiety-like behaviors (Bailey and Crawley, 2009). These anxiety-like behaviors include decreased time exploring the open arm of the elevated plus maze (EPM), increased marble burying
in the marble burying test (MBT), and decreased time exploring the center of the open field test (OFT).

Reciprocal and looping circuits between the amygdala, BNST, VTA, Hippocampus and prefrontal cortex (PFC) govern anxiety-like responses (Felix-Ortiz et al., 2013; Tye et al., 2011; Weiskrantz, 1956) (Figure I.1). These brain regions receive and integrate information from the thalamus and cortex that is assigned a positive or negative valence that then dictates a behavioral response (Li and Kirouac, 2008; Reynolds and Zahm, 2005). When mice anticipate aversive conditions, the basolateral amygdala (BLA) neurons projecting to the central amygdala (CeM) and ventral hippocampus (vHPC) respond preferentially over positive valence cues to the nucleus accumbens (NAc) (Beyeler et al., 2016). Optogenetic manipulation of these BLA efferents to the CeM and vHPC is sufficient to influence anxiety-like behaviors (Felix-Ortiz et al., 2013; Tye et al., 2011). Although the BLA projection to the CeM can drive anxiety-like behaviors, in vivo these behaviors are short-term and are present alongside an environmental cue. This suggests activity in these circuits are more akin to fear, but this does not preclude their involvement in anxiety behaviors. By contrast, the BNST promotes long-term vigilance and anxiety-like behavior which is more similar to pathological anxiety (Davis et al., 2010). The BNST receives innervation from the BLA, and can drive and suppress separable features of anxiety and fear (Kim et al., 2013). BNST afferents in the VTA promote anxiogenic behavior when glutamate is released and anxiolytic behavior when GABA is released via optogenetic stimulation (Jennings et al., 2013).
The mPFC also has reciprocal connections with the vHPC and the amygdala; these circuits are associated with fear learning and are thought to be dysregulated in anxiety disorders (Kim et al., 2011). The vHPC, in turn, projects to the lateral septum (LS) to convey an anxiety signal mainly via hypothalamic areas (Trent and Menard, 2010). These circuits are not only modulated by dopamine, norepinephrine, and serotonin, which anxiolytics target, but also by the stress associated neurotransmitter corticotropin releasing factor (CRF) (Cummings et al., 1983; Dahlstroem and Fuxe, 1964; Hall et al., 1994; Segal, 1975; Segal et al., 1973; Wang and Aghajanian, 1977). Further characterization of the receptors and connections present in these circuits can be used to develop more efficient anxiolytic therapies. However, the neuroanatomical mechanisms and circuits by which healthy anxiety becomes an anxiety disorder remain unknown. Although this is the most developed circuit for anxiety in the literature there are still room for other, likely connected, anxiety circuits in the brain.

Anxiety Circuitry of the VTA

The VTA's relationship to anxiety is particularly interesting because VTA outputs are most commonly associated with the reward system (Montague et al., 1996; Olds, 1958). This suggests the VTA is an interface between positive reinforcing and negative avoidance motivational cues. The main pathway for reward is the
mesolimbic dopamine pathway in which the VTA sends DAergic projections to the NAc (Koob and Volkow, 2010). Notably, virtually all drugs of abuse target this pathway to increase the DA signaling in the NAc (Robbins, 1976). The theory behind withdrawal states that chronic doses of drugs of abuse lead to homeostatic neuroadaptations. When these drugs are removed from the system, the resulting imbalance causes withdrawal (Koob and Le Moal, 1997). Indeed, when drugs of abuse are metabolized there is a lower than normal concentration of DA in the NAc and this is accompanied by withdrawal symptoms which include anxiety (Kasanetz et al., 2012). Therefore, one might assume that decreased release in the VTA$^{DA}$→NAc pathway would be anxiogenic. However, mesolimbic pathway stimulation and inhibition has no direct influence over baseline anxiety-like behaviors (Chaudhury et al., 2013). Instead it is thought to promote resilience against developing depression due to social stress and this may extend to social anxiety disorder in humans (Russo et al., 2012). Indeed, Chaudury et al did not test anxiety in a social context in mice, so analogs of social anxiety disorder may have been masked by the isolated anxiety test. The mesolimbic pathway stimulation can also enhance fear extinction (Luo et al., 2018). Taken together, the mesolimbic pathway may aid in resisting future emotional responses, but it is not involved in controlling anxiety-like behaviors directly. The neuroadaptations responsible for anxiety in withdrawal are likely found in a different circuit.

Optogenetic control of BNST→VTA terminals has a direct effect on anxiety (Jennings et al., 2013). Additionally, a subset of VTA DAergic neurons are
activated during anxiety and by noxious stimuli. Thus, a subset of VTA DAergic projections should modulate anxiety as well. In the literature, the only projection from the VTA that when stimulated can modify anxiety-like behaviors is the mesocortical pathway which projects to the medial prefrontal cortex (mPFC). These DAergic afferents in the mPFC are sufficient to drive anxiety-like behaviors when depolarized with high-frequency phasic optogenetic stimulation (Gunaydin et al., 2014) (Figure I.1, see red arrows). However, the VTA projects to many other areas of the brain. The mesocortical pathway should not be the only anxiety modifying output of the VTA. I hypothesize there are more VTA DAergic projections that can modulate anxiety. Furthermore, if the VTA^{DA->NAc} pathway does not generate withdrawal anxiety what pathway does?

**Anxiety in the Dorsal Diencephalic Conduction System**

Another possible anxiety circuit can be found in the evolutionarily conserved dorsal diencephalic conduction system. Cortical and limbic signals are carried by the septum and bed nucleus of the anterior commissure (BAC) to the habenula. (see Figure I.2, see blue arrows stemming from the MS, LS, BAC, TS and SFi) (Sutherland, 1982). The habenula then projects through the axon bundle known as the fasciculus retroflexus (fr) to convey descending information to the midbrain and hindbrain monoaminergic centers (Figure I.2, see red arrow) (Akagi and Powell, 1968; Hamill and Jacobowitz, 1984). This pathway is thought to be
involved in anxiety because lesion of the fr increased baseline anxiety in rats (Murphy et al., 1996). Starting at the habenula the pathway divides into the lateral habenula (lHb) and the medial habenula (MHb) (Herkenham and Nauta, 1977). The lHb primarily innervates the VTA while the MHb almost exclusively innervates the interpeduncular nucleus (IPN). Anxiety signaling DAergic neurons in the VTA have already been discussed, but there is evidence for control of anxiety in the MHb-IPN pathway as well (Qin and Luo, 2009).

To further discuss anxiety signaling from the MHb to the IPN some background information is required. The mammalian MHb is subdivided into the dorsal MHb (dMHb) and ventral MHb (vMHb) based on neuropeptide expression, the segregated septal inputs, and the IPN outputs (Qin and Luo, 2009). The neurons of the dMHb coexpress glutamate and substance P (SP), receive input from the BAC, and send efferents to the lateral IPN. The neurons of the vMHb coexpress acetylcholine and glutamate, receive input from the triangular septum (TS), and send efferents to the core of the IPN.

The IPN is an unpaired nucleus. However, the reported anatomy, number of subnuclei and their names are inconsistent, varying wildly due to differences in model species and the research groups reporting (Lenn and Hamill, 1984). These discrepancies arose from a combination of multiple labs defining the subregions around the same time and the differences in anatomy between species. In mice and rats there is a loose general consensus that the IPN is subdivided into four paired (intermediate (IPI), lateral (IPL) dorsolateral (IPDL), dorsomedial (IPDM))
and three unpaired subnuclei (rostral (IPR) central (IPC) and apical (IPA)) (Lenn and Hamill, 1984; Paxinos and Franklin, 2012). These subnuclei are determined by differences in afferents, immunoreactivity, and the timing of development. The previously mentioned projection from the cholinergic vMHb is present in all the subnuclei except for the most lateral IPL and the IPA (Contestabile and Flumerfelt, 1981). In contrast, the dMHb sends projections primarily to the IPL and is associated with fear responses and learning (Yamaguchi et al., 2013).

Ablation of the neurons responsible for the TS→vMHb projection resulted in decreased baseline levels of anxiety-like behavior in mice (Kobayashi et al., 2013, Okamoto and Aizawa, 2013, Yamaguchi et al., 2013). Therefore, the vMHb afferents arising from the septum modulate anxiety-like behaviors, and thus are likely to carry anxiogenic information. Further evidence for the role of the MHb-IPN in anxiety can be found in nicotine withdrawal anxiety.

Chronic exposure to nicotine leads to desensitization followed by upregulation of the receptor it acts upon, the nicotinic acetylcholine receptor (nAChR), throughout the brain (Langdon et al., 2016; Metaxas et al., 2010). This and other homeostatic, compensatory changes creates the neuroadaptations responsible for withdrawal symptoms, including elevated anxiety (Bakhshaie et al., 2016; Dani et al., 2011). Our lab found that nicotine withdrawal anxiety was significantly reduced by transfection of a hypersensitive nicotinic acetylcholine receptor into the MHb cholinergic neurons (Pang et al., 2016). This suggests that the signaling in the vMHb cholinergic neurons alone is able to modify anxiety-like behaviors and the
vMHb-IPN is part of the pathway responsible for nicotine withdrawal induced anxiety.

To recap the vMHb receives input from the frontal lobe and limbic forebrain through the septum and passes it on to the IPN. Disruption of the signalling along this pathway at the level of the septum or fr modifies anxiety behavior in rodents and the pathway appears to be necessary for nicotine withdrawal induced anxiety. Precise circuitry manipulations through optogenetics have yet to demonstrate control over anxiety. Furthermore, the anxiety controlling properties of this pathway have not yet been shown in the IPN. I hypothesize that manipulation of circuits in the IPN can likewise modify anxiety-like behaviors.

The IPN

To study the IPN, an understanding of its anatomy and properties is necessary. IPN neurons are primarily GABAergic (Zhao-Shea et al., 2013). However there are also glutamatergic neurons and an IPA based population of serotonergic neurons (Hemmendinger and Moore, 1984). The IPN, especially the IPR, also expresses neuropeptides like substance P (Vu and Hamill, 1988), somatostatin (Zhao-Shea et al., 2013), Leu-enkephalin (Hamill et al., 1986), galanin (Hamill et al., 1986), and atrial natriuretic factor (Kawata et al., 1985). Regardless of subregion, most IPN neurons appear to express neuronal nitric oxide synthase (Ables et al., 2017; Gotti et al., 2005; Kawaguchi, 1993; Tepper et al., 2010). The MHb-IPN is associated
with the following behaviors: reward, fear, anxiety, depression, learning and memory, spatial memory, nociception, motor activity, male behavior, maternal behavior, sleep, rapid eye movement sleep, and eating and drinking (Clark and Taube, 2009; Hammer and Klingberg, 1990; Klemm, 2004; Lisoprawski et al., 1981; Mészáros et al.; Pang et al., 2016; Sharp et al., 2006; Thompson, 1960; Valjakka et al., 1998). The vast amount of behaviors implicated fits with the idea that the MHb-IPN is part of a circuit that connects the forebrain with the midbrain and hindbrain.

The IPN has all the markings of a target region for anti-anxiety medications. Not only does it have serotonergic neurons, it has a reciprocal connection with the dorsal raphe (DR) and median raphe (MnR) nuclei (Conrad et al., 1974; Hemmendinger and Moore, 1984; Moore et al., 1978; Shibata and Suzuki, 1984). The IPN also receives presumably noradrenergic afferents from the locus coeruleus (LC) (Shibata et al., 1986). Thus, it should be a target for SSRIs, SNRIs, and MAOIs. As mentioned above the nucleus is primarily GABAergic so benzodiazepines should target it as well. This description also fits many other brain regions, but there is no reason that other sites in the brain can’t also have a modulatory effect on anxiety.
The IPN’s Connection to the Anxiety Circuitry of the Extended Amygdala

Part of this section modified from (Molas et al., 2017a).

How might the MHb–IPN circuitry interconnect with other anxiety circuitry? While there are few first-order connections between the MHb–IPN and other anxiety circuits, most afferents and efferents, particularly those that arise from and innervate the IPN, have not been functionally characterized (Figure I.2). At the level of the MHb, the majority of MHb neurons project to the IPN. However, a subpopulation of MHb neurons may innervate the lHb (Kim and Chang, 2005). The lHb consists of glutamatergic neurons that are activated by aversive or unrewarding predictive cues (Matsumoto and Hikosaka, 2008). Most lHb neurons terminate in the GABAergic tail of the VTA (Jhou et al., 2009) and provide inhibitory control over DAergic cells of the VTA (Sánchez-Catalán et al., 2016). The existence of a direct synaptic link between the MHb and lHb may be important in promoting behavioral avoidance and controlling emotional behavior, although the functional relevance of this connection has yet to be explored.

Although a direct connection between the IPN and BLA or BNST has not been reported in the literature, the IPN may affect these areas by modulating other brain regions (Figure I.3). The prime example of this is the IPN’s efferents to all serotonergic raphe nuclei, particularly the DR and MnR which are thought to innervate most of the extended amygdala and modulate anxiety. (Beyeler et al., 2016; Conrad et al., 1974; Lima et al., 2017; Marcinkiewcz et al., 2016; Moore et
Indeed, this follows the description of the dorsal diencephalic conduction system which controls and is modified by midbrain monoamines (Sutherland, 1982). It is probable that the IPN can control anxiety in the extended amygdala in this way.

Another possibility is a direct connection between the IPN and cortical regions. There is evidence that the infralimbic cortex, a subregion of the mPFC known to impose anxiolytic effects on the amygdala, innervates the IPN (Takagishi and Chiba, 1991). In addition, the IPN has efferents in the entorhinal cortex (EC) (Shibata and Suzuki, 1984). Mice undergoing contextual fear conditioning while the BLA connection to the EC is optogenetically inhibited fail to learn defensive freezing behavior in response to audio cues (Sparta et al., 2014). This circuitry relays the BLA connection to vHPC through the EC. The IPN efferents to the EC could modulate BLA control of the vHPC and ultimately elicit emotional responses.

The IPN is also known to project directly to the hippocampus (Shibata and Suzuki, 1984). Lesion of the fr in rats decreases theta power of hippocampal theta rhythms, which controls anxiety behaviors and mood (Valjakka et al., 1998). The IPN may accomplish theta rhythm modulation through its efferents in the HPC or via reciprocal connections with other areas known to affect theta rhythm. These regions include the septum, MnR, and the nucleus incertus (NI) (Ma and Gundlach, 2015).

Reciprocal projections between the IPN and midbrain areas such as the lateral dorsal tegmental nucleus (LDTg) or periaqueductal grey (PAG) may play important
roles in the expression of emotional behavior (Groenewegen et al., 1986). Optogenetic activation of either PAG glutamatergic neurons (Tovote et al., 2016) or LDTg GABAergic neurons (Yang et al., 2016) can trigger freezing behavior, which may be implicated in anxiety-related disorders. In addition, the IPN may regulate the VTA and mPFC activity via its projections to the cholinergic LDTg, although only aversive behaviors occurred when this projection was optogenetically stimulated (Wolfman et al., 2018).

In the end, the function of IPN efferents and afferents remains poorly characterized. Future experiments should focus on using opto- and chemogenetic approaches to test the neurotransmitter systems and behaviors signaled and processed by MHb–IPN neuronal connectivity.

**Dopamine and Its Receptors.**

CHAPTER III explores the idea that the novel mesointerpeduncular circuit releases DA in drug naïve conditions. DA acts on DA receptors (Kebabian et al., 1972). DA receptors are a class of G-protein couple receptors (GPCRs) found within the vertebrate CNS that have profound and far reaching effects on a range of behaviors (Berke, 2018; Brown and Makman, 1972). To bind agonists, DA receptors form dimers in the cell membrane and like other GPCRs consist of seven transmembrane spanning domains (Chien et al., 2010; Wang et al., 2017). DA receptors can be homodimers, however heterodimeric DA receptors are often reported and have slightly altered properties (Kasai et al., 2018). Activation of DA
receptors leads to signaling cascades, which amplify the signal to affect a myriad of molecules in the neuron. However, this thesis will focus on the short-term effects on cell excitability and synaptic release rather than the long-term effects on gene expression through Mitogen-Activated Protein Kinases (MAPK), cyclic AMP response element-binding protein (CREB), and long-term potentiation/depression (Liu and Graybiel, 1996; Otani et al., 1999; Sheynikhovich et al., 2013).

In humans and rodents there are five DA receptor genes which encode the five subunits D1, D2, D3, D4 and D5 although D2 has two isoforms, short and long (Lindgren et al., 2003; Missale et al., 1998). These subunits are divided into two families which share most of their signalling properties (Kebabian and Calne, 1979). These families are the D1-like DA receptors including subunits D1 and D5 and the D2-like DA receptors including subunits D2, D3 and D4 (Missale et al., 1998).

D1-like DA receptors tend to couple with Gαs or Gαolf containing G-proteins. When DA binds the receptor the Gαs/Gαolf proteins dissociate from the Gγ/Gβ complex allowing it to stimulate adenyl cyclase 5 leading to an increase in cellular cyclic AMP that activates phosphokinase A. This goes on to modulate gene expression and ion channels including Ca^{2+}, K+, Na+ and also NMDA, AMPA, and GABA receptors (Chao et al., 2002; Flores-Hernandez et al., 2000; Li et al., 1992; Mahan et al., 1990; Wang et al., 2015; Witkowski et al., 2008). There is also evidence for an alternative D1-like receptor pathway involving Gαq/11. However, there is controversy over whether these were homodimeric or heterodimeric (D1-like+D2-
like) receptors or if there were unusual conditions/tissue that allowed $\text{Go}_{q/11}$ to bind (see below) (Lezcano and Bergson, 2002).

D2-like DA receptors couple with $\text{G}_{\alpha_i}$ or $\text{G}_{\alpha_o}$ containing G-proteins. When DA binds the D2-like DA receptors the $\text{G}_{\alpha_i}/\text{G}_{\alpha_o}$ dissociates from the $\text{G}_{\gamma}/\text{G}_{\beta}$ complex allowing it to inhibit adenyl cyclase 5 and 6 leading to a decrease in cellular cyclic AMP and thus a reduction in active phosphokinase A (Stoof and Kebabian, 1981). However, there are also many reports of D2-like DA receptors acting through direct interaction with the $\text{G}_{\gamma}/\text{G}_{\beta}$ complex. For example, D2-like receptors act by direct interaction with G-protein gated inwardly rectifying K+ channels independent of the $\text{G}_{\alpha_i}/\text{G}_{\alpha_o}$ subunit (Brown et al., 1995; Lacey et al., 1988). A similar mechanism appears to effect $\text{Ca}^{2+}$ currents (Yan et al., 1997; Zamponi and Snutch, 1998). The $\text{G}_{\gamma}/\text{G}_{\beta}$ of D2-like DA receptors can also effect $\text{Ca}^{2+}$ currents through a phospholipase C to calcineurin signaling cascade (Hernandez-Lopez et al., 2000).

Heterodimers of D1-like and D2-like DA receptors tend to have an overall D1-like effect and are acted on preferentially by pharmacological substances that affect D1-like receptors (Marcellino et al., 2008). Thus, there is debate over whether the results of previous studies misidentify the composition of the D1-like receptor as homodimeric. However, D1-like/D2-like heteromers tend to bind the $\text{G}_{\alpha_{q/11}}$ containing G-proteins (Marcellino et al., 2008; Rashid et al., 2007). When DA binds the D1/D2-like DA receptors the $\text{G}_{\alpha_{q/11}}$ dissociates from the $\text{G}_{\gamma}/\text{G}_{\beta}$ complex activating phosphatidylinositol turnover leading to rapid influxes of $\text{Ca}^{2+}$. D2-like
receptor Gγ/Gβ subunits are also known to enhance the activity of D1-like receptors when expressed in the same neuron (Hopf et al., 2003).

The DAergic system is well known for its roles in neutral or positive behaviors including motivation, pleasure, positive reinforcement learning and memory, motor control, and neuroendocrine signaling. However, DA can also be either anxiogenic or anxiolytic depending on the context. For example, DA modulates medial prefrontal cortex inputs to the amygdala that when activated limit fear and anxiety. D1-like receptor agonist and antagonist infusion into the amygdala elicits anxiogenic and anxiolytic responses respectively, while D2-like receptor agonists and antagonist can regulate anxiety in a context specific manner (de la Mora et al., 2010). Another example is how D2 agonist infusion into the ventromedial PFC decreased anxiety-like behavior in the EPM (Wall, 2003). However, these infusion studies have not been reconfirmed with optogenetics. Overall the effect of DA on anxiety is mixed and under characterized.

Recent work which focus on optogenetically activating specific DAergic projections has found that VTA DA can also drive aversion (de Jong et al., 2019). This has opened up the field to the idea that there are many projection-target-defined subpopulations of VTA neurons that are activated by more unique and negative stimuli. Therefore, the mesointerpeduncular circuit should be examined as a circuit that likely falls outside the normal reward predicting population.
The Nicotinic Acetylcholine Receptor

Although this thesis does not focus on the nicotinic acetylcholine receptor, I will discuss it briefly here as it is the receptor through which nicotine acts. Nicotine acts through pentameric nicotinic acetylcholine receptors (nAChRs) (Brejc et al., 2001). When nicotine or the endogenous ligand, acetylcholine, binds it increases the probability for the channel to enter the open configuration, which allows the flow of sodium (Na+), potassium (K+) and calcium (Ca^{2+}) ions across the membrane (Miyazawa et al., 2003). There are 11 nAChR subunits expressed in the mammalian central nervous system (α2-α7, α9, α10 and β2- β4) (Albuquerque et al., 2009). Neuronal nAChR subunits α7, α9 and α10 form low affinity homomeric receptors and with the exception of α7 are expressed in non-neuronal cells (Albuquerque et al., 2009). In contrast, neuronal nAChR subunits α2-α6 form high affinity heteromeric receptors containing 2 or 3 alpha subunits that co-assemble with 2 or 3 beta subunits (Albuquerque et al., 2009; Nashmi and Lester, 2006). Neuronal nAChR subunit β3 does not physically form a ligand binding site and therefore can only be the fifth modulatory subunit. Thus, functional high affinity nAChRs must have β2, β4 or both, and functional high affinity nAChRs can be divided into β2 and β4 containing nAChRs (Nashmi and Lester, 2006). The exact properties of nAChRs are determined by the combination of subunits that compose the receptor.
**CRF and CRFR1**

CRF and one of its two receptors, CRF receptor one (CRFR1), are part of the anxiety and stress system, and this system in the BNST induces relapse to drug seeking behavior (Dedic et al., 2018). Prior to CHAPTER II, the Tapper lab noted that CRFR1 had a similar expression pattern to IPI cFos in precipitated nicotine withdrawal (Zhao-Shea et al., 2015). This led to our investigation of this receptor as a substrate for nicotine withdrawal anxiety. CRFR1 is a B1 type GPCR. Its endogenous ligands in the CNS include CRF and Urocortin 1 (Vaughan et al., 1995). Similar to D1 dopamine receptors Go_s is typically bound to the CRFR1 and, when activated, it disassociates from the Gγ/Gβ subunit to stimulate adenyl cyclase leading to an increase in cellular cyclic AMP (Wietfeld et al., 2004). Cyclic AMP goes on to phosphorylate ion channels including Ca^{2+}, K+, Na+ and also NMDA, AMPA, and GABA receptors. The overall effect is excitation of the neuron.

**Conclusion**

Anxiety disorders are the most common class of mental disorders with insufficient treatments and a strong financial incentive for specialized therapies. Anxiety is a complex behavior with separable disorders. Thus, there should be many circuits which can modulate anxiety behaviors. One such circuit is found in the aversion coding MHb-IPN axis and forms a balance point with the reward coding VTA. A comprehensive understanding of the exact molecular mechanisms that shift the dynamics of the MHb-IPN may provide valuable insights into the neurobiology of
anxiety-like behavior and therapies with less side effects. In pursuit of this aim, CHAPTER II and III will describe data derived to test the hypothesis that a subset of presumably DAergic neurons in the VTA innervate the IPN and modulate anxiety. Specifically in CHAPTER II, this circuit modulates anxiety-like behavior via the release of CRF into the IPN during withdrawal in nicotine dependent mice (Zhao-Shea et al., 2015). To focus on the broader issue of anxiety, it seemed possible to expand the nicotine withdrawal anxiety circuit to other drugs of abuse. However, cocaine withdrawal did not produce the characteristic cFos pattern. Also, in drug naïve conditions DA should be the transmitter instead of CRF. This set the stage for examining the VTA^{DA}→IPN mesointerpeduncular circuit. Thus, CHAPTER III tests the hypothesis that the VTA also releases DA into the IPN to modulate anxiety behavior.
Figure I.1. Anxiety circuitry of the extended amygdala in rodent CNS.

Arrows represent the terminal side of a projection into a region. Blunt ends indicate a monodirectional projection arises from that region. Lines with arrows on either end are reciprocal connections between regions. Red lines highlight the mesolimbic and mesocortical pathways. The dorsal raphe (DR) is thought to be the major source of serotonin in the anxiety circuitry (Marcinkiewcz et al., 2016). The locus coeruleus (LC) is the major source of norepinephrine in the CNS (Segal et al., 1973).

Abbreviations Used: BLA basolateral amygdala, BNST bed nucleus of the stria terminalis, CeA central amygdala, DR dorsal raphe, HPC hippocampus, LC locus
coeruleus, LH lateral hypothalamus, mPFC medial prefrontal cortex, NAc nucleus accumbens, PAG periaqueductal gray/central gray, VTA ventral tegmental area.
Figure I.2. Afferent and efferent connections of the MHb-IPN axis in rodent CNS. Arrows represent the terminal side of a projection into a region. Blunt ends indicate a monodirectional projection arises from that region. Lines with arrows on either end are reciprocal connections between regions. Blue lines indicate nonreciprocal connections to the MHb-IPN axis. Black lines represent reciprocal first-order connections with the MHb-IPN axis. The mHb-IPN axis is red.

Abbreviations Used: BAC bed nucleus of anterior commissure, DR dorsal raphe, DTg dorsal tegmental nucleus, HPC hippocampus, IPN interpeduncular nucleus, LC locus coeruleus, LDTg laterodorsal tegmental nucleus, LH lateral hypothalamus, IHb lateral habenula, LS lateral septum, MD medial dorsal nucleus of the thalamus, MHb medial habenula, mPFC medial prefrontal cortex (IPN may receive input from infralimbic cortex), MnR medial raphe, MS medial septum, NAc
nucleus accumbens, NDB Nucleus of the diagonal band, NI Nucleus incertus, PAG periaqueductal gray/central gray, PBG parabigeminal nucleus, SFi septofimbrial nucleus, SI substantia innominata, SUM supramammillary nucleus, TS triangular septal nucleus, VTA ventral tegmental area, VTg ventral tegmental nuclei of Gudden.
Figure I.3. Hypothetical circuitry combining the MHb-IPN axis and the anxiety circuitry of the extended amygdala in rodent CNS. Arrows represent the terminal side of a projection into a region. Blunt ends indicate a monodirectional projection arises from that region. Lines with arrows on either end are reciprocal connections between regions. Blue lines indicate nonreciprocal connections to the MHb-IPN axis. Black lines represent reciprocal first-order connections with the MHb-IPN axis. Gray lines and regions indicate anxiety/fear circuitry without first-order connection to the MHb-IPN axis. The red box highlights the hypothesized mesointerpeduncular connection.

Abbreviations Used: BAC bed nucleus of anterior commissure, BLA basolateral amygdala, BNST bed nucleus of the stria terminalis, CeA central amygdala, DR dorsal raphe, DTg dorsal tegmental nucleus, HPC hippocampus, IPN interpeduncular nucleus, LC locus coeruleus, LDTg laterodorsal tegmental
nucleus, LH lateral hypothalamus, LHb lateral habenula, LS lateral septum, MD medial dorsal nucleus of the thalamus, MHB medial habenula, mPFC medial prefrontal cortex, MnR medial raphe, MS medial septum, NAc nucleus accumbens, NDB Nucleus of the diagonal band, NI Nucleus incertus, PAG periaqueductal gray/central gray, PBG parabigeminal nucleus, SFi septofimbrial nucleus, SI substantia innominata, SUM supramammillary nucleus, TS triangular septal nucleus, VTA ventral tegmental area, VTg ventral tegmental nuclei of Gudden.
CHAPTER II

Increased CRF signaling in a ventral tegmental area-interpeduncular nucleus-medial habenula circuit induces anxiety during nicotine withdrawal.

Contributions:

Rubing Zhao-Shea performed the behavioral tests, rtPCR from single cell on samples isolated by Steven R. DeGroot, and measured blood serum cotinine levels. Andrew R Tapper edited some of the figure legends. Figures, analysis, and writing by Steven R DeGroot.

Figures 4 and 5, their legends and the methods were previously published in Zhao Shea, R, DeGroot SR <-- Needs a more complete reference.
Summary

Nicotine withdrawal anxiety is a major contributing factor to the difficulty in achieving smoking cessation. Yet, our understanding of the neuroanatomical and molecular basis for nicotine withdrawal anxiety is severely limited. Recently, studies found a role for the MHb-IPN axis in physical withdrawal symptoms. By investigating the excitability and identity of neurons in the interpeduncular intermediate (IPI), a subregion of the IPN that expresses cFos during mecamylamine precipitated withdrawal, a precise neuroadaptation that is necessary for the expression of nicotine withdrawal induced anxiety in mice was discovered. Specifically, after exposure to chronic nicotine, a subset of IPI neurons designated Type 2 begin to express functional CRFR1, the activation of which boosted glutamatergic input in these neurons but not others (Type 1). Ex vivo, the baseline firing rate of these neurons increase—switching from silent to bursting suggesting a sustained increase in neuronal excitability. Additionally, in vivo pharmacological blockade of CRFR1 by CRFR1 antagonist, antalarmin, infusion into the IPN was sufficient to reduce withdrawal-induced anxiety whereas infusion of CRF increased anxiety. These data suggest that IPI specific CRFR1 activation triggers anxiety during nicotine withdrawal.
Introduction

“...tobacco is the only legally available consumer product which kills people when it is used entirely as intended.” —The Oxford Medical Companion (1994)

Tobacco is native to the Andes around Peru/Ecuador and is believed to have been cultivated for over 5000 years (Musk and de Klerk, 2003). It was popularized in developed countries during World War I and is still the leading cause of preventable death according to the Centers for Disease Control and Prevention (CDC).

Specifically, it is estimated that seven million people around the world die from the use of tobacco related products each year (World Health Organization, 2018). In the United States, 480,000 people die prematurely from tobacco related products each year despite ample warning labels and publicity highlighting the health concerns of this habit (Jamal et al., 2018). The warning may be at least partly effective because approximately two thirds of cigarette smokers are interested in quitting (Babb et al., 2017). Quitting tobacco products has a low success rate in part because of the affective symptoms of withdrawal including nicotine withdrawal induced anxiety, nicotine being the main addictive ingredient of tobacco. (Kenny and Markou, 2001). In fact, withdrawal symptoms, especially anxiety, are a better predictor of failed smoking cessation than volume of smoke intake or dependence
Therefore, understanding and treating withdrawal symptoms is likely to produce a better rate of smoking cessation.

Withdrawal symptoms themselves can be divided into three categories of withdrawal: somatic or physical i.e. shaking, sweating, tingling sensations and increased appetite (Zhao-Shea et al., 2013), cognitive i.e. deficits in neurocognitive function including sustained attention, working memory, and response inhibition (Ashare et al., 2014), and affective i.e. irritability, anxiety and anhedonia (Zhao-Shea et al., 2015). The affective symptoms are the most salient and thought to be the most critical for facilitating relapse (De Biasi and Salas, 2008; Kenny and Markou, 2001; Koob and Le Moal, 2008; Paolini and De Biasi, 2011). However, the neuroanatomical correlates of nicotine withdrawal anxiety are largely unknown.

Most studies about nicotine addiction focus on changes in DA levels in the mesolimbic DA “reward” pathway. However infusions of nAChR antagonist mecamylamine into the VTA, cortex or hippocampus fail to evoke withdrawal symptoms while MHb-IPN infusion does (Salas et al., 2009). This suggests the neural correlates of nicotine withdrawal can be found in the MHb-IPN. Yet, the possibility that the critical withdrawal symptom “anxiety” is specific to the MHb-IPN axis remains untested.

Furthermore, the Hb-IPN axis is known to be critical for regulating nicotine intake through the aversive effects of high nicotine doses (Fowler et al., 2011; Frahm et
al., 2011). It would therefore be unsurprising if the location was involved in other aversive effects of nicotine like those found in withdrawal.

Because withdrawal symptoms appear due to the absence of nicotine acting on nAChRs, general nACHR antagonists like mecamylamine can cause rapid precipitation of nicotine withdrawal (Jackson et al., 2008). Infusion of antagonists for nAChRs into the VTA after chronic nicotine treatment evokes affective withdrawal signs such as increased reward threshold (anhedonia), a symptom associated with depression (Bruijnzeel and Markou, 2004). However, they fail to evoke anxiety suggesting that the neuroadaptations critical for nicotine withdrawal induced anxiety must be in other regions.

Our preliminary work showed that mecamylamine infusion into the MHb and IPN, but not the neighboring VTA or the dorsal striatum, are capable of inducing nicotine withdrawal anxiety in mice treated with chronic nicotine (Zhao-Shea et al., 2015). Additionally, infusion of nicotine into the IPN is sufficient to attenuate spontaneous nicotine withdrawal induced anxiety (Zhao-Shea et al., 2015). Together this suggests the MHb-IPN axis is a part of the withdrawal anxiety circuit.

To determine which part of the MHb-IPN axis is involved in signaling nicotine withdrawal anxiety, the expression of the immediate early gene cFos, a marker for increased neuronal activity, was examined in mouse brain slices from mice experiencing mecamylamine precipitated withdrawal. Distinct cFos expression
appeared as two bands in the IPN marking the interpeduncular intermediate (IPI) subnucleus (Zhao-Shea et al., 2015).

To examine how the neurons of the IPI were distinct from other IPN neurons I searched the Allen Brain Atlas for IPI specific genes and found several genes with a similar expression pattern, most notably the anxiety-associated-receptor CRFR1 (Table II.2). The CRFR1 receptor is notable because knockout mice have decreased levels of anxiety and is known to modulate drug self-administration after withdrawal (George et al., 2007; Timpl et al., 1998). Additionally, exposure to chronic nicotine leads to increased production of CRF in the DAergic neurons of the VTA and upregulation of CRFR1 mRNA in the IPI (Grieder et al., 2014; Zhao-Shea et al., 2015). These data suggest the hypothesis that neuroadaptations in the VTA and IPN could lead to anxiety signaling through the release of CRF from the VTA to CRFR1 in the IPI subnucleus of the IPN. Thus, the IPN could be the substrate of nicotine withdrawal induced anxiety.

Results

Whole cell patch clamp recordings of IPI neurons revealed a mix of heterologous physiological properties. Thus, IPI neurons were separated into two main types Type 1 and Type 2. These neurons were distinguishable by biophysical properties including firing pattern, resting membrane potential ($-62.5 \pm 0.59$ mV, $n = 68$ and $-76.1 \pm 0.97$ mV, $n = 22$, respectively; $t_{88} = 11.44$, $p<0.0001$, two-tailed t-test), membrane capacitance ($29.9 \pm 1.6$ pF, $n = 64$ and $14.8 \pm 1.4$ pF, $n = 21$,
respectively; $t_{83} = 5.17$, $p<0.0001$, two-tailed t-test), voltage-sag upon hyperpolarization, and hyperpolarization-activated currents (Figure II.1A-D). The lower membrane capacitance suggests a smaller cell surface and this correlates with the observation of the experimenter that Type 2 neurons are, in general, smaller than Type 1 neurons. However, morphological differences between the two cell types were not directly quantified. Additionally, single cell rt-PCR revealed that all cytoplasm obtained from Type 2 neurons contained mRNA for CRFR1 compared with 31.8% for Type 1 neurons (Figure II.1E-F).

To determine if the mRNA was transcribed into functional CRFR1 I used whole cell current clamp to measure any current changes. Bath application of CRF did not result in any changes in whole cell current of Type 1 neurons, but in Type 2 neurons spEPSCs frequency and amplitude reversibly increased (Figure II.2A-F, Table II.1).

To test the hypothesis that CRF is released in the IPN after exposure to chronic nicotine, mice were exposed to 3-5 weeks of 200 µg ml$^{-1}$ nicotine tartrate sweetened with 0.3% saccharin to increase palatability. This dose induced dependence in previous studies (Léna et al., 1993; Zoli et al., 1998). Control mice received and equal dose of tartrate without the nicotine and saccharin. Cotinine, the predominant metabolite of nicotine, was measured as a biomarker for nicotine exposure after 4 weeks of nicotine in the drinking water. Nicotine drinking mice had an average blood serum cotinine level of $941.8 \pm 126.1$ ng ml$^{-1}$; whereas control mice had an average of $1.54 \pm 0.1$ ng ml$^{-1}$. For reference, this is greater
than the approximate dose for a “heavy smoker” after 6 days, $300 \pm 148 \text{ ng ml}^{-1}$ (Lawson et al., 1998). Slices were taken from the chronic nicotine exposed mice and CRFR1 antagonist antalarmin was applied in whole cell patch clamp recordings. Antalarmin had no effect on Type 1 spEPSCs. However, in Type 2 cells, spEPSCs reversibly decreased in frequency and amplitude (Figure II.2g-l, Table II.1). This suggests that CRF is released and activates Type 2 CRFR1 after exposure to chronic nicotine.

Since CRFR1 appeared to boost glutamatergic signaling in Type 2 neurons, I tested if the increased glutamatergic signaling translated directly to increased baseline spAPs in Type 2 neurons. In Type 1 neurons, there was no difference in spAP frequency (Figure II.3A, C). However, Type 2 neurons significantly increased spAP frequency, and the firing pattern typically consisted of pauses and bursts (Figure II.3B, D).

These results were incorporated into our model (Figure II.4).
Figure II.1. Characterization of IPI neurons. IPI neurons can be divided into two types. Representative family of voltage traces from A. IPI Type 1 and B. IPI Type
2 neurons under current clamp. From resting membrane potential, current was injected in 20 pA steps for 1.5 s as indicated. **C.** Current-voltage relationship of Type 1 and Type 2 IPI neurons at hyperpolarized potentials between -70 and -120 mV (n = 10-13 neurons/Type). **D.** Current-voltage relationship of Type 1 and Type 2 IPI neurons from potentials between -60 and 120 mV (n = 10-13 neurons/type). **E.** Representative gel illustrating CRF1 receptor expression from a single Type 1 and Type 2 neuron measured via RT-PCR after patch-clamp recording. **F.** Bar graphs indicate percent of patch-clamped Type 1 or Type 2 neurons that expressed CRF1 receptor mRNA as measured by single cell RT-PCR. Data are expressed as mean ± SEM.
Figure II.2. CRF modulates glutamatergic activity in Type 1 but not Type 2 IPI neurons. Representative whole-cell voltage clamp recordings from Type 1 (a.) and Type 2 (d.) neurons within the IPI in response to CRF. Average sEPSC frequency and amplitude at baseline, after a 5-minute application of CRF, and after washout from Type 1 (b., c., respectively, n = 8 neurons) and Type 2 (e., f., respectively: One-way ANOVAs with repeated measures: Frequency, F2, 12 = 19.2, p=0.0002; Amplitude, F2, 12 = 10.49, p=0.0023, n = 7) IPI neurons. Representative whole-cell voltage clamp recordings from Type 1 (g.) and Type 2 (j.) nicotine-withdrawn IPI neurons in response to antalarmin. Average sEPSC frequency and amplitude at baseline, after a 5-minute application of antalarmin, and after washout from Type 1 (h., i., respectively, n = 8) and Type 2 (k., l., respectively: Frequency, F2, 14 =
17.22, $p=0.0002$; Amplitude, $F_{2, 14} = 13.66, p=0.0005, n = 8$) IPI neurons. Neurons were recorded in slices from nicotine-dependent mice. Scale bar: $y = 20$ pA, $x = 5$ s. Data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$. 
Figure II.3. Type 2, but not Type 1, neurons exhibit increased activation during nicotine withdrawal. Ex vivo cell attached recordings of IPI neurons in nicotine naive control (ctrl) mice and chronic nicotine mice experiencing withdrawal. A. Representative cell attached voltage-clamp traces from Type 1 neuron in ctrl and nicotine withdrawal. B. Representative cell attached voltage-clamp traces from Type 2 neuron in ctrl and nicotine withdrawal. C. Summary graph of cell attached recorded spAP frequencies of Type 1 neurons. Error bars
represent mean ± SEM. “n” represents the number of neurons recorded. D. Summary graph of cell attached recorded spAP frequencies of Type 2 neurons. Error bars represent mean ± SEM. “n” represents the number of neurons recorded.
Figure II.4. Model of neuroadaptations in response to nicotine in the mesointerpeduncular circuit. Chronic nicotine exposure acts on nAChRs to increase the production of CRF in the VTA neuron (purple) and CRFR1 in the Type 2 IPI neuron (gray). Then during withdrawal (red arrows), CRF is released by the VTA neuron and activates the CRFR1 on the Type 2 IPI neuron, which modulates NMADRs to increase EPSC frequency and amplitude. EPSCs come from the glutamate (glu) of the MHb neuron (blue).
Table II.1. Raw EPSC frequency data from IPI CRF pharmacology (related to Figure I.3). The treatment column defines what drug the neurons are responding to in the columns titled “response.” Slices exposed to antalarmin were from mice pretreated with chronic nicotine in the drinking water.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean sEPSC Frequency ± Standard Deviation (Hz)</td>
<td>Mean sEPSC Amplitude ± Standard Deviation (pA)</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Response</td>
</tr>
<tr>
<td>CRF</td>
<td>4.43 ± 2.58</td>
<td>4.40 ± 2.58</td>
</tr>
<tr>
<td>Antalarmin</td>
<td>2.58 ± 1.70</td>
<td>2.29 ± 1.63</td>
</tr>
</tbody>
</table>

| CRF             | 10.76 ± 4.88 | 11.47 ± 5.05 | 11.27 ± 5.13 | 10.91 ± 5.19 | 14.33 ± 5.58 | 10.86 ± 5.37 |
| Antalarmin      | 12.03 ± 3.72 | 12.13 ± 5.20 | 12.23 ± 5.15 | 12.58 ± 4.43 | 9.30 ± 2.84  | 12.57 ± 5.36 |
Table II.2. IPI specific genes found by searching Alan Brain Atlas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alias (function)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accn1</td>
<td>Amiloride-sensitive cation channel 1</td>
</tr>
<tr>
<td>CntfR</td>
<td>Ciliary neurotrophic factor receptor</td>
</tr>
<tr>
<td><strong>CRHR1</strong></td>
<td><strong>Corticotrophin Releasing Factor Receptor 1</strong></td>
</tr>
<tr>
<td>Cxcl14</td>
<td>C-X-C motif ligand 14 (Chemokine)</td>
</tr>
<tr>
<td>Cyp46a1</td>
<td>A Cytochrome oxidase (drug metabolism)</td>
</tr>
<tr>
<td>Dgkb</td>
<td>Diacylglycerol kinase beta</td>
</tr>
<tr>
<td>Dlgap2</td>
<td>DLG associated protein 2 (Membrane-associated guanylate kinase)</td>
</tr>
<tr>
<td>Epha4</td>
<td>Ephrin type-A receptor 4</td>
</tr>
<tr>
<td>Erbb4</td>
<td>Erb-B2 Receptor Tyrosine Kinase 4</td>
</tr>
<tr>
<td>Esrra</td>
<td>Estrogen related receptor alpha</td>
</tr>
<tr>
<td>Exose8</td>
<td>Exome component 8</td>
</tr>
<tr>
<td>Grin3a</td>
<td>NMDA receptor subunit 3A</td>
</tr>
<tr>
<td>Ntng1</td>
<td>NetrinG1 (Axon guidance protein)</td>
</tr>
<tr>
<td>Plekha5</td>
<td>Phosphoinositol 3 phosphate binding protein</td>
</tr>
<tr>
<td>Rab3b</td>
<td>Ras-related GTPase (vesicular budding and fusion)</td>
</tr>
<tr>
<td>Slc24a3</td>
<td>Potassium-dependent sodium/calcium exchanger</td>
</tr>
<tr>
<td>Sorcs3</td>
<td>Sortilin Related VPS10 Domain Containing Receptor 3 (Neurotrophin binding membrane protein)</td>
</tr>
<tr>
<td>Spon1</td>
<td>Spondin 1 (Extracellular matrix protein involved in axon guidance)</td>
</tr>
<tr>
<td>Tcf7l2 (Tcf4)</td>
<td>Transcription Factor 7 Like 2 (T cell specific Transcription Factor)</td>
</tr>
<tr>
<td>Zfp804a</td>
<td>Zink-finger protein 804a (transcription factor, associated with schizophrenia)</td>
</tr>
</tbody>
</table>
Discussion

To test the hypothesis that neuroadaptations increase CRF signaling in the IPN to produce anxiety-like behaviors, I examined the neurons of the IPI that appear to be activated during nicotine withdrawal. I found differences in biophysical properties that allow us to distinguish between CRFR1 expressing (Type 2) and non-expressing (Type 1) IPI neurons. CRFR1 was functionally expressed in Type 2 neurons and application of CRF resulted in an increase in EPSC frequency and amplitude. The change in amplitude is consistent with the presence of postsynaptic CRFR1. Which others in the lab showed acts through NMDA receptors (Zhao-Shea et al., 2015).

However, the change in spEPSC frequency suggests the action of presynaptic CRFR1 as well. While it is possible that the frequency change could be attributed to sub-detection threshold spEPSCs being magnified to detectable levels, this possibility is limited because of the low threshold for detection, specifically 5 pA. This leaves a few other possibilities. For example, presynaptic CRFR1 could be expressed on terminals from an unknown source that specifically targets Type 2 neurons. Alternatively, there could be retrograde signaling from a receptor like the cannabinoid receptors. Finally, the Type 2 neuron could form a local circuit that feeds back upon its inputs. Repeating the experiments in the presence of tetrodotoxin (TTX) could shed light on these possibilities because it would eliminate multi-synaptic transmission and volumetric changes in neurotransmitter released due to presynaptic action potentials. All things considered; the
electrophysiological data coupled with mRNA expression forms a convincing argument for functional CRFR1 expression by Type 2 neurons in the IPI.

Because CRFR1 antagonist, antalarmin, affects Type 2 spEPSCs in the acute slice preparation and the basal activity of Type 2 neurons from chronic nicotine treated mice is increased compared to neurons from nicotine naïve mice, CRF appears to be freely released during withdrawal conditions in slice. In vivo, I expect elevated CRF release during withdrawal without the masking anxiolytic effects of nicotine signaling. Thus, the upregulation of this CRF system appears to be a neuroadaptation driving nicotine withdrawal anxiety. This VTA-IPN CRF system could be specifically targeted in a future therapy for nicotine addiction or anxiety disorders.

It would be particularly interesting to determine what regions Type 2 neurons innervate and what neurotransmitters they release. The data did not detect a significant change in Type 1 neurons due to chronic nicotine or the application of CRF agonists and antagonists. These data suggest Type 2 neurons may be projection neurons rather than interneurons innervating Type 1 neurons. However, direct experiments are necessary to confirm this hypothesis. A strong possibility remains that Type 2 neurons do act as interneurons and the experiments performed so far are unable to detect this. For example, if the neurotransmitter released from Type 2 neurons does not directly modulate ion flow on a short timescale our experiments would not detect the Type 2 to Type 1 transmission. Additionally, Type 2 interneuron signal may require prior activation by a separate
circuit, or the ion channels modified may require a specific membrane potential not found in our experiments. This possibility could be tested by CRF application at different holding potentials. The innervation of Type 2 neurons could be tested by fluorescent labeling and tracing. Unfortunately, to the best of our knowledge no one has made a CRFR1-cre mouse line or another line capable of IPI specific expression (Table II.2). Such a mouse line would significantly ease the study of these neurons.

In chronic nicotine exposed mice but not nicotine naïve mice, CRF infusion in the IPN precipitated anxiety-like behaviors without effecting locomotor activity in the elevated plus maze (EPM) and marble burying test (MBT) (Zhao-Shea et al., 2015). This suggests activation of the interpeduncular CRF system is sufficient to produce nicotine withdrawal induced anxiety. However, the source of the CRF was unknown. Serendipitously, the Grieder lab found CRF upregulation in the VTA was necessary for nicotine withdrawal induced anxiety (Grieder et al., 2014), suggesting the source of CRF to the IPI was the VTA. This hypothesis was assessed by knockdown of CRF expression in the VTA via shRNA. This both decreased mecamylamine precipitated withdrawal and decreased IPI cFos (Zhao-Shea et al., 2015), suggesting that a mesointerpeduncular circuit was a source of interpeduncular CRF.

The lack of a behavior after CRF infusion in nicotine naïve animals was addressed by examination of the ligand and receptor mRNA expression levels. Quantification of CRFR1 and CRF mRNA by real time, quantitative reverse transcription
polymerase chain reaction (rtPCR) revealed the receptor and ligand’s mRNA is highly upregulated after nicotine exposure (Zhao-Shea et al., 2015). Thus a few possibilities for the discrepancy are apparent. The expression level of CRFR1 may be inadequate to significantly affect the IPI circuit in nicotine naïve mice. There could also be a requirement for neuroplastic adaptations through an LTP-like mechanism— strengthening the circuit’s association with anxiety.

Since enhanced EPSC amplitude appears to be the mechanism of Type 2 neuroadaptation, NMDA receptor antagonists were pre-infused into the IPN prior to Mec precipitated withdrawal. This resulted in a decrease in anxiety-like behaviors in the EPM and MBT as well as a reduced IPI cFos expression (Zhao-Shea et al., 2015). NMDA receptors are famous as the critical receptors involved in LTP (Morris, 2013). Future studies could test if the NMDA receptors in the IPI produce LTP and if these changes encourage drug seeking behaviors. LTP in the IPI may also have implications for memory as the IPN connects with the hippocampus or familiarity as familiarity increases cFos expression in the IPN (Molas et al., 2017b; Shibata and Suzuki, 1984). It should be noted that, in the hippocampus, blockade of the NMDA receptors responsible for LTP do not result in significant immediate changes in baseline EPSCs (Collingridge et al., 1983). The difference between the IPN and the hippocampus may be due to alternative NMDA receptor subunits, related cytoplasmic machinery, or the structure of the synapse granting specificity to a different neural mechanism useful for whatever process this circuit normally fulfills.
The source of the EPSCs was also investigated by enhanced halorhodopsin eNpHR inhibition of the cholinergic/glutamatergic neurons of the MHb during precipitated withdrawal. The MHb cholinergic/glutamatergic neurons primarily release glutamate and only tetanic high frequency optogenetic stimulation produced acetylcholine release (Ren et al., 2011). This also resulted in reduced anxiety-like behaviors and IPI cFos expression in chronic nicotine treated mice but not controls (Zhao-Shea et al., 2015).

Previous reports suggest that CRF acting through CRFR1 in the CeA is responsible for some nicotine withdrawal anxiety-like behaviors, specifically defensive marble burring (George et al., 2007). The current study proposes another component of the same system that may generate similar or different anxiety-like behaviors. Indeed, although there is not a direct connection with the amygdala (Molas et al., 2017a), the IPN was functionally coupled with amygdala activity in an unpublished resting state functional connectivity study by a collaborator (Zhang et al. Unpublished data).

Taken together, during nicotine exposure, CRF is produced in the VTA and CRFR1 is upregulated in Type 2 cells of the IPI. Upon withdrawal the VTA$^{\text{CRF}}\rightarrow$IPI circuit is active exciting Type 2 neurons through CRFR1 mediated increases in the MHb$^{\text{Glu}}\rightarrow$IPI Type 2 circuit signal (Figure II.4). This results in Type 2 neurons increasing anxiety-like behaviors. This is the first report of the IPN’s role in nicotine withdrawal anxiety-like behaviors and the first characterization of the CRFR1 system in the IPN. Future work on the IPI Type 2 neurons and their downstream
targets will likely be critical in developing better treatments that promote smoking cessation. Furthermore, this work reveals the possibility that the IPN could influence anxiety in other behaviors.

**Why switch away from withdrawal and Type 2 neurons in CHATPER III:**

Exploring the greater topic of anxiety became the goal. Perturbations by drugs of abuse are useful for resolving novel circuitry that modulates anxiety. After determining that the CRF system is active only after nicotine induced neuroadaptation and cocaine withdrawal fails to create the same IPI pattern of activation, testing the role of DA in non-withdrawal conditions was a logical direction for the research to turn. In most conditions the VTA will not release CRF. If the mesointerpeduncular circuit has a function outside of nicotine withdrawal, then it proves the characterization of the circuit is that much more valuable. The idea of DA modulating anxiety has been around for ages and there is a lot unknown and very few cases where there is a clear effect. Additionally, the MHb-IPN is thought to be involved with negative motivation i.e. aversion while the VTA is more associated with positive motivation i.e. reward (Kobayashi et al., 2013; Schultz et al., 1997; ZhaoShea et al., 2015). A circuit between these two regions in normal conditions could be an important anxiety balancing point.

Additionally, there are several technical difficulties involved in studying Type 2 IPI neurons. The IPI is a small subregion of a small nucleus. It is difficult to patch in the IPI consistently. Furthermore, the difficulty of patching the Type 2 neurons is
increased by their smaller cell bodies as indicated by a comparison of their membrane capacitance with Type 1 neurons. Also, Type 2 neurons do not fire many spAPs unless it is from a mouse that has been drinking nicotine for three weeks or more. The EPSC signal normally has a small amplitude and frequency as well. These properties make it more difficult to record meaningful experimental data. The requirement on exposure to nicotine makes the mice older which makes patching the neuron harder. All these factors combine to make data collection slow. With a CRFR1-Cre mouse line experiments might be easier, but I would still change my direction to investigate baseline anxiety. Together, these are my reasons for switching the research focus from withdrawal focused anxiety to drug naive focused anxiety.
Methods

Animals

Male C57BL/6J mice were purchased from Jackson Labs, bred in the UMMS animal facility, and used in biophysical experiments as indicated. Animals (mice) were housed four per cage up until the start of each experiment. Animals were kept on a standard 12-h light/dark cycle with lights on at 7:00 A.M. and off at 7:00 P.M. Mice were weaned at 21 days and were 4-6 weeks old at the start of chronic nicotine treatments. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council, as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs and drinking solution

Nicotine and control drinking solutions were prepared from nicotine hydrogen tartrate or L-tartaric acid (Sigma-Aldrich), which were dissolved in tap water with the concentrations of 200 µg ml\(^{-1}\) and 300 µg ml\(^{-1}\), respectively. Saccharin sodium (Fisher Scientific) was added at a concentration of 3 mg ml\(^{-1}\) to each solution to sweeten the taste. Nicotine doses are reported as nicotine free base.
IPN tissue collection and quantitative RT-PCR

For collection of whole IPN tissue, brains were harvested and placed on a glass slide and frozen on dry ice. Coronal sections (approximately 1 mm) were cut and the IPN was punched out using a Harris UNI-CORE (1.0 mm, Electron Microscopy Sciences). The cored IPN sample was transferred to a 1.5-ml collection tube containing lysis buffer. Laser Capture Microdissection (LCM) was performed to collect from the vIPN. The IPN serial sections were prepared as described previously (Zhao-Shea et al., 2011). Sections were fixed in ice-cold acetone followed by dehydration in a graded ethanol series. The Veritas™ Microdissection System Model 704 (Arcturus Bioscience, Inc.) was used for LCM. All ventral IPN areas were cut from each animal (from 50 slices/brain). Four mice were used per treatment. Neurons were captured on CapSure® Macro LCM caps (Arcturus Bioscience, Inc.) and stored in 100 µl of lysis solution (Ambion). Total RNA was extracted from individual samples using a Micro Scale RNA Isolation Kit (Ambion). RNA samples extracted from the IPN were reverse transcribed into cDNA using RT enzyme mix (20X) and RT buffer (2X) (Ambion). PCRs were set up in 10 µl-reaction volumes using TaqMan Gene Expression Assays (Applied Biosystems). Glyceraldehyde 3-phosphated dehydrogenase (GAPDH) was used as an internal control gene to normalize gene expression levels. PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Negative controls with no reverse transcriptase were performed for all TaqMan Assays. All reactions were performed in triplicate. Relative amplicon quantification was
calculated as the difference between $C_t$ values of GAPDH and that of the gene of interest. Relative gene expression differences in IPN between nicotine-dependent mice and nicotine non-dependent mice were calculated using the $2^{-\Delta\Delta C_t}$ method.

**Electrophysiology**

Brains were quickly removed and placed in oxygenated ice-cold high sucrose artificial cerebrospinal fluid (SACSF) containing kynurenic acid (1 mM, Sigma, St. Louis, MO). SACSF solution contained (in mM): 250 sucrose, 2.5 KCl, 1.2 NaH$_2$PO$_4$$\cdot$H$_2$O, 1.2 MgCl$_2$$\cdot$6H$_2$O, 2.4 CaCl$_2$$\cdot$2H$_2$O, 26 NaHCO$_3$, 11 D-Glucose. Coronal brain slices containing IPN brain regions (~180 µm) were made using a Leica VT1200 vibratome. Spontaneous EPSCs were obtained in the whole-cell configuration and gap-free acquisition mode in Clampex (Axon Instruments) as previously described (Zhao-Shea et al., 2013). Neurons were held at a resting membrane potential of −70 mV. For optical recordings, eYFP-positive neurons were identified under fluorescence microscopy. Trains of 593 nm (halorhodopsin, NpHR) or 470 nm (channelrhodopsin, ChR2) light pulses were applied to neurons under current clamp or voltage clamp using LEDs of the appropriate wavelength (Thor Labs), as indicated. Whole-cell NMDA currents in IPI neurons were evoked (duration of 100–200 µs, amplitude of 20–30 mA) by positioning a concentric bipolar electrode (FHC; Bowdoin, ME) in an IPN region close to the IPI. ACSF was used for bath solution and contained (in mM): 125 NaCl, 2.5 KCl, 1.2 NaH$_2$PO$_4$$\cdot$H$_2$O, 1.2 MgCl$_2$$\cdot$6H$_2$O, 2.4 CaCl$_2$$\cdot$2H$_2$O, 26 NaHCO$_3$, 11 D-Glucose. All
recordings were filtered at 1 kHz using the amplifier’s four-pole, low-pass Bessel filter, digitized at 10 kHz with an Axon Digidata 1440A interface and stored on a personal computer. Spontaneous EPSCs were detected using Mini Analysis (Synaptosoft). Briefly, root mean square noise levels were measured to set an area threshold for rejecting noise events. The program was set to detect baseline from an average of 2 ms, 25–30 ms before the event peak and detect decay time based on the default 0.37 fraction of peak within 8 ms of the event.

**Multiplex and nested scRT-PCR**

At the end of each recording, the cytoplasm of individual neurons was aspirated into the recording pipette containing electrode solution made with DEPC-treated deionized water. RNA isolation, reverse transcription, and amplification of CRF1 receptor mRNA was done as previously described (Liu et al., 2012).

**Cotinine measurements**

The Cotinine Direct ELISA Kit (CalBiotech, Inc.) was used to measure cotinine in mouse blood serum according to the manufacturer’s instructions. Mice were exposed to nicotine or control solution at least 4 weeks prior to cotinine measurements. Blood for analysis was collected in the morning, 1 hour after lights-on.
Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests or two-tailed t-tests as indicated. One-way ANOVA with repeated measures was used to analyze effects of CRF drugs on sEPSC data. Two-tailed t-tests were used for analysis of chronic treatment on gene expression. Each data set was tested for normal distribution prior to analysis. Animal numbers were determined based on previous studies with similar endpoints (Salas et al., 2009; Zhao-Shea et al., 2013). Data were analyzed and presented using GraphPad software.

Acknowledgements

This work was supported by award numbers F32DA034414 (MV), R01DA035371 (PDG and ART), and R01DA033664 (PDG, ART, and OJR) from the National Institute on Drug Abuse. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
CHAPTER III

Midbrain dopamine controls anxiety-like behaviors by engaging unique interpeduncular nucleus microcircuitry

Contributions

Rubing Zhao-Shea performed and analyzed the behavioral experiments with assistance from Paul M Klenowski and Susanna Molas. Paul M Klenowski took the confocal images and led the image analysis. Fangmiao Sun and Yulong Li created the improved version of the genetically encoded GPCR activation-based-DA sensor GRAB\textsubscript{DA1m}. Andrew R Tapper edited and wrote portions of the text and figures. Steven R DeGroot wrote and edited the text, performed the electrophysiological experiments, biocytin filling and produced the figures and analysis. Andrew R Tapper and Paul D. Gardner provided equipment and procured the funding.
Midbrain dopamine controls anxiety-like behaviors by engaging unique interpeduncular nucleus microcircuitry

Authors
Steven R. DeGroot¹, ², Rubing Zhao-Shea¹, Paul M. Klenowski¹, Fangmiao Sun³,⁴, Susanna Molas¹, Paul D. Gardner¹, Yulong Li³,⁴,⁵, Andrew R. Tapper¹

¹Brudnick Neuropsychiatric Research Institute, Dept. of Neurobiology, University of Massachusetts Medical School, Worcester, MA, 01605, USA
²Graduate Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA, 01605, USA
³State Key Laboratory of Membrane Biology, Peking University School of Life Sciences, 100871 Beijing, China
⁴PKU-IDG/McGovern Institute for Brain Research, 100871 Beijing, China
⁵Peking-Tsinghua Center for Life Sciences, 100871 Beijing, China

Abstract
Dopamine (DA) is hypothesized to modulate anxiety-like behavior. However, the precise role of DA in anxiety behaviors and the complete anxiety network in the brain have yet to be elucidated. Here I show that a circuit, consisting of ventral
tegmental area (VTA) DAergic neurons that project to the interpeduncular nucleus (IPN), controls anxiety-like behavior. Expressing GRAB\textsubscript{DA2m}, an improved version of the genetically encoded GPCR activation-based-DA sensor GRAB\textsubscript{DA1m}, in mouse IPN revealed an increase in IPN DA release as mice navigated the anxiogenic open arm of the elevated plus maze. Preventing DA release in the IPN by optogenetically silencing VTA→IPN DAergic inputs induced anxiety like behavior; whereas, activating the circuit was anxiolytic. Two neuronal populations in the ventral IPN (vIPN) robustly responded to DA via presynaptic DA receptors. These vIPN neurons are controlled by a small population of D1 receptor-expressing neurons in the caudal IPN (cIPN) that directly respond to VTA DAergic terminal stimulation. These neurons innervate the vIPN and amplify the signal from the VTA. Directly modulating this microcircuit through the D1 receptor bidirectionally controlled anxiety. Together, these data identify a DAergic circuit that mediates anxiety through unique IPN microcircuitry.

Introduction

Anxiety is a complex, multi-circuit behavioral phenomenon characterized by a prolonged sense of unease and heightened arousal in the absence of a direct threat (Lieb, 2005). Persistent uncontrolled anxiety inappropriate to the level of threat underlies anxiety disorders, which are a serious mental health issue and often comorbid with depression and other psychiatric disorders (Chisholm et al., 2016). Understanding the neurocircuitry that regulates anxiety is necessary to
inform future anxiolytic therapy development. Basal and stress-induced anxiety states are governed by brain regions that process emotions including prefrontal cortex (PFC), hippocampus, and extended amygdala, a macrostructure that includes the various subdivisions of the amygdala, the substantia innominata, the nucleus accumbens shell, and the bed nucleus of the stria terminalis (Calhoon and Tye, 2015). Each of these regions is regulated by modulatory input from dopamine (DA)-rich midbrain areas that are hypothesized to shape anxiety-like behavior (Berry et al., 2019; Jennings et al., 2013; Refojo et al., 2011; Zweifel et al., 2011), although the exact role of DA and how it drives behavior in response to anxiogenic stimuli are unknown. Emerging data implicate an additional pathway that contributes to fear and anxiety signaling, the habenulo-interpeduncular axis (Jesuthasan, 2012; Okamoto and Aizawa, 2013; Soria-Gómez et al., 2015; Zhang et al., 2016). This pathway consists of neurons in the medial habenula (MHb) that project to the interpeduncular nucleus (IPN) (Molas et al., 2017a). While the MHb receives input from the septum, the IPN transmits forebrain input to the mid- and hindbrain resulting in the regulation of behavior (Lima et al., 2017; Shibata et al., 1986) (See Figure I.2. in CHAPTER I). The majority of studies on the MHb→IPN circuit have focused on nicotine addiction-associated behaviors, where this pathway has been implicated in regulating drug intake and aversive, affective, as well as physical aspects of nicotine withdrawal (Fowler and Kenny, 2014; Fowler et al., 2011; Wolfman et al., 2018; Zhao-Shea et al., 2013, 2015). The habenulo-interpeduncular pathway also contributes to regulating baseline anxiety state
(Pang et al., 2016; Yamaguchi et al., 2013), although the mechanism(s) involved, particularly in the IPN, are not clearly understood.

We recently described a mesointerpeduncular circuit in which VTA DAergic neurons project to the neighboring IPN (Molas et al., 2017b). While the DAergic neuron-rich VTA is largely associated with increased motivation towards novelty, reinforcement, and positive affective state, the IPN is a brain region governing reduced motivation towards familiarity, as well as aversion, and negative affective state (Adamantidis et al., 2011; Chaudhury et al., 2013; Gunaydin et al., 2014; Molas et al., 2017b; Tsai et al., 2009; Tye et al., 2013; Wolfman et al., 2018). Thus, general activity in these two regions promotes opposing behaviors suggesting the mesointerpeduncular circuit could act as an important balancing point governing motivation and anxiety. Indeed, previously the data indicated that stimulating this pathway with optogenetic tools could shift the motivational aspects of familiar stimuli interactions and enhance their salience as if they were novel (Molas et al., 2017b). Provided bellow is a comprehensive understanding of the mechanistic connection between the VTA and IPN and how endogenous DA released from this circuitry contributes to anxiety-associated behaviors.

**Results**

Endogenous DA is released in the IPN during exploration of anxiogenic environments.
To test if dynamic endogenous DA release in the IPN occurs in vivo and may be involved in anxiety-like behavior, enhanced genetically encoded GPCR-activated DA sensor (GRAB$_{DA2m}$, see Methods) that changes in fluorescence intensity upon DA binding, was expressed in the IPN of C57Bl/6J mice using AAV-mediated gene delivery (Sun et al., 2018). This construct is a more direct measurement of DA release than calcium imaging. It also eliminates the possibility of cre-line related errors in terminal expression and signal can be detected for at least 30 minutes. Fiber photometry probes were implanted in the IPN for the detection of DA-mediated signals in vivo during behavior on the elevated plus maze (EPM), a common assay to assess anxiety-like behavior in rodents (Figure III.1A) (Walf and Frye, 2007). Transient changes in fluorescence normalized to baseline ($\Delta F/F_0$) revealed an increase in IPN DA signal as mice entered and explored the open arm of the EPM compared to fluorescence signal in the closed arm (Figure III.1B). In addition, IPN DA signal activity, measured as Z-scores in time-locked events, was significantly higher when mice explored the open arm as compared to the closed arm (Figure III.1C). Moreover, analysis of peaks in the fluorescence signal (fluorescence events) during arm exploration revealed that the frequency of events and the peak event area were significantly higher in the open arms compared to the closed arms, with no differences in event amplitudes (Figure III.1D). GRAB$_{DA1m}$ which GRAB$_{DA2m}$ is based on has a rise time of $\leq$ 100 ms. In Figure III, the average time between events was 1967 ms and the shortest time between any two events was 177 ms. Additionally, the maximum $\Delta F/F_0$ detected was $\sim$4% which is far from
the maximal saturating ~270% possible for this sensor. Thus, the events detected are within the accepted detectable range. These data indicate that endogenous DA in the IPN increases as animals navigate the open arms of the EPM, a phenomenon which I hypothesize is critical for the reduction of anxiety-like behavior. This hypothesis is tested in the next section.

**DA VTA afferents in the IPN bidirectionally modulate anxiety-like behaviors.**

The previous experiments demonstrated that a subpopulation of DAergic neurons in the VTA project to the IPN constituting a mesointerpeduncular pathway (Molas et al., 2017b; Zhao-Shea et al., 2015). To test if VTA→IPN axon terminals are the source of DA release that contribute to open arm exploration, Cre-dependent halorhodopsin (NpHR)-eYFP was selectively expressed in the VTA of DA transporter (DAT)::Cre mice via AAV2-mediated gene delivery and a fiber optic cannula was implanted into the IPN to deliver yellow light (593nm, constant light, 20s on, 10s off, Figure III.1E) and photo-inhibit VTADA→IPN inputs during the EPM (Gradinaru et al., 2010). VTADA→IPN photo-inhibition resulted in a decrease in open arm time in the EPM compared to light-off controls (Figure III.1F). Optogenetic stimulation was chosen over designer receptors exclusively activated by designer drugs (DREADD) so VTADA→IPN terminals could be targeted specifically without disrupting the IPN with an injection and to allow greater temporal control. VTADA→IPN photo-inhibition had little effect on total arm entries compared to control conditions, suggesting normal locomotion in these animals.
VTAD\textsubscript{DA}→IPN photo-inhibition in the open field test (OFT) resulted in a trend of decreased center time and no effect of photoinhibition on activity (Figure III.2). To test the effect of activating VTAD\textsubscript{DA}→IPN on open arm exploration, Cre-dependent channelrhodopsin (ChR2)-eYFP was selectively expressed in the VTA of DAT::Cre mice via AAV2-mediated gene delivery (Figure III.1G) (Tsai et al., 2009). A fiber optic cannula was implanted targeting the IPN for blue-light stimulation of VTAD\textsubscript{DA}→IPN inputs (473nm, 15 Hz, 20 ms/pulse, 5 s light on, 5 s light off) during behavioral testing in the EPM. Photostimulation of VTAD\textsubscript{DA}→IPN inputs significantly increased time spent in the open arms of the EPM compared to control mice receiving no light stimulation (Light-off), while having little effect on total arm entries compared to control conditions (Figure III.1H). In the OFT, photostimulation of the VTAD\textsubscript{DA}→IPN pathway increased time spent in the center compared to controls without significantly effecting total distance traveled (Figure III.2B). Together, these data indicate that the VTAD\textsubscript{DA}→IPN pathway is a critical component of anxiety circuitry that, when engaged, drives reduced anxiety-like behavior.

Two neuronal populations, Type A and Type B, in the ventral IPN differentially respond to DA via D1-like, but not D2-like, DA receptors.

If the IPN receives DAergic input from the VTA, then IPN neurons likely respond to DA through DA receptor signaling. To determine DA responses in IPN neurons, I used electrophysiology in acute coronal slices of C57Bl/6J mice. In cell-attached
mode, I measured spontaneous action potentials (spAPs) during a five-minute bath application of exogenous 10 μM DA (Figure III.3). Interestingly, none of the neurons located in the dorso-rostral part of the IPN (IPR) responded to DA (data not shown). However, in the ventral IPN (vIPN), 18 out of 39 neurons responded to DA with an increase in spontaneous action potential (spAP) frequency (designated as “Type A” neurons, Figure III.3A), while 17 out of 39 neurons responded to DA with a decrease in spAP frequency (designated as “Type B” neurons, Figure III.3B). The remaining 4 neurons exhibited no obvious responses (Figure III.3C). The effect of DA on both neuron subtypes was almost completely reversible upon wash out. To examine the physiological properties and current-voltage relationship of these two types of vIPN neurons, I injected 100 pA to -100 pA current in -20 pA steps. Type A and Type B neurons exhibited clear significant differences in their response to current injection and input resistance (Figure III.3D-G), with Type A neurons having a lower input resistance compared to Type B neurons. These results indicate that two physiologically distinct neuronal subpopulations specifically located in the vIPN have opposite responses to DA. To test which DA receptors are required for DA-induced changes in spAP frequency in the vIPN, DA was applied to Type A and Type B neurons in the absence and presence of the D1-like receptor antagonist, SCH39166 (10 μM) or the D2-like receptor antagonist, eticlopride (10 μM, Figure III.4A-C). SCH39166, but not eticlopride, significantly attenuated DA-mediated spAP frequency changes both in Type A and Type B neurons, suggesting that DA acts through D1-like but not D2-
like DA receptors in the IPN. In addition, to further rule out D2 effects, I applied a D2-like DA receptor agonist, quinpirole (1 μM) to vIPN neurons and did not observe any changes in spAP frequency, spontaneous excitatory post-synaptic current (spEPSC) frequency or amplitude (Figure III.5).

**DA modulates vIPN neurons via presynaptic DA receptors.**

To assess how D1-like DA receptors modulate vIPN neuron activity, I recorded from Type A and B neurons under voltage-clamp and measured changes in excitatory input. DA was bath applied and neurons were voltage-clamped at -70 mV to record spEPSCs. Of note, DA failed to induce obvious inward or outward post-synaptic currents under voltage-clamp (data not shown). However, DA increased spEPSC frequency in Type A neurons while decreasing spEPSCs frequency in Type B neurons, with no effect on the spEPSC amplitude in either neuron type, suggesting DA affects excitatory inputs via DA receptors that are presynaptic (Figure III.4D-I). The valence of spEPSC frequency was also consistent with the DA-induced changes in spAP frequency observed in the two vIPN neuron sub-types. In addition, when spEPSCs were blocked by NMDA and AMPA receptor antagonists (20 μM AP-5 and 10 μM CNQX), the majority of vIPN neurons ceased firing, highlighting the importance of spEPSCs in modulating firing rate and suggesting that the change in spEPSC frequency induced by DA directly causes the DA induced change in spAP frequency (Figure III.6). These findings
indicate that DA increases presynaptic excitatory transmission to Type A neurons and decreases presynaptic excitatory transmission to Type B neurons.

**Caudal IPN neurons respond to afferent VTA DAergic terminal stimulation**

To test if vIPN neurons respond to DAergic inputs from the VTA, I selectively expressed Cre-dependent ChR2-eYFP in VTA DAergic neurons of DAT::Cre mice and I recorded vIPN neuronal responses upon light-induced VTA→IPN stimulation (Figure III.7; 20 Hz, 2 ms pulse width). VTA DAergic terminals were stimulated through the microscope objective focused on the area around the recorded IPN neuron (Figure III.7A). Cell-attached mode was used to record spAPs. Interestingly, the majority of vIPN neurons failed to respond to VTA terminal optic stimulation (Figure III.8A). In contrast, consistent light evoked responses were observed in the caudal IPN (cIPN) matching the VTA→IPN innervation pattern observed in DATCre::ChR2 mice (Figure III.9). As compared to vIPN neurons, cIPN neurons exhibited a significantly higher input resistance and a different current-voltage relationship (Figure III.7B, 13B) indicating a distinct cIPN sub-type that I refer to as “Type C”. In cIPN slices, a sub-population of Type C neurons responded to light stimulation of DAergic afferents with an increase in spAP frequency that was attenuated in the presence of SCH39166 (Figure III.7C-E). To test the mechanism of light evoked changes in AP frequency in Type C neurons, I examined excitatory input, recording spEPSCs in response to light. Blue light failed to evoke a change in either spEPSC frequency or amplitude, suggesting
the effect of DA on spAP frequency in this sub-population was due to post-synaptic D1 receptor expression (Figure III.7F-H). To test this hypothesis, I sliced and imaged the IPN in mice expressing the fluorophore td-Tomato under the control of the \textit{DRD1} (the gene encoding the DA D1 receptor) promoter (Ade et al., 2011). Numerous Td-tomato cell bodies were apparent and exclusively located in the cIPN (Bregma > 3.6mm) (Figure III.10). In addition, dense Td-tomato-positive apparent terminal fields were visible in the vlIPN, suggesting the possibility that cIPN DRD1-expressing neurons may project to vlIPN neurons. Moreover, I also observed a population of Type C neurons that exhibited a light-induced decrease in spAP frequency that was likewise blocked by SCH39166 (Figure III.9D-I). In Type C neurons that decreased spAP firing in response to VTA terminal stimulation, I observed a significant reduction in spEPSC frequency that was blocked by SCH39166. Together, these data suggest that a subpopulation of Type C neurons in the cIPN that signal through D1 receptors, may amplify the VTA DAergic input to other IPN neurons, for instance, vlIPN Type A and Type B neurons, and modulate their responses.

cIPN Type C neurons project to the vlIPN to control activity of Type A and Type B neurons.

To test if D1-positive neurons in the cIPN project directly to the vlIPN, I expressed Cre-dependent ChR2-eYFP in the IPN of mice that express Cre under the control
of the DRD1 promoter (DRD1a::Cre mice) via AAV2-mediated gene delivery (Figure III.11A). As a result, eYFP signal was observed in cIPN neuronal soma and projections along the cIPN→vIPN plane (Figure III.11B). In coronal slices of the vIPN from these mice, stimulation of Type C terminals in the vIPN (20Hz, 2 ms pulse width) resulted in a significant increase of spEPSC frequency in Type A neurons and a significant decrease of spEPSC frequency in Type B neurons (Figure III.11C, D, F, G). These responses phenocopied the result of bath application of DA in 80% of vIPN neurons as predicted by their input resistances (compare with Figure III.4D-I). Experiments were repeated in the presence of 1 μM TTX and 100 μM 4-AP to block action potentials, and thus, block multi-synaptic responses (Petreanu et al., 2009). The changes in EPSC frequency upon light stimulation were maintained in both Type A and B neurons suggesting the D1 receptor-expressing Type C cIPN neurons project monosynaptically to the vIPN (Figure III.11E, H). In addition, combined GABA_A and GABA_B antagonists saclofen (10 μM) and bicuculline (20 μM) blocked the light-evoked change in spEPSC frequency in both Type A and B vIPN neurons suggesting Type C neurons release GABA to modulate excitatory synapses in the vIPN (Figure III.11I, J).

**Type A neurons receive stronger innervation from the MHb than Type B neurons.**

The MHb terminals in the IPN are known to increase excitatory transmission and therefore, glutamate release, in response to activation of GABA_B receptors (Zhang
et al., 2016). Thus, Type A and Type B neurons may be differentially innervated by the MHb, which could be partly responsible for their divergent responses to DA and to Type C neuron photostimulation. To test this idea, I recorded from vIPN neurons in coronal IPN slices from Chat::ChR2 mice where choline acetyltransferase-expressing neurons constitutively express ChR2 (Figure III.12A). Low frequency stimulation of the MHb cholinergic terminals causes the release of glutamate and not ACh (Ren et al., 2011). Thus, vIPN neurons were recorded in whole-cell mode during light stimulation for 30 s at 1 Hz (2 ms pulse widths) to induce glutamatergic transmission. Light pulses consistently evoked EPSCs in Type A neurons, but the same light pulses often failed to evoke EPSCs in Type B neurons (Figure III.12B, C, D). The average amplitude of evoked EPSCs in Type A neurons was significantly greater than in Type B neurons (Figure III.12E). When the amplitude was corrected with the input resistance to compare the peak energy of ions flowing between the two cell types, Type A amplitudes were still significantly greater than Type B amplitudes (Figure III.12F). These results indicate that vIPN Type A and Type B neurons are differentially innervated by MHb glutamatergic neurons.

To further characterize these two vIPN neuronal populations, Type A and Type B neurons were filled with biocytin internal solution. Morphology analysis demonstrated that Type A and Type B neurons represent two subpopulations with clearly distinct complexity of their dendritic branching and synaptic connectivity, as measured by dendritic spine density (Figure III.12G-J). Type A neurons were
defined by a more complex dendritic arbor with higher nodes and endings and a significantly higher total arbor length than Type B neurons (Figure III.12K-L, Tables III.1-3). In particular, whereas in Type A neurons the number of branch segments proportionally increased with higher branch orders, in Type B neurons, the number of segments remained significantly lower, particularly at branch orders of higher magnitude (Figure III.12M). In addition, Type A neurons displayed increased total spine density compared to Type B neurons (Figure III.12N). The complex dendritic arbors in Type A neurons likely interact with the spiraling MHb terminals of the IPN, consistent with the larger MHb functional input onto Type A neurons compared to Type B neurons. Thus, Type A neurons appear to receive greater innervation from the MHb cholinergic/glutamatergic terminals than Type B neurons.

Pharmacological manipulation of D1 receptors in the IPN bidirectionally modulates anxiety.

Our physiology data indicate that VTA DA activates a small population of cIPN neurons via D1 receptors, which is amplified through a microcircuit to the vIPN. To test if D1 signaling in the IPN modulates anxiety-like behavior, drug infusion cannulas were implanted to deliver either D1 receptor agonist or antagonist into the IPN prior to testing in the EPM and OFT assays (Figure III.13). In the EPM and the OFT, intra-IPN infusion of the D1 receptor agonist SKF82958 increased open arm time and increased time in the center, respectively, compared to vehicle infusion, indicating an anxiolytic effect of the drug.
Conversely, the D1 receptor antagonist SCH39166 was anxiogenic, reducing open arm time and time in the center compared to vehicle infusion (Figure III.13B, D). Neither drug affected the number of arm entries in the EPM, or distance traveled in the OFT (Figure III.13C, E). To control for a behavioral effect due to potential drug diffusion into the neighboring VTA, the D1-like receptor agonist and antagonist were infused directly into the VTA. These drugs did not result in a significant change in anxiety-like behavior in the EPM or OFT (Figure III.14). However, VTA infusion of D1 antagonist and agonist both resulted in a depression of total arm entries in the EPM (Figure III.14C). The difference in locomotor effects and the lack of a significant effect on anxiety when the VTA was infused suggests behaviors elicited from IPN infusions were not the result of off target effects from drug diffusion. Overall, these results demonstrate that endogenous DA controls anxiety-like behavior via anxiolytic D1 receptor signaling in the IPN.
Figure III.1. VTA→IPN DA input controls anxiolytic behavior.

(A) Left, schematic depicting fiber photometry recording of DA sensor-expressing neurons from the IPN of a mouse during exploration on the EPM. Right, representative micrograph depicting GRAB\textsubscript{DA}, AAV9-hSyn-DA\textsubscript{2m} (green),
expression in the IPN of a C57BL/6 mouse with optical fiber placement in cIPN outlined with white solid lines.

(B) Representative DA sensor fluorescence signal during mouse exploration on the EPM. The blue background indicates the times in which the mouse was in the open arm, the red background indicates the times in which the mouse was in the closed arm, and the white background indicates the times in which the mouse paused in the junction between arms. The black ticks above the trace highlight discrete fluorescence peaks. (C) Left, representative Z-score trace of GRAB\textsubscript{DA} sensor fluorescent signals during the last second in open (black) and closed arms (red) i.e. “0" on the x-axis corresponds to the beginning of the last second of the mouse in the arm. Right, summed average area under the curve (AUC) during the last second in open (black) and closed arms (red) (Unpaired t-test: n=6, *p< 0.05; Data presented as mean ± SEM).

(D) Analysis of discrete fluorescent event (spike) frequency, amplitude, and area during open and closed arm time. (Paired t-tests: n=6, *p<0.05, For amplitude, p=0.3314. Mean ± SEM.)

(E) Diagram of injections and placement of optic fiber for optogenetic experiments in (F) and (6A) (top). Verified optic fiber placements for experiments in (F) and (6A) (bottom).

(F) EPM activity during \textit{in vivo} NpHR inhibition (repeated 20 sec on,10 sec off) of VTA terminals in the IPN of Light-off (n=12) and Light-on groups (n=13). (Unpaired t-test: **p<0.01. Mean ± SEM.)
(G) Diagram of injections and placement of optic fiber for optogenetic experiments in (H) and (6B) (top). Verified optic fiber placements for experiments in (H) and (6B) (bottom).

(H) EPM activity during in vivo 15 Hz constant stimulation of ChR2-expressing VTA terminals in the IPN for Light-off (n=7) and Light-on (n=10) groups (top). Total arm entries between groups did not differ (bottom). (Unpaired t-test with Welch’s correction: **p<0.01. Mean ± SEM.)
**Figure III.2.**

**A**

[Images showing Heatmaps for Light Off and Light On conditions with MAX and MIN color bars.]

![Heatmap Diagram](image)

- **Center Time (sec)**
  - Light Off
  - Light On

- **Total Distance Moved (cm)**
  - Light Off
  - Light On

**B**

[Images showing Heatmaps for Light Off and Light On conditions with MAX and MIN color bars.]

![Heatmap Diagram](image)

- **Center Time (sec)**
  - Light Off
  - Light On

- **Total Distance Moved (cm)**
  - Light Off
  - Light On

*Note: The figure includes statistical data representations showing comparisons between conditions with markers indicating significance.*
Figure III.2. Optogenetic stimulation and inhibition of VTA afferents in the IPN during the OFT.

(A) OFT activity during in vivo NpHR inhibition of VTA terminals in the IPN. Representative heat map of mouse position (left). Graphs of center time (top) and total distance traveled (bottom right, n=12, 10, Light-off and Light-on, respectively). See Figure I.1G for canula placement.

(B) OFT activity during in vivo 15 Hz constant stimulation of ChR2-expressing VTA terminals in the IPN. Representative heat map of mouse position (left). Graphs of center time and total distance traveled. See Figure I.1E for canula placement. (n=13, 10 Light-off and Light-on, respectively, Unpaired t-test: *p≤0.05. Mean ± SEM.)
Figure III.3. DA modulates neuronal activity in two vIPN neuron sub-populations.

(A) Representative cell-attached trace from a Type A neuron in response to DA (top) and bar graph of averaged normalized AP frequency of Type A neurons at baseline, during the last minute of DA application, and after washout (bottom). (Friedman test: Friedman statistic (2, 34) = 28.23, p<0.0001. ****p<0.0001 compared to baseline, Dunn’s multiple comparison test. Mean ± SEM.)

(B) Representative cell-attached trace of a Type B neuron (top) in response to DA and averaged normalized AP frequency of Type A neurons at baseline, during the last minute of DA application, and after washout (bottom). (Friedman statistic (2,
32) $= 27.79$, $p\leq 0.0001$. ****$p<0.0001$ compared to baseline, Dunn’s multiple comparison test. Mean ± SEM.)

(C) Diagram of a coronal section of the IPN with approximate locations of Type A neurons (blue circles) and Type B neurons (red circles). Neurons without a response to DA are depicted as green circles. Location taken from digital images of the recording pipette in the slice after each recording. Representative traces of Type A (D) and B

(E) Current-voltage relationships determined in whole cell patch clamp in response to 20 pA current injection steps. Between each step the cell was held at 0 pA for 20-30 seconds. Traces are to scale with each other.

(F) Input resistance of Type A and B neurons calculated from the 0 to -20 pA step from traces in (D) and (E). ($n = 10$ and 12, respectively, unpaired t-test with Welch’s correction: ***$p\leq 0.001$. Data presented as mean ± SEM.)

(G) Mean current-voltage relationship determined for each cell by the lowest point per trace in the last 500ms in 2500ms of current injection in whole cell patch clamp. (Two-way ANOVA: Significant cell-type x current step interaction, $F_{(10, 218)}=5.07$, $p=0.0001$. Bonferroni’s multiple comparisons test: **$p\leq 0.01$, ***$p\leq 0.001$, ****$p\leq 0.0001$. Mean ± SEM.)
Figure III.4. vIPN neurons respond to DA through presynaptic D1-like but not D2-like DA receptors.

(A) Schematic of experiment. Dotted lines indicate approximate positions where coronal slice was cut (left). Neurons were recorded from a coronal slice of the vIPN (right).

(B) Averaged normalized spAP frequency of Type A neurons in response to DA in the absence and presence of the D1-like receptor antagonist SCH39166 (10 μM, top) or the D2-like receptor antagonist Eticlopride (10 μM, bottom). (One-way ANOVAs: (Top) n=6 cells, F(2, 10) = 19.6, p=0.0003; SCH39166: n=6 cells, F(2, 12) =
0.1435, p=0.8680; (Bottom) n=6 cells, F(2, 10) = 6.492, p=0.016; n=6 cells Eticlopride: F(2, 10) = 9.23, p=0.0054. Data presented as mean ± SEM.) *p<0.05, **p<0.01, ***p<0.001 compared to baseline.

(C) Averaged normalized spAP frequency of Type B neurons in response to DA in the absence and presence of the D1-like receptor antagonist SCH39166 (10 μM, top) or the D2-like receptor antagonist Eticlopride (10 μM, bottom). (n=6, One-way ANOVAs: (Top) F(2, 12) = 8.593, p=0.0048; SCH39166: F(2, 12) = 1.852, p=0.1991; (Bottom) F(2, 10) = 17.86, p=0.0005; F(2, 10) = 25.79, p=0.0001) ** p<0.01, ***p<0.001 compared to baseline, Mean ± SEM.

(D) Representative whole-cell patch clamp traces of spEPSCs in a Type A neuron before, during, and after DA application.

(E) Average normalized Type A spEPSC frequency at baseline, during last minute of DA application, and after washout. (n=10, Friedman test: **p≤0.01, Friedman statistic (2, 18) = 9.6., p=0.0075). ** p<0.01 Dunn’s test compared to baseline. Data are mean ± SEM.

(F) Average normalized Type A spEPSC amplitude at baseline, during last minute of DA application, and after washout. (n=10, One-way 415 ANOVA: F(2, 18) = 0.3592, p=0.7031). Data are mean ± SEM.

(G) Representative whole-cell patch clamp traces of spEPSCs in a Type B neuron before, during, and after DA application.
(H) Average normalized Type B spEPSC frequency at baseline, during last minute of DA application, and after washout. (n=7, One-way ANOVA: $F_{(2, 12)} = 14.47$, $p=0.0008$). *$p < 0.05$ compared to baseline. Data are mean ± SEM.

(I) Average normalized Type A spEPSC amplitude at baseline, during last minute of DA application, and after washout. (One-way ANOVA: $F_{(2, 12)} = 0.6047$, $p=0.5621$). Data are mean ± SEM.
Figure III.5. D2-like DA receptor agonist does not significantly affect vIPN spAPs.

(A) Representative cell-attached traces of a vIPN neuron before, during, and after 1 μM quinpirole application.

(B) spAP frequency does not significantly change after quinpirole application. (One-way ANOVA: F(2, 8) = 0.3414, p=0.7206. Data presented as mean ± SEM.)

(C) Representative whole-cell trace of a vIPN neuron before, during, and after 1 μM quinpirole.

(D) spEPSC frequency was not significantly affected by quinpirole application. (One-way ANOVA: F(2, 12) = 0.5191, p=0.6078. Mean ± SEM.)

(E) spEPSC amplitude was not significantly affected by quinpirole application. (One-way ANOVA: F(2, 12) = 0.1251, p=0.8836. Mean ± SEM.)
Figure III.6. vIPN neurons spAPs are blocked by exposure to glutamate receptor antagonists CNQX and AP-5.

(A) Representative trace of spAP in the vIPN.

(B) Representative trace of spAP in the vIPN after bath application of CNQX and AP-5. Representative trace of vIPN spEPSCs before (C) and after (D) application of CNQX and AP-5.
Figure III.7. Optogenetic stimulation of VTA→IPN DAergic terminals modulate cIPN neurons via D1 receptors.

(A) Schematic of experiment. Cre-dependent ChR2-eYFP was expressed in putative DAergic neurons of the VTA in DAT::Cre mice via AAV2-mediated gene delivery (left). Neurons in the cIPN were recorded in coronal slices while optogenetically stimulating the DRD1 expressing terminals (right).
(B) Representative whole-cell current-clamp traces from a cIPN neuron in response to 20 pA current injection steps from +100 to -40 pA. Compare to Figure III.3A and B.

(C) Representative traces of a cIPN neuron that responded to VTA terminal stimulation with an increase in firing rate.

(D) Normalized spAP frequency of Type C neurons that responded to light stimulation with an increase in spAP frequency. The data in figures (C-H) were collected from the same neurons. \( n=6, \) One-way ANOVA: \( F(2, 10) = 13.68, \) \( p=0.0014 \). **\( p<0.01 \) compared to Light-off control. Data presented as mean ± SEM.

(E) spAP frequency of cIPN neurons from (D) during 10 uM SCH39166 application. (Friedman statistic(2, 6) = 3.6, \( p=0.1852 \)). Data presented as mean ± SEM.

(F) Representative trace of EPSC frequency from a cIPN neuron that increased its spAP frequency in response to VTA terminal stimulation.

(G) In cIPN neurons that increased their spAPs, spEPSC frequency was not significantly affected. (One-way ANOVA: \( F(2, 10) = 0.1732, \) \( p=0.8435 \)). Data presented as mean ± SEM.

(H) In cIPN neurons that increased their spAPs, spEPSC amplitude was not significantly affected. (One-way ANOVA: \( F(2, 10) = 2.106, \) \( p=0.1725 \)). Data presented as mean ± SEM.
Figure III.8.

**(A)** Quantification of vIPN response to DAergic VTA terminal stimulation. (One-way ANOVA with repeated measures: $F_{(2, 10)} = 0.1575$, $p=0.8563$. Data presented as mean ± SEM.)

**(B)** Comparison of input resistance between cIPN neuron types. (One-way ANOVA with repeated measures: $F_{(2, 35)} = 9.116$, $p=0.0007$). *$p≤0.05$, ***$p≤0.001$. Mean ± SEM.

**(C)** Comparison of input resistances between cIPN neurons that either increased or decreased spAP frequency when DAergic VTA terminals were optogenetically stimulated. (Unpaired t-test: $p=0.5493$. Mean ± SEM.)
(D) Representative trace of spAP decrease in the cIPN in response to light stimulation blocked by 10 \( \mu \text{M} \) SCH39166.

(E) Summary of vIPN neurons with decreasing spAP frequency in response to DAergic VTA terminal stimulation. (One-way ANOVA with repeated measures: \( F_{(2, 18)} = 11.48, p=0.0006 \). ***\( p\leq0.001 \) compared to Light-off control. Mean ± SEM.

(F) The decreased spAP frequency was blocked by 10 \( \mu \text{M} \) SCH39166. (One-way ANOVA with repeated measures; \( F_{(2, 6)} = 1.425, p=0.3116 \). Mean ± SEM.)

(G) Representative traces of spEPSC frequency where cIPN neurons had decreased frequency in response to DAergic VTA terminal stimulation.

(H) Summary of cIPN neuron spEPSC frequency decrease in response to VTA DAergic terminal stimulation. (One-way ANOVA with repeated measures: \( F_{(2, 12)} = 15.79, p=0.0004 \). ***\( p\leq0.001 \) compared to Light-off control. Mean ± SEM.

(I) spEPSC amplitude was unaffected in cIPN neurons inhibited by VTA DAergic terminal stimulation. (One-way ANOVA with repeated measures: \( F_{(2, 12)} = 0.08637, p=0.9178 \). Mean ± SEM.

(J) spEPSC frequency change in (H) was blocked by 10 \( \mu \text{M} \) SCH39166. (One-way ANOVA with repeated measures: \( F_{(2, 10)} = 0.09674, p=0.9086 \). Mean ± SEM.
Figure III.9. VTA DAergic predominantly innervate the cIPN.

Cre-dependent ChR2-eYFP was expressed in putative DAergic neurons of the VTA in DAT::Cre mice via AAV2-mediated gene delivery. Confocal images of a slice through the vIPN (left) and the cIPN (right). White arrows point to projections into the cIPN. Scale bar represents 100 μm.
Figure III.10. D1 DA receptor expression in the IPN.

Fluorescent tdTomato (red) signal in 20 μm coronal slices from the IPN of a DRD1-tdTomato mouse. Slices of the IPN are arranged from ventral to caudal. IPR refers to the rostral subnucleus of the IPN, IPC refers to the central subnucleus of the IPN. The DRD1-tdTomato mouse expresses tdTomato under the DA receptor 1 gene promoter. Scale bar represents 50 μm. Beneath each image is a schematic of the approximate slice position in a sagittal view of the IPN. Note (presumed) terminals are found in the vIPN but (presumed) soma are only present in the cIPN.
Figure III.11. cIPN Type C putative D1 receptor-expressing neurons project to the vIPN and modulate Type A and Type B neuronal activity via GABA.
(A) Schematic of experiment. Cre-dependent ChR2-eYFP was expressed in putative DRD1-expressing neurons of the cIPN in DRD1::Cre mice via AAV2-mediated gene delivery (left). Neurons in the vIPN were recorded in coronal slices while optogenetically stimulating the DRD1 expressing terminals (right).

(B) Sagittal slice showing Cre-dependent eYFP (green) from a (DRD1)::Cre mouse. cIPN neurons send projections rostrally to the vIPN.

(C) Representative whole-cell patch clamp traces of Type A neuron EPSCs before, during and after 1 min of constant 20 Hz stimulation of cIPN terminals in the presence of TTX and 4-AP.

(D) Average normalized Type A EPSC response to 20 Hz terminal stimulation. (n=8, One-way ANOVA: F(2, 14) = 12.29, p=0.0008). **p<0.01 compared to Light-off control. Data presented as mean ± SEM.

(E) Type A response to DRD1-Cre terminal stimulation in the presence of AP blockers. The response was “monosynaptic”. (n=14, Friedman test: Friedman statistic(2,26) = 24.57, p<0.0001). **p<0.01 compared to Light-off control. Data presented as mean ± SEM.

(F) Representative whole-cell patch clamp traces of Type B neuron EPSCs before, during and after stimulation of cIPN terminals in the presence of TTX and 4-AP.

(G) Type B response to 20 Hz terminal stimulation. (n=8, One-way ANOVA: F (2, 14) = 23.22, p<0.0001). ****p<0.0001 compared to Light-off control. Data presented as mean ± SEM.
(H) Type B response to 20 Hz DRD1-Cre terminal stimulation in the presence of AP blockers. The connection was monosynaptic. \( n=14, \) One-way ANOVA: \( F(2, 24) = 5.717, \) \( p=0.0093 \). *\( p<0.05 \) compared to Light-off control. Data presented as mean ± SEM.

(I) Normalized EPSC frequency of a Type A neuron before, during, and after cIPN DRD1-Cre terminal stimulation in the presence of 1 μM TTX and 100 μM 4-AP. The experiment was repeated with the addition of bath-applied 20 μM Bicuculline and 100 μM Saclofen to block GABA\(_A\) and GABA\(_B\) receptors, respectively. \( n=7, \) One-way ANOVAs: \( F(2, 12) = 10.08, \) \( p=0.0027 \); GABA\(_A+B\) receptor antagonists: \( F(2, 12) = 1.539, \) \( p= 0.2542 \). **\( p<0.01 \) compared to Light-off control. Data are presented as mean ± SEM. (J) Normalized EPSC frequency of a Type B neuron before, during, and after cIPN DRD1-Cre terminal stimulation in the presence of 1 μM TTX and 100 μM 4-AP. The experiment was repeated with the addition of bath-applied 20 μM Bicuculline and 100 μM Saclofen to block GABA\(_A\) and GABA\(_B\) receptors, respectively. \( n=5, \) One-way ANOVA: \( F(2, 8) = 7.437, \) \( p=0.015 \); GABA\(_A+B\) receptor antagonists: \( F(2, 8) = 3.458, \) \( p= 0.0827 \). *\( p<0.05 \) compared to Light-off control. Data are presented as mean ± SEM.
Figure III.12. Type A neurons receive stronger innervation from MHb cholinergic/glutamatergic neurons than Type B neurons.

(A) Schematic of experiment. Neurons in the vIPN were recorded in coronal slices (right) while optogenetically stimulating ChAT-expressing terminals in the IPN (left) which are dominated by MHb glutamatergic/cholinergic terminals.
(B) Representative EPSC’s evoked in a Type A neuron. Blue vertical bars indicate time of light flashes.

(C) Representative EPSC’s evoked in a Type B neuron.

(D) Percentage of successfully evoked EPSCs in a batch of 30 (1 Hz) light pulses over 30 s in Type A (n=8) and Type B (n=16) neurons. Mann Whitney test: **p≤ 0.01. Data presented as mean ± SEM.

(E) Average amplitude of successfully evoked EPSCs in a batch of 30 (1 Hz) light pulses over 30 s in Type A neurons and Type B neurons (Unpaired t-test: ****p≤ 0.0001. Mean ± SEM.)

(F) Potential energy of evoked EPSCs. The average amplitude was corrected for input resistance differences. (Unpaired t-test: **p≤ 0.01. Mean ± SEM.)

(G) Representative image of a biocytin filled Type A neuron. Scale bar is 50 μm. Image generated from a confocal image z-stack obtained with a 40x objective.

(H) Representative image of a biocytin filled Type B neuron to scale with (G). Image generated from a confocal image z-stack obtained with a 40x objective.

(I) Representative images of spines on proximal and distal dendrites of a biocytin filled Type A neuron. Image obtained with a 63x objective at 2.5 zoom. Scale bar is 5 μm.

(J) Representative images of spines on proximal and distal dendrites of a biocytin filled Type B neuron to scale with I. Image obtained with a 63x objective at 2.5 zoom.
(K) Quantification of total arbor length between Type A and B neurons. (Unpaired t-test: **p≤ 0.01. Mean ± SEM.)

(L) Quantification of dendritic nodes and endings. (Unpaired t-test: ***p≤ 0.001. Mean ± SEM.)

(M) Branch segments per branch order. Demonstrating the significantly more complex dendritic arbors present in Type A compared to Type B neurons. (Two-way ANOVA: Significant cell type X branch interaction. F(8,144)=6.50, ****p≤0.0001). Bonferroni’s multiple comparisons test: *p≤0.05, ***p≤0.001, ****p≤0.0001. Data are presented as mean ± SEM.

(N) Quantification of total spine density. (Unpaired t-test: ***p≤ 0.05. Mean ± SEM.) For additional general morphology parameters measured and multiple comparisons of branch order analyses see tables III.1-3.
Figure III.13. Manipulation of D1 receptors in the IPN controls anxiety-like behaviors.

(A) Diagram of experiment (left). Image of guide canula track in slice. Locations of infusion cannula placements within the IPN (right). Black scale bar represents 50 μm (middle).
(B) Quantification of open arm time in the EPM between D1-like DA receptor agonist (n=10, SKF82598 0.7 μg/μl, infused 0.3 μl, 210 ng), antagonist (n=13, SCH39166 35 ng/μl, infused 0.3 μl, 10.5 ng), or saline control (n=15). (One-way ANOVA with repeated measures: F_{2, 35}= 11.43, p=0.0002). *p≤0.05, ***p≤0.001. Data presented as mean ± SEM.

(C) Total arm entries in the EPM after drug infusion. (One-way ANOVA with repeated measures: F_{2, 35} = 0.6541, p=0.5261). Data presented as mean ± SEM.

(D) Representative heat map of mouse position in the OFT after infusion of drug into the IPN (left). Quantification of center time in the OFT (right). (n=10, 9, 8 for saline, agonist, antagonist, respectively, One-way ANOVA with repeated measures: *p≤0.05, **p≤0.01, F_{2, 24} = 8.558. Mean ± SEM.)

(E) Quantification of total distance moved in the OFT. There was no significant difference between groups. (One-way ANOVA with repeated measures: F_{2, 24} = 1.437, p=0.2574). Data are presented as mean ± SEM.
Figure III.14. Effects of VTA D1 drug infusion on anxiety-like behavior.
(A) Diagram of experiment (left). Image of guide canula track in slice. Black scale bar represents 50 μm (middle). Locations of infusion cannula placements in the VTA (right) (B) Time in open arms of the EPM was not significantly different. (n=8, 13, 9 for saline, agonist, antagonist, respectively, One-way ANOVA: \(F_{(2, 27)} = 1.936, p=0.1638\)). Data presented as mean ± SEM.

(C) Total arm entries in the EPM after a VTA infusion of Saline, D1-like DA receptor agonist (SKF82958), or D1-like receptor antagonist (SCH39166). (One-way ANOVA: \(F_{(2, 27)} = 7.917, p=0.002\)). **p≤0.01 Mean ± SEM.

(D) OFT of VTA infusion of D1 agonist (SKF82958) and antagonist (SCH39166). No significant differences were found between treatment groups. (n=9, 8, 8, respectively, One-way ANOVA: \(F_{(2, 22)} = 0.8463, p=0.4425\)). Mean ± SEM.

(E) Total distance moved in OFT. (One-way ANOVA: \(F_{(2, 22)} = 0.7456, p=0.4861\)). Mean ± SEM
Figure III.15. Circuit model for DA signal amplification in the IPN.

Circles represent neurons, the lines originating from the circles represent axons and the triangles represent terminals. The terminals are set so that the side of the triangle opposite the axon faces its presumed target. Each color represents a unique population of neurons.

Anxiety-like Behaviors
Table III.1: General morphology parameters of Type A and B neurons within the IPN. All data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type A (n)</th>
<th>Type B (n)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma Volume (µm³)</td>
<td>681 ± 97 (11)</td>
<td>498 ± 51 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.31</td>
</tr>
<tr>
<td>Total dendritic length (µm)</td>
<td>1888 ± 223 (11)</td>
<td>810 ± 186 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.002**</td>
</tr>
<tr>
<td>Mean tree length (µm)</td>
<td>595 ± 103 (11)</td>
<td>354 ± 99 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.11</td>
</tr>
<tr>
<td>Max terminal length (µm)</td>
<td>252 ± 42 (11)</td>
<td>196 ± 38 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.35</td>
</tr>
<tr>
<td>Nodes and endings</td>
<td>122 ± 18 (11)</td>
<td>34 ± 10 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.0009***</td>
</tr>
<tr>
<td>Total spine density (spines/100µm)</td>
<td>56 ± 5 (11)</td>
<td>38 ± 7 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.04*</td>
</tr>
<tr>
<td>Proximal spine density (spines/100µm)</td>
<td>61 ± 7 (11)</td>
<td>40 ± 7 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.06</td>
</tr>
<tr>
<td>Distal spine density (spines/100µm)</td>
<td>55 ± 5 (11)</td>
<td>32 ± 7 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.02*</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, *** p<0.001, Two-tailed unpaired Student’s t-test.
Table III.2: Branch order characteristics of Type A and B neurons within the IPN. All data presented as mean ± SEM

<table>
<thead>
<tr>
<th>Branch Order Properties</th>
<th>Type A (n)</th>
<th>Type B (n)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st order branch segments</td>
<td>3.5 ± 0.2 (11)</td>
<td>2.6 ± 0.2 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>1st order mean branch segment length (µm)</td>
<td>30.5 ± 5.6 (11)</td>
<td>23.2 ± 3.4 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>1st order branch spine density</td>
<td>61.2 ± 8.4 (11)</td>
<td>40.6 ± 10.6 (9)</td>
<td>0.40</td>
</tr>
<tr>
<td>2nd order branch segments</td>
<td>5.9 ± 0.5 (11)</td>
<td>4.4 ± 0.5 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>2nd order mean branch segment length (µm)</td>
<td>29.8 ± 3.2 (11)</td>
<td>28.2 ± 6.7 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>2nd order branch spine density</td>
<td>58.9 ± 7.4 (11)</td>
<td>34.7 ± 6.6 (9)</td>
<td>0.18</td>
</tr>
<tr>
<td>3rd order branch segments</td>
<td>8.7 ± 0.7 (11)</td>
<td>5.1 ± 1.0 (9)</td>
<td>0.85</td>
</tr>
<tr>
<td>3rd order mean branch segment length (µm)</td>
<td>28.2 ± 4.7 (11)</td>
<td>37.1 ± 13.6 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>3rd order branch spine density</td>
<td>51.0 ± 8.2 (11)</td>
<td>23.3 ± 4.3 (9)</td>
<td>0.08</td>
</tr>
<tr>
<td>4th order branch segments</td>
<td>11.2 ± 0.7 (11)</td>
<td>4.9 ± 1.0 (9)</td>
<td>0.04*</td>
</tr>
<tr>
<td>4th order mean branch segment length (µm)</td>
<td>20.2 ± 2.9 (11)</td>
<td>29.4 ± 5.7 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>4th order branch spine density</td>
<td>51.2 ± 5.4 (11)</td>
<td>37.3 ± 7.9 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>5th order branch segments</td>
<td>13.9 ± 1.6 (11)</td>
<td>3.4 ± 1.2 (9)</td>
<td>0.0001***</td>
</tr>
<tr>
<td>5th order mean branch segment length (µm)</td>
<td>13.8 ± 1.1 (11)</td>
<td>12.2 ± 4.5 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>5th order branch spine density</td>
<td>59.3 ± 8.8 (11)</td>
<td>22.4 ± 9.3 (9)</td>
<td>0.006**</td>
</tr>
<tr>
<td>6th order branch segments</td>
<td>16.6 ± 2.4 (11)</td>
<td>3.2 ± 1.5 (9)</td>
<td>0.0001***</td>
</tr>
<tr>
<td>6th order mean branch segment length (µm)</td>
<td>18.8 ± 3.3 (11)</td>
<td>10.1 ± 4.4 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td>6th order branch spine density</td>
<td>54.3 ± 5.8 (11)</td>
<td>22.0 ± 10.4 (9)</td>
<td>0.02*</td>
</tr>
<tr>
<td>7th order branch segments</td>
<td>13.3 ± 2.5 (11)</td>
<td>2.9 ± 1.9 (9)</td>
<td>0.0001***</td>
</tr>
<tr>
<td>7th order mean branch segment length (µm)</td>
<td>11.0 ± 1.0 (11)</td>
<td>7.7 ± 4.1 (9)</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>p A VS B</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>7th order and above branch spine density</td>
<td>56.4 ± 3.7 (11)</td>
<td>10.1 ± 6.1 (9)</td>
<td>p = 0.0004***</td>
</tr>
<tr>
<td>8th order branch segments</td>
<td>11.8 ± 2.9 (11)</td>
<td>2.2 ± 1.6 (9)</td>
<td>p = 0.0001***</td>
</tr>
<tr>
<td>8th order mean branch segment length (µm)</td>
<td>10.3 ± 2.0 (11)</td>
<td>5.4 ± 3.6 (9)</td>
<td>p &gt; 0.9999</td>
</tr>
<tr>
<td>9th order and above branch segments</td>
<td>6.1 ± 1.3 (11)</td>
<td>1.4 ± 0.9 (9)</td>
<td>p = 0.27</td>
</tr>
<tr>
<td>9th order and above mean branch segment length (µm)</td>
<td>10.2 ± 2.0 (11)</td>
<td>3.2 ± 2.1 (9)</td>
<td>p &gt; 0.9999</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, two-way ANOVAs with Bonferroni’s post tests.
Table III.3: Sholl analysis differences for Type A and B neurons within the IPN. All data presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Distance from soma</th>
<th>Type A (n)</th>
<th>Type B (n)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean dendrite length 30-40 µm</td>
<td>163.1 ± 18.7 (11)</td>
<td>71.2 ± 11.1 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.005**</td>
</tr>
<tr>
<td>Mean dendrite intersections 30-40 µm</td>
<td>11.5 ± 1.4 (11)</td>
<td>5.6 ± 0.9 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.003**</td>
</tr>
<tr>
<td>Mean dendrite length 40-50 µm</td>
<td>185.6 ± 27.9 (11)</td>
<td>70.1 ± 9.3 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &lt; 0.0001****</td>
</tr>
<tr>
<td>Mean dendrite intersections 40-50 µm</td>
<td>11.3 ± 1.9 (11)</td>
<td>3.4 ± 0.6 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &lt; 0.0001****</td>
</tr>
<tr>
<td>Mean dendrite length 50-60 µm</td>
<td>177.0 ± 28.4 (11)</td>
<td>46.9 ± 8.0 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &lt; 0.0001****</td>
</tr>
<tr>
<td>Mean dendrite intersections 50-60 µm</td>
<td>11.5 ± 1.5 (11)</td>
<td>3.4 ± 0.6 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &lt; 0.0001****</td>
</tr>
<tr>
<td>Mean dendrite length 60-70 µm</td>
<td>167.8 ± 24.4 (11)</td>
<td>60.8 ± 16.5 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.0004***</td>
</tr>
<tr>
<td>Mean dendrite intersections 60-70 µm</td>
<td>10.1 ± 1.8 (11)</td>
<td>4.7 ± 1.1 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.01*</td>
</tr>
<tr>
<td>Mean dendrite length 70-80 µm</td>
<td>179.6 ± 34.9 (11)</td>
<td>73.8 ± 25.8 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.0004***</td>
</tr>
<tr>
<td>Mean dendrite intersections 70-80 µm</td>
<td>11.5 ± 3.2 (11)</td>
<td>4.4 ± 1.3 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.0001***</td>
</tr>
<tr>
<td>Mean dendrite length 80-90 µm</td>
<td>194.1 ± 50.7 (11)</td>
<td>62.1 ± 19.4 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &lt; 0.0001****</td>
</tr>
<tr>
<td>Mean dendrite intersections 80-90 µm</td>
<td>10.5 ± 3.2 (11)</td>
<td>4.3 ± 1.3 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.002**</td>
</tr>
<tr>
<td>Mean dendrite length 90-100 µm</td>
<td>146.9 ± 42.6 (11)</td>
<td>66.3 ± 30.8 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; = 0.03*</td>
</tr>
<tr>
<td>Mean dendrite intersections 90-100 µm</td>
<td>7.2 ± 2.5 (11)</td>
<td>4.2 ± 2.1 (9)</td>
<td>p&lt;sub&gt;A VS B&lt;/sub&gt; &gt; 0.9999</td>
</tr>
</tbody>
</table>

* p<0.01, ** p<0.001, **** p<0.0001, two-way ANOVAs with Bonferroni’s post tests.
Discussion

DA signaling has long been implicated in anxiety-like behavior presumably through midbrain DA projection areas to the hippocampus, extended amygdala, and prefrontal cortex, among other brain regions (Jennings et al., 2013; Kim et al., 2013; Liu et al., 2011; Refojo et al., 2011; Zhong et al., 2018; Zweifel et al., 2011). However, precise DA dynamics during anxiety-like behavior have not been investigated in detail. Our data combining fiber photometry with GRAB_{DA} sensor expression in the IPN revealed that IPN DA increases as an animal explored the anxiogenic open arm of the elevated plus maze. Preventing this increase by silencing the VTA→IPN input reduced exploration of the open arm while activating the input increased time spent in the open arm, suggesting that this IPN DA signal controls anxiety-like behavior specifically by driving anxiolysis. Assays used to evaluate anxiety-like behavior in mice including the EPM are multimodal and integrate two opposite motivational drives: 1) behavioral avoidance and 2) novelty seeking (Walf and Frye, 2007). Mice are driven to explore the open arms of the EPM because they are novel but also avoid exploration because they are elevated and without protection from predation. Thus, the read-out or expression of anxiety-like behavior relies upon the strength of these two motivational drives. Interestingly, previous studies implicate the habenula-interpeduncular pathway in behavioral avoidance and aversion (Fowler and Kenny, 2014; Soria-Gómez et al., 2015; Wolfman et al., 2018); whereas, the Tapper lab has also discovered that the
IPN and associated circuitry is critically involved in signaling familiarity, reducing motivation to explore novelty to control novelty preference (Molas et al., 2017b). Thus, my data indicate that VTA input and IPN DA may provide a signal that either reduces avoidance behavior to allow expression of reduced anxiety-like behavior or increase motivation to explore novelty. Future studies will focus on how the IPN integrates anxiety and novelty signals to drive exploratory behavior. Interestingly, excitation of DAergic IPN inputs stimulates a small sub-population of dopaminoceptive neurons expressing the D1 receptor located predominantly in the caudal portion of the IPN. Remarkably, these neurons, through a microcircuit spanning the vIPN, amplify the DA signal ultimately controlling anxiety-like behavior. Indeed, the vast majority of vIPN neurons respond to exogenous DA in midbrain slices (35 out of 39) presumably through D1 receptor-expressing Type C terminals which modulate excitatory input to vIPN neurons. The effect of exogenous DA application on vIPN neuronal activity is phenocopied by direct optogenetic electrical excitation of D1-expressing terminals in the vIPN. This data supports the theory that there is a micro-circuit amplifying DA signal. Thus, what at first glance would appear to be a modest connection between VTA and cIPN, through this amplification step, transmits activity to the majority of neurons in the ventral portion of the nucleus to control behavior.

The microcircuit controlling activity of vIPN neurons is unique in that it consists of two morphologically distinct neurons, Type A and Type B, which both receive GABAergic input from cIPN Type C neurons but act oppositely in response to
GABA. Type A neurons are excited by activation of Type C terminals via increased glutamate release; whereas Type B neurons are inhibited by activation of Type C terminals via decreased glutamate release (Figure III.15). Interestingly, Type A neurons are robustly controlled by MHb excitatory inputs that are activated by GABA via excitatory GABA<sub>B</sub> receptors on MHb terminals (Ren et al., 2011; Zhang et al., 2016). Type B neurons, on the other hand, are weakly innervated by the MHb, thus, it is likely that GABA reduces excitatory input from other, unidentified excitatory afferents that express inhibitory GABA receptors. Our morphology analysis supports divergent habenula inputs between these two neuron sub-populations: Type A neurons exhibit complex dendritic arbors and increased spine densities compared to Type B neurons, which may more readily synapse with MHb excitatory afferents that have a similarly complex “tornado” pattern input (Molas et al., 2017a). Thus, the morphological data supports the electrophysiological data. Specifically, Type A neurons have a functional connection to the MHb and complex dendrites— a form which could support a strong connection to the cholinergic glutamatergic vMHb efferent “tornado.” Whereas these structures are dramatically reduced in Type B neurons which have a much weaker functional connection.

Ultimately, engaging this microcircuit either through optogenetic stimulation of VTA→IPN inputs or through infusion of D1 receptor agonist increases Type A neuronal activity while decreasing Type B neuronal activity to reduce anxiety-like behavior. Future studies will functionally map Type A and Type B neuron projections in detail to determine their contributions to anxiety and behavior.
In summary, our data indicate that VTA DAergic input to the IPN mediates anxiety-like behavior by activating D1-expressing neurons in the cIPN. This small population of dopaminoceptive neurons amplify VTA DA input by projecting to and innervating vlPN through MHb glutamatergic inputs to bidirectionally control anxiolysis. Thus, I have identified a critical component of the neural network contributing to an affective state through dopaminergic signaling that engages a unique interpeduncular microcircuit. Neuroadaptation in the circuit may contribute to anxiety disorders. Thus, manipulation of this microcircuit may be therapeutically beneficial for those with anxiety disorders.

Author Contributions
S.R.D., R.Z, P.M.K., and S.M. conducted the experiments. Y.L. provided the GRABDA sensors. S.R.D., R.Z., P.M.K., F.S., P.D.G. Y.L. and A.R.T. designed the experiments. S.R.D. and A.R.T. wrote the paper with input from all co-authors.

Acknowledgments
Thank you to Dr. Karl Deisseroth for optogenetic plasmids and Dr. Guangping Gao for viral plasmid packaging. Thank you to Anthony Sacino for the technical support. This work was supported by the National Institute on Drug Abuse award number DA041482 (A.R.T.), DA035371 (P.D.G. and A.R.T.) and by a NARSAD Independent Investigator Grant from the Brain & Behavior Research Foundation
(A.R.T.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of Interests

The authors declare no competing interests.
Methodology

Animals

All experiments followed the guidelines for care and use of laboratory animals provided by the National Research Council, and with approved animal protocols from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. C57Bl/6J (#000664), GAD2-Cre (#010802), Chat-Cre (#006410), DAT-Cre (#006660), Chat-ChR2 (#014546), DRD1-Cre (#028298), and Drd1a-tdTomato (#016204) mice were obtained from The Jackson Laboratory, bred in the UMMS animal facility and used in behavioral, optogenetic and biophysical experiments as indicated. Cre lines were crossed with C57Bl/6J mice and only heterozygous animals carrying one copy of the Cre recombinase gene were used for experimental purposes. Mice were housed together in cages of no more than five animals and kept on a standard 12 h light/dark cycle (lights ON at 7 A.M.) with ad libitum access to food and water. Three to four weeks before experimentation, subject mice were kept under a reverse 12 h light/dark cycle (lights ON at 7 P. M.) for at least 5 days before any behavioral testing.

Viral Preparation

Optogenetic plasmids were packaged into AAV serotype 2 (AAV2) viral particles by the UMMS Viral Vector Core. AAV2 was used as the serotype for optogenetic viral-mediated gene deliveries as AAV2 preferentially infects neurons with minimal spread and retrograde infection (Shevtsova et al., 2005). GRAB_{DA2m} is derived
from GRABDA1m (Sun et al., 2018), with additional mutations in cpEGFP. GRABDA2m has ~3-fold improvement in the maximal ΔF/F₀ and similar apparent affinity (EC₅₀~90 nM). GRABDA1m has a rise time of ≤ 100 ms which indicates the fluorescent events detected were within the proper range for detection. Detailed characterization of GRABDA2m will be published elsewhere (DeGroot et al. in revision). GRABDA2m was packaged into AAV9 by Vigene Biosciences. Viral titrations consisted of 8.5 × 10¹² genome copies per ml for pAAV-Ef1a-DIOeNpHR3.0-eYFP, 2.5 × 10¹² viral particles per ml for pAAV-Ef1a-DIO-ChR2-eYFP, 5 × 10¹² viral particles per ml for pAAV-Ef1a-DIO-eYFP, and 2.12 × 10¹³ genomic copies per ml for pAAV-hSyn-DA2m. All viral injections were performed 4–6 weeks before experiments to allow for sufficient time for transgene expression.

**Stereotaxic injections**

Surgeries were performed under aseptic conditions. Male mice (6 – 8 weeks old) were deeply anaesthetized via intraperitoneal (IP) injection of a 100 mg/kg ketamine (VEDCO) and 10 mg/kg xylazine (LLOYD) mixture. Ophthalmic ointment was applied to maintain eye lubrication. Following anesthesia, the surgical area was shaved and disinfected with iodine before the scull bone was exposed. Mice were then placed on a stereotaxic frame (Stoelting Co.) where bregma and lambda landmarks were used to level the skull along the coronal and sagittal planes. A 0.4-mm drill was used for craniotomies at the target Bregma coordinates. Mice were microinjected at a controlled rate of 30 nl/min using a gas-tight 33G 10-μl
neurosyringe (1701RN; Hamilton) in a microsyringe pump (Stoelting Co). After injection, the needle remained unmoved for 10 min before a slow withdrawal. Injection coordinates were (in mm, Bregma anteroposterior (AP), mediolateral (ML), dorsoventral (DV) and angle): IPN (−3.4, −0.5, −4.82, 6°), VTA (−3.4, ±0.5, −4.2, 0°). Viral volumes for unilateral IPN injections were 300 nl. Bilateral VTA injections were 800 nl total.

**Implantation of cannulas and optic fibers**

Optogenetic behavioral experiments were performed 4-6 weeks post-injection of the viral construct. A unilateral optic fiber implant (200-μm core diameter; 0.53 NA, Doric Lenses) held in a magnetic aluminum receptacle (Doric Lenses) was placed above the IPN (−3.4, −0.5, −4.8, 6°) and secured into the skull using adhesive (C&B Metabond cement, Parkell Inc.) followed by dental cement (Cerebond, PlasticsOne).

The ChR2 light stimulation paradigm was continuous light pulses at 5 Hz with 500 mA current and 25 % duty cycle. Conversely, the NpHR photo-inhibition paradigm was constant light 20 sec on, 10 sec off repeated. Tests were recorded immediately after the first light pulse and were measured over 5 min in the EPM assay and 10 min in the OFT.

For pharmacological experiments, a stainless-steel guide cannula (26 gauge with 4-mm pedestals, PlasticsOne) was inserted above the IPN (−3.4, 0, −4.5, 0°) and secured to the skull with Cerebond.
Post-surgery procedures

After surgeries, all mice were placed on heating pads until recovery from anesthesia. Following recovery, mice received subcutaneous injections of the analgesic ketoprofen (Zoetis; 1 mg/kg). Mice were allowed to recover in their home cages for 5 days before any behavioral testing. Injection sites and viral expression were confirmed for all animals by experimenters blinded to behavioral outcome. Animals showing no viral expression or off-target site viral expression or incorrect optic fiber placement were excluded from analysis.

Intra-cerebral Infusion

Mice were anesthetized with 2% isoflurane via a nose cone adaptor at a flow rate of 800 ml/l. An internal infusion cannula (33 gauge) designed to reach IPN coordinates (−4.75 mm) was inserted into the guide cannula and vehicle (2% dimethyl sulfoxide, DMSO and 98% sterile saline) or D1 antagonist SCH39166 (70 ng/μl dissolved in 2% DMSO and 98% sterile saline) was infused at a rate of 0.3 μl/min for 1 min. After 674 infusion, the injection cannula was left in place for an additional 2 min before removal. SKF82958 (0.2 μg) was dissolved in sterile saline and also administered at a rate of 0.3 μl/min. The drug solutions were infused 10 min before behavioral testing. After experiments, mice were culled and brains were removed for coronal sectioning into 25-μm slices with a cryostat (Leica Microsystems, Inc.). Sections were mounted on glass slides (Sigma) to visualize
cannula placement. Animals showing incorrect cannula placement were excluded from analysis by experimenters blinded to treatment.

**Fiber Photometry**

Recordings were obtained with Doric Instruments Fiber Photometry System. An LED driver was used to deliver excitation light from LEDs at 465 nm and at 405 nm, which was used as an isosbestic wavelength for the indicator (Doric Lenses). The light was reflected into a 200-μm 0.53 N.A. optic fiber patch cord via the Dual Fluorescence Minicube (Doric Lenses). The patch cord was connected to the optic fiber implanted in the IPN prior to the start of the EPM session. Recording began after the mouse was placed into the EPM apparatus. DA sensor fluorescence was collected through the same patch cord. The DA-dependent (525 nm) and isosbestic control (430 nm) fluorescence signals were projected onto a femtowatt photoreceiver (Newport, 2151) through the Fluorescence Minicube (Doric Lenses). Sampling (12 kHz) and lock-in demodulation of the fluorescence signals were controlled by Doric Neuroscience Studio software with a decimation factor of 50.

**Fiber Photometry Analysis**

Demodulated fluorescence signals were converted into $\Delta F/F_0$ using the photometry analysis module of the Doric Neuroscience Studio software. The least mean squares method was used for the $F_0$ calculation. The $\Delta F/F_0$ of the 465 nm and 405 nm signals were calculated independently and were subtracted. Areas
under the curve of the normalized $\Delta F/F_0$ curves were calculated in GraphPad Prism using the trapezoidal method. For Z-scores and AUC (Figure I.1C), the Z-score was calculated over the entire trace and the last second in each arm was averaged to create the representative open and closed arm traces. AUC was calculated in GraphPad Prism from the average z-score over the entire duration the mouse spent in each respective arm of the EPM. Discrete florescence events (Figure I.1D) were detected from entire traces in Mini Analysis Program (Synaptosoft) by automated detection with the following parameters: amplitude and area thresholds were set to 5 times the root means square calculated noise level, the fraction of amplitude used to determine decay time was set to 0.1, baselines were calculated by averaging 200 ms of trace 800 ms before the event peak, and three data points were averaged to determine peaks. After automated detection, the start, end and peak of each event was manually verified in a blinded fashion. Overlapping or erroneous events were excluded or corrected. After event verification, event data were grouped by mouse position in the EPM.

**BEHAVIORAL ASSAYS**

**Elevated Plus Maze**

Implanted mice were plugged into a corresponding patch cord by magnetic force before the beginning of the session and subsequently placed in an EPM. The apparatus consisted of four arms connected by a central axis ($5 \times 5$ cm) and was elevated 45 cm above the floor. Two of the arms were enclosed with plastic black
walls (5× 30 × 15 cm) while the other two remained open (5 × 30 ×0.25 cm). At the start of each session, mice were placed at the intersection of the maze facing into an arm with no walls and allowed 5 min of free exploration. Optical stimulation occurred in animals during the entire 5-min session (ChR2: 473 nm, 500 mv 15 Hz 30% duty cycles, 5 s on 5 s off; NpHR: 593 nm constant light, 20 sec on, 10 sec off). Canula-implanted mice were tested 10 min after drug infusion. The number of entries into the open and closed arms and the total time spent in the open and closed arms were measured by MED-PC IV software. The time spent in open arms was considered an index of anxiety-like behavior and the total number of entries as an index of locomotor activity. The apparatus was cleaned between animals with Micro-90 solution.

**Open Field Test**

After EPM tests, individual mice were placed facing the walls of a Plexiglass open-field (42 × 38 × 30 cm). Mice explored the open field for 10 minutes without interaction with the experimenters. The time spent occupying the center of the chamber, as compared to time along the outer areas, was tracked automatically via EthoVision XT 11.5 (Noldus Apparatus). For tests with drug infusion, the OFT proceeded immediately after the EPM test. For optogenetic tests, mice had a 5-10 min break between tests. Control mice received the virus and surgery but received no light stimulation.
ELECTROPHYSIOLOGY

Slice preparation

Mice received a fatal IP injection of 200 mg/kg pentobarbital before perfusion with oxygenated ice-cold cutting solution consisting of (in mM): 2.5 KCl, 1.25 NaH₂PO₄, 20 HEPES, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 92 NMDG, 30 NaHCO₃, 25 D-Glucose, 0.5 CaCl₂, 10 MgSO₄ set to pH 7.4 with HCl. Brains were swiftly removed and placed in oxygenated ice-cold cutting solution. Brains were cut coronally to provide a clean mounting surface and mounted with Loctite 454 instant adhesive gel then placed in a Leica VT 1200 vibratome filled with ice-cold cutting solution. The brains were sliced into coronal sections (~200 μm) each containing the IPN. Slices were removed from the vibratome and percolated at 31°C in oxygenated cutting solution for 15 min before being moved to room temperature oxygenated ACSF consisting of (in mM): 125 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂. Oxygenation was maintained by bubbling solutions with 95% oxygen 5% carbon dioxide.

Recordings

Recordings were made using glass pipettes (1B150F-4 World Precision Instruments) pulled using a standard patch pipette protocol in a Sutter Instrument Model P-1000. Pipettes had an opening that provided a resistance of 3-7 MΩ. Pipettes were filled with internal solution containing the following (in mM): 121 KCH₃SO₃, 4 MgCl₂, 11 EGTA, 1 CaCl₂, 10 HEPES, 2 ATP, 0.2 GTP set to pH 7.2
with 1M KOH. Patch clamp recordings were held at -70 mV to obtain capacitive transients from spAPs. Pipettes were held in a micromanipulator (Scientifica) and visualized with a Bx51W 763 microscope (Olympus). spEPSCs were recorded in the whole-cell configuration and gap-free acquisition mode in Clampex (Axon Instruments). The internal solution was balanced to eliminate the driving force of chloride ions at the holding potential -70 mVs. Neurons were held at a resting membrane potential of −70 mV. Trains of 470 nm 1 hz or 20 Hz 2 ms pulse width (ChR2) light pulses were applied to neurons under current clamp or voltage clamp using LEDs of the appropriate wavelength (Thor Labs), as indicated. All recordings were filtered at 1 kHz using the amplifier’s four-pole, low-pass Bessel filter, digitized at 10 kHz with an Axon Digidata 1440A interface and stored on a personal computer.

**Data processing**

spAPs were detected using a threshold search in pClamp Software, Clampfit, (Molecular Devices.) EPSCs were detected using Mini Analysis Program (Synaptosoft). A digital LoPass Elliptic Filter with a cut-off-frequency of 50 Hz was applied. Automated detection had the following parameters: amplitude and area thresholds were set to 5 times the root means square calculated noise level, the fraction of amplitude used to determine decay time was set to 0.37, baselines were calculated by averaging 20 ms of trace 55 ms before the event peak. Automated detection was followed by time blinded manual verification. Electrophysiological
event data were averaged into one-minute bins. Due to the extreme variation in frequency and amplitude between cells, data were normalized to the bin prior to the baseline bin. Thus, the baseline group more accurately represents the spontaneous fluctuation between bins, and its mean is not necessarily equal to zero.

MICROSCOPY

Microscopy was performed as described previously (Zhao-Shea et al., 2011, 2013). In brief, mice received an euthanizing dose of sodium pentobarbital (200 mg/kg via intraperitoneal injection, and were transcardially perfused with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 10 ml of cold 4% (W/V) paraformaldehyde (PFA) in 0.1 M PBS. Brains were post-fixed for 2 h in 4% PFA and submerged in 30 % sucrose for 48 h. Coronal sections (25 μm) were obtained using a freezing microtome (HM430; Thermo Fisher Scientific, MA, USA) to assess viral expression and to perform immunohistochemistry. After washes in 0.1 M PBS, sections were mounted, air-dried and coverslipped with Vectashield mounting medium (Vector Laboratories). All slices were imaged using a fluorescent microscope (Zeiss, Carl Zeiss MicroImmagine, Inc., NY, USA) connected to computer associated image analyzer software (Axiovision Rel., 4.6.1).
Biocytin filling

Neurons were whole-cell patch clamped as described above except 0.5% biocytin (Sigma) was added to the internal solution. After current injection protocol to identify neuron type based on input resistance, neurons were filled for 10 min using whole-cell current clamp square wave (1 Hz, 500 ms pulse width, 300 pA pulses.) The pipettes were removed from the slice using the return to home position function of the Scientifica micromanipulator. After filling, slices were fixed in 4% paraformaldehyde in 0.1 M (PBS) at 4°C for 30 min then washed three times with PBS. Slices were then incubated overnight in blocking solution (PBS, 4%BSA, 0.5% Triton X-100) at 4°C. Slices were then incubated in blocking solution with Streptavidin-Alexa Fluor 595 conjugate (1:500, Invitrogen) before being washed three additional times with PBS. Labeling quality was determined with a standard fluorescence microscope (Axiovert 200M Zeiss) before slices were mounted for confocal imaging in Vectashield mounting medium (Vector Laboratories) and coverslipped.

Confocal Imaging and Morphologic Quantification

Morphological properties of the biocytin filled neurons were analyzed from confocal image stacks obtained at 40x magnification in a manner similar to previous studies (Klenowski et al., 2015). Images stacks containing total, untruncated dendritic arbors were acquired on a Zeiss LSM 700 confocal microscope using a Zeiss 40x/1.4 numerical aperture (NA) water immersion objective at z-separation of 0.5
μm. For spine quantification, images were obtained using a 63x/1.2 NA oil immersion objective at 1.5 zoom and z-step size of 0.3 μm. Representative spine images were obtained using the same objective at 2.5 zoom. Morphological properties (dendritic branching, length and dendritic spines) and Sholl analysis (Sholl, 1953) of filled cells were performed using Neurolucida™ software (MBF 301 Bioscience Inc) in a manner identical to previous reports (Klenowski et al., 2015, 2016). For Sholl analysis, the center of concentric spheres was defined as the center of the soma and a 10-μm radius interval was used. All neurons included for analysis exhibited high quality filling with intact dendritic arbors. Dendritic processes were classified as spines if their lengths were less than 6 μm (Harris and Kater, 1994).

STATISTICS
Statistical tests were performed in GraphPad Prism 7 Software (Graphpad Software Inc.). Data sets were tested for normal distribution and equal variances prior to analysis. Normality was assessed by D'Agostino & Pearson normality tests or Shapiro-Wilk normality tests to determine if parametric or nonparametric tests were appropriate. Equal variance was determined by the F-test. For statistical tests of Gaussian data, significance was determined by paired or unpaired two-tailed t-tests for two column analysis, one-way ANOVA for three or more columns as indicated, or a two-way ANOVA. Unequal variation was corrected with Welch's correction. Tukey's multiple comparisons test and Bonferroni's multiple
comparisons test were used for post hoc analysis when appropriate. In cases of Non-Gaussian data, Mann Whitney test was used in place of t-tests and Friedman test was used in place of one-way ANOVAs. No statistical methods were used to pre-determine sample sizes, but n numbers representing either animals in behavioral tests or cells in electrophysiological or morphological analysis were determined with reference to previous studies. Data are presented as the mean ± standard error of the mean (SEM). For ANOVAs and Friedman Tests, F values and Friedman statistics are reported as “F (degrees of freedom for interaction, total degrees of freedom) = F-value.”
CHAPTER IV

Discussion
Summary

This thesis provides direct, optogenetic evidence for a novel VTA→IPN, mesointerpeduncular, circuit capable of influencing anxiety-like behaviors in mice. The mesointerpeduncular circuit connects the classical fear/anxiety circuits with the emotion coding MHB-IPN axis critical in the modulation of nicotine intake and withdrawal. Signaling in this circuit is both critical for the expression of nicotine withdrawal-induced anxiety and the modulation of baseline anxiety in drug naïve mice. In nicotine withdrawal, the Type 2 neurons of the IPI subregion of the IPN express functional CRFR1. In acute brain slices from chronic nicotine treated mice, CRF activates CRFR1 to increase the amplitude of the MHB→IPN glutamatergic EPSCs of Type 2 neurons. Presumably, activation of CRFR1 activates a Gαs signaling cascade to modulate the glutamatergic receptors. In acute brain slices taken from chronic nicotine treated mice, this increased stimulation resulted in the normally silent Type 2 neurons firing action potentials in a dramatic bursting pattern. Theoretically the Type 2 neurons innervate extra-IPI circuits to produce anxiety and this idea could be tested further with a yet-to-be-generated CRFR1-CRE mouse line.

Our collaborators in the George lab found that, after chronic nicotine treatment, the VTA DAergic neurons begin to express CRF (Grieder et al., 2014). Knockdown of the mRNA responsible for CRF expression with shRNA in the VTA ablated precipitated withdrawal anxiety, suggesting the VTA may be the source of CRF in the IPN necessary for expressing nicotine withdrawal anxiety.
However, mRNA expression levels suggest the VTA only produces CRF after exposure to chronic nicotine (Zhao-Shea et al., 2015). What behavior could the presumed VTA DAergic terminals elicit through the circuitry for nicotine withdrawal anxiety in the drug naïve state?

The hypothesis that DA is released in the mesointerpeduncular circuit and this is tied to anxiety-like behaviors was tested in free moving mice in the EPM. DA release was measured by expressing GRAB$_{DA2m}$, an improved version of the genetically encoded GPCR activation-based-DA sensor GRAB$_{DA1m}$ in the IPN. Through DA binding induced GFP florescence, DA release was detected in the IPN and found that DA release increased as mice navigated the anxiogenic open arm of the elevated plus maze. The timing of DA release suggests that the mesointerpeduncular circuit is activated to suppress anxiety and allow the animal to enter and explore the arms avoided by anxious mice. This is opposed to the idea that the mesointerpeduncular circuit is a simple signal for environmental danger.

Next, the mesointerpeduncular circuit was manipulated to test if activity in this circuit is sufficient to drive or prevent anxiety-like behavior in the EPM. NpHR and ChR2 manipulation of VTA terminals in the IPN resulted in an anxiolytic and angiogenic response respectively in the EPM. Furthermore, downstream interpeduncular circuitry through which the VTA DAergic signal is propagated was assessed in drug naïve mice. Using cell-attached patch-clamp electrophysiology in acute mouse midbrain slices, two neuronal populations of the vIPN were defined
by input resistance and response to exogenous application of DA. “Type A” neurons displayed low input resistance and responded to DA with an increase in spAP frequency, while “Type B” neurons exhibited a higher input resistance and responded to DA with a decrease in spAP frequency. The location of D1 receptors and their mechanism were assessed by whole-cell patch clamp recordings. DA application elicited changes in spEPSC frequency, but not amplitude in vIPN neurons. These data suggest DA acts on vIPN Type A and B neurons through presynaptic D1-like DA receptors.

Next, the ability of the VTA to release dopamine in the IPN was tested by ChR2 based VTA terminal stimulation. Using viral mediated gene delivery and CRE-Lox technology, channelrhodopsin-2 (ChR2) was expressed specifically in putative DAergic VTA neurons of DA transporter (DAT)-CRE mice. Optogenetic stimulation of VTA terminals in the IPN resulted in spAP frequency changes prominently in the caudal IPN. These responses were blocked by D1-family DA receptor (DRD1) antagonists and were localized to areas containing putative DRD1-expressing cell bodies. Dopaminoceptive cIPN neurons exhibited significantly higher input resistance compared to Type A or B neurons suggesting a third neuronal subtype, “Type C”. Could these Type C DRD1 expressing neurons transmit the VTA based signal to the vIPN?

Optogenetic stimulation of putative DRD1-expressing Type C terminals in the presence of TTX and 4AP in the vIPN of DRD1-Cre mice resulted in EPSC frequency changes in Type A and Type B neurons that phenocopied the response
to exogenous DA application. Thus, it appears the sparse DAergic connection with the VTA is amplified by the DRD1 expressing Type C interneurons.

The neurotransmitter released from DRD1 expressing Type C neurons was then tested via whole cell EPSC recordings combined with pharmacology. Combined, GABA-A and GABA-B receptor antagonists were sufficient to block mEPSC frequency changes suggesting Type C neurons are primarily GABAergic and the signal is propagated to glutamatergic terminals through both GABA receptor subtypes. This mode of signaling where a single signal results in two opposite outcomes could be explained by a difference in the characteristics of the glutamate terminals in the IPN. Indeed, unlike most glutamatergic terminals in the CNS, MHb terminals are excited by GABA (Zhang et al., 2016).

The two opposing responses could be explained if GABA-activated, MHb terminals preferentially innervate Type A neurons, while non-MHb, glutamatergic terminals (that are inhibited by GABA) preferentially innervate Type B neurons. This hypothesis was tested by stimulation of terminals in the Chat-ChR2 mouse. The major source of chat positive terminals in the IPN is the MHb (Mata et al., 1977). Additionally, cholinergic/glutamatergic terminals preferentially release only glutamate under low frequency stimulation (Ren et al., 2011). Therefore, low frequency terminal stimulation in this mouse line should only stimulate glutamate from vMHb terminals. The experiment supported the hypothesis because stimulation of the MHb terminals resulted in stronger more consistent glutamatergic activation in Type A vIPN neurons. In Type B neurons light
stimulation resulted in much weaker currents and sometimes failed to evoke EPSCs. A similar result was obtained by fr stimulation in cats (Lake, 1973).

If the differential innervation hypothesis is correct, the dendrites of Type A and B neurons may be morphologically distinct as form accommodates function. To further characterize the Type A and B neurons and to provide support of their different innervation, vlPN neurons were filled with biocytin and their dendrites were traced. Type A neurons showed significantly more complex, spiraling dendritic arbors. The spiraling pattern is reminiscent of the “tornado”-like MHb terminals in the IPN (Bianco et al., 2008; Pang et al., 2016). In contrast, the Type B neurons showed much less complexity and simple, thin dendrites. The difference in dendritic arbors is suggestive of differences in innervation.

Finally, the effect of this circuit on anxiety-like behaviors was tested by pharmacological manipulation of the D1-like DA receptor in the IPN. Infusion of the D1 antagonist into the IPN resulted in increased anxiety as measured by the EPM, while D1 agonist decreased anxiety in the same test. Together, these results identify a novel microcircuit by which the VTA controls activity of the IPN to potentially modulate affective behavior.

Relating Types 1 and 2 with Types A, B, and C.

Chapter II divides IPI neurons into Type 1 and Type 2. Chapter III divides vlPN neurons into Type A and Type B, and I designate the heterologous clPN neurons “Type C”. However, because the IPI is part of the vlPN, the Type 1 and 2 neurons
overlap anatomically with the vIPN Type A and B neurons. Thus, an exploration of the possibility of overlap between the neuron types in these two chapters is necessary.

Differences in neuronal properties between the types in Chapters II and III can be tested by examining the physiological properties of the neurons. First, I will recap the properties that defined the neuronal types. Type 2 neurons were distinguished from Type 1, by their more hyperpolarized resting membrane potential, lower membrane capacitance, smaller hyperpolarization activated (Ih) current, and the presence of functional CRFR1 expression. Because Type 1 neurons consist of any IPI neuron that is not Type 2 and IPI neurons do respond to DA, I hypothesize that Type 1 neurons may also be classified as Type A or B neurons.

Type A and B were distinguished via their input resistance, dendrite morphology, and the EPSC and spAP changes in response to DA. Finally Type A and B were distinguished from Type C by the anatomical location of their soma in the vIPN rather than the cIPN and further through their input resistance.

Unfortunately, there is insufficient data to compare some of these properties between Chapter II and III neurons. First, there are no stable recordings of a Type 2 neuron responding to bath application of DA. The absence of this data is due to a combination of not actively seeking it, the low firing rate of nicotine naïve Type 2 neurons being inappropriate for cell attached experiments, the scarcity of the neuron, and its smaller cell body size increasing recording difficulty. In one case
Type 2 EPSCs were examined during DA application. However, the observed increased frequency did not washout and the baseline frequency was too unstable to form a conclusion. Furthermore, although the CRFR1 pattern clearly correlates with the IPN I do not have single cell RTPCR data and electrophysiology data for this receptor in all cell types. Lastly, there is insufficient morphology data for Type 2 neurons.

In contrast, there is sufficient data for drawing conclusions from membrane capacitance, resting membrane potential, Ih current, and input resistance for all cell types (Figure IV.1, Table IV.1).

Comparing Type 1 to Type A, B and C reveals a significant difference in at least one physiological property between groups (Table IV.1). In contrast, there was no significant difference in any parameter between Type 1 and the combination of Type A and B. This suggests that Type 1 is not the same as any other pure cell type. However, Type 1 could be a mix of Type A and B. If it is, we may expect a near equal ratio of A and B as seen in the vIPN. Alternatively, the proposed clustering of neurons in Chapter III suggests that the IPI has a higher portion of Type A neurons. Indeed 17/32 or 53% of Type 1 neurons fall within two standard deviations of the Type A neuron input resistance. In addition, 8/11 or about 73% of “Type 1” neurons exhibit a Type A like DA response. The remainder exhibit Type B-like DA response. However, the bias towards a Type A response may be a coincidence due to the small sample size. While inconclusive, this evidence does not rule out the possibility that Type 1 neurons are a mix of Type A and B neurons.
Because there is no evidence Type 1 neurons are distinct from a mix of Type A and B neurons, I consider Type 1 neurons to be a mixture of Type A and B in the current hypothetical model (Figure IV.2).

The expression pattern of CRFR1 in the IPI and the presence of functional CRFR1 suggests Type 2 neurons are a unique cell type (CHAPTER II). Indeed, Type 2 neurons are significantly different in at least one physiological parameter from every other cell type including the mix of Type A and B (Table IV.1). Indeed, Type 2 neurons have the most distinct physiological properties, except for input resistance, compared to the other proposed neuronal types (Figure IV.1). Together these data suggest that Type 2 neurons are a distinct neuronal type independent of any other neuronal type.

Optogenetic inhibition of vMHb cholinergic neurons resulted in decreased Type 2 cFos expression and anxiety behaviors during precipitated nicotine withdrawal (Zhao-Shea et al., 2015). This result suggests the Type 2 neurons receive the majority of their glutamatergic input from the vMHb, similar to Type A neurons. However, the possibility that this is an indirect connection remains. I cannot conclude with certainty that Type 2 neurons have Type A style or Type B style glutamatergic afferents. Also, the electrophysiology data suggests that most Type B neurons receive at least partial input from the MHb. Without evidence to the contrary my hypothetical model will suggest the simplest explanation, that MHb dominated inputs innervate Type 2 neurons (Figure IV.2).
The conclusion that Type 2 neurons are strongly enervated by the vMHB has interesting implications. While there is no data to suggest that Type C neurons innervate the glutamatergic afferents to Type 2 neurons, it is possible that they do. In which case DA would likely excite Type 2 neurons. Because DA release in the IPN is anxiolytic, this possibility does not mesh with the simple model of Type 2 neurons signaling anxiety. Therefore, there may be room for a more complex anxiety circuit. Perhaps inputs to Type 2 neurons act like Type B neurons in the absence of certain signals including CRF. Or maybe Type 2 neurons have postsynaptic DA receptors of their own or do not respond to DA at all.

Future experiments could fill in the gaps by examining Type 2 response to DA application and dendritic arbors. Additionally, examining differences in expression of mRNA and proteins, especially receptors could help resolve the cell types further.
Figure IV.1. Comparison of physiological properties across proposed neuronal types of the IPN. Bars represent mean ± SEM. 

A) Membrane capacitance  n=21-100.  

B) Resting membrane potential  n=22-90  

C) Hyperpolarization activate current (Ih)  n=10-41  

D) Input resistance  n=10-83.  

For tests of significance see Table IV.1. For methods refer to the methods sections in CHAPTER II and III.
Table IV.1. Statistics for Figure IV.1.

<table>
<thead>
<tr>
<th>Membrane capacitance</th>
<th>Resting Membrane Potential</th>
<th>Ih current</th>
<th>Input Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vs 2</td>
<td>**** &lt;0.0001</td>
<td>** 0.0012</td>
<td>**** &lt;0.0001</td>
</tr>
<tr>
<td>1 vs A</td>
<td>Ns 0.5371</td>
<td>ns</td>
<td>**** &lt;0.0001</td>
</tr>
<tr>
<td>1 vs B</td>
<td>Ns &gt;0.9999</td>
<td>ns &gt;0.9999</td>
<td>** 0.0054</td>
</tr>
<tr>
<td>1 vs A+B</td>
<td>Ns &gt;0.9999</td>
<td>ns &gt;0.9999</td>
<td>* 0.0181</td>
</tr>
<tr>
<td>1 vs C</td>
<td>Ns &gt;0.9999</td>
<td>Ns 0.1093</td>
<td>ns &gt;0.9999</td>
</tr>
<tr>
<td>2 vs A</td>
<td>**** &lt;0.0001</td>
<td>**** &lt;0.0001</td>
<td>**** &lt;0.0001</td>
</tr>
<tr>
<td>2 vs B</td>
<td>** 0.0032</td>
<td>** 0.0032</td>
<td>ns &gt;0.9999</td>
</tr>
<tr>
<td>2 vs A+B</td>
<td>**** &lt;0.0001</td>
<td>**** &lt;0.0001</td>
<td>* 0.0181</td>
</tr>
<tr>
<td>2 vs C</td>
<td>**** &lt;0.0001</td>
<td>**** &lt;0.0001</td>
<td>ns &gt;0.9999</td>
</tr>
<tr>
<td>A vs B</td>
<td>** 0.0086</td>
<td>** 0.0086</td>
<td>0.4682</td>
</tr>
<tr>
<td>A vs A+B</td>
<td>Ns 0.9517</td>
<td>ns 0.9517</td>
<td>** 0.0045</td>
</tr>
<tr>
<td>A vs C</td>
<td>Ns 0.7659</td>
<td>Ns 0.7659</td>
<td>** 0.0056</td>
</tr>
<tr>
<td>B vs A+B</td>
<td>Ns 0.5082</td>
<td>Ns 0.5082</td>
<td>* 0.0107</td>
</tr>
<tr>
<td>B vs C</td>
<td>Ns &gt;0.9999</td>
<td>Ns &gt;0.9999</td>
<td>ns &gt;0.9999</td>
</tr>
<tr>
<td>A+B vs C</td>
<td>Ns &gt;0.9999</td>
<td>Ns &gt;0.9999</td>
<td>* 0.0422</td>
</tr>
</tbody>
</table>

Table IV.1. Significance between groups in Figure IV.1. For each property, the column lists its significance as a star value and its calculated p-value. Ns p>0.05, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, one-way ANOVAs with Dunn’s post tests.
**Figure IV.2.**

*Control of Anxiety-like Behaviors*

*Figure IV.2. Expanded schematic diagram of IPN anxiety circuitry*

Circles represent neurons, the lines originating from the circles represent axons and the triangles represent terminals. The terminals are set so that the side of the triangle opposite the axon faces its presumed target. Each color represents a unique population of neurons. Dotted lines indicate more uncertain connections. Symbols represent neurotransmitters and their receptors. They are labeled in the legend on the top right and are placed in their presumed locations within the circuit diagram. Note that Type 1 is considered a mix of Type A and B neurons.
Anxiety and fear vs exploration of novelty in the behavioral tests

The emotions felt by a rodent cannot be directly measured nor can the animal directly communicate its feelings, and thus, anxiety tests measure “anxiety-like behaviors.” The OFT and EPM tests used in this thesis are both considered tests of anxiety-like behaviors because they have been repeatedly verified to represent anxiety by pharmacology (Choleris et al., 2001; Pellow et al., 1985). Administration of anxiolytic and anxiogenic substances consistently changed these anxiety-like behaviors. Additionally, exploration of the open space increases levels of the stress hormone corticosterone (Rodgers et al., 1999). Both the OFT and EPM present a choice: either to hide in cover or explore the novel open area. The height of the EPM and the open space of the OFT are thought to produce stress and unconditioned fear in mice (Walf and Frye, 2007). This suggests that the anxiety level of the rodent determines how it will react to stress and unconditioned fear. Therefore, anxiety and fear are interconnected emotions each enhancing the other and they inhibit exploration of novel environments.

Caveats and future directions

The conclusions in chapters II and III could be strengthened by better visualization of the DAergic terminals in the IPN. The limited visualization may be a result of poor representation in the subpopulation of DAergic VTA neurons projecting to the IPN in the DAT-Cre mouse line. Indeed, the terminals appear denser in the TH-cre
mouse and after chronic nicotine treatment than in the nicotine naïve DAT-Cre mouse (Zhao- Shea et al., 2015) (CHAPTER III Figure III.9 and unpublished observations). However, the alternative Cre line for DAergic neurons, the TH-Cre mouse, adds a caveat to the experiments. There are approximately a half-dozen TH mRNA positive but TH protein negative neurons found in the ventral IPL of the IPN (Choi et al., 2012). Leaking virus to these neurons could result in incorrect identification of VTA terminals and optogenetic stimulation of IPN neurons rather than DAergic VTA neurons. The DAT-Cre mouse is also thought to cover more DAergic neurons and have less chance of infecting GABA positive neurons in the VTA (Lammel et al., 2015).

Future studies can investigate the subpopulation of VTA neurons that project to the IPN. This would not only provide more details for manipulating the mesointerpeduncular circuit, but also help define the population of VTA neurons involved in suppressing anxiety. This subpopulation may also project to other areas to suppress additional forms of anxiety. These details may be important clues for resolving anxiety circuits involved in anxiety disorders and provide inspiration for future therapies. Indeed, activation of the mesointerpeduncular circuit itself may one day be used to treat anxiety disorders or drug addiction.

The VTA subpopulation projecting to the IPN can theoretically be determined by a precise infusion of a retrograde tracer. However, due to the proximity of the VTA which covers the dorsal surface of the IPN, this experiment has strict requirements for the injection volume, location of injection, angle of approach, control injections,
and microscopy to verify the infusion. The experiment may be more successful with nanoliter volumes or RetroBeads to prevent viral leak. The angle of approach is important due to the tendency of virus or tracers to backfill the needle track. Perhaps smaller needles and a specific angle could aid in this regard. Angling the injection from the caudal side of the brain to the IPN lowers the amount of VTA DAergic neurons encountering the needle track. However, in my experience such approaches tend to be lethal, presumably due to the needle track disrupting the autonomic functions of the hindbrain necessary for survival (Ghali, 2017). With a large enough data set the varied angles of approach may be informative for eliminating false positive neurons.

The VTA terminals visualized in my experiments are limited to a few small areas in the mouse brain making precision even more necessary. Utilizing a model organism with a larger brain could lower the precision requirements and provide information about the evolutionary conservation of the circuit. If the VTA subpopulation is genetically distinct, identification of the subpopulation could inform development of a new Cre line with better specificity for VTA neurons that innervate the IPN. Additionally, a technique to infuse the IPN from the ventral side of the skull would help alleviate this caveat because there would be no needle track running through the VTA.

Reliance on Cre lines also adds caveats to data interpretation. More often than not Cre lines have erroneous Cre expression confounding experiments (Stuber et al., 2015). My conclusions could be strengthened by a meticulous characterization and
screening of Cre lines in the target region. Future studies of the genetic identities of IPN and VTA neurons could lead to better conditional Cre lines and strategies for specific cell type targeting.

Although the current study “passes the torch” of anxiety along the dorsal diencephalic conduction system from Yamaguchi’s ablation of the septum to the current studies of IPN microcircuitry, future research should clarify which IPN projection(s) are important for anxiety. Could the IPN’s control of serotonergic regions feed back to the VTA, hippocampus, and amygdala? Could the IPN connect to the amygdala centric anxiety circuit by feedback to VTA through regions like the LDTg? There are many possibilities previously covered in the introduction chapter.

Another question remains, is the action of both Type A and B necessary or is one more important than the other? What about Types 2 and C? These questions could be assessed by precise manipulation of cell types through genetic constructs like cre lines. Selecting lines for these experiments requires better characterization of the mRNAs expressed in these neuron types. Alternatively, fluorescent retrograde tracers and viruses can mark neurons based on their projections to specific targets. Recording from the fluorescently marked neurons may reveal a cell type bias. Then retrograde optogenetics or DREADDs can be used to manipulate behavior (Roth, 2016).
Further characterization of IPN activity in vivo is necessary for clues on how it processes information and what the differential excitation of its neuronal types encodes. It is possible that neuroadaptations could be discovered in more anxious mice that give insight into types of anxiety disorders. Manipulation of these circuits could provide mouse models for studying anxiety disorders.

Future experiments should also reexamine the mesointerpeduncular circuit’s role in other drugs of abuse. The activity of Type 2 IPI neurons could be more closely examined in other drugs of abuse. Specifically, production of a CRFR1-Cre line could provide an invaluable tool for examining both withdrawal and drug naïve anxiety. Additionally, as an anxiety inducing neuroadaptation this circuit is closer to the theories behind anxiety disorders themselves. If this line was created, in vivo optogenetic stimulation and inhibition of the Type 2 IPN neuron should be examined in a battery of behavioral tests such as EPM, MBT, OFT, conditioned place preference/aversion, tail suspension test for depression, free moving behavior in the home cage, social interaction tests etc. Also, characterization of Type 2 morphology and efferents could add another level to our understanding of the circuitry in the IPN. A whole new thesis could be produced by experiments stimulating and inhibiting Type 2 projections. Additionally, the CRFR1-Cre line could be used to test if optogenetic stimulation is sufficient to create withdrawal symptoms in chronic nicotine drinking mice that still have nicotine in their system or nicotine naïve mice. Sufficiency suggests Type 2 neurons may be more downstream than Type A and B neurons if Type 2 neurons are not interneurons. If
Type 2 neurons are insufficient to generate anxiety in nicotine naïve mice, it may indicate the presence of other critical nicotine induced neuroadaptations. Another experiment could utilize in vivo calcium imaging to investigate if Type 2 neurons are differentially activated by stressors in chronic nicotine vs nicotine naïve animals. This could provide insight into why stressful situations tend to incite relapse to drug seeking behaviors (Mantsch et al., 2016).

Research could also examine other IPI specific or even vIPN specific genes for example: Grin3a is an IPI specific gene encoding NMDA receptor subunit 3A with a similar expression pattern to CRFR1 suggesting it could be colocalized to the Type 2 neurons (Table II.2). NMDA receptors containing this subunit may be regulated by CRFR1 and thus, be responsible for Type 2 neuronal excitability and nicotine withdrawal induced anxiety. In human studies, Grin3a is heavily associated with addiction especially to nicotine and opioids (Chen et al., 2018, 2019; Ma et al., 2010). Examination of NMDA receptors containing this subunit in the IPN could lead to further discoveries about the function of Type 2 neurons. If the addiction promoting effects of Grin3a polymorphisms are neuroanatomically located in Type 2 neurons, it would reinforce the circuit as a target for the treatment of nicotine addictions and expand the possibilities to other drugs of abuse.
Major Questions Remaining in the Anxiety Field

Anxiety opposes exploratory behaviors. It is kept in balance by the circuitry of the CNS to help an organism choose behavioral outcomes favorable for survival. Although the data in this thesis and others expose several balance points in the anxiety system, the wide range of anxiety inducing and inhibiting situations suggest a complex system. The question remains: Are there other important balance points that have yet to be discovered? The mesointerpeduncular circuit was found by perturbing the brain with withdrawal anxiety inducing nicotine. However, the IPN might not be the substrate of anxiety for all drugs of abuse. Future studies examining other drugs of abuse should pay special attention to the neuroadaptations behind withdrawal-induced anxiety. Not only can this resolve circuits to curb addiction, it may also lead to the discovery of other novel balancing points for anxiety behaviors. The mesointerpeduncular circuit is an intersection between the “rewarding” VTA and the “aversive” MHb-IPN. Other circuits where positive reinforcing and negative affective signals merge may similarly be balancing points of the anxiety system.

We have shown that the IPN is critical for nicotine withdrawal and unpublished data from our lab shows that cocaine withdrawal does not produce the same IPI cFos pattern. However, it remains to be seen if other drugs of abuse create withdrawal-induced anxiety through the IPN. The specific, cFos-inducing activation of Type 2 neurons may not be a consistent feature in the activation of the interpeduncular anxiety circuit.
The field has yet to establish which brain areas are involved in which features of anxiety or anxiety disorders. The differential effect of anxiolytics on anxiety make it clear that different circuits should govern different anxiety disorders. How does the anxiety of the dorsal diencephalic conduction system differ from amygdala centric and cortical anxiety circuits?

The specific neuroadaptations behind anxiety behavior in anxiety disorders have not been demonstrated. Do the neural correlates of specific anxiety disorders correlate with their own specific anxiety circuits? While the data in this study highlight adaptations responsible for nicotine withdrawal induced anxiety-like behavior, I have not expanded this to commonly diagnosed anxiety disorders. Some of the failure to answer this question may be due to the limitations of mouse models. However, the questions we can ask with animal models are nowhere near tapped out. There are still many important experiments that can inspire human studies and ultimately locate, describe, and therapeutically modify human physiology.

In conclusion, the field of anxiety-associated behaviors has reached an exciting point. The missing extra amygdala circuits that separate fear from anxiety and the various types of anxiety can now be resolved. This work brings to the forefront one such circuit, the mesointerpeduncular pathway. Decoupling of negative emotions in this circuit and others are likely causal to the many different kinds of anxiety disorders. Therapies targeting these circuits could provide more potent and effective treatments with less side effects for anxiety disorders in humans.


Bandelow, B., Zohar, J., Hollander, E., Kasper, S., Möller, H.-J., and World Federation of


Chaudhury, D., Walsh, J.J., Friedman, A.K., Juarez, B., Ku, S.M., Koo, J.W., Ferguson,


Nature 496, 224–228.


Klemm, W.R. (2004). Habenular and interpeduncularis nuclei: shared components in


increase activated by GABAB and dopamine D2 receptors in rat substantia nigra neurones. J. Physiol. 401, 437–453.


Liu, J., Perez, S.M., Zhang, W., Lodge, D.J., and Lu, X.-Y. (2011). Selective deletion of the leptin receptor in dopamine neurons produces anxiogenic-like behavior and increases


Pomerleau, O.F., Pomerleau, C.S., Mehringer, A.M., Snedecor, S.M., Ninowski, R., and


and diversity of striatal GABAergic interneurons. Front. Neuroanat. 4, 150.


Wang, S., Wacker, D., Levit, A., Che, T., Betz, R.M., McCorvy, J.D., Venkatakrishnan,


Yan, Z., Song, W.J., and Surmeier, J. (1997). D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-


channels in the ventral tegmental area regulate behavioral responses to chronic stress.

Elife 7, 1–27.
