THE IPN REGULATES ANXIETY INDEPENDENTLY OF DRUG ADDICTION

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THE INTERPEDUNCULAR NUCLEUS REGULATES ANXIETY INDEPENDENTLY OF DRUG ADDICTION

Dedication page

This work is devoted to my parents, without whom I'd never have developed the curiosity to sustain the stamina necessary to keep pushing the following boulder up this hill.

To my wife, Bo, whose enthusiasm, brilliance, ambition, humor, and patience have been an example to which I aspire.

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ABSTRACT

THE INTERPEDUNCULAR NUCLEUS REGULATES ANXIETY

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Dr. Mariella De Biasi

This work is devoted to better understanding how a component of one of the principle anatomical intersections of cognitive, emotional, and motivational signaling regulates anxiety and addiction. The majority of my work focused on the interpeduncular nucleus (IPN). The IPN and its principle source of afferent signals, the medial habenula (MHb), comprise a junction of signaling within the diencephalic conduction system (DDC). Along with the medial forebrain bundle, the DDC is a highly conserved pathway by which signals from the limbic forebrain reach the midbrain and hindbrain. Work in our lab and others has implicated the MHb-IPN axis in the aversive affective and somatic withdrawal syndrome that manifests following cessation of chronic exposure to habit-forming drugs, including alcohol, nicotine, and opioids. Given that functional role, I sought to establish if this pathway regulates affect independently of chronic drug exposure.

In addition to my primary work, additional experiments I performed contributed to the growing understanding of how this pathway functions within the context of addiction. In particular, I contributed to studies focused on how the presence of the α5 nicotinic acetylcholine receptor subunit (nAChRs) in the IPN and its neighboring structure, the ventral tegmental area (VTA), influences predispositions to alcohol and nicotine addictions in animal models.

Using *in vivo* chemogenetics, viral tracing and receptor subunit rescue, microdialysis, and behavioral analyses, I worked to evaluate how perturbations of signaling within the IPN or VTA affected mouse behavior.

Overall, I have found that the IPN does indeed regulate affect independently of chronic drug exposure, and that signaling via the α5 nAChR subunit within both the IPN and VTA significantly regulates drug-taking behavior.

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18-MC	**18-methoxycoronaridine**
5-HT	**5-hydroxytryptamine, serotonin**
AAV	**Adeno-associated virus**
ACh	**Acetylcholine**
ACSF	**Artificial cerebral spinal fluid**
AMPA	**α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**
AMPA	**Amygdala**
ATP	**Adenosine triphosphate**
BAC	**Bed nucleus of the anterior commissure**
BBB	**Blood brain barrier**
BD	**Bipolar disorder**
BF	**Basal forebrain**
BNST	**Bed nuclei of the stria terminalis**
CNO	**Clozapine n-Oxide**
CNQX	**6-cyano-7-nitroquinoxaline-2,3-dione**
CP	**Caudate putamen**
CPP	**Conditioned place preference**
CRF1	**Corticotropin-releasing factor**
DA	**Dopamine**
DCC	**Dorsal diencephalic conduction system**
DHβE	**Dihydro-β-erythroidine**
dMHb	**Dorsal medial habenula**
DREADD	**Designer receptor exclusively activated by designer drugs**
DTg	**Dorsal tegmentum**
EPM	**Elevated plus maze**
EPSCs	**Excitatory post synaptic currents**
FC	**Frontal cortex**
fr	**Fasciculus retroflexus**
GABA	**Gamma-aminobutyric acid**
GAD	**Generalized anxiety disorder**
GnRH	**Gonadotropin-releasing hormone**
GPCR	**G protein-coupled receptor**
HA	**Hypothalamic nuclei**
HCN	**Hyperpolarization-activated cyclic nucleotide-gated channels**
hM3Dq	**Human M3 muscarinic receptor**
HPC	**Hippocampus**
ICSS	**Intracranial self-stimulation**
IP	**Intraperitoneal**

IPN	**Interpeduncular nucleus**
LC	**Locus coeruleus**
LDTg	**Lateral dorsal tegmentum**
LHb	**Lateral habenula**
MDMA	**3,4-methylenedioxymethamphetamine**
MHb	**Medial habenula**
miRNA	**microRNA**
MRI	**Magnetic resonance imaging**
MS	**Medial septum**
NAcc	**Nucleus accumbens**
nAChR	**Nicotinic acetylcholine receptor**
NBM	**Nucleus basalis of Meynert**
NE	**Norepinephrine**
NI	**Nucleus incertus**
Nic	**Nicotine**
NK1	**Neurokinin-1**
NK2	**Neurokinin-2**
NK3	**Neurokinin-3**
NKA	**Neurokinin A**
NKB	**Neurokinin B**
NMDA	**N-methyl-D-aspartate receptor**
NMDG	**N-methyl-D-glucamine**
OB	**Olfactory bulb**
OFA	**Open field arena**
PAG	**Periaqueductal gray**
PBN	**Parabrachial nuclei**
PNS	**Peripheral nervous system**
PPT	**Pre-pro-tachykinin**
SAC	**Saccharine**
SC	**Superior colliculus**
SEP	**Septum**
Sfi	**Septofimbrial nucleus**
SI	**Substantia innominata**
SNP	**Short nucleotide polymorphism**
SNP	**Substantia nigra**
SP	**Substance P**
SST	**Somatostatin**
SUDs	**Substance use disorders**
Tac1	**Mouse Tachykinin 1 gene**
TAC1	**Tachykinin 1 gene**
Tac2	**Mouse tachykinin 2 gene**

TAC2	**Tachykinin 2 gene**
TAC3	**Tachykinin 3 gene**
TAC4	**Tachykinin 4 gene**
TH	**Thalamus**
TS	**Triangular septum**
vMHb	**Ventral medial habenula**
VTA	**Ventral tegmental area**
WD	**Withdrawal**
WRA	**Wheel running activity**
WT	**Wild-type**
ZI	**Zona incerta**
α5*	**α5-containing nicotinic acetylcholine receptor**

CHAPTER 1: GENERAL INTRODUCTION

Portions of this section are derived from a published article:

McLaughlin I, Dani JA, De Biasi M. The medial habenula and interpeduncular nucleus circuitry is critical in addiction, anxiety, and mood regulation. J Neurochem. 2017 Aug;142 Suppl 2:130-143. doi: 10.1111/jnc.14008. Review. PubMed PMID: 28791703.

Anxiety & Addiction

In the United States, anxiety and substance use disorders (SUDs) rank among the most commonly diagnosed psychiatric conditions, with lifetime rates of 28.8% and 14.6%, respectively (J. P. Smith & Book, 2008). In 2017, over 70,000 individuals were lost to drug overdoses (Hedegaard, Minino, & Warner, 2018), representing an unprecedented number of drug related mortalities, with drug overdoses causing more injury fatalities than both guns and traffic accidents (Abuse, 2014; Services, 2019) in the United States. Perhaps unsurprisingly, SUDs have been identified as among the most commonly comorbid psychiatric disorders among those diagnosed with generalized anxiety disorder (GAD) (Wittchen, Zhao, Kessler, & Eaton, 1994). Epidemiological studies have shown that GAD is associated with accelerated progression from first ingestion of habit-forming drugs to the onset of dependence, and comorbid GAD and SUD significantly reduces the prospects of recovery from GAD (Conway, Compton, Stinson, & Grant, 2006). Complicating successful treatment of either is the tendency of withdrawal symptoms exhibited by individuals ceasing chronic use of habit-forming drugs to emulate some GAD symptoms (Back & Brady, 2008). In addition to GAD, a variety of other mental health disorders share symptomatology with withdrawal syndromes, including depression and suicidality, borderline personality, conduct, and anti-social personality disorders (Merikangas et al., 1998; Patriquin, Bauer, Soares, Graham, & Nielsen, 2015;

Trull et al., 2018). Further complications arise from the variability in the duration and severity of withdrawal symptoms, determined by the unique pharmacologies of the habit-forming drugs used by each individual (Back & Brady, 2008). Additionally, unique pharmacogenomic effects arising between individuals and specific drugs may result in distinct contingents of patients who warrant targeted therapeutic interventions. Currently, medications applied in psychiatric settings fail to reduce symptoms in 30-40% of individuals (Haile, Kosten, & Kosten, 2009; Patriquin et al., 2015). Accordingly, a basic understanding of the neurophysiology underlying predispositions to addiction, both generally and to specific drugs, will help to improve therapeutic approaches to this rapidly growing challenge. Furthermore, given the high comorbidity of SUDs with anxiety-associated conditions, a more complete understanding of the neurophysiology underlying anxiety will likely reveal shared mechanisms, perhaps yielding novel opportunities to target both therapeutically.

Among the constellation of CNS regions that regulate anxiety, components of the dorsal diencephalic conduction system (DDC) have been implicated in animals and humans (Boulos, Darcq, & Kieffer, 2017; Stamatakis & Stuber, 2012; Torrisi et al., 2017), as well as the withdrawal syndrome from habit-forming drugs (Dani & De Biasi, 2013; Dani, Jenson, Broussard, & De Biasi, 2011; McLaughlin, Dani, & De Biasi, 2015, 2017; E. Perez, Quijano-Carde, & De Biasi, 2015a; Zhao-Shea et al., 2015)

The Dorsal Diencephalic Conduction System

The dorsal diencephalic conduction system (DDC) is a highly evolutionarily conserved neural pathway, identified in species including fish, mammals, and reptiles (Epstein, Hurley, & Taber, 2018). The majority of research has been devoted to a similar pathway, the medial forebrain bundle, which originates in anterior olfactory areas, sending

posterior projections via the lateral preoptic, lateral hypothalamic, and ventral tegmental area (VTA). Extending dorsally, the DDC consists of projections that travel along the epithalamus, with the stria medullaris and inferior thalamic peduncle representing the primary conduits of signaling, arriving at the epithalamic habenular complex (Sutherland, 1982). There are three central components of the DDC: the stria medullaris, the habenular complex (Hb), and the fasciculus retroflexus (Bianco & Wilson, 2009), with the medial habenula (MHb) representing the most dorsal component of the diencephalon (Okamoto & Aizawa, 2013) – the anatomy of which is represented in Fig 1.

Figure 1. Afferents & efferents of the MHb-IPN axis and broader DDC.

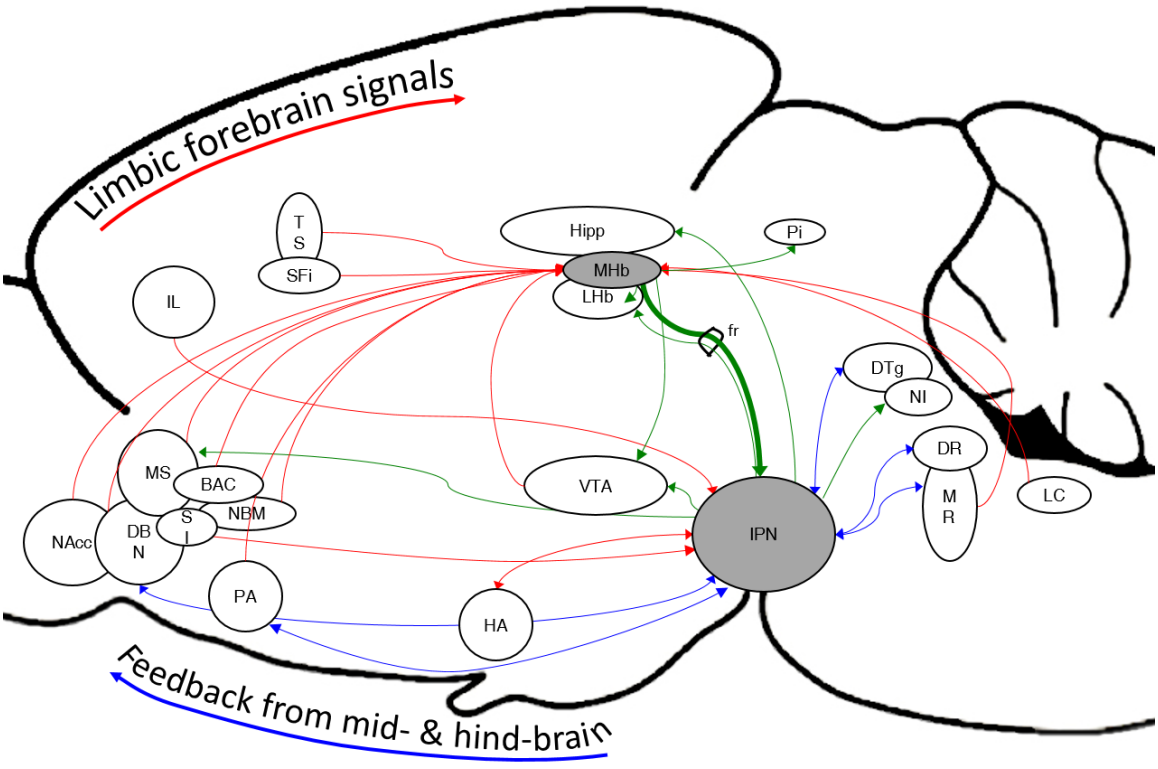

Figure 1. The medial habenula-interpeduncular nucleus pathway unites forebrain limbic with midbrain & hindbrain motivation & reward signaling. The medial habenula receives afferent inputs from a wide variety of forebrain limbic structures, and the interpeduncular nucleus sends afferent projections to a variety of midbrain & hindbrain structures implicated in the neurophysiology underlying addiction and a variety of mood-related psychiatric conditions. Accordingly, this pathway is a junction of signaling which regulates both addiction and mood-related conditions.

Overall, the DDC represents a principal pathway by which signaling in the forebrain is sent to, and received from, the mid- and hind-brain, and serves as a junction of cognitive, emotional, and sensory signaling, regulating motivation and reward (Gardon et al., 2014). Anatomically, the DDC is among the most densely populated regions with μ-opioid receptors in the central nervous system, with the MHb-interpeduncular nucleus (IPN) axis hosting expression to the exclusion of the lateral habenula (LHb) (Gardon et al., 2014). Studies of its functional properties by multiple groups have identified a role of the MHb-IPN pathway in the manifestation of the withdrawal syndrome following cessation of chronic intake of habit-forming drugs, including alcohol, nicotine, opioids, and stimulants (Boulos et al., 2019; J. Carlson, Armstrong, Switzer, & Ellison, 2000; De Biasi & Salas, 2008; Ellison, 2002; Molas, DeGroot, Zhao-Shea, & Tapper, 2017; Salas, Sturm, Boulter, & De Biasi, 2009; Zhao-Shea et al., 2015; Zhao-Shea, Liu, Pang, Gardner, & Tapper, 2013). While the habenular subnuclei remain prohibitively small to resolve using neuroimaging in humans, resting state functional connectivity reveals associations between the Hb and a variety of distal structures associated with the regulation of anxiety, including the medial prefrontal cortex, posterior insula, bed nucleus of the stria terminalis (BnST), hippocampus, and raphe nuclei (Torrisi et al., 2017).

The Medial Habenula

The Hb is an asymmetrical bilateral structure (Bianco & Wilson, 2009), phylogenetically conserved (Aizawa, Amo, & Okamoto, 2011; Torrisi et al., 2017), and broadly divided into two primary subdivisions, medial and lateral, though some studies have identified properties of both that might indicate further sub-anatomical distinctions due largely to the diversity of neurochemicals present within the structures (Aizawa, Kobayashi, Tanaka, Fukai, & Okamoto, 2012). Both the LHb and MHb send efferent projections

through the fasciculus retroflexus (FR), and some studies suggest that those originating in the LHb travel through the sheath of the bundle, while those from the MHb comprise the core (Bianco & Wilson, 2009). The LHb is comparatively larger, but the MHb hosts the synthesis of, and receptivity to, a surprisingly broad array of neurotransmitters. Those synthesized within the MHb include glutamate, acetylcholine (ACh), neurokinins, interleukin-18 (IL-18), and substance P (SP) (Aizawa et al., 2012). Neurotransmitters identified as likely being released within the MHb include ACh, substance P, adenosine triphosphate (ATP), γ-aminobutyric acid (GABA), and glutamate, as well as a variety of monoamines, including dopamine (DA), and norepinephrine (NE) from the locus coeruleus and superior cervical ganglion (Gottesfeld, 1983; Viswanath, Carter, Baldwin, Molfese, & Salas, 2013). Projections from the median raphe nucleus (Herkenham & Nauta, 1977), nucleus accumbens (NAcc), and ventral tegmental area (VTA) (Sutherland, 1982) have been identified within the MHb. The MHb also hosts among the highest levels of expression of the $GABA_B$ receptor in the brain (Bischoff et al., 1999; Charles et al., 2001; Durkin, Gunwaldsen, Borowsky, Jones, & Branchek, 1999; D. G. Wang, Gong, Luo, & Xu, 2006), as well as a variety of nicotinic acetylcholine receptors (nAChRs), with the majority of MHb neurons estimated to express nAChRs that include the α3, α4, β2, and/or β4 nAChR subunits (Fonck et al., 2009; Paolini & De Biasi, 2011; Sheffield, Quick, & Lester, 2000; Viswanath et al., 2013). The vast majority of efferent projections emerging from the MHb are conveyed via the FR, arriving at the interpeduncular nucleus (IPN) (Herkenham & Nauta, 1977).

The Interpeduncular Nucleus

While the MHb receives inputs from a broad array of forebrain nuclei, the interpeduncular nucleus (IPN) has a more limited afferent repertoire, with the MHb

providing the majority of inputs. However, the IPN sends efferent projections to a broader set of targets, spanning the forebrain to the hindbrain, many of which have been implicated in regulating affect, motivation, and reward (Lima et al., 2017; McLaughlin et al., 2017). Much like the MHb, the IPN is a rather dense structure, receptive to a variety of neurotransmitters. Anatomically, the IPN is oval-shaped, with only some bilateral organization (Bianco & Wilson, 2009). Neurons within the IPN tend to be rather small, hosting limited cytoplasmic space, and have been described as exhibiting neurosecretory characteristics in non-human species, containing large dense granules and beaded varicosities that were not observed to form synapses with neurons, but rather terminate in the interpeduncular cistern (Kemali, 1977; Morley, 1986). Similarly, other cells within the IPN appear to form connections with the pial vasculature of the IPN in humans (Kemali & Casale, 1982).

Similar to the MHb, despite being a relatively small and dense structure, studies have identified subdivisions of the IPN according to afferent and efferent distinctions, as well as the neurochemicals present (Kemali, 1977; Kemali & Casale, 1982; Lenn & Bayer, 1986; Quina, Harris, Zeng, & Turner, 2017). Two elegant tracing studies conducted recently demonstrate the topographical relationship between subnuclei within the MHb and IPN, as well as probable distinctions in efferent targets among IPN subnuclei (Lima et al., 2017; Quina et al., 2017). It is critical to be mindful of these anatomical nuances when interpreting the data collected over the course of my graduate work, as the technical limitations of methods currently available render precise functional sub-anatomical distinctions with behavioral relevance quite challenging. Regardless, a modulatory role relevant to a variety of behavioral, motivational, emotional, and endocrine activities played by the nucleus seems likely, given these anatomical features.

While the LHb has been implicated in the dopaminergic mechanisms underlying reward and addiction, the MHb-IPN axis has also been identified as playing a regulatory role. Signaling within the MHb-IPN axis has been implicated in the manifestation of aversive symptoms of withdrawal following chronic exposure to habit-forming drugs, including alcohol (Hwang, Suzuki, Lumeng, Li, & McBride, 2004; E. Perez et al., 2015a; Roux et al., 2015; D. G. Smith et al., 2001), nicotine (Dani & De Biasi, 2001; Dao, Perez, Teng, Dani, & De Biasi, 2014; De Biasi & Dani, 2011; Fowler, Lu, Johnson, Marks, & Kenny, 2011; Fowler, Tuesta, & Kenny, 2013; Gorlich et al., 2013; Jackson, Muldoon, De Biasi, & Damaj, 2015; Lotfipour et al., 2013; McLaughlin et al., 2015; Salas et al., 2009; Zhao-Shea et al., 2015; Zhao-Shea et al., 2013), psychomotor stimulants (J. Carlson et al., 2000; Ellison, 2002; Hussain, Taraschenko, & Glick, 2008; James, Charnley, Flynn, Smith, & Dayas, 2011), and opioids (Bajic, Soiza-Reilly, Spalding, Berde, & Commons, 2015; Darcq et al., 2012; Gardon et al., 2014; Neugebauer et al., 2013). Furthermore, infusion of 18-methoxycoronaridine, an α3β4 nAChR antagonist, into either the MHb or IPN has been shown to influence drug-taking behavior and symptoms of withdrawal from multiple habit-forming drugs (Glick, Maisonneuve, & Dickinson, 2000; Glick, Ramirez, Livi, & Maisonneuve, 2006; Glick, Sell, & Maisonneuve, 2008; Panchal, Taraschenko, Maisonneuve, & Glick, 2005; Rho & Glick, 1998; Taraschenko, Shulan, Maisonneuve, & Glick, 2007).

In addition to addiction, the MHb-IPN axis has been identified as relevant to several mood-related psychiatric conditions, either in humans or animal models, including depression (Faron-Gorecka et al., 2016; Hsu et al., 2014; Mirrione et al., 2014; Padilla, Shumake, Barrett, Sheridan, & Gonzalez-Lima, 2011; Ranft et al., 2010; Shumake, Edwards, & Gonzalez-Lima, 2003; Svenningsen et al., 2016), anxiety- and fear-associated behaviors (Agetsuma et al., 2010; Cirulli, Pistillo, de Acetis, Alleva, & Aloe,

1998; Facchin, Duboue, & Halpern, 2015; Jesuthasan, 2012; A. Lee et al., 2010; Mathuru & Jesuthasan, 2013; Sugama et al., 2002; Thompson, 1960; Yamaguchi, Danjo, Pastan, Hikida, & Nakanishi, 2013), and bipolar disorder (Savitz et al., 2011).

Role of the DDC in Addiction to Specific Drugs

Alcohol

The pharmacological activity of ethanol encompasses a broad range of targets in the central nervous system, and studies have indicated that the MHb-IPN circuit represents a significant component of its affective and behavioral effects. Local cerebral glucose utilization rates in alcohol-preferring rats are significantly elevated in both divisions of the habenular complex relative to non-preferring rats (D. G. Smith et al., 2001). Data from our lab have identified an interaction between ethanol and nicotine withdrawal, as well as a role played by nAChRs in the MHb or IPN in ethanol withdrawal. Specifically, intraperitoneal injection of a non-selective nAChR antagonist, mecamylamine, is capable of precipitating a withdrawal syndrome in mice chronically treated with ethanol. Withdrawal symptoms are also exhibited when mecamylamine is infused into the MHb or IPN, but not the ventral tegmental area (VTA) or hippocampus (E. Perez et al., 2015a), indicating that nAChR blockade in the MHb-IPN circuit is sufficient to precipitate the ethanol withdrawal syndrome. Another recent finding has identified changes in signaling molecules associated with apoptosis, inflammation, neurodegeneration, and senescence in the habenula, among other structures, following chronic treatment with ethanol (Roux et al., 2015). Receptor knock-out studies have shown that neuropeptide Y (NPY) acts as an important regulator of alcohol intake, with knock-out mice exhibiting increased alcohol ingestion relative to wild-type mice (Thiele, Koh, & Pedrazzini, 2002). More recently, it

was shown that alcohol-preferring rats exhibit an absence of NPY mRNA in the MHb while non-preferring rats do have a signal of mRNA for the neuropeptide (Hwang et al., 2004). Taken together, the literature suggests that activity in the MHb-IPN circuit likely modulates the effects and ingestive behavior of alcohol, as well as its withdrawal symptoms. Variations between individuals in the signaling within this circuit may underlie predispositions to pathological intake patterns.

Opioids

One of the densest regions of opioid receptor expression is the DDC, and the MHb, FR, and IPN are particularly rich with expression (Gackenheimer et al., 2005; Gardon et al., 2014; Sim-Selley, Daunais, Porrino, & Childers, 1999; Zhu, Hsu, & Pintar, 1998). Additionally, there is a diversity of neurotransmitters that the MHb-IPN circuit synthesizes or is sensitive to, including acetylcholine, neurokinins, interleukin-18 (IL-18) (Sugama et al., 2002; Viswanath et al., 2013), and purines (Kanjhan et al., 1999; Pankratov, Lalo, Krishtal, & Verkhratsky, 2009; Pankratov, Lalo, Verkhratsky, & North, 2006) that may be modulated by the activities of opioids. Given the broad efferent targets of the IPN that are known to regulate affect and substance use, including the raphe nuclei, nucleus incertus, lateral septum, lateral dorsal tegmentum (LDTg), and hypothalamus (Bianco & Wilson, 2009; Gardon et al., 2014; Morley, 1986; Ryan, Ma, Olucha-Bordonau, & Gundlach, 2011; Sutherland, 1982), the MHb-IPN circuit is likely an anatomical node, centrally involved in the signaling underlying both acute effects of, and withdrawal from, opioids. Evidence over the past several decades has corroborated such a role. For example, lesions of the MHb have been observed to induce hyperalgesia and increase the analgesic efficacy of morphine (Meszaros, Gajewska, & Tarchalska-Krynska, 1985), and morphine is capable of inducing analgesia when infused directly

into the habenular complex (Cohen & Melzack, 1985). When evaluating the effects of intracranial self-stimulation of the VTA on opioid release, a unique reduction of endogenous opioid binding was observed in the MHb (E. A. Stein, 1993). Following chronic morphine administration, altered acetylcholinesterase (AChE) activity is observed in the MHb, and precipitation of withdrawal with the opioid receptor antagonist, naloxone, resulted in altered AChE activity in the IPN (Neugebauer et al., 2013). Additionally, chronic morphine administration induced a trend towards increased nAChR expression in the MHb (Neugebauer et al., 2013). Acute administration of 18-methoxycoronaridine (18-MC), an α3β4 nAChR antagonist, reduced signs of naltrexone-precipitated withdrawal from morphine (Rho & Glick, 1998), an effect that appears to be mediated by activity in the MHb and IPN (Panchal et al., 2005; Taraschenko et al., 2007). 18-MC was also observed to reduce morphine self-administration upon intracranial infusion into the MHb or IPN (Glick et al., 2006). In the MHb, RSK2, a component of the ribosomal S6 kinase 90kDa family, which act as substrates of extracellular-regulated kinases 1 & 2 to regulate cytosolic and nuclear targets, has been identified as critical to morphine-induced analgesia (Darcq et al., 2012). Finally, the LDTg, an efferent target of the IPN, has been shown to exhibit significant increases in vesicular ACh transporter markers following chronic morphine administration (Bajic et al., 2015; Gardon et al., 2014). Altogether, these data implicate the DDC, and MHb-IPN circuit in particular, in the signaling underlying some of the acute effects of opioids, as well as aspects of their addictive properties. Furthermore, adaptations in cholinergic components may represent a significant facet of these changes.

Nicotine and Psychomotor Stimulants

The role of the LHb in regulating dopaminergic activity in the VTA via the rostromedial tegmental nucleus has been established, and represents an important mechanism by which aversion and addiction are modulated (Barrot et al., 2012; Jean-Richard Dit Bressel & McNally, 2014; Quina et al., 2015; Sanchez-Catalan et al., 2017). Both the LHb and MHb send dense efferent projections through the fasciculus retroflexus, and – as previously discussed - anatomical studies suggest that projections emerging from the MHb course through its core and those from the LHb through its sheath (Bianco & Wilson, 2009). Degeneration of dopaminergic fibers in the caudate following chronic exposure to psychomotor stimulants like amphetamines was observed decades ago (Ellison, Eison, Huberman, & Daniel, 1978). In addition to dopaminergic fibers, similar degeneration has been observed in axons populating the sheath of the FR following chronic exposure to cathinone, cocaine, amphetamine, methamphetamine, and MDMA (J. Carlson et al., 2000; Ellison, 2002). In rats treated with cocaine, increased expression of Fos-protein, a marker of neuronal activation, was observed in the MHb associated with cue-induced reinstatement (James et al., 2011). Additionally, similar to its interference with opioid-derived reward, 18-MC administration results in reduced methamphetamine self-administration in rats (Glick et al., 2000). Once again, direct infusions into the MHb and/or IPN induced similar reductions of self-administration, with what appears to be greater efficacy when infused into the IPN, suggesting that activity in the MHb-IPN circuit likely mediates this effect (Glick et al., 2008). Finally, methamphetamine and cocaine have been shown to increase extracellular concentrations of ACh in the IPN, with cocaine inducing a dose-dependent biphasic effect (Hussain et al., 2008).

Probably the best-characterized activity of a drug in the MHb-IPN pathway is that of nicotine, perhaps attributable to the considerable density of a variety of nAChRs that populate the structure (Mugnaini et al., 2002). Studies have suggested that up 90-100% of MHb neurons express nAChRs, with the majority containing the α3, α4, α5, β2, and/or β4 subunits (Sheffield et al., 2000; Viswanath et al., 2013). Some data suggest that approximately 20% of nAChRs in the MHb expressed by neurons that project to the IPN contain the α5 subunit (Grady et al., 2009; Picciotto & Kenny, 2013). In the IPN, high levels of α2 subunit-containing nAChRs can be found (De Biasi & Salas, 2008; Grady et al., 2009), and the distributions of nAChRs composed of specific subunit combinations can help distinguish subnuclei within both the MHb and IPN (Shih et al., 2014).

As many reviews of the effects of nicotine in this circuit have been written over the years (Dani & De Biasi, 2001; Dao et al., 2014; De Biasi & Dani, 2011; De Biasi et al., 2014; Jackson et al., 2015; McLaughlin et al., 2015, 2017), this section will focus on recent advances in characterizing the effects of chronic use and withdrawal from nicotine in the MHb-IPN pathway. For example, it was recently shown that, during withdrawal from nicotine, the spontaneous action potential frequencies in MHb cholinergic neurons are doubled after mice are administered nicotine relative to mice in withdrawal treated with saline (Gorlich et al., 2013). Further, these studies demonstrated that the pacemaking activities of MHb cholinergic neurons are determined by the activities of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, and pharmacological inhibition of these HCN channels resulted in the manifestation of nicotine withdrawal-associated behaviors, including both somatic and anxiety-associated symptoms. A study from our lab showed that nicotine enhances the intrinsic excitability of MHb neurons by activating α5-containing nAChRs, which results in the facilitation of

neurokinin release onto NK1 and NK3 receptors (Dao et al., 2014). Notably, pharmacological blockade of NK1 & NK3 receptors in the MHb of mice chronically treated with nicotine resulted in the precipitation of somatic symptoms of nicotine withdrawal. Further implicating the α5-containing nAChRs in the MHb in the physiology of nicotine addiction and withdrawal, mice lacking the expression of the subunit have been observed to self-administer doses of nicotine at levels that are aversive to wild-type mice, and virus-mediated re-expression of the subunit in the MHb rescues self-administration to levels resembling those consumed by wild-type mice (Fowler et al., 2011). Additionally, mice lacking the α5 nAChR subunit exhibit reduced IPN activation following exposure to nicotine relative to wild-type mice, suggesting a significant role played by this subunit in the MHb-IPN pathway in determining the range of nicotine doses capable of facilitating activity in brain reward circuitry (Fowler et al., 2011; Fowler et al., 2013). Mice lacking the α2 nAChR subunit do not manifest nicotine withdrawal signs (Salas et al., 2009). Furthermore, α2 null mice exhibit elevations of both glutamate and GABA in the IPN, suggesting that α2-containing nAChRs may participate in the signaling underlying the effects of nicotine (Lotfipour et al., 2013). Studies have also demonstrated a significant role played by IPN signaling in somatic symptoms of nicotine withdrawal, with GABAergic neuronal activation enhanced by increased glutamate release, perhaps from the MHb (Zhao-Shea et al., 2013). This group also showed that pharmacological inhibition of NMDA receptor activation reduced symptoms of withdrawal, suggesting a glutamatergic signal from the MHb playing a significant role in the nicotine withdrawal syndrome. Following chronic nicotine exposure, mecamylamine infusion into the MHb or IPN has been observed to induce anxiety-associated behaviors and symptoms of nicotine withdrawal (Salas et al., 2009; Zhao-Shea et al., 2015). Given that mecamylamine infusion into nicotine-naïve mice does not result in significant

changes in anxiety-associated behavior, the MHb-IPN axis represents a node of neuroplastic adaptations resulting from chronic nicotine exposure that underlie the affective and somatic symptoms of nicotine withdrawal upon cessation.

Accordingly, while not necessarily a direct pharmacological target of most psychomotor stimulants apart from nicotine, the MHb-IPN circuit likely represents a system that modulates the acute effects of psychomotor stimulants, and contributes to signaling underlying withdrawal and relapse.

Role of the DDC in Emotional States

Anxiety, fear, and stress

While anxiety and fear have been associated for quite some time with anatomical structures including the amygdala, hippocampus, hypothalamus, and periaqueductal gray, recent research has implicated the DDC in these behaviors as well (Okamoto & Aizawa, 2013). A genetic lesion study indicates that selective elimination of MHb afferents arriving from two forebrain structures, the triangular septum and bed nucleus of the anterior commissure, results in disrupted anxiety- and fear-associated behaviors (Yamaguchi et al., 2013). In particular, lesions of the projections from the triangular septum, which terminate in the ventral subnucleus of the MHb, disrupted anxiety-associated behavior. Conversely, lesions of projections arriving from the bed nucleus of the anterior commissure, which terminate in the dorsal subnucleus of the MHb, disrupted fear-associated behavior. Following both acute and chronic restraint stress in rats, significant elevations of the pro-inflammatory cytokine, IL-18, have been observed in the dorsal MHb (Sugama et al., 2002). Mast cells are another immune system-associated signaling component that appears to be sensitive to environmental stressors and aversive mood states (Frenzel & Hermine, 2013; Georgin-Lavialle et al., 2016; Nautiyal,

Ribeiro, Pfaff, & Silver, 2008; Silver & Curley, 2013). Mice treated with a 3-week behavioral subordination paradigm, either via exposure to an aggressor or placement in a clean cage, exhibited increased numbers of mast cells in the habenula, thalamus, and hypothalamus (Cirulli et al., 1998).

When characterizing fear-associated behavior in zebrafish, inhibition or lesion of the dorsal habenula (analogous to MHb in mammals) resulted in elevated freezing behaviors in response to a conditioned fear stimulus (Agetsuma et al., 2010; A. Lee et al., 2010). Both control and habenula-disrupted fish froze upon first exposure to an electric shock, but as subsequent shocks were administered, control fish exhibited reduced freezing behavior while those with disrupted habenular function exhibited no such behavioral adaptation. Another study identified behavioral signatures of elevated baseline anxiety in zebrafish following dorsal habenula lesions, including responses to novel environments and alarm substance secretion in response to overhead shadows (Mathuru & Jesuthasan, 2013). This has led to the suggestion that reciprocal connectivity between the IPN, raphe nuclei, and dorsal tegmental region may be critical to behavioral responses to stressors. Furthermore, activity in the habenular complex may be an upstream determining factor in selection of behavioral strategies to cope with stressors (Jesuthasan, 2012; Okamoto, Agetsuma, & Aizawa, 2012). A unique characteristic of the habenular complex is its asymmetry, with the left habenula larger than its right counterpart (Ahumada-Galleguillos et al., 2017; Hetu et al., 2016). When this asymmetry is lost in zebrafish, behavioral assays indicated elevated manifestations of anxiety, as well as elevated cortisol levels in response to stressors (Facchin et al., 2015).

While the size of the habenular complex renders current neuroimaging technology incapable of distinguishing the LHb from the MHb in human studies (Salas, Baldwin, de

Biasi, & Montague, 2010), some studies indicate a role played by the habenular complex in the pathophysiology of bipolar disorder (BD). In a study using high-resolution magnetic resonance imaging (MRI), it was found that patients diagnosed with BD who had either never been medicated, or had been un-medicated for at least two months, exhibited smaller habenular volumes than healthy controls (Savitz et al., 2011).

Studies have also implicated the IPN in regulating anxiety and fear. Early studies of the IPN identified a role for the structure in the retention of avoidance conditioning. Rats were trained to perform a jumping response following a visual stimulus to avoid a shock. After a learning period, a group of trained rats were treated with electrolytic lesions of the IPN. Following recovery from the procedure, rats re-learned the task and retention of the response was compared to controls. Rats with IPN lesions exhibited comparatively inferior retention of the response, though exhibited other signatures of a fear reaction, implying a role played by IPN signaling in specific components of fear learning (Thompson, 1960). Following chronic nicotine administration, increased corticotropin releasing factor (CRF) synthesis is observed in dopaminergic neurons in the VTA, which appear to send efferents to the ventral IPN (Zhao-Shea et al., 2015). This is accompanied by an increase in CRF1 receptor expression in a particular subnucleus of the ventral IPN, and withdrawal induces release of CRF by the VTA onto ventral IPN neurons. Blockade of CRF1 receptor binding in the IPN was shown to reduce anxiety-associated behavior generated during withdrawal from nicotine (Zhao-Shea et al., 2015).

Serotonin Signaling & Anxiety

Serotonin signaling has long been implicated in regulating anxiety-associated behavior, and defensive behavior more broadly, in animals. However, pharmacological and behavioral studies indicate that this role is complex, with serotonergic pharmacological

agents both facilitating and inhibiting behaviors associated with anxiety and panic in animal models (Francisco S. Guimarães, 2010; Viana, Graeff, & Loschmann, 1997). Further, while serotonergic signaling has been implicated in a broad range of behavioral and physiological functions, most can still operate in the absence of serotonergic signaling (Lucki, 1998). Serotonin can bind at least 14 receptor subtypes, resulting in a diversity of signaling both pre- and post-synaptically, rendering it among the most complicated of neurotransmitter systems (Grandjean et al., 2019; Hoyer, Hannon, & Martin, 2002). Evidence in humans – from discoveries of the mechanisms and efficacies of serotonergic medications (M. S. Eison, 1990; Goldberg & Finnerty, 1979; Kahn et al., 1986; D. F. Klein, 1964) to neuroimaging (Lanzenberger et al., 2007) - implicates serotonin signaling in anxiety-associated psychiatric conditions, including generalized anxiety, social phobia, and panic disorders (Lucki, 1998).

Serotonin & Anxiety in Humans

A broad set of symptoms, as well as a responsivity to pharmacological therapeutics, characterizes anxiety-associated psychiatric disorders. Anxiety ranks among the most common sources of psychiatric distress (Bandelow, Michaelis, & Wedekind, 2017; O. J. Robinson, Pike, Cornwell, & Grillon, 2019). It is also central to the most common psychiatric disorders – with generalized anxiety disorder and panic disorders the most prevalent in the United States, and regularly among the top, internationally (Sansone & Sansone, 2010). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), anxiety disorders all share features of excessive fear and anxiety – and it is the anticipation of a future threat that distinguishes anxiety from fear (*Diagnostic and statistical manual of mental disorders : DSM-5*, 2013). This distinction is

reflected in some of the ethological frameworks proposed over time that are useful to interpreting animal behavior in pursuit of mechanistic explanations of human psychiatric conditions. These frameworks will be discussed in later sections, after a review of proposed models of the role serotonin plays in anxiety.

While almost certainly oversimplifications, several heuristics have been proposed over time to model the relationships between anxiety and serotonin signaling. One example is the “see-saw model”, wherein increased serotonin signaling promotes anxiety while deficient serotonin signaling produces depression. As described by Stein & Stahl (D. J. Stein & Stahl, 2000), this model would propose that anxious states arise from elevated serotonin release – resulting in a neuroplastic compensatory downregulation of presynaptic 5-HT_{1A} autoreceptors. Ultimately, treatment with selective serotonin reuptake inhibitors (SSRIs) or 5-HT_{1A} receptor agonists, following a period of adaptation, results in a “normalization” of 5-HT_{1A} receptor signaling. Conversely, in conditions of depression, the 5-HT_{1A} receptor exhibits upregulation due to reduced serotonin release. Similarly, pharmacological therapeutics act to elevate serotonin signaling and downregulates the 5-HT_{1A} receptor.

An alternative model focuses on serotonergic modulation of specific neuroanatomical structures implicated in anxiety. This model arises, in part, from evidence that diminished serotonin levels over time is correlated with increased sensitivity to threatening stimuli (Handley, 1995; D. J. Stein & Stahl, 2000). Within this framework, the efficacy of SSRI treatments of anxiety can be explained by modulation of afferent amygdalar signaling arriving from the raphe nuclei, modifying the output from amygdalar subnuclei to regions that regulate defensive responses, such as the periaqueductal gray (PAG), locus coeruleus, and hypothalamic-pituitary-adrenal (HPA) axis (D. J. Stein & Stahl, 2000).

Among the initial recognitions of a role played by serotonergic signaling in human anxiety were assessments of pharmacological therapeutics for the treatment of psychiatric conditions. One example was an evaluation of the potential psychiatric utility of the tricyclic antidepressant, imipramine, by Klein & Fink (D. F. Klein & Fink, 1962), following an early trial conducted by Kuhn in 1957 (Brown & Rosdolsky, 2015), ultimately followed by a publication in English in 1958 (Kuhn, 1958).

Initially developed in pursuit of antipsychotic alternatives to chlorpromazine, the efficacy of imipramine in managing anxiety-associated distress – as opposed to depression - was highlighted by Kahn et al. (Kahn et al., 1986). However, in addition to serotonin, imipramine exerts reuptake inhibition upon multiple monoaminergic neurotransmitters (L. Zhang & Barrett, 1991). Accordingly, the discovery of the efficacy of buspirone – a serotonin 5-HT_{1A} receptor partial agonist (Loane & Politis, 2012) - in managing anxiety, underscored the role of monoaminergic signaling in the anxiety experienced by humans (A. S. Eison & Temple, 1986; Goldberg & Finnerty, 1979). Additionally, early pharmacological evaluations of fluoxetine were published, demonstrating activity as a selective serotonin reuptake inhibitor (SSRI), in 1974 (Wong, Horng, Bymaster, Hauser, & Molloy, 1974). While initially associated with the treatment of depression, SSRIs have since become first-line treatments for anxiety-associated conditions like GAD due to a side effects profile of comparatively lower severity (D. S. Baldwin & Polkinghorn, 2005; Bandelow et al., 2017).

Further confirmation of the critical role serotonin signaling plays in the therapeutic effect of SSRIs is derived from serotonin depletion studies performed in patients diagnosed with panic disorder, an anxiety-associated psychiatric condition. Fourteen patients (7 male, 7 female), who had undergone treatment with the SSRI, paroxetine, for panic disorder were evaluated in a double-blind, placebo controlled study. As the endogenous

synthesis of serotonin relies upon consumption of the essential amino acid, tryptophan (Moja, Cipolla, Castoldi, & Tofanetti, 1989), its deprivation was anticipated to affect anxiety-associated behavior when challenged with the panicogenic agent, flumazenil, due to consequential serotonin depletion (Bell et al., 2002). To test the necessity of serotonin availability to the therapeutic efficacy of the prescribed SSRI, the test group were administered either a tryptophan-free drink to induce serotonin depletion, or a drink including tryptophan. Indeed, the anxiolytic efficacy of SSRIs was shown to depend upon serotonin signaling. Similar studies have been performed, yielding similar effects on mood in participants with family histories of mood-associated conditions, though suggesting that the relationship between dietary monoamine amino acid precursors may not exert a direct effect on mood (Ruhe, Mason, & Schene, 2007).

Additional evidence derived from human studies was identified by Lesch et al., indicating that a polymorphism in the 5'-regulatory region of the serotonin transporter (SERT) was associated with anxiety-associated traits (Lesch et al., 1996), though subsequent studies suggest this correlation may be less clear (Owens & Nemeroff, 1998). However, when evaluating responsivity of children diagnosed with anxiety disorders to cognitive behavioral therapy (CBT), Lester et al. observed epigenetic changes in regions associated with serotonin signaling. Children diagnosed with an anxiety disorder – exhibiting a clinical severity rating of 4 or higher, using the Anxiety Disorders Interview Schedule for DSM-IV (ADIS-IV-C/P) - were evaluated for their DNA methylation patterns upstream of the serotonin transporter (SERT) promoter region, both before and after treatment with cognitive behavioral therapy (CBT). Children who exhibited a therapeutic effect of CBT exhibited significant elevations of DNA methylation at a particular CpG site located within the SERT promoter region, while those exhibiting no therapeutic response exhibited reductions in DNA methylation at the same site (Roberts et al., 2014).

Similarly, Stein et al. demonstrated that patients diagnosed with generalized social anxiety disorder, who participated in randomized controlled trials of SSRIs and received either paroxetine or fluvoxamine, appeared to respond favorably according to variants of the serotonin transporter gene. Specifically, patients expressing the same allele of the gene studied by Stein et al. - (the S, or “short” allele) - exhibited comparatively poorer response to treatment (M. B. Stein, Seedat, & Gelernter, 2006). This allele confers reduced efficiency of transcription (Goldman, Glei, Lin, & Weinstein, 2010), and therefore lower production of serotonin transporters, suggesting that differences in serotonin signaling may be involved in the differences in treatment responsivity. Similarly, the presence of a short allele for the 5-HTT was identified in a majority of individuals diagnosed with social anxiety disorder, and was associated with elevated trait anxiety (Furmark et al., 2004). Interestingly, Lenze et al. found genetic variation in the promoter region of the serotonin transporter correlated with differences in treatment efficacy of SSRIs for the treatment of GAD in adulthood – though results of the study suggested that the source of this variability was likely due to an effect on symptom variability rather than a pharmacogenetic effect of treatment (Lenze et al., 2010). While not an indication that serotonergic signaling is the sole source of pathological activity, these data indicate that it plays an integral role in at least the clinical confrontation of anxiety-associated conditions.

Neuroimaging studies have also implicated serotonin signaling in anxiety-associated conditions. Using SPECT imaging with a tracer capable of visualizing the serotonin transporter (5-HTT), I-β-CIT, van der Wee et al. identified higher 5-HTT binding in patients diagnosed with GAD (van der Wee et al., 2008). In patients with social anxiety disorder, Frick et al. found elevated rates of serotonin synthesis and/or transporter

availability in a network of brain regions that includes the amygdala, raphe nuclei, hippocampus, and insular cortex (Frick et al., 2015).

While this is not an exhaustive review of studies in humans implicating serotonergic signaling in anxiety-associated conditions, the balance of evidence indicates that these processes are critical to understanding their underlying neurophysiologies – and pharmacogenomic analyses may aid in explaining why some patients respond to current therapeutics more favorably than others. In particular, while reviews have been conducted (D. S. Baldwin & Polkinghorn, 2005; McGowan, 2019), pharmacogenomic studies focused on therapeutic interventions for anxiety-associated conditions may yield greater insights regarding patient response variability. This approach has been already applied in studies such as The Sequenced Treatment Alternatives to Relieve Depression (STAR*D), Munich Antidepressant Response Signature (MARS), and Genome-based Therapeutic Drugs for Depression (GEN-DEP) – all large genome-wide association studies (GWAS) assessing anti-depressant pharmacogenomics for the treatment of major depressive disorder.

Ethological Approaches to Anxiety

A substantial challenge to translating preclinical investigations of the neurophysiology of anxiety is interpreting the ethological relationships between defense- and survival-associated behavior exhibited by rodents and human anxiety. In particular, distinguishing anxiety- and fear-driven behaviors in assays of animal behavior is critical to efforts at mapping the governing neurophysiologies. For example, animal models of these behaviors often derive from responses to threat, while human research generally concentrates on analyses of psychiatric conditions (D. C. Blanchard, Hynd, Minke,

Minemoto, & Blanchard, 2001; Davis, Walker, Miles, & Grillon, 2010). Nevertheless, a foundation of literature suggests that the mechanisms underlying both fear and anxiety are sufficiently analogous to justify analyses of both in animal models in pursuit of an understanding of processes in humans (Tovote, Fadok, & Luthi, 2015).

Theoretical frameworks have been proposed over time that can aid in this effort. One example is provided by early analyses of predator-elicited behaviors by Caroline & Robert Blanchard (D. C. Blanchard & Blanchard, 1988; R. J. Blanchard, Griebel, Henrie, & Blanchard, 1997), describing the concept of "defensive distance". These models were later updated by McNaughton & Corr who iterated upon Gray & McNaughton's "Neuropsychology of Anxiety" (Jeffrey A. Gray, 1982) to craft a model of animal behavior. Particularly insightful to the work described in this dissertation is the characterization of survival-association behaviors, articulated by the Blanchards, associating anxiety with risk assessment of the potential presence of a predator, rather than those directly elicited by predators, as is characteristic of fear (R. J. Blanchard et al., 1997; McNaughton & Corr, 2004).

At the core of Gray's original framework is a tension between two primary motivational systems that govern behavior: a Behavioral Inhibition System (BIS) and Behavioral Activation System or Behavioral Approach System (BAS) (Carver & White, 1994; Jeffrey A. Gray, 1982, 2000). Briefly, catecholaminergic signaling was proposed to drive behavioral activation, driving animals to approach or engage in behaviors in pursuit of reward. A septo-hippocampal network, along with monoaminergic signaling from caudal and ventral brainstem regions to the cortex and rostral structures, were proposed to underlie the BIS (Carver & White, 1994). The BIS fundamentally operates to drive the evaluation of risk in conflict situations. Both anxiety and fear can be contextualized within the BIS, though – as McNaughton & Corr argue – operate in opposition with regards to

behavioral outputs when an animal is confronted with danger. Most notably, they argue that fear operates to motivate an animal to move away from danger, while anxiety ultimately motivates an animal to move toward danger (McNaughton & Corr, 2004). Another insightful framework is described by Deakin & Graeff in 1991, proposing that distinct pathways of serotonergic neurons – originating in the midbrain raphe nuclei - regulate both behavioral and physiological responses to aversive or threatening stimuli (Deakin & Graeff, 1991). Further, they hypothesized that particular serotonergic circuits regulate discrete components of the repertoire of behavioral and physiological responses to negative stimuli, potentially translating to specific categories of anxiety-associated psychiatric conditions (Paul, Johnson, Shekhar, & Lowry, 2014). Principally concerned with the neurophysiology of anxiety, fear, and panic, they enumerated a network of anatomical structures that studies had determined to have roles in these behavioral and physiological responses. They highlighted the amygdala, hippocampus, medial hypothalamus, and periaqueductal grey – the dorsal subregion of which in particular (dPAG) - as central components of this anatomical network. The network they described comprises a system wherein serotonergic signaling from the dorsal raphe nucleus (DRN), arriving in the dPAG, inhibits escape-associated behavior, whereas DRN serotonin signaling arriving at the amygdala facilitates anxiety-associated responses. Finally, they proposed, serotonin signaling in the median raphe nucleus (MRN) – sending substantial efferent projections to the hippocampus - increases resilience to stress and are critical to anti-depressant effects (Deakin & Graeff, 1991; Paul et al., 2014).

While certainly abridged representations of the regulatory neurophysiology, these frameworks facilitate more effective ethological interpretations of in vivo pharmacological and viral perturbations of neurophysiology in behavioral assays, particularly when

parsing behavioral responses to aversive stimuli and environments. For example, while behaviors associated with fear and anxiety can appear similar, nuances have been revealed by lesion and pharmacological examinations.

Involvement of Serotonin in Anxiety

In addition to clinical observations, early indications of serotonergic regulation of anxiety were derived from animal models. Studies had demonstrated that anxiolytic medications used to treat anxiety-associated psychiatric conditions in the 1960s and 1970s influenced responses to reinforcing stimuli in animal operant conditioning models (Francisco S. Guimarães, 2010). Approach-avoidance responses, wherein appetitive stimuli are paired with an aversive stimulus, were effectively released by the administration of anxiolytics, such as barbiturates and benzodiazepines (Francisco S. Guimarães, 2010). While not principally serotonergic in its pharmacological action, Wise, Berger, and Stein identified an important effect of the benzodiazepine, oxazepam, causing reduced serotonin turnover in the rat midbrain – as well as in releasing punished responses in a conflict behavioral test (Francisco S. Guimarães, 2010; Wise, Berger, & Stein, 1972). The results from studies conducted by Wise et al. indicated that part of the anxiolytic action exerted by benzodiazepines was a reduction of serotonin release in regions associated with behavioral responses to punishment, the periaqueductal gray (PAG) and forebrain. The corollary interpretation of these data was a putative enhancement of anxiety by serotonin release in these two brain regions. Indeed, the evidence that antidepressant medications effectively confront anxiety disorders in many patients suggests that these conditions arise from activity beyond GABAergic signaling (D. J. Stein & Stahl, 2000). In short, several classes of anxiolytics seem to exert their

effects either by reduction of serotonin release via GABAergic inhibition of serotonergic neurons, as is the case with benzodiazepines, or by activation of 5-HT_{1A} autoreceptors presynaptically, as is the case with buspirone (Lucki, 1998).

Further, studies by Robichaud & Sledge demonstrated that selective reductions in serotonin concentrations in the brain with para-chlorophenylalanine (PCPA) influenced approach-avoidance conflict tests (Kathleen R. Bailey, 2009), reducing foot-shock punishment-driven avoidance in favor of pursuing sweetened milk (Robichaud & Sledge, 1969). Subsequently, the non-selective serotonin receptor antagonists, methysergide and bromolysergic acid, were shown to exert similar effects in a similar approach-avoidance behavioral test (Graeff & Schoenfeld, 1970).

Later efforts have expanded our understanding of serotonergic regulation of anxiety-associated behaviors. Both the neural circuitry and repertoire of neurotransmitters with regulatory roles in survival-associated behavior, and anxiety in particular, have broadened. As mentioned earlier in this section, a consistent network of anatomical regions has been associated with the regulation of anxiety-associated behavior in animal models. Notable structures relevant to the thesis work described in this dissertation within the network – all of which being serotoninoceptive - are the amygdala, periaqueductal gray, and septo-hippocampal system.

Regulation of Anxiety by Serotoninoceptive Anatomical Structures

Periaqueductal Gray

In animal models, electrical stimulation of dorsal regions of the PAG (dPAG) have been shown to induce defensive behaviors (Hunsperger, 1956). Attesting to the aversive

quality of dPAG stimulation, animals readily learn to mitigate stimulation of the region (Hunsperger, 1956). Subsequently, methysergide was shown to reduce behavioral responses to deactivate electrical stimulation of the dPAG, while chlordiazepoxide administration failed to result in similar behavioral reactions (Schenberg & Graeff, 1978). Interestingly, recent studies indicate that animal diabetic models exhibit a predisposition to panicogenesis, and this was effectively blocked by infusion of a 5-HT_{1A} receptor agonist, 8-OH-DPAT, into the dPAG (Gambeta et al., 2017). For additional evidence implicating the regulation of anxiety- and panic-associated behavior by serotonin in the PAG, see the review by Frederico Graeff (Graeff, 2004).

A fascinating experimental probe into the neuroanatomical basis of aversive emotional experience was performed by Nashold et al. in 1969. Worth reading for its breadth of neurophysiological functions explored, the group evaluated patients with intractable central pain who agreed to implantation of electrodes into the dorsolateral mesencephalic tegmentum prior to therapeutic lesioning – enabling stimulation while the patients were awake. Severely aversive emotional reactions, including fear and panic, were reported among the variety of subjective responses experienced (Nashold, Wilson, & Slaughter, 1969). While principally interested in delineating the neurophysiology of pain arising from pathologies of the central nervous system, the electrophysiological exploration in this study implicates the dorsal periaqueductal grey in the regulation of anxiety-associated experiences.

Amygdala

Among brain regions implicated in anxiety, the amygdala has consistently been highlighted. Due to its role in regulating both anxiety- and fear-associated signaling,

however, parsing its participation in each can be challenging. Further complicating these studies is the heterogeneity of signaling contributions of different subnuclei that comprise the structure. Some researchers even suggest that, rather than representing a unitary anatomical structure, the amygdala may be better understood as a set of separate structures with distinct – even divergent – contributions to anxiety and fear (McNaughton & Corr, 2004; Swanson & Petrovich, 1998). Regardless, the amygdala has been associated with the regulation of both anxiety and fear, evidenced in part by studies of local anxiolytic infusions resulting in reduced anxiety-associated behavior in animal behavioral models (Davis, 1992; Scheel-Kruger & Petersen, 1982; K. Shibata, Kataoka, Gomita, & Ueki, 1982). Further evidence of the involvement of the amygdala in regulating anxiety is derived from fMRI studies in humans, showing abnormal functional connectivity between two subnuclei within the structure, the basolateral amygdala (BLA) and central nucleus (CeA), in patients diagnosed with GAD (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009; Janak & Tye, 2015). Serotonergic signaling within the amygdala has been shown to be involved in the physiology of panic disorder. Patients diagnosed with social anxiety disorder were found to exhibit lower 5-HT_{1A} receptor binding potential in the amygdala, as well as the dorsal raphe nuclei (Lanzenberger et al., 2007). While no direct afferent or efferent connections between the amygdala and MHb-IPN axis have been identified, investigations of the role the MHb-IPN axis plays in seizure physiology found that lesions of the IPN resulted in a suppression of subsequent amygdalar kindling in rats, suggesting that kindling in the amygdala – and limbic seizure susceptibility more broadly - may be modulated by the IPN (Chiba & Wada, 1995).

Septo-hippocampal system

The observation of similarities between the behavioral repercussions of hippocampal lesions and anxiolytic pharmacological agents served as a foundation for the implication of the septo-hippocampal pathway in anxiety-associated signaling (McNaughton & Corr, 2004). Conceptually, signaling within the septo-hippocampal pathway has been proposed to underlie the execution of behaviors pursuant of risk assessment, including exploration to determine if approach or avoidance represents the optimal strategy. A variety of potential mechanisms by which hippocampal activity influences these determinations has been proposed, including the reduction of both positive and negative associations (Ito & Lee, 2016). Recent human studies have suggested that behavioral inhibition – measured by scores on the Sensitivity to Punishment subscale of the Sensitivity to Punishment and Sensitivity to Reward questionnaire – is positively associated with hippocampal volume, suggesting a role played by the structure in regulating anxiety (Levita et al., 2014). Multiple dynamics within the hippocampus have been implicated in anxiety-associated behavior, ranging from efferent signaling to neurogenesis. For example, inhibition of the latter has been observed to block the effects of serotonergic anxiolytic drugs in mice (Santarelli et al., 2003). Interestingly, multiple antidepressant medications have been shown to enhance neurogenesis in the dentate gyrus of the hippocampus, including the SSRI, fluoxetine (Malberg, Eisch, Nestler, & Duman, 2000).

The septal area is a forebrain structure, located ventral to the corpus callosum, that separates the lateral ventricles. The region has been studied from many perspectives over the years, some of which are somewhat disquieting in retrospect (Moan & Heath, 1972). Early studies of the functional output of signaling in the septum noted that lesions to the structure resulted in hyperirritability, a behavioral profile that came to be termed

"septal rage", characterized by indiscriminate defensive responses (Anthony et al., 2014; Harrell & Balagura, 1975). Increased emotional reactivity was observed in animals following septal lesions (J. V. Brady & Nauta, 1953). Conversely, animals will readily self-stimulate the septal area via lever-presses through implanted electrodes, indicating substantially rewarding responses (Olds & Milner, 1954). Studies in humans have also indicated that stimulation of the region is similarly pleasant (Heath, 1963; Moan & Heath, 1972). Pharmacological studies have also revealed a role played by the septal region in regulating anxiety-associated behavior. Microinfusion of the benzodiazepine, midazolam, into the septum resulted in elevations of activity in the open arms of the elevated plus maze (De Almeida, Giovenardi, Charchat, & Lucion, 1998), indicative of anxiolysis. In the same study, infusion of 8-OH-DPAT, a 5-HT_{1A} receptor agonist, yielded anxiogenesis in the elevated plus maze at specific doses. Pharmacological studies have also revealed septo-hippocampal – and ventral hippocampal in particular – regulation of open-arm exploration in the elevated plus maze. Infusion of muscimol, a selective $GABA_A$ receptor agonist derived from the *Amanita muscaria* mushroom, into the lateral septum and ventral hippocampus was anxiolytic (Trent & Menard, 2010). Interestingly, however, anxiolysis was only observed when muscimol was infused into the regions contralaterally; ipsilateral infusion, or infusion into either structure alone, both failed to produce this effect. Tetrodotoxin (TTX), a toxin produced by multiple animals of the order Tetraodontiformes, most notably in the pufferfish, potently inhibits voltage-gated sodium channels, resulting in the blocking of action potential firing (Narahashi, 2008). When infused into the septum, TTX increased open arm exploration in the EPM, and also reduced burying behavior in the shock-probe test, another model of anxiety-associated behavior (Degroot & Treit, 2004; Fucich & Morilak, 2018).

Ventral Hippocampus & Anxiety

As discussed above, the hippocampus has long been implicated in the regulation of anxiety. The ventral subregion of the structure, and serotonergic signaling within the structure specifically, appears to be especially involved. When infused into the ventral hippocampus, tertatolol – a 5-HT_{1A} receptor and β-adrendergic receptor antagonist – has been shown to produce anxiolysis in the EPM (File & Gonzalez, 1996). Rats treated with ventral hippocampal lesions exhibited lower anxiety-associated behavior in multiple assays, and interestingly exhibited no signs of spatial learning impairments in the hidden-platform water maze test (McHugh, Deacon, Rawlins, & Bannerman, 2004). These findings corroborate prior studies of hippocampal lesions resulting in anxiolysis (Deacon, Bannerman, & Rawlins, 2002), and the ventral hippocampus specifically (Bannerman et al., 2002). Absence of the 5-HT_{1A} receptor has been shown to result in elevated anxiety associated behavior (Heisler et al., 1998). *In vivo* recordings of neuronal activity in the hippocampus of 5-HT_{1A} receptor knock-out mice revealed elevations of theta-frequency oscillations in anxiogenic environments, while familiar environments were associated with no such elevations (Gordon, Lacefield, Kentros, & Hen, 2005). In the study described above, exploring TTX-induced lesions of the septum, TTX was found to produce anxiolysis when infused into the ventral hippocampus as well (Degroot & Treit, 2004). Interestingly, the group also found that TTX lesions of the dorsal hippocampus failed to result in similar reductions of anxiety, indicating that septo-hippocampal regulation of anxiety-associated behavior may involve more granular anatomical hippocampal sub-regions. Withdrawal from chronic administration of the habit-forming psychomotor stimulant, amphetamine – which results in elevated anxiety (H. Li et al., 2014) - has been shown to result in reduced extracellular serotonin concentrations in the ventral hippocampus (Barr et al., 2013). Restraint stress has been

shown to correlate with elevations of ventral hippocampal serotonin concentrations, though rats undergoing amphetamine withdrawal did not exhibit this elevation – which the researchers interpreted as potentially resulting in a vulnerability to drug relapse as a byproduct of elevated stress sensitivity (H. Li et al., 2014).

Additional investigations of the effect of ventral hippocampal lesions found that rats exhibited a significantly higher percentage of open arm visits compared to those with dorsal lesions or sham-operations in the EPM (Kjelstrup et al., 2002). Among the targets of direct ventral hippocampal efferent projections is the medial prefrontal cortex (mPFC), the substructures of which have also been implicated in the regulation of anxiety (Gonzalez et al., 2000). *In vivo* recording of neuronal activity in the mPFC, as well as both the dorsal and ventral hippocampus, of freely-moving mice revealed increased theta-frequency synchrony between the mPFC and ventral hippocampus in anxiogenic environments (Adhikari, Topiwala, & Gordon, 2010). Further impressive examinations of this circuit by this group suggest that the mPFC, influenced by signals arriving from the ventral hippocampus, operates to construct a cognitive model of anxiogenic environments – guiding the tension between the drive to explore and avoid potential threats (Adhikari, Topiwala, & Gordon, 2011).

Elegant *in vivo* optogenetic experiments demonstrated that activation of somata within the BLA was anxiogenic, while more selective activation of afferent terminals arriving in the lateral CeA produced the opposite effect (Felix-Ortiz et al., 2013). Further, and pertinently to the experiments I conducted in this dissertation, inhibition of terminals arriving in the ventral hippocampus from the BLA proved to be anxiolytic (Felix-Ortiz et al., 2013). Additionally, activation of the same terminals yielded anxiogenesis, suggesting that signaling within the ventral hippocampus plays a critical role in regulating anxiety-associated behavior. Similarly, activation of granule cells in the dentate gyrus of

the ventral hippocampus resulted in anxiolysis in both the open field test and EPM (Kheirbek et al., 2013). Granule cells in the dentate gyrus are notably sensitive to elevated stress hormone levels, whereby stressful experiences can inhibit neurogenesis (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; McEwen, 1999).

In summary, interpretations of the regulation of defensive behavior by serotonin are complicated by contradictory effects in different anatomical structures. Behaviorally, serotonergic signaling appears to facilitate anxiety-associated behaviors while inhibiting those associated with panic (Francisco S. Guimarães, 2010). In the septo-hippocampal system and amygdala, serotonin release appears to promote behaviors ethologically associated with anxiety while, in the PAG, serotonin signaling appears to inhibit these behaviors (Francisco S. Guimarães, 2010). Accordingly, interpretations of animal behavior observed following circuit-specific perturbations, such as the experiments described in this dissertation, are best served by a consideration of these potential nuances.

Relationship of the IPN with Serotoninoceptive Structures that Regulate Anxiety

One of the earliest reviews of the afferent and efferent connectivity of the interpeduncular nucleus (IPN) was composed by Barbara Morley in 1986 (Morley, 1986), referenced throughout this dissertation – most comprehensively in (McLaughlin et al., 2017). Several anatomical structures noted by Morley, some of which have been corroborated in subsequent studies, are implicated in both serotonergic signaling and anxiety-associated behavior. The periaqueductal gray (PAG), locus coeruleus, and raphe nuclei are among the relevant afferent pathways. Evidence of direct projections

from the infralimbic region of the mPFC to dorsal regions of the IPN has been reported, though not subsequently corroborated (Takagishi & Chiba, 1991). Among the relevant efferent pathways are the septum, hippocampus, and lateral hypothalamus (Baisden, Hoover, & Cowie, 1979; Groenewegen, Ahlenius, Haber, Kowall, & Nauta, 1986; Quina et al., 2017). In addition to tracing studies, lesions of the fasciculus retroflexus – the conduit of afferent projections from the MHb to the IPN – have been shown to reduce the power of hippocampal theta rhythms, as well as influence rapid eye movement (REM) sleep, in rats (Valjakka et al., 1998). As discussed above, theta rhythm oscillations within the ventral hippocampus have been associated with anxious states, indicating one potential mechanism by which the MHb-IPN axis might regulate anxiety arising from hippocampal activity (Adhikari et al., 2010). Evidence of noradrenergic projections from the locus coeruleus to the MHb-IPN axis is derived from earlier neuroanatomical studies which have yet to be corroborated using contemporary methods (Gottesfeld, 1983).

While the MHb-IPN axis has not been shown to receive direct afferent projections from the bed nucleus of the stria terminalis (BnST), the caudal lateral habenula (LHb) – lateral complement to the medial subnucleus of the habenular complex - has been shown to. Further, fMRI studies, while unable to achieve sufficient resolution to distinguish the medial and lateral habenular subnuclei, demonstrated functional connectivity between the habenular complex and the BnST (Torrisi et al., 2017).

Early reviews of the afferent and efferent connectivity of the IPN suggested that it sends projections to the LHb, but only recently have they been corroborated (Morley, 1986). Unpublished viral tracing experiments I performed identify direct projections from the IPN to the LHb – and this has been confirmed by recent elegant tracing performed by Lely Quina in the Turner lab (Quina et al., 2017). Additionally, the LHb may be among the

handful of anatomical structures that receives efferent projections directly from the MHb (Kim & Chang, 2005).

Accordingly, given the projections to the LHb from the BnST, the MHb-IPN axis contributes to the downstream recipients of BnST signaling. The septal nuclei have been shown to receive innervation from the IPN (Morley, 1986; Quina et al., 2017), and additional tracing experiments I performed corroborates the existence of these projections. A recent study by Yamaguchi et al. provided a detailed anatomy of septal projections to the MHb, along with a parsing of the regulation of fear and anxiety by septohabenular pathways (Yamaguchi et al., 2013).

Overall, the role of serotonin in the regulation of anxiety is complex. Defensive behaviors, and anxiety in particular, arise from a distributed network of anatomical structures. It is therefore prudent, when interrogating specific structures involved in the orchestration of behaviors driven by anxiety, to consider their functional relationships within the broader circuitry involved in anxiety-associated signaling. While this section certainly hasn't exhaustively reviewed all regions or signaling pathways associated with anxiety, many prominent structures implicated in its underlying neurophysiology are associated with the MHb-IPN axis, and therefore its functional analysis is warranted in pursuit of a more thorough understanding of the neurophysiology of anxiety.

Central Hypotheses

Central Hypothesis: the IPN regulates anxiety-associated behavior independently of withdrawal from habit-forming drugs.

Secondary hypotheses: chemogenetic activation of glutamatergic IPN neurons is anxiogenic, and broad IPN activation will correlate with altered serotonergic signaling in the ventral hippocampus.

These hypotheses arise from two primary theoretical bases. First, the anatomy of the dorsal diencephalic conduction system (DDC) positions the IPN as a junction of signaling associated with cognition, mood, and motivation. As the principal recipient of medial habenular output, and given the diversity of its efferent targets, the IPN represents a source of signaling of multiple anatomical structures involved in affect regulation.

The second basis is derived from pharmacological drug withdrawal experiments that have implicated signaling within the MHb-IPN axis in the manifestation of both affective and somatic symptoms of withdrawal from alcohol, nicotine, and opioids. I anticipated that this signaling pathway, and the IPN in particular, participates in the regulation of baseline affective and somatic states independently of chronic drug exposure.

Secondary hypotheses are based on the anatomical connectivity between the IPN, raphe nuclei, and ventral hippocampus. Projections from the IPN to the raphe nuclei have long been established, and the raphe nuclei represent a major source of serotonergic signaling in the brain. As the raphe nuclei have been shown to send

projections to the hippocampus, I hypothesized that general activation of the IPN would result in changes in the concentration of serotonin in the ventral hippocampus.

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CHAPTER 2: THE INTERPEDUNCULAR NUCLEUS REGULATES BASELINE ANXIETY LEVELS

The Interpeduncular Nucleus Regulates Baseline Anxiety Levels

This chapter presents work that is under review for final publication:

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Abstract

The interpeduncular nucleus (IPN) and its principle source of afferent signals, the medial habenula (MHb), comprise much of the dorsal diencephalic conduction system (DDC). Along with the medial forebrain bundle, the DDC is a highly conserved pathway by which signals from the limbic forebrain reach the midbrain and hindbrain. This circuit has been implicated in the aversive symptoms – both affective and physical – that follow cessation of chronic use of habit-forming drugs, including alcohol, nicotine, and opioids. In this study, we identified a role played by IPN signaling in regulating affect independently of chronic drug exposure and withdrawal. Using an *in vivo* chemogenetic approach, we observed significant anxiolysis following chemogenetic IPN stimulation, induced by administration of 5 mg/kg of clozapine N-oxide (CNO). We next evaluated the role played by glutamatergic signaling in this change of behavior, infusing a virus expressing the chemogenetic receptor, hM3Dq, in the IPN of transgenic mice expressing Cre recombinase under the control of the vesicular glutamate transporter 3 (VGLUT3-Cre). Stimulation of glutamatergic subpopulations within the IPN resulted in no significant change in anxiety-associated behavior. Using a combinatorial viral approach, we also confirmed a direct projection from the IPN to the ventral hippocampus (VH), and microdialysis experiments revealed significant elevations of serotonin (5-HT) concentrations in the ventral hippocampus (VH) following chemogenetic stimulation of

the IPN. Taken together, these data suggest that IPN signaling regulates anxiety independently of chronic exposure to addictive drugs, and places the IPN in a broader neuronal circuit that implicates signaling in the IPN and VH in the regulation of anxiety-associated behavior.

Introduction

Anxiety is a symptom that is consistently observed after chronic use of essentially any recreational drug is terminated (McLaughlin et al., 2017). This proves true both in humans undergoing withdrawal from chronic drug use, and in animal models of addiction and withdrawal. The dorsal diencephalic conduction system (DDC) is a pathway shown to be highly evolutionarily conserved, observed in a variety of species including mammals, reptiles, and fish (Epstein, Hurley, & Taber, 2018). Along with the medial forebrain bundle, the DDC is a principle means by which the forebrain conveys and receives signals to and from the mid- and hind-brain. Given this anatomy, it is not surprising that the pathway has been identified as an important regulator of motivation and reward signaling in higher vertebrates, and a junction of cognitive, emotional, and sensory signaling (Gardon et al., 2014).

Over the past two decades, our group and others have identified a substantial regulatory role played by two primary components of the DDC, the medial habenula (MHb) and the predominant recipient of its efferent signaling, the interpeduncular nucleus (IPN), in the maintenance of chronic drug use and the manifestation of aversive symptoms of withdrawal (Dani & De Biasi, 2013; De Biasi & Dani, 2011; Fowler et al., 2011; Glick et al., 2006; McLaughlin et al., 2015, 2017; Molas et al., 2017; E. Perez et al., 2015a; Salas et al., 2009; Taraschenko et al., 2007; Viswanath et al., 2013; Zhao-Shea et al., 2013). In particular, chronic use of nicotine and/or ethanol results in neuroplastic adaptations

within these structures which, upon cessation of use, leads to the manifestation of withdrawal symptoms. Furthermore, the DDC expresses high levels of the μ-opioid receptor, and the MHb-IPN axis in particular has been shown to be remarkably dense with expression (Gardon et al., 2014). Consequently, the functional properties of the DDC and MHb-IPN axis, both independent and in the context of addiction, are alluring components of our understanding of addiction and mood-associated distress that remain uncharacterized.

In this study, we used chemogenetics coupled with behavioral and viral tracing methods to characterize how the IPN acutely regulates baseline anxiety-associated behaviors in mice. Chemogenetics exploits designer receptors exclusively activated by designer drugs (DREADDs), which are G protein-coupled receptors engineered to no longer bind their cognate ligand, that can be expressed in neuronal populations. This enables acute perturbations of neuronal activity using an agonist, clozapine n-Oxide (CNO), in freely-behaving mice *in vivo*. hM3Dq, the DREADD used in this study, is an engineered variant of the muscarinic M3 receptor coupled to the G_q signaling cascade. Activation of the receptor results in robust enhancements of neuronal firing that are sustained for longer than the duration of behavioral assays we used to assess anxiety in this study, the open field arena (OFA) and elevated plus maze (EPM). This approach enables acute and remote activation of the IPN in a minimally invasive fashion, attenuating any unintended anxiogenesis derived from the experiment as much as possible.

While a foundation of research has been growing that places the MHb-IPN axis within the broader context of the neurobiology of addiction, comparatively less work has been done that elucidates the functional properties of this pathway independently of chronic drug exposure and resulting neuroplastic adaptations. Therefore, we set out to understand how the IPN modulates anxiety-associated behavior, using acute activations

of the nucleus to perturb the system, in behavioral assays that have been long established as effective evaluations of anxiety-associated behaviors in rodents (Crawley, 1985; Crawley et al., 1997). Recent studies of the signaling contributions from the MHb-IPN axis to affective distress have identified signatures of elevated neuronal signaling within the pathway in rodent models of depression (Xu et al., 2018). Additionally, past studies in zebrafish indicated that genetic reductions in activity within the anatomical structure that has been proposed to be orthologous to the human MHb attenuated fear-associated behavior (Agetsuma et al., 2010), while subsequent studies showed disproportionately severe fear-associated behavior in fish possessing the same lesion in response to an otherwise mild stimulus (Mathuru & Jesuthasan, 2013).

Given the anatomical context of the MHb-IPN axis, we evaluated if the circuit regulates anxiety independently of chronic drug exposure.

Materials and Methods

Animals

We studied 2- to 6-month-old C57BL/6J mice, both male and female. Weaning was performed 21 days following birth, and same-sex littermates were housed in cages composed of a maximum of 5 animals and a minimum of 2 with *ad libitum* access to food pellets and water. All mice were maintained in a 12-h light/dark cycle, temperature-controlled room (24±2 °C, relative humidity 55±10%). All behavioral tests were performed during their light cycle, and were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania, according to the guidelines for intramural animal research provided by the National Institutes of Health in an animal care facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

Drugs

Clozapine N-oxide (CNO) was obtained from Abcam (ab141704), and aliquoted to be stored at -20°C until use. The stock solution was diluted in sterile saline at 1 mg/mL and filtered through a 0.2 μm syringe filter before injection. CNO was administered via intraperitoneal injection (i.p.) at 5 mg/kg for all *in vivo* experiments.

Behavioral Tests

To control for environmental confounds attributable to variations in animal husbandry and transportation, habituation to transportation from holding room to testing room was achieved by performing everything but injection and testing for 3 days. Prior to testing day, mice were transported using an identical cart and allowed to rest in home cages for a minimum of 2 hours in the testing room used for behavioral experiments. Ambient conditions during habituation were identical to those of testing days. Following habituation, mice were treated with either 5 mg/kg CNO or saline control injections, and permitted to habituate in a separate cage for approximately 15 minutes before placement in the testing apparatuses to provide sufficient time for CNO to take effect.

The open field arena (OFA) was used to measure changes in anxiety-associated behavior. Dimensions of the maze were 40 cm x 40 cm x 40 cm, and tests were run for 30 minutes while behavior was tracked and quantified using the computer software, ANY-maze (Stoelting). Distance traveled, and time spent, in the center region (10 cm x 10 cm) of the maze was tracked and compared between treatment groups.

The elevated plus maze (EPM) was used to further assess changes in anxiety-associated behavior following CNO administration. EPM testing was performed at least 3 days after OFA testing. The maze consisted of four black arms forming a cross shape,

with walls surrounding the sides of two of the arms, elevated from the ground by >30 cm. Time spent in open arms, as well as numbers of open arm entries, were tracked using ANY-maze.

All tests were conducted at ambient light levels empirically determined to have no effect on anxiety-associated behavior with a separate group of C57BL/6J in both mazes (8-10 lux for OFA, 7-16 lux for EPM).

Virus Use & Production

Initially, AAV8-hSyn-hM3Dq-mCherry was acquired from the UNC Vector Core. All other viruses (pAAV-hSyn-DIO-hM3D(Gq)-mCherry; retroAAV2-CreGFP) were produced within our lab. HEK293 cells were transfected with 3 plasmids using a Helper-Free system. The first plasmid contains the genes of interest and promoter sequence, the second plasmid contains most of the adenovirus gene products required for efficient infection, and the third plasmid contains the replication and capsid genes to establish the serotype of the virus. Following transfection, viral particles were purified and concentrated using iodixanol gradients, spun at 80,000 RPM/1.5 hrs at 4°C, and concentrated using Centricon columns (100,000 MWCO, Millipore UFC0910024). Volume was reduced to 200ul HBSS, aliquoted, and stored at -80°C until use.

Stereotactic Surgery & Viral Infusions

All intracranial infusions were performed with mice approximately 2 months old under general isoflurane (1-2%) anesthesia coupled with meloxicam (2 mg/kg). After fur removal and disinfection, and following exposure of the skull, small holes were drilled to enable stereotactic targeting with a 10 µL syringe with removable needle tips lacking bevels (Hamilton; Reno, Nevada). Virus-containing fluid was infused at 0.01 µL/min with

a microinfusion pump (kdScientific, Holliston, MA) attached to the stereotactic apparatus (Kopf, Tujunga, CA). Total volumes infused varied by brain region, with 110-140 nL in the IPN and 1 µL in the VH.

Stereotactic targeting of the IPN and VH used the following coordinates. IPN: A/P: -3.46 - -3.48 mm, M/L: -1.70 mm, D/V: -4.84 mm at a 20° angle. VH: A/P: -3.2 mm, M/L: 3 mm, D/V: 3.5 mm. To allow sufficient time for viral expression, behavioral experiments were performed following a minimum of 6 weeks.

For general IPN stimulation we used AAV2/8-hSyn-hM3D-mCherry. For stimulation of VGLUT3^{+} neurons, we used AAV2/8-hSyn-DIO-hM3D-mCherry. For retrograde expression of Cre recombinase in IPN neurons that project to the VH, retroAAV2-CreGFP was infused into the VH.

Microdialysis Probe Implantation and Dialysate Collection

CMA/12 microdialysis guide cannulae were stereotactically implanted, targeting the VH (A/P: -3.2 mm, M/L: 3 mm, D/V: 3.5 mm). Following recovery, on the day prior to dialysate collection, CMA/12 microdialysis probes (inner diameter, 0.5 mm; length, 4 mm, membrane, polycarbonate; cutoff, 12,000 DA) were perfused with artificial cerebral spinal fluid (147 mM NaCl, 3.99 mM KCl, 1.7 mM $CaCl_2$, 0.90 mM $MgCl_2$). A minimum of 14 hours prior to collection, probes were lowered slowly through the guide cannula while mice were anesthetized with 2% isoflurane, and mice were placed in a modified cage similar to their home cages with a counterbalance arm equipped with a swivel permitting liquid flow. Flow rate of probe perfusion was reduced to 0.5 µL/min overnight, followed by an increase to 2µL/min at least 1 hour prior to establishing baseline 5-HT concentrations. Following collection of 5 baseline samples, mice were injected with 5 mg/kg of CNO IP, and samples were collected every 20 minutes for just over 3 hr while

mice remained in modified cages. Dim ambient light levels were maintained in the sample collection room to reflect those that mice encountered in OFA and EPM experiments (~15 lux). Vials containing dialysates were changed manually and immediately stored at -80°C until analyzed by high-performance liquid chromatography (HPLC). Dummy microdialysis probes were dipped in a solution containing DiI ((2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylindole; perchlorate), Invitrogen, D282, Carlsbad, CA), and inserted back into the microdialysis cannulae at the end of each experiment to verify successful targeting of the VH. Mice were then perfused with phosphate-buffered saline (PBS) under anesthesia, followed by 10% formalin, and brains were collected, fixed overnight in 10% formalin followed by cryoprotection with 10%, 20%, and finally 30% sucrose solutions in PBS over the course of 3 days. Brains were then embedded in clear Tissue-Plus Optimal Cutting Temperature Compound (OCT; Fisher Scientific, Hampton, NH, 23-730-571), and stored at -20 to -80°C. Brains were cryosectioned at 25-40 μm, and both accurate viral targeting and probe placement were verified using epifluorescence microscopy.

Immunohistochemistry

For verification of viral expression, 25-40 μm sectioned brain slices were collected via cryosectioning following anesthetic overdose and the transcardial perfusion protocol described previously. Slices were either mounted directly onto slides, or were permeabilized in 0.1% Triton X-100, and blocked in a 5-8% combination of normal goat and donkey serums for 2 hours in free floating wells. Next, slices were incubated with primary antibodies against GFP and mCherry (ThermoFisher, Waltham, MA, GFP Monoclonal Antibody, A-11120; abcam, Cambridge, United Kingdom, Anti-mCherry,

ab167453) overnight. Slices were incubated with secondary antibodies (abcam Goat Anti-Rabbit, ab150080; abcam Goat Anti-Mouse, ab150113) or streptavidin conjugated to AMCA (Jackson ImmunoResearch Laboratories Inc., 016-150-084, West Grove, Pennsylvania) for slices used in electrophysiological experiments. Stained slices were mounted in either VECTASHIELD Antifade Mounting Medium with DAPI (Fisher Scientific, NC9524612), or ProLong Diamond Antifade Mountant (ThermoFisher Scientific, P36965). Slides were left to cure for a minimum of 12 hours prior to imaging and imaging was carried out on an epifluorescence microscope (Olympus, BX63) under 4X and 20X magnifications.

Slice Preparation

Following behavioral experiments, mice infused with a virus to induce expression of the hM3Dq variant of DREADD were deeply anesthetized with a combination of ketamine and xylazine. Following anesthesia, transcardial perfusion was performed as described previously (Broussard et al., 2016; Yang et al., 2017) with ice-cold N-methyl-D-glucamine (NMDG)-based artificial cerebrospinal fluid (aCSF) composed of the following (in mM): 92 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, and 10 $MgSO_4$, pH 7.3-7.4 with concentrated hydrochloric acid (Ting, Daigle, Chen, & Feng, 2014). Brains were quickly extracted, placed in ice-cold NMDG solution saturated with 95% O_2 and 5% CO_2. Horizontal slices (230 μM) that contained the lPN were collected using a vibratome (Leica VT 1200s) in ice-cold NMDG solution. Slices were collected and placed in NMDG solution at 32 °C for 13 minutes, then transferred to HEPES-based holding aCSF (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5

Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2$, and 2 $MgSO_4$, and kept at room temperature for a minimum of 1 hour prior to recording.

Electrophysiology

Slices containing the IPN were placed in a home-made recording chamber and were continuously bathed in well oxygenated standard recording aCSF, containing (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $CaCl_2$, and 2 $MgSO_4$. The bath solution was maintained at 32–34°C using an inline heater system (TC-324B, Warner Instrument Corp, Hamden, CT). Responses to puff and bath application of CNO were recorded using glass recording electrodes (~2-3 MΩ), which were pulled using a micropipette puller (Narishige PC-10, Tokyo, Japan) from borosilicate glass capillaries (TW 150-4, World Precision Instruments, Inc., Sarasota FL). Electrodes were filled with a K-gluconate-based intracellular solution composed of (in mM): 140 K-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine, pH 7.3 with KOH. Brief, gentle suction was applied to first achieve a tight-seal patch-clamp recording configuration, and under tight-seal patch-clamp mode, baseline firing activities in fluorescently labeled, DREADD-expressing IPN neurons were recorded. Subsequently, responses to either puff- or bath-application of 500 nM CNO, the DREADD agonist, using a Picospritzer II (Parker Instrumentation, Fairfield, NJ) were recorded. Whole-cell recording were performed only in cells with access resistance (Ra) of less than 10 MΩ. Ra was continuously monitored throughout recordings. During patch-clamp recording, neurons were labeled with neurobiotin to further ensure that recorded neurons were indeed $hM3Dq^+$ by immunohistochemical confirmation with streptavidin conjugated to the fluorescent dye, 7-amino-4-

methylcoumarin-3-acetic acid (AMCA, Jackson ImmunoResearch Laboratories Inc., 016-150-084) using an epifluorescence microscope (Olympus, BX63).

Data analysis

All analyses were presented as mean ± SEM. Paired/unpaired Student's *t* test or Single-Factor ANOVA were used for the statistical analysis of behavioral and electrophysiological data. Statistical significance was identified by $p<0.05$. Microdialysis data were reported as percentage of baseline values that were determined by the average of five samples collected prior to drug treatments. Repeated measure ANOVA was used to assess treatment effects on 5-HT levels with repeated measures over time after injection, and then compared at individual time points following drug treatments with Tukey's test. All assessments considered $p<0.05$ to be statistically significant, and data were expressed as mean ± SEM.

Results

Stimulation of IPN neurons is anxiolytic

To identify some of the behavioral effects of stimulating IPN neurons, we stereotactically infused a virus expressing the hM3Dq DREADD variant into the IPN of C57BL/6J mice (Fig. 1A & 1B). Following 6-8 weeks of expression time, mice underwent behavioral testing in the open field arena (OFA) and elevated plus maze (EPM). For the OFA, time spent and distance travelled in the center zone were quantified and compared between groups of mice receiving either saline or 5 mg/kg CNO (Fig. 1C & 1D) for the 30 min. duration of the test. We observed a significant reduction of anxiety-associated behavior in mice treated with CNO, exhibited by more time spent, and greater distance traveled,

in the center zone, relative to saline-treated controls (n= 11, 9, 10 [$F(2, 27) = 5.714$, $p = 0.0085$]; [$F(2, 27) = 4.6339$, $p = 0.0186$]). A minimum of 3 days later, the same mice were assessed in the EPM for 15 min in a counterbalanced treatment design (Fig. 1E & 1F). Total entries and distance traveled in open arms of the maze were quantified. CNO administration resulted in reduced anxiety-associated behavior, exhibited by a greater number of entries and more distance traveled in open arms relative to saline-treated control mice (n= 12, 6, 4 [$F(2, 19) = 5.276$, $p = 0.015$]; [$F(2, 19) = 25.772$, $p < 0.0001$]). CNO administration to wild-type mice lacking DREADD expression resulted in no change in anxiety-associated behavior.

Stimulation of glutamatergic IPN neurons does not affect anxiety-associated behavior.

Given that general stimulation of the IPN was observed to be anxiolytic, and the IPN is a structure that hosts the synthesis of a variety of neurotransmitters, we assessed whether glutamatergic output from the nucleus may mediate this change in behavior following general chemogenetic stimulation. Following 8-10 weeks of expression time, VGLUT3-Cre mice infused with AAV8-DIO-hSyn-hM3Dq-mCherry (Fig. 2A & 2B) were evaluated in the OFA for 30 min. and the EPM for 15 min. to assess any changes in anxiety-associated behavior following administration of 5 mg/kg CNO. In the OFA, there was no significant change in anxiety-associated behavior exhibited by mice administered CNO compared to mice receiving saline (Fig. 2C & 2D). Similarly, 5 mg/kg CNO administration did not result in any significant change in anxiety-associated behavior observed in the EPM (Fig. 2E & 2F).

Behavioral responses to activation of glutamatergic IPN neurons are not sexually dimorphic

At the suggestion of a member of my thesis committee, we evaluated whether male and female mice might respond differently to chemogenetic activation of IPN glutamatergic neurons. This guidance was very well received, as past studies have indicated that modulation of anatomical structures associated with the MHb-IPN axis is indeed associated with sexually dimorphic behavioral responses (K. Li, Nakajima, Ibanez-Tallon, & Heintz, 2016). Further, α5-containing nAChRs – expressed at a uniquely high density in the IPN – have been shown to mediate sex-dependent differences in anxiety-associated behavior (Gangitano, Salas, Teng, Perez, & De Biasi, 2009). However, while interesting trends were observed, no significant dimorphism between female and male mice was observed in the OFA (Fig. 2G – 2N) or EPM (Fig. 2O – 2V), suggesting that glutamatergic IPN signaling in particular does not appear to mediate any differences in anxiety-associated behavior among female or male mice.

The Interpeduncular Nucleus Sends Direct Projections to the Ventral Hippocampus.

C57BL/6J mice at 2-4 months of age were infused with retroAAV2-hSyn-CreGFP in the VH, and AAV2/8-hSyn-DIO-hM3Dq-mCherry in the IPN during the same stereotactic surgery (Fig 3A & 3B). After 6-8 weeks, brains were cryosectioned, stained for both fluorescent markers, and evaluated for the expression products of both viruses. As expected, dense expression of CreGFP was observed at the site of injection within the VH, as well as sparsely within the IPN. Cre-dependent expression of hM3Dq-mCherry was observed throughout the IPN, with the densest fluorescence observed within

specific substructures, including the intermediate (IPI) and lateral (IPL) subnuclei, and comparatively sparser levels in the caudal and rostral subnuclei (Fig 3C).

Activation of IPN neurons results in elevated serotonin concentrations in the VH.

Given the identification of projections arriving in the VH from the IPN, and previous reports of the presence of serotoninergic neurons in the IPN of rats (Montone, Fass, & Hamill, 1988), we evaluated changes in 5-HT concentrations in the VH following chemogenetic stimulation of IPN neurons. At 2 months of age, male and female C57BL/6J mice were infused with AAV2/8-hSyn-hM3D-mCherry into the IPN according to the protocol described in prior experiments (Fig. 4C). Subsequent to behavioral evaluations, mice were then implanted with microdialysis probes targeting the VH (Fig. 4A & 4B). Dialysate samples were collected in 20-minute increments. At 180 min. post-injection of 5 mg/kg CNO, we observed a significant increase in 5-HT concentrations that initiated rapidly and remained elevated for approximately 20 minutes until returning to a rather variable baseline approximately 40 minutes following CNO injection (Fig. 4D) (df = 7.10, F = 3.19, p = 0.004).

Discussion

The DDC, and the MHb-IPN axis in particular, has been identified as an important regulator of midbrain motivation and reward circuitry (Bianco & Wilson, 2009; Lecourtier & Kelly, 2007; Sutherland, 1982). In addition to evidence derived from studies with simple model organisms, anatomical studies position the MHb-IPN axis within a broader network of structures historically associated with the regulation of anxiety. The IPN has been shown to send efferent signals to both the dorsal and median raphe nuclei, receive

signaling from the locus coeruleus (Molas et al., 2017; H. Shibata, Suzuki, & Matsushita, 1986), and recent studies have demonstrated direct projections to the HPC (Lima et al., 2017). To our knowledge, however, direct projections from the IPN to the ventral hippocampus (VH) have yet to be confirmed using contemporary viral techniques until this study. Past work has suggested the presence of both serotonergic and non-serotonergic efferent signaling from apical neurons in the IPN arriving in the dorsal hippocampus (Montone et al., 1988; Wirtshafter, Asin, & Lorens, 1986).

Given that role, and that studies have implicated activity in the IPN in the manifestation of aversive symptoms of withdrawal from alcohol and nicotine, among other drugs (E. Perez et al., 2015a; Salas et al., 2009; Taraschenko et al., 2007; Zhao-Shea et al., 2015; Zhao-Shea et al., 2013), we hypothesized that increased signaling within this circuit may acutely regulate affect independently of chronic drug exposure. In particular, we hypothesized that general activation of the nucleus would result in increased anxiety-associated behavior.

Using the same behavioral paradigms that have been used to characterize affective symptoms of withdrawal symptoms in the past (Damaj, Kao, & Martin, 2003; De Biasi & Salas, 2008; Irvine, Cheeta, & File, 2001), we observed that the IPN does indeed acutely regulate affect following chemogenetic activation of IPN neurons. However, to our surprise, activation of the IPN resulted in reduced anxiety-associated behavior in the OFA and EPM, rather than increased anxiety as reported during drug withdrawal. Due to the neurochemical diversity present in this dense nucleus, we further explored which efferent signals may mediate this regulatory role. We focused on glutamatergic outputs, given the results obtained with the expression of hSyn-hM3D-mCherry. However, upon chemogenetic stimulation of glutamatergic IPN neurons in VGLUT3-Cre, we observed no significant change in anxiety-associated behavior in the OFA or EPM.

While many brain regions are involved in the regulation of affect, recent studies have identified a role played by the VH in playing a modulatory role (Jimenez et al., 2018; Kheirbek et al., 2013; Kheirbek & Hen, 2011). Both past and recent studies have shown direct projections from the IPN to the HPC, and decades ago, neuroanatomical studies proposed efferent projections from the IPN arriving in the VH, but this anatomy has yet to be verified using contemporary methods (Montone et al., 1988; Quina et al., 2017; Wirtshafter et al., 1986). Using a combinatorial viral approach, resulting in projection-dependent hM3Dq-mCherry expression, we have confirmed the existence of these IPN-VH projections in mice. By infusing an adeno-associated virus (AAV) with efficient retrograde functionality (Tervo et al., 2016) into the VH, along with a Cre-dependent virus driving hM3Dq-mCherry expression, we observed robust hM3Dq-mCherry expression within the IPN, as well as hM3Dq-mCherry positive terminals within the VH. Interestingly, Cre-dependent hM3Dq-mCherry expression patterns within the IPN exhibit anatomical specificity, with the densest expression observed in ventral regions of the structure, including the intermediate and lateral subnuclei, with sparser expression in the rostral subnucleus and along the inner periphery of the structure. This indicates that projections from the IPN to the VH likely arise only from specific subnuclei within the IPN, corroborating the suggestions made by Lima et al. (Lima et al., 2017).

Taken together, these data suggest that the IPN does indeed regulate anxiety-associated behavior independently of chronic drug exposure. However, the mediatory signals of this anxiolytic effect that arise from the IPN remain to be identified. Given the diverse repertoire of neurotransmitters that are synthesized and received by neurons within this nucleus, there are numerous possible candidates that may convey this anxiolytic effect. Past studies have identified both serotonergic and non-serotonergic efferents arising from the IPN (Montone et al., 1988). An intriguing future study will be to

inhibit serotonergic subpopulations of the median and dorsal raphe nuclei while exciting the IPN to evaluate whether direct or indirect IPN-VH pathways mediate the anxiolysis observed in this study, as well as elevations of 5-HT concentrations in the VH.

The limitations of interrogating IPN function warrant attention when considering behavior arising from chemogenetic, or any virus-mediated, perturbation of the structure. Given the complexity and density of this structure, achieving viral expression throughout its entirety while avoiding diffusion into neighboring structures is a substantial technical challenge. As a result, viral expression targeting the IPN necessarily excludes certain neuronal populations due to the avoidance of this diffusion. Furthermore, studies have shown that different AAV serotypes exhibit variable tropisms for neuronal subtypes (Aschauer, Kreuz, & Rumpel, 2013; Hammond, Leek, Richman, & Tjalkens, 2017), and given the variety of cell subtypes present within this nucleus, it remains possible that the use of any one serotype may result in poor or restricted expression within components of the structure.

Conclusions & Future Directions

The majority of research characterizing the functional properties of the MHb-IPN axis in mammals has emerged from studies focused on the neurobiology of addiction and withdrawal. Recent studies have revealed a regulatory role played by the pathway in modulating affect, including conditions like depression in animal models (Xu et al., 2018), independently of chronic drug exposure. Given that reductions of signaling within the IPN have been shown to alleviate aversive symptoms of withdrawal, diminish fear-associated reactions, and reduce conditioned avoidance (Agetsuma et al., 2010; Salas et al., 2009; Thompson, 1960; Vincenz, Wernecke, Fendt, & Goldschmidt, 2017), we

anticipated that acute activation of the nucleus would amplify anxiety-associated behaviors. However, a robust anxiolytic response was observed, indicating that the role of neuroplastic adaptation to chronic drug administration or behavioral conditioning plays a crucial role in determining the signaling within the nucleus and consequential behavioral outputs. Recent studies have demonstrated shifts towards excitatory $GABA_A$ receptor signaling within the ventral tegmental area following exposure to stressful stimuli, influencing dopaminergic signaling associated with increased ethanol self-administration in rodents (Aschauer et al., 2013; Thomas et al., 2018). Accordingly, exploring how the effects of pharmacological treatment and environmental contexts, including chronic drug administration and chronic stress exposure, influence signaling within the IPN such that differential behavioral outputs emerge may be revealing. Further, given the complexity and density of the nucleus, and the diversity of neurotransmission occurring within, and emerging from, the IPN, genetic tools enabling expression of DREADDs or opsins within specific subnuclei will enable a more precise dissection of the functional properties that distinguish them. It is likely that activation of distinct neuronal populations within the nucleus results in opposing modulatory activity, promoting or inhibiting aversive affective output. Similarly, while the VH has been shown to regulate affect in mouse models, it represents just one component of a diversity of efferent targets emanating from the IPN, arriving at distal anatomical structures that have been shown to regulate affect. Accordingly, projection-specific excitation/inhibition studies will undoubtedly yield a more comprehensive understanding of how this pathway modulates affect.

Moving forward, it will prove fruitful to investigate the behavioral effects of IPN GABAergic activation at baseline to further elucidate the role of IPN signaling in the regulation of anxiety-associated behavior, as well as identifying the broader circuit

components that contribute signaling that mediates this regulatory effect including the raphe nuclei, lateral habenula, and septal structures.

Supplementary Methods

Depolarization of IPN neurons by the DREADD agonist clozapine N-Oxide (CNO)

To directly examine effects of DREADD on IPN neurons, we performed patch-clamp recordings in fluorescently labeled, DREADD-expressing neurons in brain slices containing the IPN in some animals after behavioral tests. As shown in supplementary figure 1, two types of DREADD positive IPN neurons were recorded. Under control conditions, 62.5% of recorded neurons are type 1 neurons (Supp Fig. 1A), which have much greater hyperpolarized rest membrane potential (RMP) than type 2 (Supp Fig. 1B, 37.5%) neurons (-67 ± 3.27 mV vs. -50.2 ± 3.79 mV, n= 10, 6, $p<0.005$). No spontaneous action potential firings were overserved under baseline recording conditions because of the very hyperpolarized RMP, and transient burst firings were observed after puff application of 500 nM CNO. Bath-application of 500 nM CNO significantly depolarized the type 1 neurons (RMP = -44.8 ± 1.65, n=5, $p< 0.01$ compared with baseline) and resulted in consistent action potential firings at 9.28 ± 1.01 (Hz, n=5, Supp Fig. 1A). Spontaneous action potential firings were recorded in type 2 neurons (Supp Fig 1B). Bath application of 500 nM CNO dramatically depolarized the RMP from -50.2 ± 3.79 mV to -37.2 ± 1.49 mV (n=5, $p< 0.05$) and partially inactivated action potential firing of the recorded neurons. As shown in Supp Fig 1C, a brief puff application of 500 nM CNO onto a type 2 neuron only caused brief depolarization and transient burst firings followed by inactivation of action potential firings. Our data suggest that 500 nM CNO can effectively depolarize DREADD-expressing IPN neurons in slice.

Figures & Legends

Figure 1.

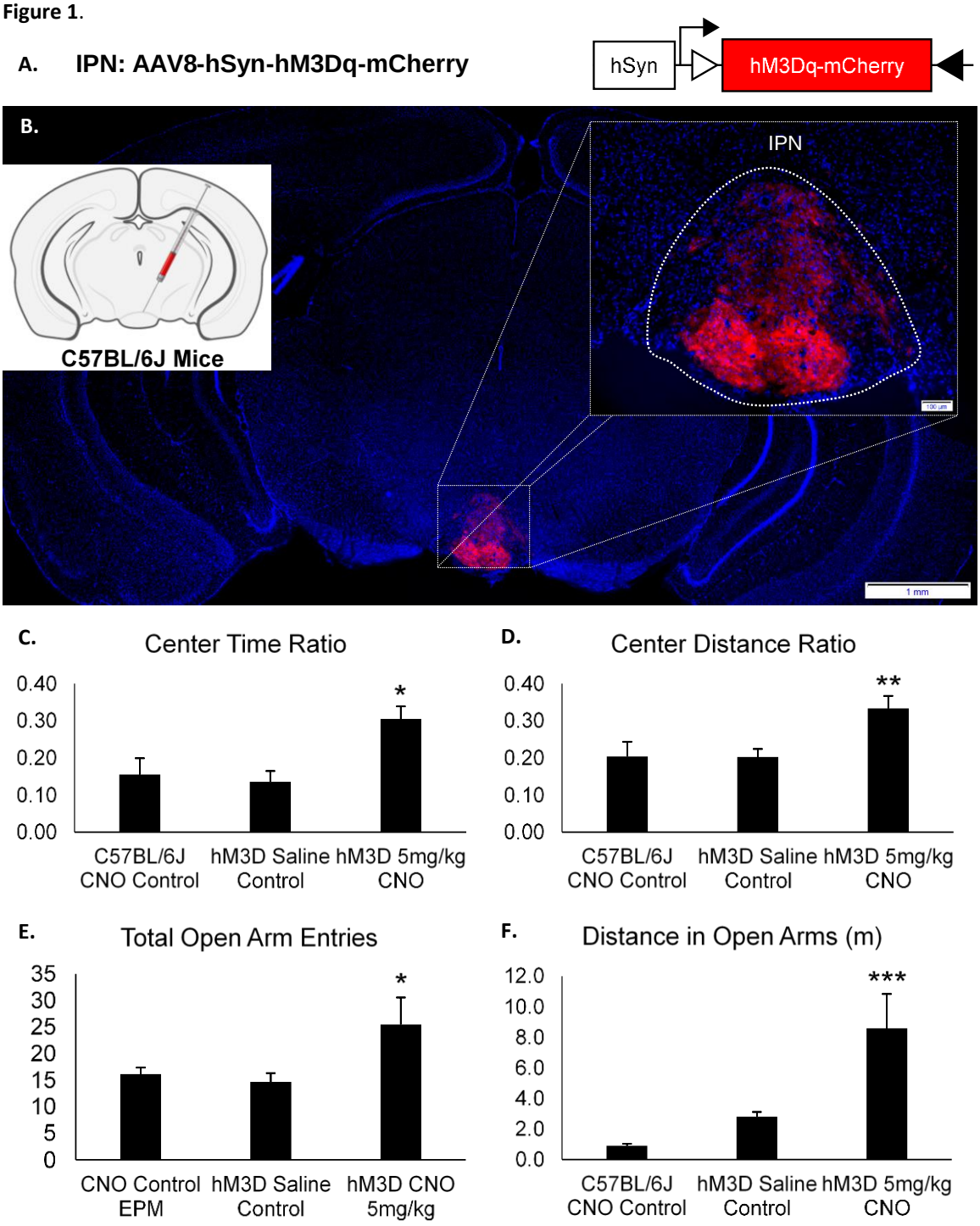
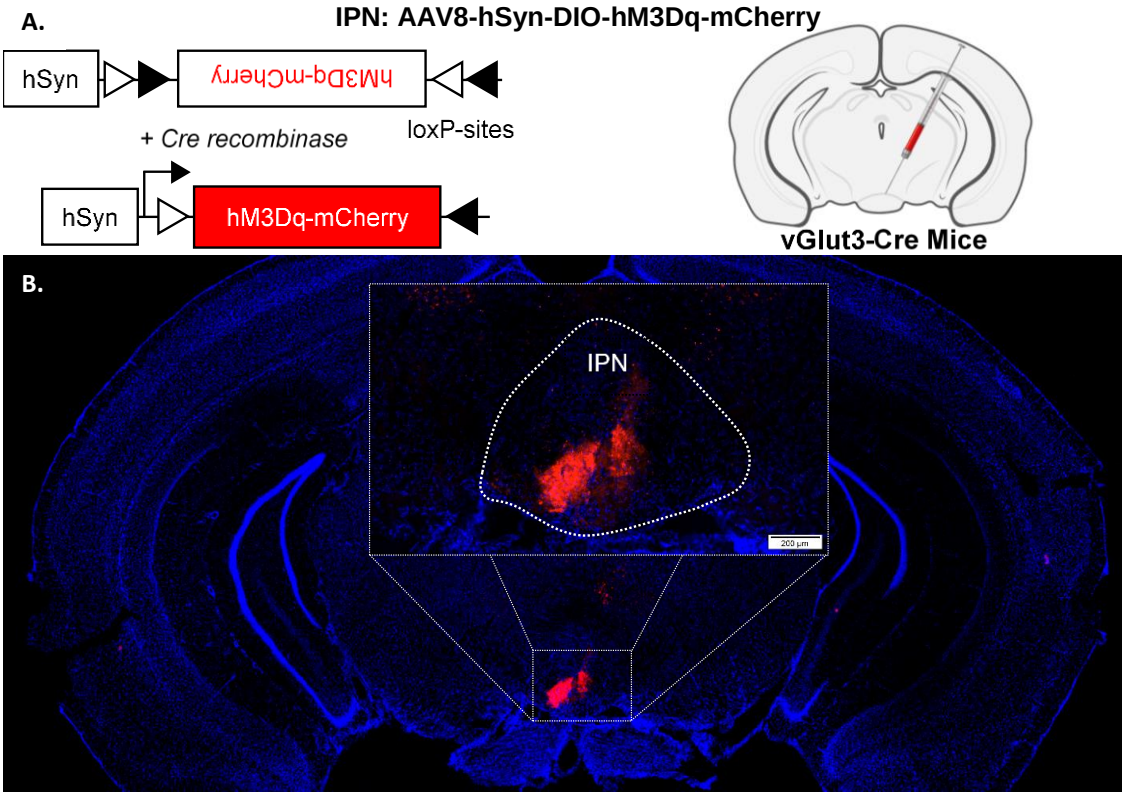
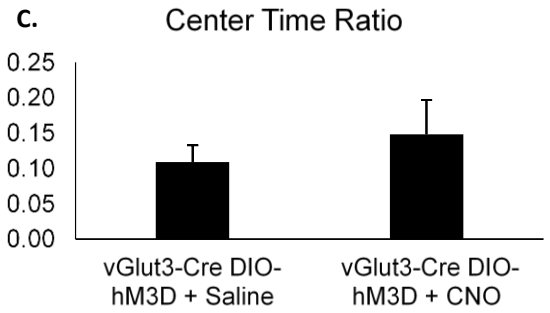
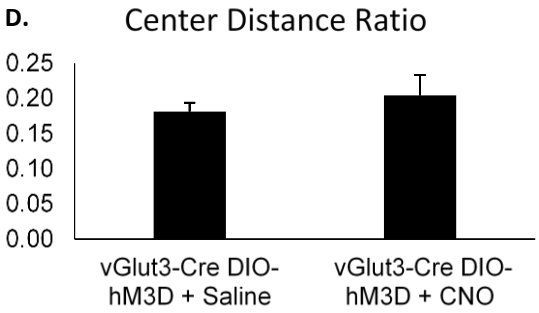
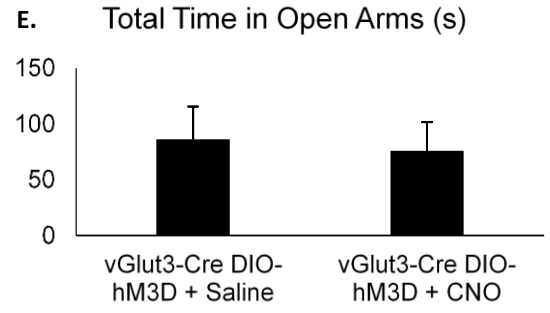


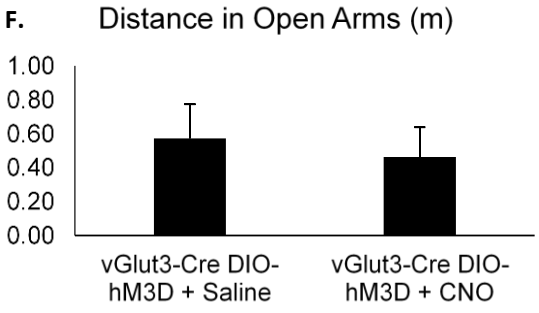
Figure 2.

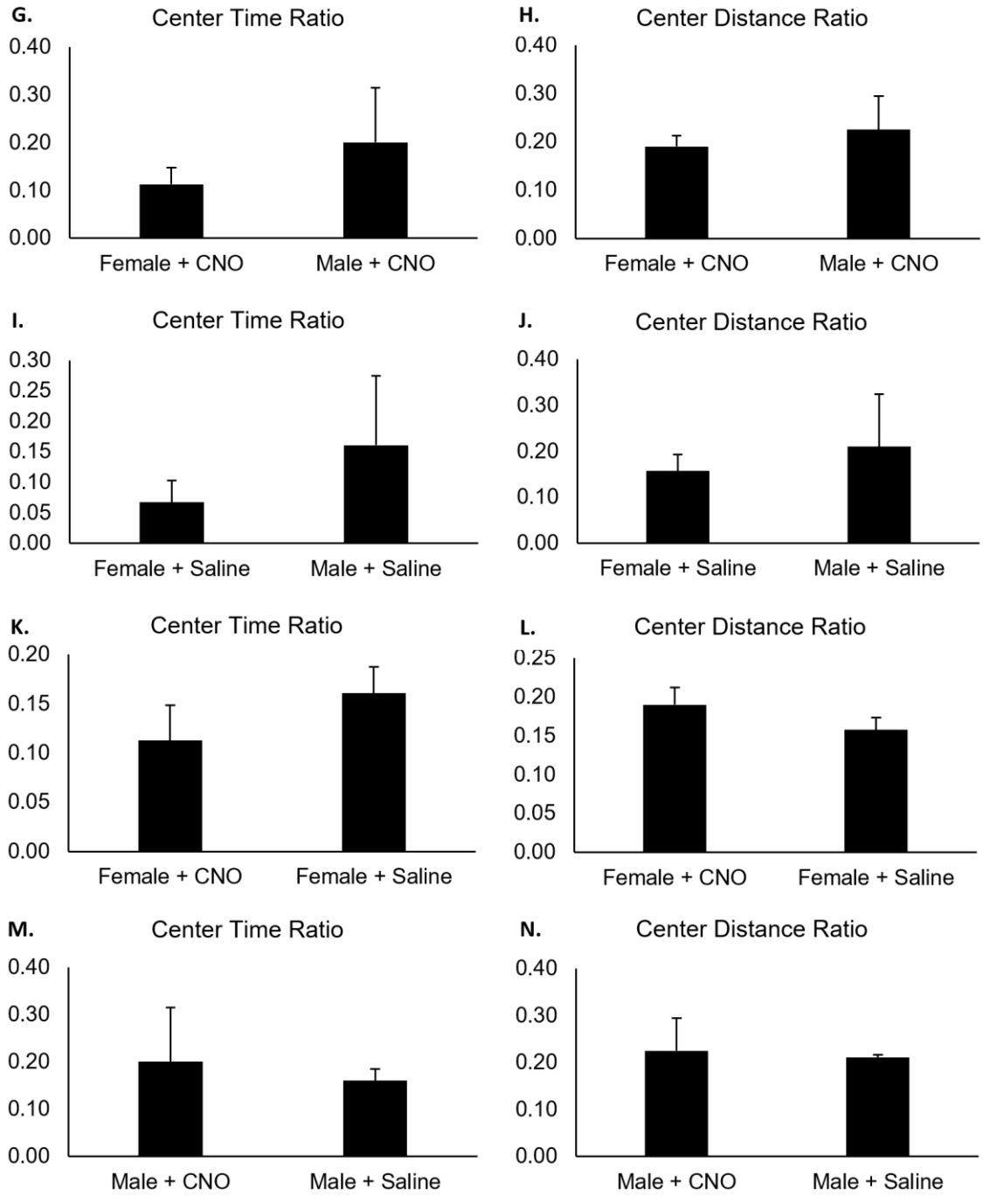

A.
IPN: AAV8-hSyn-DIO-hM3Dq-mCherry
hSyn
hM3Dq-mCherry
loxP-sites
+ Cre recombinase
hSyn
hM3Dq-mCherry
vGlut3-Cre Mice
B.
IPN

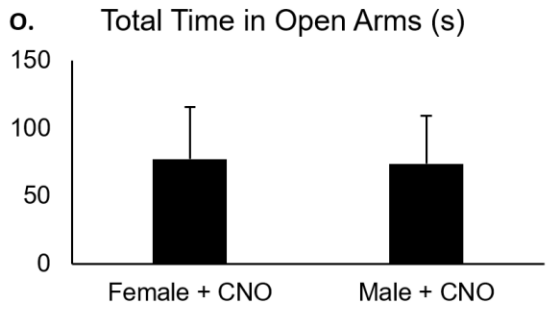

C.
Center Time Ratio
0.25
0.20
0.15
0.10
0.05
0.00
vGlut3-Cre DIO-hM3D + Saline
vGlut3-Cre DIO-hM3D + CNO

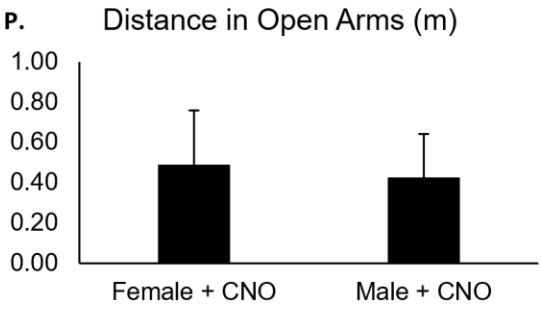

D.
Center Distance Ratio
0.25
0.20
0.15
0.10
0.05
0.00
vGlut3-Cre DIO-hM3D + Saline
vGlut3-Cre DIO-hM3D + CNO

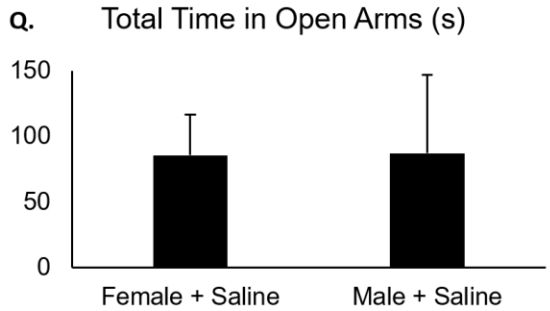

E.
Total Time in Open Arms (s)
150
100
50
0
vGlut3-Cre DIO-hM3D + Saline
vGlut3-Cre DIO-hM3D + CNO

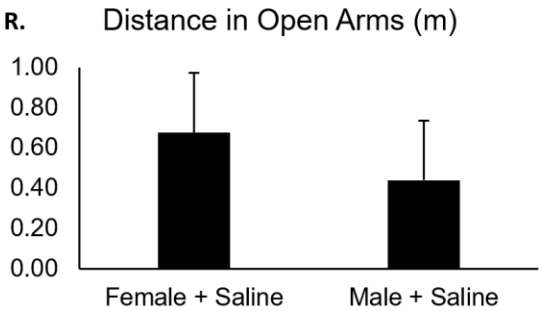

F.
Distance in Open Arms (m)
1.00
0.80
0.60
0.40
0.20
0.00
vGlut3-Cre DIO-hM3D + Saline
vGlut3-Cre DIO-hM3D + CNO

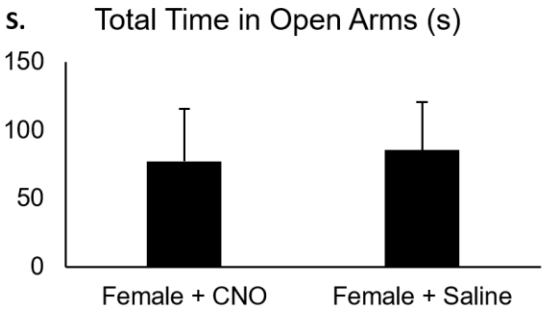

G.
Center Time Ratio
0.40
0.30
0.20
0.10
0.00
Female + CNO
Male + CNO
H.
Center Distance Ratio
0.40
0.30
0.20
0.10
0.00
Female + CNO
Male + CNO
I.
Center Time Ratio
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Female + Saline
Male + Saline
J.
Center Distance Ratio
0.40
0.30
0.20
0.10
0.00
Female + Saline
Male + Saline
K.
Center Time Ratio
0.20
0.15
0.10
0.05
0.00
Female + CNO
Female + Saline
L.
Center Distance Ratio
0.25
0.20
0.15
0.10
0.05
0.00
Female + CNO
Female + Saline
M.
Center Time Ratio
0.40
0.30
0.20
0.10
0.00
Male + CNO
Male + Saline
N.
Center Distance Ratio
0.40
0.30
0.20
0.10
0.00
Male + CNO
Male + Saline

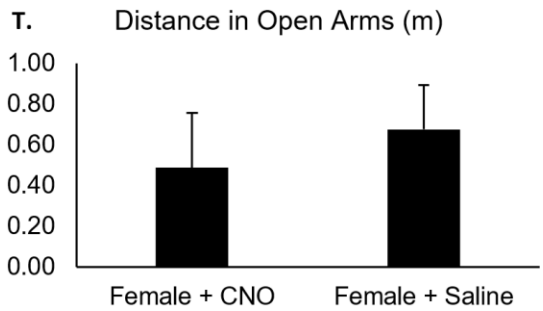

O.
Total Time in Open Arms (s)
150
100
50
0
Female + CNO
Male + CNO

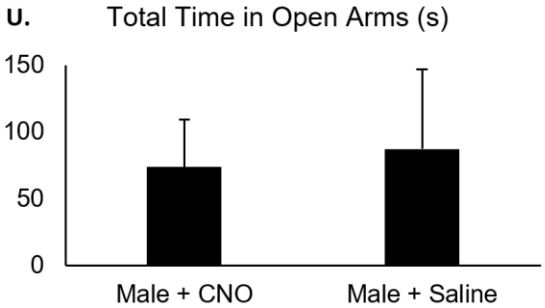

P.
Distance in Open Arms (m)
1.00
0.80
0.60
0.40
0.20
0.00
Female + CNO
Male + CNO


Q.
Total Time in Open Arms (s)
150
100
50
0
Female + Saline
Male + Saline


R.
Distance in Open Arms (m)
1.00
0.80
0.60
0.40
0.20
0.00
Female + Saline
Male + Saline


S.
Total Time in Open Arms (s)
150
100
50
0
Female + CNO
Female + Saline


T.
Distance in Open Arms (m)
1.00
0.80
0.60
0.40
0.20
0.00
Female + CNO
Female + Saline


U.
Total Time in Open Arms (s)
150
100
50
0
Male + CNO
Male + Saline

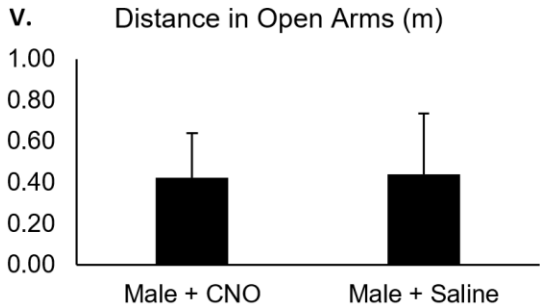

V.
Distance in Open Arms (m)
1.00
0.80
0.60
0.40
0.20
0.00
Male + CNO
Male + Saline

Figure 3.

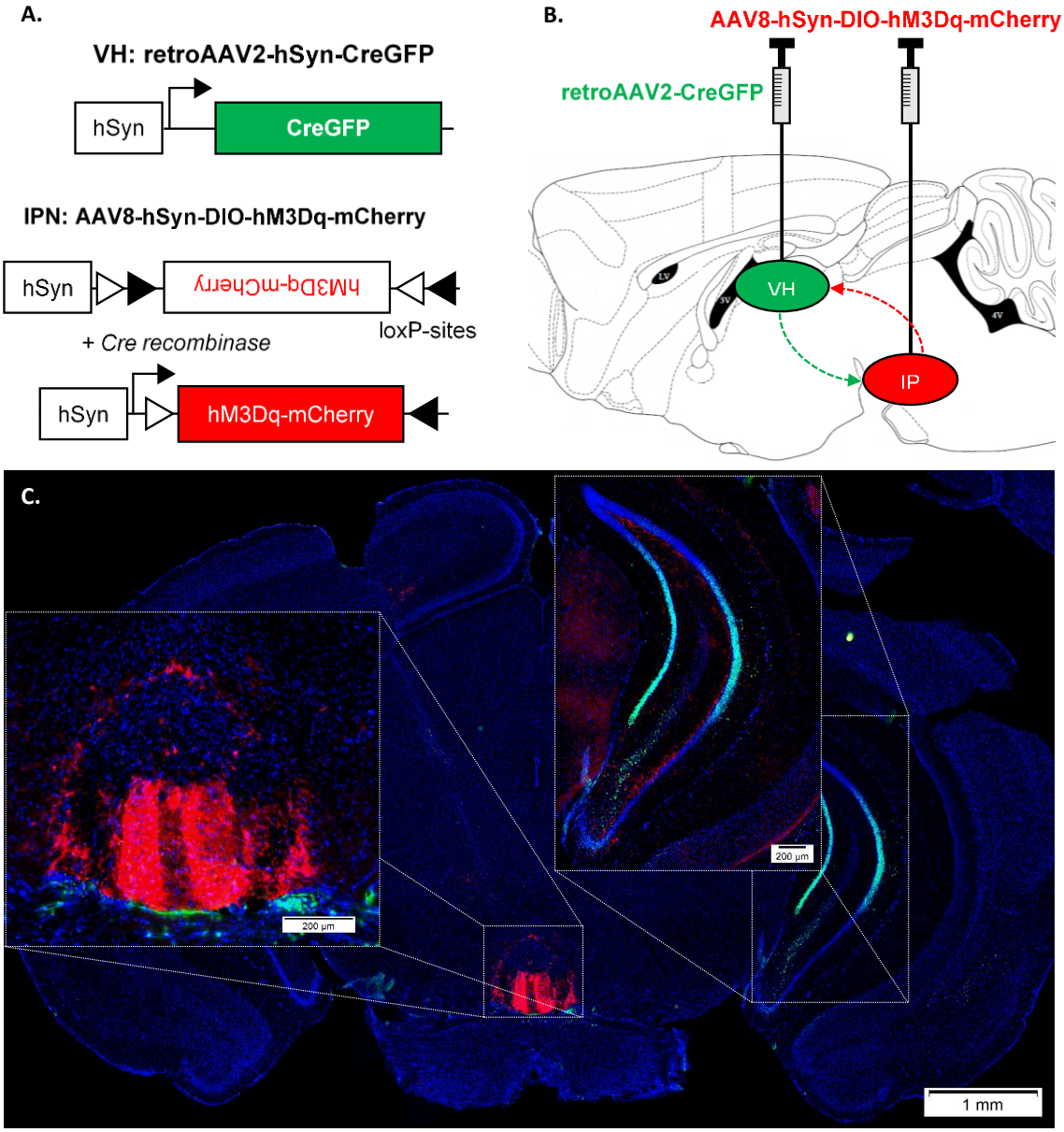
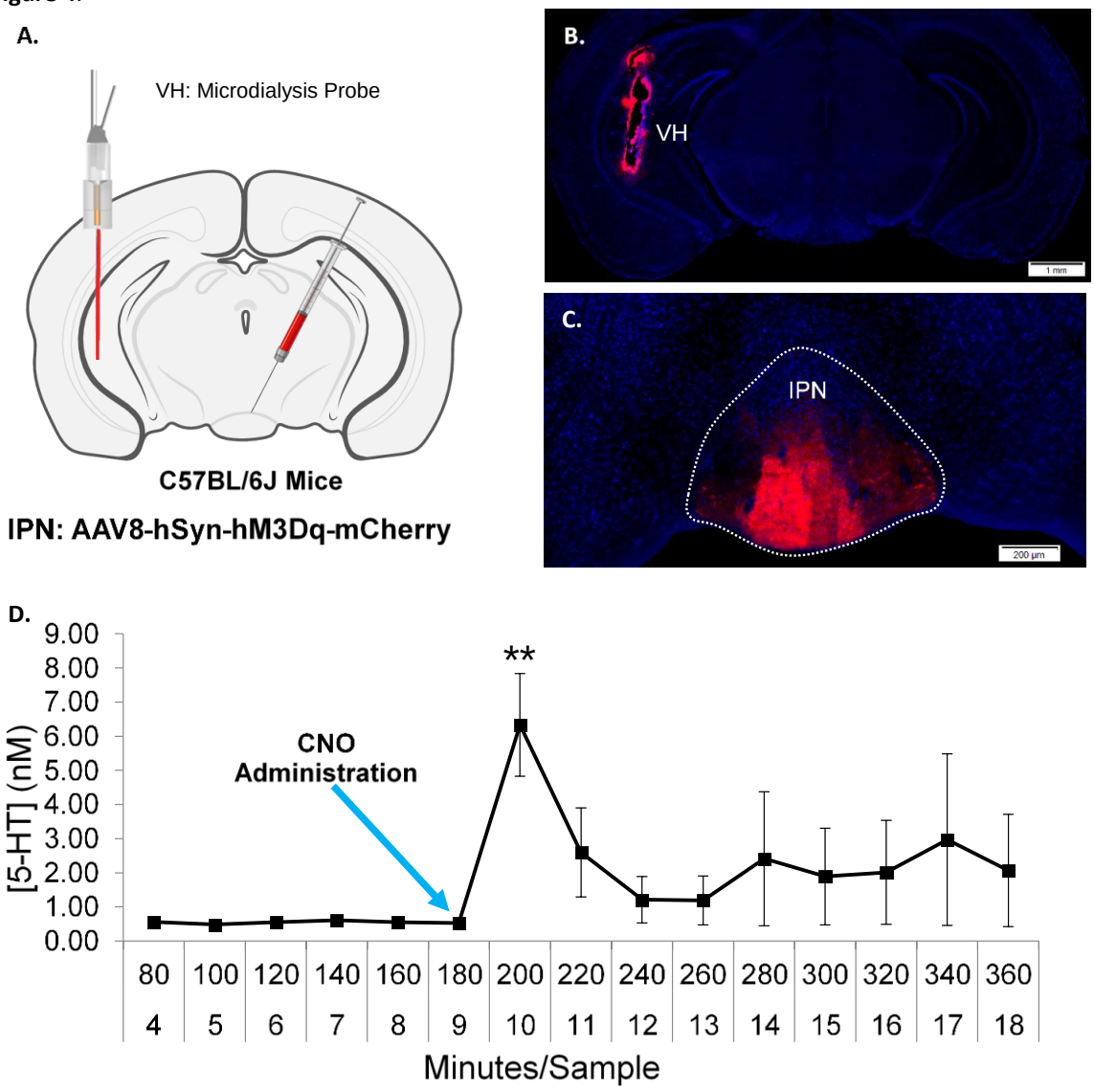

A.
VH: retroAAV2-hSyn-CreGFP
hSyn
CreGFP
IPN: AAV8-hSyn-DIO-hM3Dq-mCherry
hSyn
hM3Dq-mCherry
loxP-sites
+ Cre recombinase
hSyn
hM3Dq-mCherry
B.
AAV8-hSyn-DIO-hM3Dq-mCherry
retroAAV2-CreGFP
VH
IP
LV
3V
4V
C.
200 µm
200 µm
1 mm

Figure 4.


A.
VH: Microdialysis Probe
C57BL/6J Mice
IPN: AAV8-hSyn-hM3Dq-mCherry
B.
VH
1 mm
C.
IPN
200 µm
D.
[5-HT] (nM)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
**
CNO Administration
80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Minutes/Sample

Supplementary Figure 1.

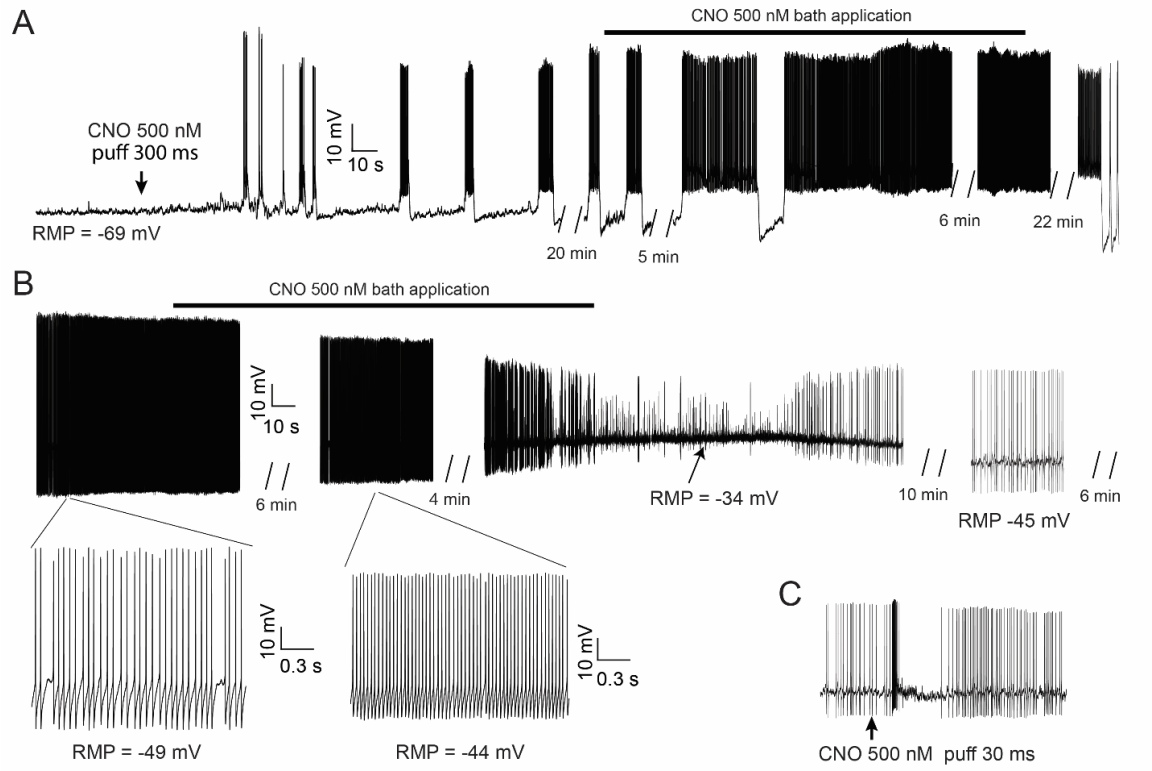


Figure Legends

Figure 1. Chemogenetic stimulation of the IPN in C57BL/6J mice was evaluated for changes in anxiety-associated behavior. Representative image of AAV2/8-hSyn-hM3Dq-mCherry (A) infused into the IPN at low and high magnifications (B). In the Open Field Arena, administration of 5 mg/kg CNO resulted in increased time spent, and distance travelled, in the center zone, indicating a reduction of anxiety-associated behavior in C57BL/6J mice expressing the excitatory designer receptor, hM3Dq (C & D). However, neither wild-type C57BL/6J administered 5 mg/kg CNO, nor C57BL/6J expressing hM3Dq in the IPN administered saline, resulted in reduced anxiety-associated behavior. Similarly, in the Elevated Plus Maze, 5 mg/kg CNO administered to C57BL/6J mice expressing hM3Dq in the IPN exhibited reduced anxiety-associated behavior, indicated by greater time spent, and distance traveled, in the open arms, while control groups exhibited no change (E & F). (OFA n= 11, 9, 10 [$F(2, 27) = 5.714$, $p = 0.0085$]; [$F(2, 27) = 4.6339$, $p = 0.0186$]; EPM n= 12, 6, 4 [$F(2, 19) = 5.276$, $p = 0.015$]; [$F(2, 19) = 25.772$, $p < 0.0001$]).

Figure 2. Chemogenetic stimulation of vGlut3-Cre mice was evaluated for altered anxiety-associated behavior. Representative image of AAV2/8-hSyn-DIO-hM3Dq-mCherry infused into the IPN (A) at low and high magnifications (B). In the Open Field Arena, administration of 5 mg/kg CNO resulted in no significant difference in time spent, and distance travelled, in the center zone (C & D). Upon examination of potential sex-dependent dimorphism, no significant effect was detected (G-N). Similarly, 5 mg/kg CNO administration had no significant effect on time spent, nor distance travelled, in the open arms of the elevated plus maze (E & F). No significant sexual dimorphism was detected in either measure (O-V) (OFA n=9, 10 $p = 0.4808$, $p = 0.4802$; EPM n=9, 10, $p = 0.792$, 0.687).

Figure 3. The IPN sends direct projections to the ventral hippocampus. C57BL/6J mice were infused with retro-AAV2-hSyn-CreGFP in the ventral hippocampus, and AAV2/8-hSyn-DIO-hM3Dq-mCherry in the IPN (A & B). Cre-dependent expression of hM3Dq was observed in regionally specific ventral subnuclei of the IPN, including the IPC, IPI, IPL, as well as sparse expression within the periphery of the structure and terminals within the VH (C).

Figure 4. Microdialysis of serotonin in the ventral hippocampus following chemogenetic stimulation of the IPN. C57BL/6J mice were infused with AAV2/8-hSyn-hM3Dq-mCherry into the IPN (C), and then implanted with a microdialysis probe targeting the ventral hippocampus (A). Probe was coated in DiI to facilitate localization of probe locations (B). Dialysate samples were collected to establish baseline 5-HT concentrations, followed by I.P. injection of 5 mg/kg CNO, resulting in significantly increased 5-HT concentrations (D). (n=3, DF=14, $F=3.19$, $p=0.004$)

Supplementary Figure 1. Electrophysiological characterization of chemogenetic stimulation of virally-infected IPN neurons. C57BL/6J mice were infused with AAV2/8-hSyn-hM3Dq-mCherry into the IPN. Following behavioral evaluations, horizontal sections containing the IPN were evaluated for responses to CNO. After obtaining stable baseline recordings, 300 ms of 500 nM CNO was puffed onto neurons causing robust brief increases in firing (A & C). Bath application of 500 nM led to dramatic

depolarization, which resulted in robust increases in firing frequencies in type 1 neurons (A), while partially inactivation of action potential firing in type 2 neurons (B).

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CHAPTER 3: OTHER CONTRIBUTIONS & PUBLICATIONS

Nicotine and Neurokinin Signaling

This section has been published:

De Biasi, M., McLaughlin, I., Klima, M. Nicotine and Neurokinin Signaling. In: The Neuropathology of Drug Addictions and Substance Misuse. Volume #1: Tobacco, Alcohol, Cannabinoids and Opioids. Chapter 18, Pages 189-200 IVictor Preedy editor, from Elsevier, 2016 ISBN: 9780128002131

ABSTRACT

Substance P (SP) belongs to the family of tachykinins that also contains neurokinins A (NKA) and B (NKB). The three neuropeptides and their respective receptors are expressed in several of the brain regions that regulate stress, anxiety, and addiction-related behaviors. Although there is extensive evidence for a role of the SP/NK1R system in opiate and alcohol dependence, very little is known regarding the role that tachykinins play in the mechanisms of nicotine dependence. This contribution provides an overview of the molecular and circuit-based mechanisms that provide a rationale for the effects of neurokinins on drug abuse. In particular, we provide a summary of the evidence for the interactions with the nicotinic cholinergic system and how these systems represent a mechanistic link between stress, anxiety, and the symptoms of nicotine withdrawal. More studies are needed to determine whether NK receptors are promising drug targets that might complement current smoking cessation strategies.

Abbreviations

ACh, acetylcholine;
AM, amygdala;
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;
BF, basal forebrain;
BNST, bed nuclei stria terminalis
CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione;
CNS, central nervous system;
CP, caudate putamen;
CPP, conditioned place preference;
DA, dopamine;
dMHb, dorsal medial habenula;
DR, dorsal raphe;
EKA, endokinin A,
EKB, endokinin B;
EKC, endokinin C;
EKD, endokinin D;
EPSCs, excitatory post synaptic currents;
FC, frontal cortex;
GABA, gamma-aminobutyric acid;
GnRH, gonadotropin-releasing hormone
HC, hippocampus;
HK-1, hemokinin-1;
HTH, hypothalamus;
5-HT, 5-hydroxytryptamine, serotonin;
ICSS, intracranial self-stimulation paradigm;
IPN, interpeduncular nucleus;
LC, locus coerulus;
MHb, medial habenula;
NAcc, nucleus accumbens;
nAChR, nicotinic acetylcholine receptors;
NK1, neurokinin 1 receptor;
NK2, neurokinin 2 receptor;
NK3, neurokinin 3 receptor;
NKA, neurokinin A;
NKB, neurokinin B;
NMDA, N-Methyl-D-aspartate;
OB, olfactory bulb;
PAG, periaqueductal gray;
PBN, parabrachial nuclei;
PNS, Peripheral nervous system
PPT, pre-pro-tachykinin;

SC, superior colliculus;
SEP, septum;
SN, substantia nigra;
SP, substance P;
Tac1, mouse Tachykinin 1 Gene;
TAC1, Tachykinin 1 Gene;
Tac2, mouse Tachykinin 2 Gene;
TAC2, Tachykinin 2 Gene;
TAC3, Tachykinin 3 Gene;
TAC4, Tachykinin 4 Gene;
TH, thalamus;
vMHb, ventral medial habenula;
VTA, ventral tegmental area;
ZI, zone incerta;

INTRODUCTION

Neurokinins belong to the tachykinin family of proteins, which comprises a series of structurally related neuropeptides found in many species, from amphibians to mammals. Tachykinins are expressed throughout the nervous and immune systems, and participate in a variety of physiological processes, including inflammation, nociception, smooth muscle contractility, epithelial secretion, and proliferation. They also contribute to various disease processes, such as acute and chronic inflammation and pain, fibrosis, bladder and intestinal disorders, infection, and cancer. In addition, pharmacological and biochemical evidence implicates the tachykinin signaling system in the mechanisms underlying aversive affective states, as well as several pathological brain states. Animal models have shown long-lasting effects on neurokinin receptor regulation by stress, and pharmacological experiments have demonstrated an ability of neurokinin receptor agonists and antagonists to modulate anxiety levels. Coupled with the distribution of peptides and receptors in the CNS, pre-clinical and clinical experiments are identifying the tachykinin system as a clear regulator of aversive affect and addiction.

Tachykinin genes and gene products. In humans, there are three tachykinin genes: *TAC1* and *TAC3*, which are equivalent to *Tac1* and *Tac2* in the mouse, respectively, and *TAC4* (Fig. 1). These genes encode precursor proteins, called pre-pro-tachykinins which undergo post-translational proteolytic cleavage to yield the active peptides (Steinhoff, von Mentzer, Geppetti, Pothoulakis, & Bunnett, 2014). *TAC1* encodes for neurokinin A (NKA; also known as neurokinin α and neuromedin L), neuropeptide gamma, neuropeptide K, and substance P (SP). Three splice variants of *TAC1* are known and produce different sets of peptides. All three splice forms of *TAC1* produce SP, but only the beta and gamma

forms produce the other three peptides. *TAC2* was initially assigned to the gene encoding the NKA precursor, but was subsequently found to be identical to *TAC1*. *TAC3* encodes neurokinin B (NKB; also known as neurokinin β and neuromedin K). Finally, *TAC4* encodes for hemokinin-1 (HK-1) and its N-terminal extended forms, endokinin A (EKA), and EKB (Fig. 1). EKA, and EKB have biological actions similar to SP and can interact with NKRs while EKC and EKD lack the tachykinin sequence, have minimal tachykinin-like actions, and show negligible affinity for the NKRs (Steinhoff et al., 2014).

Neuropeptide distribution in the CNS. Neurokinins and their receptors are found throughout the brain, where they have both overlapping and distinct patterns of expression. High levels of SP-immunoreactivity are found in many forebrain, midbrain, and brainstem areas. Those areas include the cingulate cortex, caudate putamen, nucleus accumbens, septum, hippocampus, amygdala, various hypothalamic nuclei, periaqueductal gray, dorsal raphe nucleus, locus coeruleus, various parabrachial nuclei, and the nucleus of the tractus solitarius (Hökfelt, Kuteeva, Stanic, & Ljungdahl, 2004). In general, the distribution of SP in the human brain is quite similar to that in the rat brain, with particularly dense distributions of immunoreactive fibers or SP-positive neurons in cortex, hypothalamus, hippocampus, substantia nigra, and brain stem (Hökfelt et al., 2004).

The immunoreactivity for NKA is highly co-localized with that of SP due to the shared precursor gene (Fig. 1 and Fig. 2). However, despite being co-localized, NKA and SP may be expressed at different levels in a given tissue. For example, SP is more abundant than NKA in striatum and substantia nigra, while NKA levels are higher in the hippocampus (Arai & Emson, 1986).

The distribution of NKB-positive neurons and fibers is, in general, distinct from that of other neuropeptides. NKB is highly expressed in the olfactory bulb, certain hypothalamic nuclei, the basal forebrain, and the habenulo interpeduncular tract. The bed nucleus of the stria terminalis presents one of the highest immunoreactivities and mRNA levels for both NKB and SP (Warden & Young, 1988). The concentration of NKB is also high in the dorsal and ventral portions of the medial habenula (MHb) (Marksteiner, Sperk, & Krause, 1992). This is in contrast with the distribution of SP immunoreactivity and mRNA, which are mainly present in the dorsal part of the MHb. MHb neurons send projections to the interpeduncular nucleus (IPN) that form an extremely dense triangular network of NKB immunoreactive fibers, visible especially in the ventrolateral portion of the rostral IPN. At a more caudal level, this dense immunoreactive plexus separates into the intermediate and lateral nuclei. No NKB mRNA is observed within IPN neurons (Marksteiner et al., 1992). The IPN also receives SP-rich projections from the MHb (Cuello & Kanazawa, 1978). The striatum and substantia nigra pars reticulata contain very few NKB-positive neurons, in contrast with the prominent presence of SP-positive neurons (Burgunder & Young, 1989). Conversely, the hippocampal formation expresses very low levels of SP but contains rather high concentrations of NKB mRNA and immunoreactivity (Ribeiro-da-Silva & Hokfelt, 2000).

Receptor distribution in the CNS. NK1, NK2, and NK3 are the three neurokinin receptor subtypes found in mammals. SP binds preferentially to NK1R, NKA to NK2R, and NKB to NK3R. Despite binding with high affinity to one receptor subtype, each tachykinin can activate all three receptors, a feature that may contribute to pathophysiological conditions characterized by increased tachykinin release (Ebner, Sartori, & Singewald, 2009).

NK1Rs are widely distributed in the CNS, the PNS, and various peripheral organs. They are highly expressed in brain areas known to regulate emotions and the response to stress, such as the amygdala, hypothalamus, hippocampus, frontal cortex, raphe, and locus coeruleus. In those areas, SP and NK1Rs are co-localized. However, in regions such as the substantia nigra and the lateral interpeduncular nuclei, the intense SP staining does not always match the signal for NK1Rs (Mantyh, 2002). In those areas, substance P may bind to closely related NK2 or NK3 receptors (Saria, 1999).

NK2Rs have a limited distribution in the CNS and are most abundant in peripheral tissues. In the CNS, NK2Rs are found in some cortical areas, the hippocampus, the nucleus accumbens, parts of the thalamus, and the lateral septum (Hökfelt et al., 2004; Saffroy, Torrens, Glowinski, & Beaujouan, 2003). As discussed for NK1Rs, the presence of NK2Rs in several limbic structures is consistent with a role of NKA in the processing of emotions (Hökfelt et al., 2004).

NK3Rs are mostly found in the CNS. They are expressed in the cerebral cortex, the zona incerta, the MHb, the amygdala, the superior colliculus and the IPN, as well as the septum, dorsal raphe, periquaductal gray, and locus coeruleus (Ebner et al., 2009). Despite considerable anatomical overlap of NK1Rs and NK3Rs, there are marked differences of distribution patterns in certain brain areas. For example, strong NK1R signals are found in the medial and cortical portions of the amygdala, while NK3R expression is mainly localized to basomedial and basolateral nuclei. Distinct patterns of expression for the two receptors are also found in septum, hypothalamus, and prefrontal cortex (Ebner et al., 2009; Hökfelt et al., 2004; Saffroy et al., 2003).

Neurokinin-mediated mechanisms in the CNS. Tachykinins are found in the axons of many neuronal populations, including those that release glutamate, GABA, serotonin, and acetylcholine (Commons, 2010). In addition to axons, neurokinin-containing large dense-core vesicles are found in somata, dendrites, and neuronal varicosities. Neuropeptides are packaged in large dense-core vesicles that are distinct from the small synaptic vesicles containing the co-existing neurotransmitter (Keegan, Woodruff, & Pinnock, 1992; Mantyh, 2002). The neuropeptides present in various neuronal classes are co-released with classical neurotransmitters or neuromodulators, especially upon strong activation of the parent cell. The mechanisms that regulate neurokinin release are different from those that regulate fast synaptic transmission, as neurokinin-mediated responses have a slower time course of activation compared to those triggered by ionotropic neurotransmitters. Due to these characteristics, neurokinins provide a spatially and temporally defined refinement of neuronal circuits.

Dopamine. SP is prominently expressed in striatal structures and has the ability to increase extracellular DA levels (Ribeiro-da-Silva & Hokfelt, 2000). In particular, there seems to be a positive feedback loop involving the striatum and the mesencephalon in which the DA released from VTA projections can induce the expression of *TAC1* mRNA coding for SP in the striatum (Campbell & Walker, 2001). Conversely, the SP released from striatal projections onto the VTA can increase DA release from midbrain neurons (Kovács, Steinmann, Magistretti, Halfon, & Cardinaux, 2006). Given that DA release is associated with reward and reward prediction, such a positive feedback loop is likely to play an important role in motivation and addiction. Besides NK1Rs, the VTA and substantia nigra pars compacta express NK3Rs, and application of the selective NK3R agonist senktide to those two areas can also enhance the firing of DA neurons (Keegan et al., 1992) and trigger DA release in projection sites (Marco et al., 1998).

Serotonin. A number of studies suggest that neurokinins may induce serotonin (5-HT) – mediated behaviors by enhancing the release of endogenous 5-HT through the increase of serotonin cell firing in the raphe nuclei (Gradin, Qadri, Nomikos, Hillegaart, & Svensson, 1992). Electrophysiology studies in brain slice preparations showed that both NK1 and NK3 receptor agonists, rather than acting directly on 5-HT neurons, activate them indirectly, by increasing excitatory postsynaptic currents (EPSCs) (R. Liu, Ding, & Aghajanian, 2002). The EPSCs are blocked by the AMPA/kainate glutamate receptor antagonist CNQX and can be prevented by tetrodotoxin, suggesting that neurokinins enhance local glutamatergic neuronal afferents (R. Liu et al., 2002).

Norepinephrine. Locus coeruleus (LC) neurons are innervated by SP-containing fibers (Baker et al., 1991), express NK1 receptors (Chen, Wei, Liu, & Rao, 2000), and are activated by SP (Cheeseman, Pinnock, & Henderson, 1983). Acute exposure to SP increases LC firing rates and chronic, but not acute, treatment with a SP receptor antagonist changes firing properties from tonic to phasic burst firing (Maubach et al., 2002). This effect resembles that of the conventional antidepressant, imipramine. Phasic burst firing activity in the LC occurs during focused attention and orienting behavior, while tonic firing is increased during acute stress (Aston-Jones, Rajkowski, & Cohen, 1999). Based on this evidence, it has been hypothesized that SP might participate in the processing of stressful experiences, and that the use of NK1R antagonists might facilitate adaptations to stress (Maubach et al., 2002).

Acetylcholine. In addition to modulating DAergic activity, tachykinins exert a profound influence on cholinergic activity. This effect has been documented in both the striatum and the septum (Schable, Huston, & Silva, 2012). In the striatum, tachykinins (SP and NKA) and GABA have opposite effects on the regulation of ACh release from cholinergic

interneurons (Blanchet et al., 2000). While GABA has a facilitatory effect on ACh release, tachykinins inhibit it, especially under maximal NMDA receptor activation.

In the medial septum, which sends cholinergic projections to the hippocampus and the amygdala, tachykinins increase ACh release. The medial septum has among the highest levels of NK2Rs (Saffroy et al., 2003), and expresses both NK1Rs and NK3Rs, which are located on the cholinergic population of septal neurons (Schable et al., 2011). Injection of either NK1R, NK2R, or NK3R agonists into the medial septum increases ACh levels in the hippocampus, and NK2Rs have specifically been implicated in stress-induced hippocampal ACh release (Schable et al., 2012). It has been proposed that these tachykinin-mediated alterations of septal cholinergic activity might underlie the reported effects of neurokinins on anxiety and learning processes (Schable et al., 2012). It should also be noted that intraseptal injection of NKA and NKB increases ACh release in the amygdala but not in the frontal cortex (Schable et al., 2012).

The medial habenula (MHb) is another brain area where tachykinins modulate cholinergic activity. MHb neurons are glutamatergic, but the nucleus can subdivided into two principal subnuclei based on the use of ACh as a co-transmitter in the ventral MHb (vMHb) and the expression of SP in the dorsal MHb (dMHb) (Dao, Salas, & De Biasi, 2015). MHb neurons respond to NK1R and NK3R stimulation with a rapid and concentration-dependent increase in firing rates, but no effect is seen following NK2R activation (Norris, Boden, & Woodruff, 1993).

Tachykinins and Addiction Mechanisms

Tackykinins and their receptors are expressed in areas that are prominently involved in addictive processes such as the nucleus accumbens and the habenula (Luthi & Luscher,

2014). Drug abuse often occurs in subjects experiencing mood and anxiety-related symptoms, and stress is a major cause of drug seeking and relapse. Therefore, when considering the regulation of behaviors that lie at the intersection of stress and addiction, the ability of neurokinins to modulate neurotransmitter function is particularly relevant. For example, norepinephrine mediates stress-induced reinstatement and escalates self-administration of multiple addictive drugs (Erb et al., 2000; Gilpin & Koob, 2010; Leri, Flores, Rodaros, & Stewart, 2002; Mantsch et al., 2010; Vranjkovic, Hang, Baker, & Mantsch, 2012). NK1R ligands, by modulating the firing patterns of the LC, can affect norepinephrine release. Dopaminergic activity, while best known for its role in reward-related mechanisms, is also increased during exposure to some stressors. Interestingly, NK1R antagonists can prevent DA release in the prefrontal cortex induced by immobilization stress (Hutson, Patel, Jay, & Barton, 2004).

Neurokinins might be directly involved in the mechanisms of drug abuse as systemic and intracranial administration of SP (Hasenohrl et al., 2000) and the NK3R-selective agonist, aminosenktide is sufficient to produce conditioned place preference (CPP) (Ciccocioppo et al., 1998). The effects of SP are likely mediated by its active C-terminal fragment, which exhibits greater affinity for NK3Rs than NK1Rs (Hasenohrl, Gerhardt, & Huston, 1992), suggesting that NK3Rs contribute to the reinforcing effects of SP.

Neurokinins and nicotine: cellular mechanisms. Most of what is known about the interactions between neurokinins and the nicotine contained in tobacco comes from studies conducted on peripheral organs. Those studies established that SP and NKA, expressed in sensory neurons and immune-system cells such as macrophages and dendritic cells, participate in the mechanisms of bronchoconstriction and inflammation produced by smoking (Joos, De Swert, & Pauwels, 2001). Initially, smoke activates

bronchopulmonary C-fiber afferents, at least in part by binding to nAChRs expressed in nearby epithelial cells (L. Y. Lee et al., 2007). This produces a vagal reflex that leads to the release of neurokinins (Hong, Rodger, & Lee, 1995). Neurokinins, in turn, facilitate ACh release from postganglionic parasympathetic neurons, thereby increasing the contractility of airway smooth muscle cells. SP and NK1Rs also promote the accumulation of macrophages and dendritic cells in the airways following exposure to cigarette smoke. Through this mechanism, they contribute to the development of smoking-induced emphysema and chronic obstructive pulmonary disease (Joos et al., 2001).

In the CNS, nicotine acts through multiple, intricate mechanisms to facilitate the release of DA and several other neurotransmitters (Li Wang et al., 2014). In the VTA and substantia nigra, chronic nicotine treatment causes a decrease in SP immunoreactivity, consistent with enhanced SP release and consequent tissue depletion of neuropeptide (Alburges, Frankel, Hoonakker, & Hanson, 2009). These nicotine-mediated changes in SP immunoreactivity can be blocked by the non-selective nAChR antagonist, mecamylamine, as well as a dopamine D1 or D2 receptor antagonist. This observation, considered together with the fact that SP injection into the VTA increases neuronal firing and dopamine turnover (Kelley & Delfs, 1991), suggests that part of the effects of nicotine on mesolimbic function could be derived from an interaction with the neurokinin system.

The MHb is another area of interaction between the cholinergic and tachykinins systems that is involved in nicotine addiction. All MHb neurons are cholinoceptive and express high levels of nAChRs (Dao et al., 2015). In addition, the vMHb contains neurons that synthesize and release ACh onto the IPN and other projection sites. Our lab examined the effects of nicotine on the cholinoceptive and cholinergic neurons located in the vMHb (Fig. 3A) and showed that activation of nAChRs by nicotine enhances the intrinsic excitability of MHb neurons in a fashion similar to that produced by tachykinins (Fig. 3B) (Dao et al.,

2014). We found that enhancement of excitability by nicotine does not require postsynaptic nAChR function, nor ionotropic glutamatergic or GABAergic transmission. Rather, nicotine's enhancement of MHb excitability is abolished by blockade of either NK1Rs or NK3Rs. Thus, under basal conditions, SP and NKB release onto MHb neurons modulates their intrinsic excitability, but this effect can be further amplified by local release of ACh or by nicotine. Our work suggests that nAChRs containing the α5 subunit modulate MHb excitability, largely by facilitating NKB release onto MHb neurons, with additional facilitation of SP release. Because of the anatomical expression, we suspect that the source of NKB onto the cholinoceptive/cholinergic MHb originates from local neurons, in addition to neurons throughout the MHb. Conversely, the source of possible SP release onto the vMHb might be from the dorsal one-third of the MHb (Dao et al., 2015).

Neurokinins and nicotine: regulation of mood and affect

Due to their expression in the limbic circuits that control stress and anxiety, tachykinins participate in the regulation of mood and affect (Ebner et al., 2009). Most of the information available focuses on the roles played by NK1R and its preferred ligand, SP, although more recent animal studies have revealed that NK3R and its cognate ligand, NKB, can also contribute to aversive emotional states. NKA and NK2Rs appear to play a modulatory role as well.

SP/NK1R. SP infusion induces anxiety-like behavior in rodents that is NK1R-dependent (Ebner et al., 2009). Early studies with guinea pigs, characterizing the effects of systemic SP or NK1R agonist injections, reported behaviors associated with anxiety, such as reduced food intake (Hasenohrl, Schwarting, Gerhardt, Privou, & Huston, 1994), grooming behaviors (Takahasi et al., 1987), and increased vocalizations. These manifestations were abolished by pretreatment with NK1R antagonists (Kramer et al., 1998). Acute

administration of an NK1R antagonist to rats is also anxiolytic when tested in the social interaction test (File, 1997). In addition, mice with genetic deletion of the NK1R exhibit decreased anxiety in behavioral paradigms that provoke anxious states, such as the elevated plus maze and novelty-suppressed feeding test, and produce fewer ultrasonic vocalizations during maternal separation (Santarelli et al., 2001). Additionally, transgenic mice that lack *Tac1*, and therefore do not express SP or NKA, exhibit significantly lower levels of anxiety relative to WT controls in several behavioral paradigms (Bilkei-Gorzo, Racz, Michel, & Zimmer, 2002).

Although systemic injection of SP is typically anxiogenic, and NK1R antagonism is anxiolytic, there is evidence for variable regulation of anxiety by the SP/NK1R system in different anatomical regions. For example, selective ablation of NK1R-expressing neurons within the amygdala generates increased anxiety-associated behavior in the EPM (Gadd, Murtra, De Felipe, & Hunt, 2003). Immobilization stress and the EPM test elevate SP levels in the medial sub-nucleus of the amygdala, an effect that can be blocked by microinfusion of NK1R antagonists into the nucleus (Ebner et al., 2009). Additionally, direct infusion of SP into the central or medial - but not basolateral sub-nuclei of the amygdala - is anxiogenic in the EPM, suggesting distinct contributions of amygdalar sub-nuclei to the behavior (Bassi, de Carvalho, & Brandao, 2014).

In addition to the amygdala, other studies have shown that the levels of SP in the lateral septum increase in response to the forced swim test – with NK1R antagonists enhancing intraseptal serotonergic transmission, which increases active stress-coping strategies (Ebner et al., 2009). The LC, paraventricular nucleus (PVN) of the hypothalamus, and lateral septum may also contribute to the behavioral effects of SP, as NK1R antagonists counteract stress-induced elevations of c-Fos expression in those three brain areas (Vendruscolo, Takahashi, Bruske, & Ramos, 2003). Further, speaking to the complex

effects of neurokinins in the CNS, while some anxiety-provoking behavioral tests elevate SP in anatomical structures associated with anxiety and stress, a 20 minute foot-shock paradigm reduced SP levels in the VTA and interpeduncular nucleus (IPN) (Lisoprawski, Blanc, & Glowinski, 1981).

Several studies in humans have validated the pre-clinical work described above. fMRI imaging highlighted altered signaling in limbic structures following NK1R blockade in healthy human volunteers (McCabe, Cowen, & Harmer, 2009), and the administration of a different NK1R antagonist for 6 weeks in patients diagnosed with social phobia resulted in significant symptom alleviation (Furmark et al., 2005). An early NK1R antagonist yielded significant anxiolytic activity in a population of depressed patients exhibiting high levels of anxiety (Kramer et al., 1998). Since this study, additional human studies have not yielded consistently encouraging outcomes (McCabe et al., 2009), possibly because of pharmacokinetic and genetic variables that were not considered in experimental designs.

NKB/NK3R. More recent efforts have revealed signaling contributions by NKB and its preferred receptor, NK3R, in areas of the brain associated with anxiety and stress. For example, NKB is highly expressed in the amygdala. Fear conditioning, a paradigm that depends on intact amygdalar function, increases NKB expression significantly, and the infusion of Osanetant, an NK3R antagonist, impairs the consolidation of fear memories (Andero, Dias, & Ressler, 2014). Interestingly, a loss-of-function approach to establishing the role of NKB/NK3R signaling in modulating affect revealed a nuanced story. Absence of the NK3R impairs both the acquisition of conditioned avoidance and performance in the Morris water maze (Siuciak et al., 2007), further suggesting a particular involvement of NKB/NK3R in aversive emotional memory.

Other peptides might also contribute to the effects of SP on mood and affect. Restraint stress results in altered trafficking of NK3Rs in vasopressin-expressing cells of the PVN

(Miklos, Flynn, & Lessard, 2014). Vasopressin and the PVN are part of a signaling system that regulates social behavior and stress, the release of ACTH, and the hypothalamic-pituitary-adrenal axis (HPA) (Stevenson & Caldwell, 2012). In addition, an NK3R agonist could inhibit gonadotropin releasing hormone (GnRH) secretion in mice (Navarro et al., 2009), pointing to another possible mechanism for the interaction between neurokinins and anxiety/stress via GnRH secretion (S. N. Umathe, Bhutada, Jain, Dixit, & Wanjari, 2008). Indeed, the administration of an NK3R antagonist resulted in anxiolysis (Salome, Stemmelin, Cohen, & Griebel, 2006), performing similarly to diazepam and buspirone – both of which are used to treat anxiety. Contrary to the lack of effect upon systemic injection of an NK3R agonist, anxiolysis in the EPM was observed following intracerebroventricular (ICV) infusion in the mouse. Pretreatment with naloxone enhanced this effect, while naloxone alone did not alter behavior, suggesting a possible interaction between NK3R and the opioid signaling systems (S. J. Ribeiro & De Lima, 1998). Taken together, these data suggest that NKB/NK3R also participate in anxiety-associated signaling.

NKA/NK2R. Perhaps due to the restricted expression profile of NKA/NK2 in the CNS, relatively few studies have evaluated the role of NK2R activation in anxiety and stress. However, the administration of NK2R antagonists has proven to be consistently anxiolytic. Saredutant, an NK2R antagonist, was anxiolytic in gerbils undergoing the social interaction test (Salome et al., 2006), mice tested in the light-dark box paradigm, and primates evaluated by the human intruder response test (Walsh, Stratton, Harvey, Beresford, & Hagan, 1995). Additionally, NK2R antagonists reduce defensive biting and escape attempts in mice (Griebel, Perrault, & Soubrie, 2001), and reduce anxiety in the EPM in both mice (Teixeira et al., 1996) and rats (Griebel et al., 2001). NK2R agonists are anxiogenic in the EPM (Teixeira et al., 1996), and are capable of inhibiting the anxiolytic

effects of diazepam (R. L. Ribeiro & De Lima, 2002). An investigation of the long-term consequences of footshock treatment in rats revealed significantly changed *Tac1* mRNA in a variety of anatomical structures associated with the regulation of emotional states, and autoradiography studies revealed reduced NK2R binding in the amygdala two weeks after treatment. While the mechanisms of this receptor down-regulation are unclear, the authors posit that it may result from an adaptive response to limit excessive SP release (de Lange, Wiegant, & Stam, 2008).

Neurokinins and the symptoms of nicotine withdrawal.

Although reward significantly contributes to the addictive properties of nicotine, smoking is often motivated by the urge to alleviate the affective, cognitive, and somatic symptoms of withdrawal (McLaughlin et al., 2015). Affective and cognitive signs of withdrawal are produced by CNS mechanisms. Anxiety is a prominent affective symptom, and it acts as a potent negative reinforcer that promotes smoking. Somatic signs reflect mainly peripheral, "bodily" mechanisms such as decreased heart rate and constipation. However, besides a peripheral nAChR component, somatic signs have a central component that is thought to translate to a dysphoric state of heightened irritability. These withdrawal symptoms are effectively recapitulated in animal models; upon nicotine withdrawal, both rats and mice exhibit stereotypies, including excessive grooming, chewing, tremors, 'wet-dog shakes', yawns, and teeth chattering – as well as behaviors that have been associated with anxiety, anhedonia, depression, dysphoria, and hyperalgesia (McLaughlin et al., 2015). Despite the abundance of data implicating neurokinin signaling in anxiety and stress, to our knowledge, no studies have been conducted so far to precisely characterize the role of neurokinins and their receptors in the anxiety-related symptoms of nicotine withdrawal. Besides the areas in which tachykinin expression overlaps with anatomical structures that synthesize and release serotonin, norepinephrine, and dopamine, the MHb

and IPN are potential sites for interactions between signaling systems. This hypothesis is derived from the high levels of SP/NK1R, NKB/NK3R and nAChRs in the MHb, and the fact that the MHb-IPN circuit plays a prominent role in anxiety in both humans and rodents (Dao et al., 2015). In addition, the interplay between cholinergic and neurokinin signaling within the MHb may represent a critical anatomical region wherein neuroadaptations occur, resulting in the signaling that causes anxiety exhibited during withdrawal from the chronic use of nicotine and other drugs (Dao et al., 2015).

The MHb-IPN circuit has an established role in the somatic symptoms of nicotine, ethanol and likely, morphine abstinence (Dao et al., 2015; Muldoon et al., 2014; E. Perez, Quijano-Carde, & De Biasi, 2015b). Interestingly, intraperitoneal and ICV infusions of SP in rats elicit physiological and behavioral responses such as increased heart rates, wet-dog shakes, and increased grooming (Tschope, Jost, Unger, & Culman, 1995). Those symptoms are observed during stress as well as nicotine withdrawal (McLaughlin et al., 2015). To that end, we were able to show that administration of NK1R and NK3R antagonists in mice chronically treated with nicotine can precipitate somatic symptoms of withdrawal (Fig. 3C) – supporting a model wherein nicotine directly modulates neurokinin signaling in the MHb to promote intrinsic neuronal excitability (Dao et al., 2015).

Applications to Other Addictions and Substance Misuse

Several groups have examined the involvement of tachykinins in drug abuse. Most studies addressed the role of SP and NK1Rs and have shown their involvement in the mechanisms of dependence from opioids, cocaine, and alcohol. The following sections describe the involvement of neurokinin signaling in addictions to other substances.

Opiates. Substance P and NK1Rs modulate the rewarding effects of opiates (Commons, 2010). NK1R antagonism suppresses heroin self-administration in rats (Barbier et al., 2013). In addition, NK1R null mice display morphine reward deficits in the CPP paradigm (Gadd et al., 2003; Murtra, Sheasby, Hunt, & De Felipe, 2000) and self-administer less morphine than their control littermates, without any change in natural reward (Ripley, Gadd, De Felipe, Hunt, & Stephens, 2002). Morphine's ability to potentiate reward is also attenuated by NK1R antagonism in the intracranial self-stimulation paradigm (ICSS) (J. E. Robinson et al., 2012). Based on lesion studies, the amygdala, rather than the nucleus accumbens, is where the presence of NK1Rs affects morphine reward (Gadd et al., 2003). Lack of NK1Rs also attenuates morphine-induced locomotor activation and psychomotor sensitization (Murtra et al., 2000; Ripley et al., 2002). There are also preclinical data indicating that NK1R blockade might attenuate the symptoms of opioid withdrawal (Maldonado, Girdlestone, & Roques, 1993), and that administration of SP or its N-terminal peptide can prevent the development of morphine withdrawal symptoms (Kreeger & Larson, 1996). A small study conducted in methadone-maintained patients showed that the FDA approved NK1R antagonist, aprepitant, might have utility for alleviating opioid withdrawal and craving (Jones et al., 2013).

Besides the influence of SP on the brain circuits underlying morphine-related behaviors, NK1Rs and μ-opioid receptors (MOR) may interact at the cellular level. Specifically, SP prevents morphine-induced internalization and acute desensitization of MORs (Yu, Arttamangkul, Evans, Williams, & von Zastrow, 2009). This phenomenon was observed in both amygdala and LC neurons, which can co-express NK1Rs and MORs. Finally, chronic morphine administration results in NK1R upregulation (Wan et al., 2006). Overall, these data point to a rich interaction between the opioid and tachykinin systems and suggest

that NK1R blockade could be one approach to reduce opiates abuse by limiting their rewarding and reinforcing properties, as well as mitigating withdrawal symptoms.

Cocaine. The effects of SP on cocaine-induced behaviors differ from those observed for morphine. When cocaine-induced locomotion and DA release in the dorsal striatum were examined, it was found that NK1R blockade attenuated both responses (Loonam, Noailles, Yu, Zhu, & Angulo, 2003). However, lack of NK1Rs did not affect locomotor sensitization to cocaine in mice (Ripley et al., 2002), nor did it affect cocaine CPP (Murtra et al., 2000). Furthermore, in contrast with what was found for morphine, targeted lesions of the amygdala did not affect CPP (Gadd et al., 2003). Cocaine self-administration rates were not affected by NK1R deletion in mice (Ripley et al., 2002) or NK1R antagonism in rats (Schank et al., 2014). Interestingly, NK1R blockade could prevent stress-induced reinstatement but had no effect on cocaine seeking triggered by a cocaine priming injection (Placenza, Vaccarino, Fletcher, & Erb, 2005).

Other studies showed that NK3Rs are involved in cocaine-induced hyperlocomotion and DA release within the nucleus accumbens, but not in cocaine-induced CPP (Jocham, Lauber, Müller, Huston, & De Souza Silva, 2007). In summary, although neurokinins might be involved in some aspects of cocaine dependence, there is no evidence that they influence its rewarding properties.

Alcohol. Pre-clinical and clinical data, which are discussed in detail elsewhere in this volume, indicate that neurokinins are involved in the mechanisms of alcohol abuse. Briefly, similar to morphine, NK1R null mice do not display alcohol CPP (Thorsell, Schank, Singley, Hunt, & Heilig, 2010). They also consume less alcohol in the two-bottle choice drinking paradigm (George et al., 2008), a phenomenon also observed when using both NK1R antagonists (Thorsell et al., 2010) and NK1R knockdown (Baek et al., 2010). In addition, NK1R null mice do not display increased alcohol consumption after repeated

cycles of deprivation, suggesting that SP influences the neuroadaptations that occur during repeated withdrawal and lead to drinking escalation (Thorsell et al., 2010). Interestingly, blockade of NK1R does not affect baseline operant alcohol self-administration in the rat, but, similarly to what was found for cocaine, it suppresses stress-induced reinstatement of alcohol seeking (Schank et al., 2014).

Information on the effects of NK1R blockade is also available for humans. A clinical trial of the NK1R antagonist, LY686017, in detoxified alcoholic inpatients demonstrated suppression of spontaneous alcohol cravings and improved overall well-being (George et al., 2008). Subsequently, a case–control association study conducted in Caucasian subjects concluded that polymorphisms of the NK1 receptor are significantly associated with the development of alcohol dependence (Seneviratne et al., 2009). Based on this evidence, NK1R antagonists may be a treatment strategy for some subpopulations of alcohol abusers, such as those with certain genetic variants of the *TACR1* gene.

Conclusions

Tachykyins and their receptors are expressed in brain areas and circuits that control stress and anxiety. These areas also participate in the mechanisms of drug abuse, and there is evidence for the involvement of tachykinins in the abuse of opiates, cocaine, and alcohol. For opiates, NK1Rs affect baseline reward and reinforcement, as well as escalated self-administration rates. For cocaine and alcohol, NK1Rs mediate stress-induced reinstatement of drug seeking and escalated drug self-administration, but do not affect baseline self-administration. Very little is known regarding the role of tachykinins in the manifestations of withdrawal, except for reports that link SP to some of the symptoms of opiate abstinence.

Surprisingly, despite a clear role of tachykinins in many peripheral effects of nicotine, there have been no studies on their roles in nicotine reward and seeking. As for the mechanisms of withdrawal, our lab was the first to report a link between SP and NKB and the manifestations of physical dependence to nicotine, highlighting the MHb-IPN circuit as a major site for those interactions. Therefore, the field is ripe for further investigations into mechanisms and the potential application of NK1R antagonists to facilitate smoking cessation. Even less is known about the role of NK2R and NK3R in drug addiction. However, given the growing availability of NKR subtype–specific antagonists, genetically engineered pre-clinical models, and many additional molecular and genetic tools, future studies should be able to address the specific role of NKA and NKB in nicotine addiction. As for the potential use in the clinic, NK1R antagonists are generally well tolerated, warranting further investigation into their effectiveness for the treatment of nicotine and other drug abuse disorders. The clinical trials examining the effect of NK1R antagonists on alcohol abuse have yielded mixed results, possibly due, among other factors, to relatively small sample sizes and variable drug pharmacokinetics. As pharmacogenetic analyses become cheaper and more easily incorporated into clinical trials, investigators will be able to determine the impact of genetic variations in neurokinin genes on both the risk and the response to treatment in addicted subjects. The use of empirically derived sub-phenotypes (e.g. co-morbid anxiety) will be particularly helpful in identifying the subjects that would benefit the most from the addition of neurokinin antagonists to their treatment strategies.

Mini Dictionary of Terms

Cholinergic neuron: A neuron that possesses the intracellular machinery for the synthesis and release of acetylcholine

Cholinoceptive neuron: A neuron that expresses receptors for acetylcholine

Electrophysiology: A technique that allows measurement of the electrical activity of neurons.

Feedback loop: A mechanism through which a neuronal system can self-adjust to optimize function and maintain equilibrium

Immunoreactivity: Measure of the signal for a particular protein that can be detected in the brain by antibodies that bind with high specificity to that protein.

Intracranial self-stimulation: an experimental paradigm in which animals self-stimulate certain brain regions through electrodes surgically implanted into the brain. The stimulation activates the brain reward system.

Mecamylamine: Nicotinic receptor antagonist used to precipitate nicotine withdrawal in mice chronically exposed to nicotine

Somatic signs of withdrawal. The physical symptoms associated with nicotine abstinence. The nicotine abstinence syndrome comprises affective, cognitive, and somatic signs.

Ultrasonic vocalizations: Calls of distress inaudible to humans. They are thought to reflect negative affective states.

Key Points

Tachykinin signaling is involved in anxiety, stress, and addiction.

- Tachykinin signaling plays a modulatory role in the signaling of dopamine, serotonin, norepinephrine, and acetylcholine – all of which are known to play roles in the pathophysiology of mood-related disorders and addiction
- Systemic injection of SP is generally anxiogenic, while antagonists targeting SP's cognate receptor are anxiolytic
- NK2R antagonists have proven to be anxiolytic in pre-clinical models, and stress induces long-lasting alterations of tachykinin gene product levels
- Pharmacological manipulations of the NK3R result in modified anxiety-associated behavior in animal models, depending upon the particular CNS structure into which they are infused.
- The MHb-IPN circuit, which is critically involved in anxiety and symptoms of withdrawal from nicotine and other drugs, hosts considerable tachykinin signaling. Pharmacological manipulations of NK1R & NK3R-dependent signaling in this circuit are sufficient to induce withdrawal symptoms in mice chronically treated with nicotine.
- Many questions remain unanswered concerning the functional roles played by each receptor type, as well as the roles of other tachykinin signaling peptides
- Clinical applications of neurokinin receptor-targeting drugs have not consistently proven superior to the standards of care for mood-related disorders or addiction.
- More nuanced approaches to clinical trials, such as genomic screening, combinatorial pharmacological strategies, and increased receptor specificity for

more targeted action may yield more positive results for the treatments of mood-related disorders and addiction

Summary Points

- Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are all members of the tachykinin family of signaling peptides, and are expressed and released in areas of the brain associated with anxiety, stress, and drug addiction
- NK1r and NK3r are predominantly expressed in the CNS, while NK2 is most densely expressed in the PNS
- NK1 receptors (NK1R) bind SP, NK2R bind NKA, and NK3R bind NKB with the greatest affinities – but each of the peptides is capable of binding all of the receptors
- While there is substantial overlap in anatomical distribution of expression and release, each peptide and its receptor also occupy distinct anatomical regions
- The receptors for all three receptors are G-protein coupled, and have been shown to modulate dopamine, serotonin, and acetylcholine signaling
- Tachykinin signaling has been implicated in the effects of nicotine and other drugs, as well as the neuroadaptations that occur as a result of chronic use
- Pharmacological manipulation of neurokinin receptors has been shown to alter anxiety-, depression-, and stress-associated behavior in pre-clinical and clinical studies

FIGURES

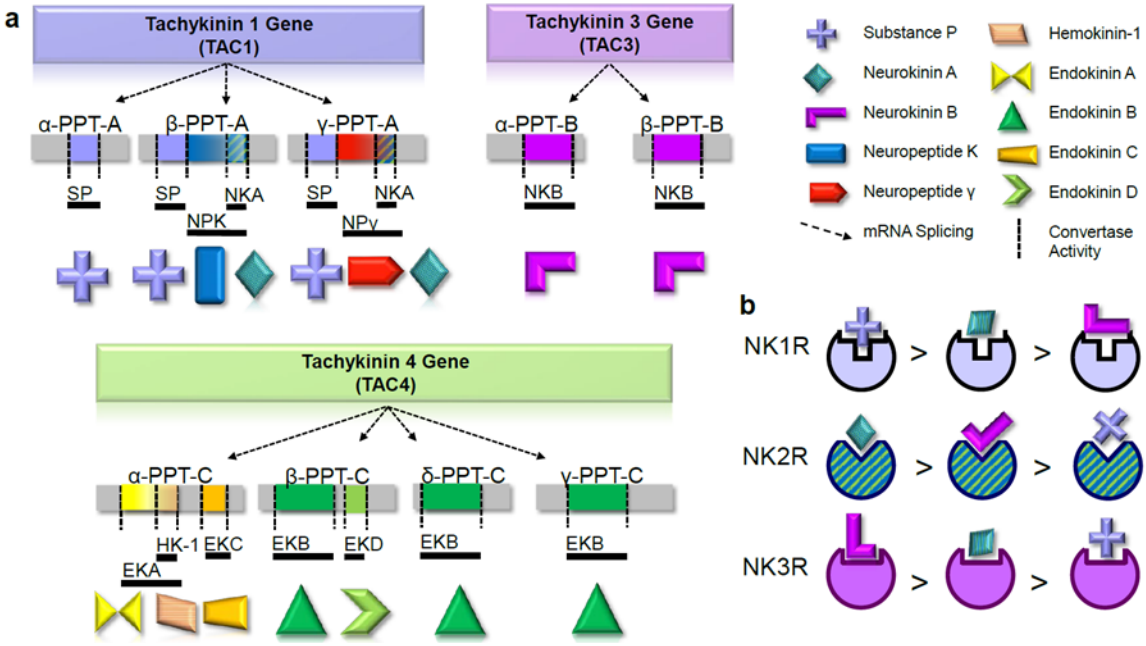

Figure 1. **Coding and processing of tachykinin peptides**. **a**. Neurokinins are the product of three genes: Tachykinin 1 Gene (*TAC1*), *TAC3*, and *TAC4*. The mRNA from these genes is spliced to form precursor proteins, pre-protachykinin (PPT) A, B, and C, which are further cleaved by convertases to give the final peptide products. Substance P, Neurokinin A, and Neurokinin B are the major neurokinins produced. **b**. Neurokinins bind to three G-protein coupled receptors called Neurokinin 1 Receptor (NK1R), NK2R, and NK3R. Although all neurokinins can bind to these receptors, each neurokinin has a preferential affinity for one NKR.

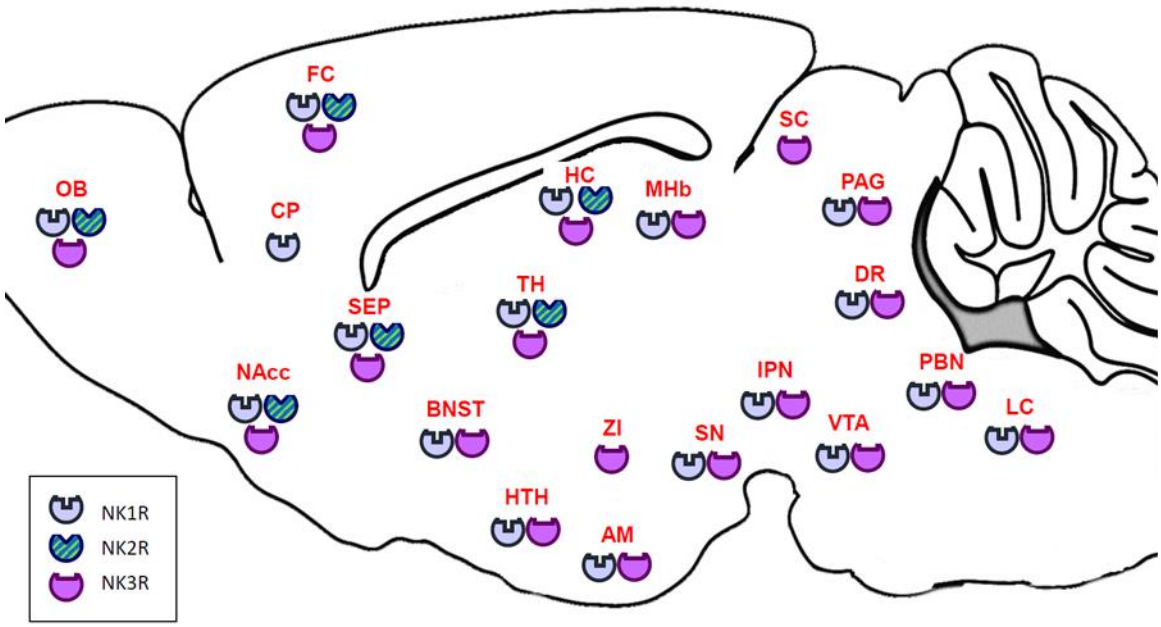

Figure 2. NK1R, NK2R, and NK3R are located throughout the CNS. The receptors are predominantly found on limbic structures and often overlap with serotonergic and noradrenergic pathways implicating neurokinins in affective behavior and mood regulation. OB, olfactory bulb; FC, frontal cortex; CP, caudate putamen; SEP, septum; NAcc, nucleus accumbens; BNST, bed nuclei of stria terminalis; HTH, hypothalamus; AM, amygdala; TH, thalamus; ZI, zone incerta; HC, hippocampus; MHb, medial habenula; SC, superior colliculus; PAG, periaqueductal gray; DR, dorsal raphe; PBN, parabrachial nuclei; LC, locus coerulus; VTA, ventral tegmental area; IPN, interpeduncular nucleus; SN, substantia nigra.

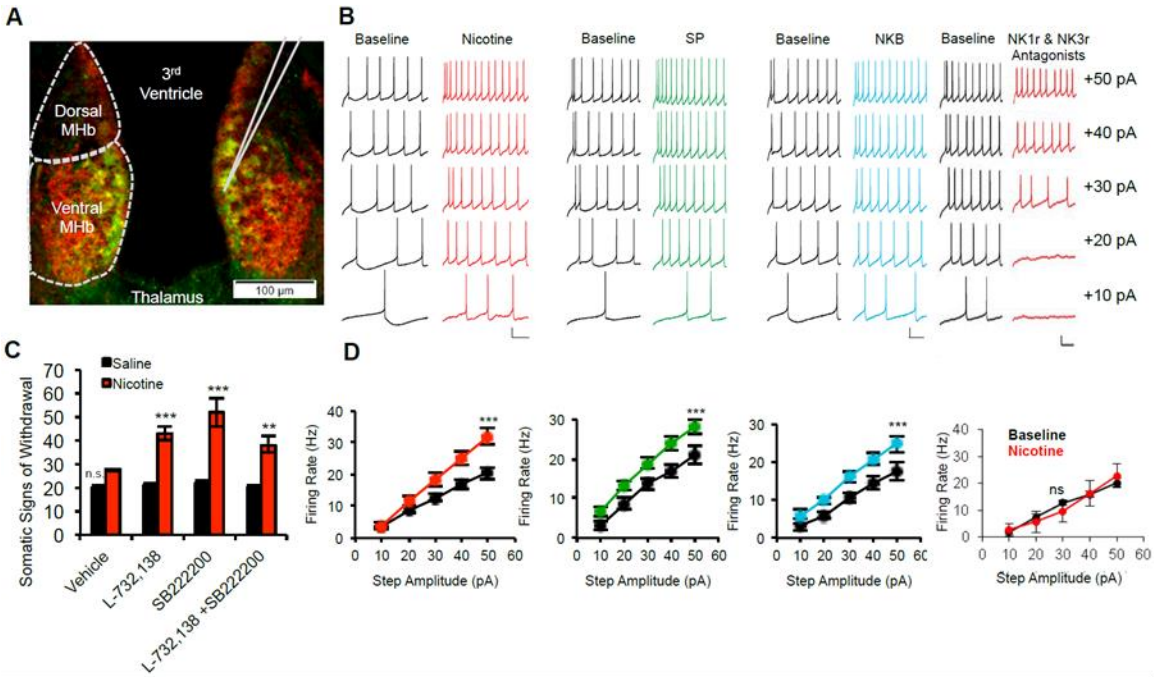

Figure 3. SP and NKB increase MHb neuronal firing rates similar to the effect of nicotine, and neurokinin receptor antagonists inhibit nicotine-induced facilitation of excitability. A. A brain slice from a mouse expressing EYFP (green) driven by the choline acetyltransferase (ChAT) promoter, stained with antibodies targeting the NK1R (red), reveals a dense presence of both ChAT and the NK1R in the ventral sub-region of the MHb. Lines indicate the placement of recording electrodes. **B**. Cholinoceptive neurons locate in the vMHb held at -70mV in current-clamp were stimulated with 2 s depolarizing current steps of increasing amplitudes (10-50 pA) to evaluate action potential behavior. Action potential frequency elicited by each current step increased relative to baseline, suggesting increased intrinsic excitability. Bath application of nicotine (1µM), as well as both SP and NKB augmented the increase in firing frequencies produced by cell depolarization. This potentiation was abolished by exposure to antagonists targeting NK1R (L-732138, 10µM) and NK3R. Relative frequencies quantified and graphed in **D**. **C**. Infusion of NK1R and NK3R antagonists into the MHb of mice chronically treated with nicotine precipitated somatic signs of withdrawal. Following 2 weeks of 8.4 mg/kg/day delivery of nicotine via osmotic minipump, infusions of L-732138 (NK1R antagonist), SB222200 (NK3R antagonist), or both simultaneously, increased the number of somatic signs over the course of a 20-minute examination period relative to vehicle-microinjected controls. *Figure partially adapted from (Dao et al., 2014).*

Nicotine Withdrawal

This section has been published

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1. Withdrawal syndrome in humans

1.1. Symptoms

1.2. Genetic influences

2. Withdrawal syndrome in mice

2.1. Behavioral Manifestations and Testing Paradigms.

3. Receptors and nAChR Subunits Underlying Withdrawal Symptoms

3.1. Insight from nAChR Subunit KO mice

4. Molecular mechanisms involved in the nicotine withdrawal syndrome

4.1. nAChR Upregulation

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5. Anatomical Structures and Circuits Implicated in Nicotine Withdrawal

5.1. Dopaminergic System.

5.2. Habenular Complex and Interpeduncular Nucleus

5.3. Hippocampus

5.4. Extended amygdala

6. Summary and Research Moving Forward

Abstract

An aversive abstinence syndrome manifests 4 to 24 hours following cessation of chronic use of nicotine-containing products. Symptoms peak on approximately the 3rd day and taper off over the course of the following 3-4 weeks. While the severity of withdrawal symptoms is largely determined by how nicotine is consumed, certain short nucleotide polymorphisms (SNPs) have been shown to predispose individuals to consuming larger amounts of nicotine more frequently – as well as to more severe symptoms of withdrawal when trying to quit. Additionally, rodent behavioral models and transgenic mouse models have revealed that specific nicotinic acetylcholine receptor (nAChR) subunits, cellular components, and neuronal circuits are critical to the expression of withdrawal symptoms. Consequently, by continuing to map neuronal circuits and nAChR subpopulations that underlie the nicotine withdrawal syndrome – and by continuing to enumerate genes that predispose carriers to nicotine addiction and exacerbated withdrawal symptoms – it will be possible to pursue personalized therapeutics that more effectively treat nicotine addiction.

1. Withdrawal Syndrome in Humans

1.1. Symptoms

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) reports 7 primary symptoms associated with nicotine withdrawal: irritability/anger/frustration, anxiety, depressed mood, difficulty concentrating, increased appetite, insomnia, and restlessness (Association 2013). The syndrome might also include constipation, dizziness, nightmares, nausea, and sore throat. For practical purposes, nicotine withdrawal symptoms are classified as affective, somatic, and cognitive. Affective symptoms include

anxiety, anhedonia, depression, dysphoria, hyperalgesia, and irritability. Somatic manifestations include tremors, bradycardia, gastrointestinal discomfort, and increased appetite. Cognitive symptoms manifest as difficulty concentrating and impaired memory (Heishman, Kleykamp et al. 2010). This constellation of symptoms reflects the brain-wide influence of cholinergic transmission. Studies characterizing the participation of specific nAChRs in signaling during particular manifestations of withdrawal are helping to reveal their underlying mechanisms (Paolini and De Biasi 2011).

1.2. Genetic Influences.

Nicotine addiction is influenced by both genetic and environmental factors. Depending on the parameters used to define dependence, and the population considered, heritability contributes 50%-75% of the risk for dependence (Dokal, Pagliuca et al. 1989, Hall, Madden et al. 2002, Lessov, Martin et al. 2004, Furberg, Kim et al. 2010, Nugent, Million-Mrkva et al. 2014). Risk factors for nicotine dependence can be identified using genetic methods such as linkage and candidate gene analyses, as well as genome wide association studies (GWAS). Linkage analysis assesses the presence of a phenotype in large, high-risk families to map the location of disease-causing loci in relation to a known genetic marker. Candidate gene analysis assesses the association between a particular gene allele (or alleles) potentially involved in the disease and the disease itself. GWAS, in contrast to candidate gene approaches, do not limit analyses to relationships between specific genes and a phenotype, and aim to identify loci for novel susceptibility genes. An example of how such genetic approaches can be successfully utilized in nicotine research is the gene cluster on chromosome 15q25 that encodes the α5, α3, and β4 nAChR subunits. Gene variants within the cluster have been repeatedly shown to affect nicotine dependence and smoking quantity (Saccone, Saccone et al. 2009). A non-

synonymous nucleotide polymorphism (SNP), rs16969968, which substitutes an aspartic acid for an asparagine (D398N) in the CHRNA5 region of the cluster, has been associated with reduced receptivity to nAChR agonists in vitro, reduced neuronal calcium permeability, and more extensive nAChR desensitization (Jackson, Marks et al. 2010). Individuals who are homozygous for this SNP are more likely to progress to heavy smoking and nicotine dependence (Hartz, Short et al. 2012), and it has been suggested that the reduction in agonist responsivity and increased desensitization may contribute to these increased propensities (Jackson, Marks et al. 2010). Apart from the missense rs16969968 variant, there are other SNPs within the CHRNA5-CHRNA3-CHRNB4 cluster that are relevant to nicotine addiction and withdrawal, and further biological characterizations are needed to establish the functional consequences of those mutations. Other nAChR genes have also been shown to influence smoking quantity and nicotine dependence, such as common variants in the chromosome 8p11 region that contains the genes encoding the α6 and β3 nAChR subunits (Thorgeirsson, Gudbjartsson et al. 2010). Overall, linkage analyses have highlighted 13 regions on 11 chromosomes that include genes with potential influences on nicotine dependence (Leeb and Tamse 1985, Li 2008). Genes involved in nicotine metabolism are also likely important to the nature of nicotine withdrawal. Cytochrome P450 2A6 (CYP2A6) is the enzyme mainly responsible for the conversion of nicotine to cotinine, which typically accounts for 70%–80% of nicotine metabolism (Hecht, Hochalter et al. 2000). In subjects of European descent, GWAS meta-analyses identified SNPs in the region of CYP2A6 associated with the number of cigarettes smoked per day, as well as other smoking behavior phenotypes (Thorgeirsson, Gudbjartsson et al. 2010).

Genetic factors may also account for 29-53% of the variance in withdrawal symptoms and approximately 50% of the variance in quitting success (Xian, Scherrer et al. 2003, Xian, Scherrer et al. 2005, Pergadia, Heath et al. 2006). In a linkage analysis study, a sample of Australian and Finnish smokers was queried about withdrawal symptoms within the context of a smoking cessation attempt that they recalled well. The study revealed a linkage signal that meets genome-wide significance on chromosome 11p15 in the Finnish families (Pergadia, Agrawal et al. 2009). Four strong candidate genes lie within or near the peak area on chromosome 11: dopamine receptor 4 (DRD4), TPH tryptophan hydroxylase 1 (TPH), TH (tyrosine hydroxylase), and CHRNA10 (nAChR subunit 10). A second region identified by linkage on chromosome 11q23 includes HTR3A (hydroxytryptamine receptor 3A), HTR3B (hydroxytryptamine receptor 3B), DRD2 (dopamine D2 receptor gene), ANKK1 (ankyrin repeat and kinase domain containing 1), and GRIK4 (ionotropic kainate glutamate receptor 4 gene). An earlier study reported that DRD2 TaqI-B polymorphisms influence abstinence and withdrawal symptoms (Robinson, Lam et al. 2007). Smokers carrying the DRD2 TaqI-B1 risk allele (B1/B1 or B1/B2) reported significantly more symptoms of daily smoking withdrawal compared to smokers homozygous for the TaqI-B2 allele (B2/B2). In addition, while withdrawal symptoms - measured 14 days pre-quit and 42 days post-quit - decreased significantly in Taql-B2 homozygous over time, smokers with the TaqI-B1 allele reported little improvement in self-reported withdrawal symptoms.

Several other pharmacogenetic studies, including a couple of genome wide association study (GWAS) analyses, have examined genetic influences on smoking cessation and response to therapy (Leeb and Tamse 1985, Uhl, Liu et al. 2007, Furberg, Kim et al. 2010, Gold and Lerman 2012, King, Paciga et al. 2012, Bloom, Martinez et al. 2013,

Chen, Bloom et al. 2014). SNPs in CHRNB2 and the CHRNA5-CHRNA3-CHRNB4 cluster seem to influence smoking cessation, although the reported effects are not always reproducible (Conti, Lee et al. 2008, Baker, Weiss et al. 2009, Perkins, Lerman et al. 2009, Gold and Lerman 2012, Hartz, Short et al. 2012). The influence of CHRNA5-CHRNA3-CHRNB4 haplotypes on tolerance, craving, and loss of control seems greatest in individuals who began smoking early in life, suggesting that the risk associated with those genes is greatest with early tobacco exposure (Baker, Weiss et al. 2009). The rs8192475 variant in CHRNA3 was shown to predict withdrawal symptoms and craving after quitting (Sarginson, Killen et al. 2011). The major G allele was associated with stronger but relatively short-lived symptoms, while the minor A allele resulted in a relatively constant level of symptoms and craving during the acute phase of withdrawal. The rs8192475 α3 SNP leads to an R37H change in the amino acid sequence that increases agonist sensitivity of heterologously expressed α3β4 nAChRs (Haller, Druley et al. 2012). Rare missense variants at conserved residues in CHRNB4 (T375I and T91I) are associated with reduced numbers of cigarettes per day and fewer signs of withdrawal (Haller, Druley et al. 2012). Similar to the α3 SNP rs8192475, expression of the two β4 mutations in HEK 293 cells leads to larger currents in response to ACh stimulation, which has been suggested to increase the aversive properties of nicotine.

Similar to what was found for phenotypes related to nicotine dependence, there are also associations of CYP2A6 enzyme activity and nicotine metabolic rates with smoking cessation and smoking cessation strategies (Lee, Jepson et al. 2007, Chen, Bloom et al. 2014). Individual carrying the null activity allele, CYP2A6*2, are twice as likely to quit smoking as subjects without that allele (Gu, Hinks et al. 2000). Conversely, smokers with

high activity alleles (CYP2A6*1/*1B) experience more severe withdrawal symptoms when trying to quit (Kubota, Nakajima-Taniguchi et al. 2006).

Currently, the literature suggests that multiple genetic loci influence the severity of withdrawal symptoms and the ability to abstain from smoking. Such polygenic contributions overlap, at least in part, with those associated with vulnerability to nicotine dependence. Because the majority of published studies was conducted on relatively small samples, examined different cessation strategies, and did not always address withdrawal symptoms directly, additional work is needed to yield more robust and reproducible associations between genes and withdrawal phenotypes. Once putative genes are identified, it will be critical to assess the potential for genetic variations to alter biological function and, consequently, nicotine-related behaviors. Pre-clinical studies that address both behavior and molecular mechanisms can help to explain how the changes in DNA sequence are associated with the behavioral phenotypes observed in smokers. Rodents, and particularly mice, offer unique opportunities to explore and characterize the relationships between gene function and nicotine withdrawal. Given the difficulty of effectively characterizing gene products and circuits responsible for the symptomatology of nicotine withdrawal in humans, genetic manipulations can be carried out in mice to directly address the circuits and molecular mechanisms involved.

2. Withdrawal Syndrome in Mice.

Many symptoms of nicotine withdrawal described in humans can be recapitulated in rodent models of addiction and withdrawal under experimental conditions. After at least a week of chronic nicotine administration in mice, cessation of nicotine exposure or administration of a nAChR antagonist induces affective, somatic, and cognitive signs of

withdrawal (Malin, Lake et al. 1994, De Biasi and Salas 2008). The withdrawal syndrome exhibited can be alleviated by nicotine administration – akin to nicotine replacement strategies such as nicotine patches or gum – or by administration of pharmacological agents used as cessation aids in humans, such as buproprion or varenicline (Rennard and Daughton 2014). Because mice express neuroadaptions that result in the nicotine withdrawal syndrome, and because nAChR-subunit knockout mouse lines are readily available, researchers have been able to study the functional contributions of particular nAChR subunits to specific symptoms of withdrawal.

2.1. Behavioral Manifestations and Testing Paradigms.

The nicotine withdrawal syndrome can be studied in mice by observing the frequencies of certain stereotypies, or by evaluating changes in behavior during withdrawal, relative to baseline behaviors exhibited by control mice naïve to nicotine. As in humans, the behavioral manifestations of nicotine withdrawal in mice can be categorized as somatic, affective, and cognitive.

Somatic symptoms in mice include excessive grooming, chewing, tremors, ‘wet-dog’ shakes, yawns, and teeth chattering (Paolini and De Biasi 2011). The occurrence of these symptoms tends to increase according to the severity of withdrawal. Affective symptoms are somewhat subtler in mice than somatic symptoms; their evaluation requires a series of behavioral assays to reveal changes in affect that are associated with withdrawal. Just as humans undergoing nicotine withdrawal experience anxiety, anhedonia, depression, and hyperalgesia, behavioral paradigms applied to mice expose analogous affective states. A challenge researchers face when evaluating rodent affect is that no single paradigm effectively isolates any one particular affective state. Each

measure likely evokes multiple affective states, unintentionally recruiting off-target neuronal circuits that are outside the scope of a given study. However, several behavioral paradigms applied as a set can help reveal relative differences in affect following experimental treatments. The emergence of anxiety-like symptoms following nicotine abstinence can be measured in the open field test (OFT) and the elevated plus maze (EPM), two of the most commonly employed paradigms in studies evaluating emotional states of rodents (Crawley, Belknap et al. 1997). The OFT is conducted by placing a mouse or rat in a square arena and evaluating the ratio of time rodents spend in the center of the environment to time spent along its perimeter. As rodents experience increased anxiety, they tend to avoid the center and spend more time along the walls of the environment, a behavior termed thigmotaxis. As rodents undergo nicotine withdrawal, they tend to exhibit increased levels of thigmotaxis. The open field test also enables researchers to evaluate rearing behavior, overall locomotion, freezing, and changes in defecation during withdrawal. As a further source of experimental control, levels of ambient illumination can be adjusted to modulate baseline levels of anxiety. The EPM consists of two enclosed arms with walls running along their periphery, and two open arms with no walls that present an environment resembling elevated cliffs (Crawley, Belknap et al. 1997). The arms are arranged in the shape of a plus sign, and anxious mice tend to make fewer entries into the open arms and spend less time in open arms. Rodents undergoing nicotine withdrawal exhibit both of these anxiety-associated behaviors, and anxiolytic drugs have been observed to increase open-arm entries (Crawley, Belknap et al. 1997).

Conditioned place aversion (CPA) can be used to measure the dysphoric manifestations associated with withdrawal. The paradigm involves repeated pairing of distinct

environmental cues with negative stimuli (i.e. withdrawal), such that when given a choice, mice avoid the withdrawal-paired cues relative to neutral cues. The time mice spend avoiding environments paired with withdrawal serves as an indicator of the severity of aversion (Kenny and Markou 2001). The emergence of depression-like symptoms during withdrawal can be assessed with the Forced Swim Test (FST), which assesses learned helpness by monitoring passive coping strategies such as immobility (Cryan, Markou et al. 2002, Thanos, Delis et al. 2013). During withdrawal, immobility is increased, indicating a depression-like state.

Intra-cranial self-stimulation (ICSS) is used to investigate the “reward” circuitry, and can be useful to evaluate anhedonia during withdrawal. Electrodes are implanted into the rodent brain targeted to the medial forebrain bundle, which includes the mesolimbic DA pathway associated with hedonia or reward. The rodent is first trained to perform the operant task of self-stimulation, and then is allowed to self-administer small electrical stimulations to the targeted pathway. As rodents perform this operant conditioning, the stimulus intensity is experimentally adjusted to determine the baseline stimulation threshold at which self-stimulation is consistently achieved and retained. Following the establishment of this threshold, researchers can explore the effects various stimuli have on brain reward and hedonic signaling. A lowered threshold resulting from a treatment is suggested to represent increased reward signaling along the pathway, while a heightened threshold is interpreted as indicating a state of greater anhedonia (O'Dell and Khroyan 2009). Animals experiencing nicotine withdrawal display elevated thresholds for ICSS.

The effects of withdrawal on cognition, especially hippocampal-dependent learning and memory, can be examined with contextual fear conditioning (FC). Subjects must learn

contextual information and form an association between the context and an aversive electric shock (Fanselow and Poulos 2005, Sigurdsson, Doyere et al. 2007). Acute nicotine administration enhances contextual fear conditioning while nicotine withdrawal impairs it (Davis and Gould 2008).

The five-choice serial reaction time task (5-CSRTT), also called operant signal detection, is a behavioral test used to characterize the effects of treatments on sustained and divided attention (Robbins 2002). The task consists of a maze that presents five holes, each of which may be paired with a reward. A brief flash of light inside one of the holes indicates the one that contains a reward, and rodents must poke the correct hole to receive the reward. This task quantifies the capability of rodents to maintain spatial attention that has been split among five locations over the course of many trials by dividing the proportion of correct nose-pokes (pokes into the lit hole) by total nose-pokes. While nicotine administration has been observed to generate cognitive enhancements, rats withdrawing from nicotine have been observed to exhibit attention deficits in the 5-CSRTT paradigm (Shoaib and Bizarro 2005), reflecting some of the symptoms that human smokers report while undergoing withdrawal.

While these rodent behaviors may reflect symptoms of the human withdrawal syndrome, the neuronal circuits recruited during experimental paradigms remain largely unknown. Differences in the behavioral outputs of animals undergoing withdrawal, relative to control animals, can help reveal the circuits nicotine withdrawal impinges upon, as each behavioral test offers unique environmental stimuli to which animals must respond, and engages specific neuronal circuits (Wahlsten 2010). The integration of mice genetically modified to carry nAChR null (knock-out, KO) or SNP-related mutations into behavioral research has introduced a finer level of resolution to the characterization of the neuronal

basis of withdrawal. By examining how eliminating signaling contributions from specific receptor subunits affects behavioral outcomes, researchers can determine which receptor subunits are necessary for the expression of certain nicotine withdrawal symptoms (Brown, Stanford et al. 2000). Additionally, it is important to consider the behavioral repercussions of the genetic differences that distinguish each inbred strain of mice, as each strain is characterized by a unique repertoire of consistent behavioral traits (Crawley 1996, Crawley 2008, Krackow, Vannoni et al. 2010, Matsuo, Takao et al. 2010). As these differences can translate to increased or decreased detectability of behavioral changes induced by withdrawal, it is prudent to establish which strain is most sensitive to the hypothesized behavioral changes in question (Bailey, Rustay et al. 2006, Crawley 2008, Lalonde and Strazielle 2008, Wahlsten 2010).

Ultimately, the development of enhanced therapeutics that effectively promote smoking cessation with minimal side effects will result from the identification of particular receptor subunit compositions that are necessary for the expression of withdrawal symptoms.

3. Receptors and nAChR Subunits Underlying Withdrawal Symptoms

3.1. Insight from nAChR Subunit KO mice.

In rodents, α2 mRNA levels are highest in the interpeduncular nucleus (IPN), with more restricted expression in scattered neurons within the amygdala, hippocampus, cortex, retina, spinal cord, and cerebellum (Lotfipour, Byun et al. 2013). Analyses of withdrawal symptoms in α2 KO mice have shown that the physical manifestations of nicotine abstinence are context-dependent, as α2 KO mice exhibit more somatic symptoms of withdrawal in novel environments (Lotfipour, Byun et al. 2013), but display no somatic signs of withdrawal in habituated environments (i.e. the home cage) (Salas, Sturm et al.

2009). Additionally, the lack of α2 is sufficient to abolish somatic symptoms of precipitated nicotine withdrawal (Salas, Sturm et al. 2009). In male mice, Chrna2 deletion also produces nicotine withdrawal-induced deficits of cued fear conditioning (Lotfipour, Byun et al.). α2 KO mice also self-administer higher doses of nicotine than WT controls (Lotfipour, Byun et al. 2013). It should be noted that in a study of European Americans and African Americans, CHRNA2 showed a strong association with the Fagerström Test for Nicotine Dependence (FTND) after correction for multiple testing (Wang, A et al. 2014). The rs2472553 SNP, which seems to have the strongest association with nicotine dependence, encodes a functional variant in the signal peptide, which leads to a threonine-to-isoleucine amino acid substitution at residue 22. In oocytes, the T22I mutation changes the sensitivity to nicotine of α2β4-containing nAChRs (Dash, Lukas et al. 2014).

The α3 nAChR subunit is encoded by CHRNA3, one of the genes in the chromosome 15q25 cluster that has shown the most robust association with smoking behavior and nicotine dependence. α3 forms functional nAChR complexes with α5 and β4, the other two subunits encoded by CHRNA5-CHRNA3-CHRNB4. These subunits are densely expressed in the medial habenula (MHb) and IPN (Grady, Moretti et al. 2009). Due to developmental abnormalities - including bladder enlargement and infection, urinary stones, and difficult urination - α3 KO mice survive birth, but exhibit severely impaired growth and perinatal mortality (Xu, Gelber et al. 1999). This phenotype has rendered evaluation of the subunit's potential involvement in withdrawal symptomatology impractical in α3 null mice. However, pharmacological data suggest that receptors comprising the α3 and β4 nAChR subunits influence both nicotine reward and somatic manifestations of withdrawal (Jackson, Sanjakdar et al. 2013). AuIB, an α-conotoxin

peptide that potently blocks α3β4 nAChRs (Luo, Kulak et al. 1998), dose-dependently inhibits nicotine elicited reward as measured in the conditioned place preference paradigm. The α-conotoxin also reduces somatic signs of withdrawal and withdrawal-induced hyperalgesia, while it has no effect on the aversive motivational component of withdrawal as measured in the CPA paradigm (Jackson, Sanjakdar et al. 2013).

α5-containing nAChRs are expressed in various brain areas implicated in the key effects of nicotine, including ventral tegmental area (VTA), MHb, IPN, hippocampus and cortex (Salas, Orr-Urtreger et al. 2003). α5 KO mice do not display physical symptoms associated with both spontaneous and mecamylamine-precipitated nicotine withdrawal (Salas, Sturm et al. 2009) nor withdrawal-induced hyperalgesia (Jackson, Martin et al. 2008). The MHb/IPN pathway plays a crucial role in mediating the physical manifestations of nicotine withdrawal and is the likely location of the effects of α5-containing nAChR on this phenotype (Salas, Sturm et al. 2009). Acute nicotine application to MHb slices enhances the intrinsic excitability of MHb neurons (Dao, Perez et al. 2014). This dynamic depends on the presence of α5-containing nAChRs within the MHb and the release of neurokinins (Dao, Perez et al. 2014), as such enhancement of excitability was prevented by bath application of neurokinin 1 (NK1) or NK3 receptor antagonists. In addition, infusion of the same NK receptor antagonists into the MHb of mice chronically treated with nicotine precipitated somatic signs of nicotine withdrawal (Dao, Perez et al. 2014). Microinjection of neurokinin receptor antagonists into adjacent anatomical structures, including the lateral habenula (LHb), failed to elicit behavior resembling withdrawal. Similarly, microinjection of the NK1 and/or NK3 antagonists into the MHb of nicotine-naïve mice failed to generate somatic symptoms of withdrawal. It was concluded that interactions between cholinergic and neurokininergic systems

contribute to the emergence of nicotine withdrawal symptoms (Dao, Perez et al. 2014). The α5 null mutation does not appear to influence affective symptoms such as withdrawal-induced CPA (Jackson, Martin et al. 2008). The same study reported that α5 KO mice do not display increased anxiety levels in the EPM during withdrawal (Jackson, Martin et al. 2008). However, interpretation of those data is not straightforward, as Chrna5 deletion reduces anxiety in the EPM in basal conditions independent of nicotine treatment (Gangitano, Salas et al. 2009). It has also been shown that α5-containing nAChRs do not regulate the reward-inhibiting effects induced by nicotine withdrawal in the ICSS paradigm (Fowler, Tuesta et al. 2013), suggesting that the receptors do not influence anhedonia.

The α6 subunit also appears to play a role in the nicotine withdrawal syndrome. DA release in the NAcc following nicotine administration is regulated in part by α6-containing (α6*) nAChRs indicating receptors containing on DAergic terminals in the dorsal and ventral striatum (Exley, Clements et al. 2008) and DAergic somata in the VTA (Grady, Salminen et al. 2007, Zhao-Shea, Liu et al. 2011). Intracerebral infusion of a selective antagonist of α6β2* nAChR blocks CPA and withdrawal-precipitated anxiety-like behavior in the EPM (Jackson, McIntosh et al. 2009). There was no influence on the somatic symptoms of withdrawal, suggesting a selective role for α6 nAChR subunits in the affective manifestations of withdrawal (Jackson, McIntosh et al. 2009).

Unlike most other subunits, α7 nAChR subunits are capable of forming homomeric receptors that are broadly distributed in the brain. The hyperalgesia symptoms that emerge during mecamylamine-precipitated withdrawal are reduced in α7 KO mice (Grabus, Martin et al. 2005). In contrast to wild-type mice, α7 null mice failed to exhibit elevated ICSS thresholds during nicotine withdrawal between 3 and 6 hours after their

last nicotine exposure (Stoker, Olivier et al. 2012). When the anhedonic affective state was evaluated between 8 and 100 h after nicotine withdrawal, ICSS thresholds were equally elevated in α7 WT and null littermates, indicating that a lack of α7 nAChR subunits delays, rather than abolishes, withdrawal symptoms. As for the physical signs of abstinence, α7 null mice exhibit significantly reduced withdrawal symptoms immediately after precipitation of withdrawal by mecamylamine injection (Salas, Main et al. 2007). However, their physical signs are indistinguishable from those of WT mice when measured at later times, up to 48 h after withdrawal (Jackson, Martin et al. 2008, Stoker, Olivier et al. 2012).

β2 KO mice exhibit levels of somatic signs of withdrawal and abstinence-induced hyperalgesia comparable to those of WT mice (Salas, Pieri et al. 2004). However, the mutant mice do not exhibit anxiety-related behaviors normally associated with withdrawal from chronic nicotine exposure (Jackson, Martin et al. 2008). Overall, these results suggest participation of β2 nAChR subunits in the signaling responsible for affective, but not somatic, symptoms of nicotine withdrawal. α6β2* nAChRs are expressed in the VTA and ventral striatum and are associated with reward and addiction, but they are not expressed peripherally. Therefore, the α6β2* nAChRs may represent viable targets for the treatment of affective symptoms experienced during nicotine withdrawal. Indeed, a non-nicotine pharmaceutical currently FDA approved for the treatment of smoking cessation, varenicline, acts as a partial agonist at α6β2* nAChRs (Grady, Drenan et al. 2010, Bordia, Hrachova et al. 2012).

While sequence variants associated with smoking behavior lie within the regions that harbor the CHRNB3-CHRNA6 genes on chromomsome 8p11 (Thorgeirsson, Gudbjartsson et al. 2010), no pre-clinical data are available on the effects of the β3 null

mutation on nicotine-related behavior. However, it has been determined that β3 KO mice exhibit lower baseline levels of anxiety-related behavior in three different paradigms (Booker, Butt et al. 2007). As reported for the α5 KO mice (Gangitano, Salas et al. 2009), β3 KO mice have altered hypothalamic-pituitary-adrenal axis responses (Booker, Butt et al. 2007). Changes were also reported for locomotor activity and prepulse inhibition of acoustic startle, behaviors that are controlled, at least in part, by nigrostriatal and mesolimbic dopaminergic activity (Cui, Booker et al. 2003). As β3 mRNA is detected in the substantia nigra, ventral tegmental area, and medial habenula (Cui, Booker et al. 2003), it is tempting to attribute the anxiolytic phenotype to MHb mechanisms and the locomotor phenotype to nigrostriatal mechanisms.

β4 KO mice exhibit no somatic signs of nicotine withdrawal or hyperalgesia following mecamylamine-induced withdrawal (Salas, Pieri et al. 2004). Similar results were found for β4 KO mice undergoing spontaneous withdrawal from chronic nicotine administration (Stoker, Olivier et al. 2012). In addition, β4 KO mice do not display anhedonia-like symptoms during withdrawal, as identified by unchanged intracranial self-stimulation thresholds (Stoker, Olivier et al. 2012). The reported phenotypes likely reflect an involvement of different subtypes of β4* nAChRs. α3β4* nAChRs are the most likely contributors to physical components of withdrawal given the high levels of expression of α3 and β4 mRNA in the MHb, and the fact that the α3β4-selective α-conotoxin AuIB blocks the emergence of somatic signs of withdrawal (Jackson, Sanjakdar et al. 2013). Because AuIB does not interfere with the affective symptomatology of withdrawal in the EPM and CPA paradigms, α6β4* nAChRs might be involved. Indeed, a transgenic mouse model that overexpresses β4 nAChR subunits exhibits altered nicotine consumption and conditioned place aversion (Frahm, Ślimak et al. 2011), and there is a

documented role for α6* nAChRs in withdrawal-induced CPA (Jackson, McIntosh et al. 2009).

4. Molecular mechanisms involved in the nicotine withdrawal syndrome.

4.1. nAChR Upregulation

Long term exposure to nicotine leads to an increase, or upregulation, of nicotine binding sites in the brains of smokers (Benwell, Balfour et al. 1988) and rodents subjected to repeated nicotine administrations (Marks, Burch et al. 1983). The increased pool of nAChRs arising from chronic nicotine exposure may drive symptoms associated with the nicotine withdrawal syndrome (Turner, Castellano et al. 2011, Gould, Portugal et al. 2012) and might impact the ability to maintain abstinence in the clinical population (Staley, Krishnan-Sarin et al. 2006). Using a radioligand with specificity for β2-containing nAChRs, which enabled the use of single-photon emission computed tomography (SPECT) (Staley, Krishnan-Sarin et al. 2006), it was found that chronic smokers have more cortical, striatal, and cerebellar β2* nAChRs than non-smokers (Staley, Krishnan-Sarin et al. 2006). Furthermore, β2* nAChRs in the anterior cingulate and frontal cortex were significantly correlated with the number of days following cessation of smoking. This was interpreted as a progressive increase of the number of β2* nAChRs in proportion to the number of consecutively abstinent days. In addition, a significant negative correlation was observed between β2* nAChR availability in the post-central gyrus or somatosensory cortex and the urge to relieve withdrawal symptoms by smoking (Staley, Krishnan-Sarin et al. 2006). Similarly, in rodents, nAChR upregulation in the dorsal hippocampus was associated with withdrawal-related deficits in hippocampal learning (Gould, Portugal et al. 2012, Portugal, Wilkinson et al. 2012).

A variety of cellular processes influence receptor upregulation (Govind, Vezina et al. 2009, Rezvani, Teng et al. 2009, Rezvani, Teng et al. 2010, Henderson, Srinivasan et al. 2014). Receptors containing the β2 subunits are particularly sensitive to nicotine-induced upregulation. If β4 replaces β2 subunits in either α3* or α4* nAChRs, receptor upregulation is significantly reduced (Wang, Nelson et al. 1998, Sallette, Bohler et al. 2004). As replacement of β2 by β4 is sufficient to increase nAChR levels at the plasma membrane in the absence of nicotine, the proposed “chaperoning” function of nicotine might not be as effective (Srinivasan, Pantoja et al. 2011, Henderson, Srinivasan et al. 2014). The presence of an accessory nAChR subunit in the receptor complex can also influence nicotine-induced upregulation. For example, the presence of α5 renders α4β2* nAChRs insensitive to nicotine-induced upregulation (Mao, Perry et al. 2008). Conversely, co-expression of β3 increases α6β2 and α6β4 receptor levels and enhances nicotine-induced upregulation of α6β2β3 receptors compared to α6β2 receptors (Tumkosit, Kuryatov et al. 2006). The effects of β3 on receptor trafficking and upregulation may help explain why in the striatum α6-containing receptors without β3 are downregulated by nicotine, while those containing β3 are unaffected (Perry, Mao et al. 2007).

4.2. Desensitization of nAChRs

While some combinations of nAChR subunits render receptor complexes more prone to upregulation than others, desensitization is a prominent mechanism that contributes to this upregulation (Fenster, Whitworth et al. 1999). As nicotine has a half-life in humans of 2 hours or more, nicotine accumulates during a day of regular smoking to reach steady-state plasma concentrations typically ranging between 10 and 50 ng/mL (Allan W. Graham May 5, 2007). This long-lasting level of nicotine ensures that high affinity

nAChRs recurrently bind and unbind nicotine throughout the day (Picciotto, Addy et al. 2008). Consequently, receptors undergo transitions between different conformations in response to ligand binding and dissociation, and agonists will tend to stabilize particular conformations. Because desensitized nAChR conformations have a higher affinity for agonist, nAChRs will increasingly adopt desensitized conformations in response to chronic nicotine exposure (Quick and Lester 2002, Picciotto, Addy et al. 2008). It has been suggested that receptor desensitization may be a major contributor to the upregulation observed in chronic users of nicotine-containing products (Benowitz 2008). As the usage patterns of regular smokers result in persisting levels of circulating nicotine over the course of the day, nAChR desensitization occurs in response to ongoing occupancy of CNS nAChRs. For example, working with 11 tobacco-dependent individuals using a radiotracer developed to image α4β2* nAChRs with positron emission tomography (PET), it was found that regular smokers approach complete saturation of CNS α4β2* nAChRs throughout the day (Brody, Mandelkern et al. 2006). After 2 days of abstaining from smoking, participants' cravings were reduced only once nAChRs were again nearly saturated. It is important to note that rates of desensitization are not necessarily equivalent across all nAChR subunit combinations. For example, inclusion of the α5 subunit into α4β2* nAChRs decreases the extent of desensitization (Bailey, De Biasi et al. 2010).

When considering the implications of receptor upregulation and desensitization for symptoms of nicotine withdrawal, kinetics characterizing the two dynamics may present critical processes by which withdrawal signaling occurs. In fact, nAChRs recover from desensitization on the scale of seconds to hours (Gentry and Lukas 2002). Additionally, studies have demonstrated that upregulation of nAChR expression can persist for

several days following termination of chronic nicotine (Pietila, Lahde et al. 1998, Staley, Krishnan-Sarin et al. 2006) Accordingly, as the rates of desensitization and recovery from receptor upregulation during nicotine withdrawal are incongruent, a physiological landscape is established in which there are more receptors expressed than would be present in nicotine-naïve individuals, and these receptors are less responsive to agonist binding. When nicotine levels are low or absent, the nAChRs recover from desensitization, leading to potentially overactive cholinergic signaling. This process represents a major factor in the neurobiology of nicotine withdrawal (Dani and Heinemann 1996). Considering this dynamic, Gould and colleagues (Gould, Portugal et al. 2012) have proposed a pharmacological strategy of treating hypersensitive nAChR systems with compounds that maintain receptor desensitization, or diminish cholinergic signaling while avoiding upregulation and activation, to alleviate the negative impact of withdrawal.

5. Anatomical Structures and Circuits Implicated in Nicotine Withdrawal

Given the diversity of symptoms manifested during nicotine withdrawal, a constellation of anatomical structures is likely involved in its etiology. Different anatomical structures in the CNS express distinct populations of nAChRs. The variety of nAChRs, combined with the specificity determined by afferent/efferent projections, produces the distinct neurochemistry and signaling that underlies the withdrawal syndrome. Evaluating changes in behavior relative to WT controls upon removal of particular nAChR subunits has been crucial to characterizing the contributions that each subunit makes to the withdrawal syndrome. If a withdrawal symptom exhibited by wild-type mice is absent in mice that lack functional expression of a specific nAChR subunit in a particular CNS structure, the specific subunit is likely critical to the withdrawal symptom in question. By

performing these kinds of experiments, neuronal circuits contributing to the withdrawal syndrome will continue to be defined, and viable targets to enable the development of more specific therapeutics will be identified. Thus far, several brain structures have been already implicated in the withdrawal syndrome.

5.1. Dopaminergic System.

The mesocorticolimbic dopamine (DA) system has been identified as critical to the development of addictive behaviors, and DA signaling is involved in reward-based reinforcement of drug-derived behaviors. While the DAergic ventral tegmental area (VTA) has been widely implicated in the rewarding aspects of addictive drugs, it has also been shown to participate in the signaling of aversion and lack of expected reward (Schultz, Tremblay et al. 1998, Ungless, Magill et al. 2004, Tobler, O'Doherty et al. 2007, De Biasi and Dani 2011). Dopaminergic deficits in the mesolimbic pathway, particularly in the nucleus accumbens, are among the neurochemical mechanisms underlying the symptoms of nicotine withdrawal (Hildebrand, Nomikos et al. 1998, Carboni, Bortone et al. 2000, Rada, Jensen et al. 2001). Such deficiencies in accumbal dopaminergic transmission are believed to contribute to the aversive anhedonic or dysphoric state experienced during nicotine abstinence (Koob and Le Moal 2008, Zhang, Dong et al. 2012). A reduction in basal DA levels was observed to linger for at least 5 days in mice following 12 weeks of chronic nicotine treatment (Zhang, Dong et al. 2012). This change in DA activity during withdrawal was correlated with a reduced modulatory influence by β2* nAChRs over DA release in the NAcc. What causes the hypodopaminergic state associated with withdrawal is not yet clear. However, increased inhibitory input to the VTA is a potential mechanism, as GABA input is sufficient to suppress burst firing even with excitatory inputs intact (Lobb, Wilson et al. 2010, Jalabert, Bourdy et al. 2011).

GABAergic inputs arrive at the VTA from the substantia nigra pars reticulata, nucleus accumbens, ventral pallidum/globus pallidus, laterodorsal tegmentum, pedunculopontine nuclei, diagonal band of Broca, bed nucleus of the stria terminalis, and the caudal tip of the VTA, termed the rostromedial tegmental nucleus (RMTg) (Geisler and Zahm 2005, Jhou, Fields et al. 2009, Kaufling, Veinante et al. 2009). Activity in the RMTg increases upon exposure to aversive stimuli and decreases upon exposure to reward stimuli, representing inversely correlated signaling patterns (Hong, Jhou et al. 2011).

5.2. Habenular Complex and Interpeduncular Nucleus

The habenular complex, comprising the lateral (LHb) and medial (MHb) nuclei, is a diencephalic, epithalamic structure located ventrally along the dorsal third ventricle (Dani and De Biasi 2013). The habenula has been demonstrated to participate in the signaling underlying fear, anxiety, depression, and stress (Viswanath, Carter et al. 2013). The LHb sends excitatory glutamatergic projections to the RMTg and is a major component of the circuit underlying negative reward (Dani and De Biasi 2013). Additionally, the cholinoceptive cellular population within the medial habenula (MHb) has been associated with the aversive effects of nicotine (Fowler, Lu et al. 2011). The most prominent efferent output from the MHb is to the IPN via the fasciculus retroflexus, forming the MHb-IPN axis. Through this projection, the MHb releases acetylcholine (ACh), substance P (SP), and glutamate onto the IPN (Zhao-Shea, Liu et al. 2013). The MHb also hosts the expression of norepinephrine, serotonin, ATP, and several neuropeptides (Dao, Perez et al. 2014). The IPN is located in the ventral midbrain, with the VTA and median raphe nucleus located dorsally.

Mice chronically treated with nicotine exhibit symptoms of withdrawal upon microinjection of mecamylamine into the MHb and IPN, but fail to manifest withdrawal symptoms when mecamylamine is injected to the cortex, hippocampus, or VTA (Salas, Sturm et al. 2009). Furthermore, lidocaine infusion into the MHb to inhibit signaling significantly blocks the manifestations of somatic symptoms resulting from both mecamylamine-precipitated and spontaneous induction of nicotine withdrawal (Zhao-Shea, Liu et al. 2013). Following precipitated nicotine withdrawal in WT mice, there are elevated markers of neuronal activation in GABAergic IPN cells (Zhao-Shea, Liu et al. 2013). The involvement of the IPN in the manifestation of nicotine withdrawal symptoms was demonstrated by expression of channelrhodopsin (ChR2), a light-driven excitatory ion channel, in the GABAergic cells of the IPN (Zhao-Shea, Liu et al. 2013). Additionally, infusion of an α3β4* nAChR-selective antagonist into the IPN elicited somatic withdrawal signs in nicotine-naïve mice, and an even greater number of total symptoms in mice chronically treated with nicotine (Zhao-Shea, Liu et al. 2013). Considered together, these data strongly implicate the MHb-IPN circuit in the nicotine withdrawal syndrome. As previously discussed, the α5, α3, and β4 nAChR subunits participate in withdrawal symptomatology. They are each densely expressed in the MHb and/or IPN (Salas, Pieri et al. 2003, Salas, Pieri et al. 2004) and are potential targets for treatments of the nicotine withdrawal syndrome (Grady, Moretti et al. 2009).

5.3. Hippocampus

The hippocampus is a crucial structure for the manifestation of cognitive deficits during nicotine withdrawal. Contextual fear conditioning is dependent upon hippocampal signaling (Davis, James et al. 2005), and during spontaneous withdrawal from nicotine, mice exhibit deficient contextual fear conditioning relative to saline-treated controls

(Davis, James et al. 2005). Administration of nicotine to mice undergoing withdrawal ameliorates this deficit (Davis, James et al. 2005). This effect was not attributable simply to a lowered threshold of fear responses inherent to the effects of nicotine because cued fear conditioning was equivalent between nicotine-treated and nicotine-naïve groups. Similar deficits of contextual fear learning were observed when a nAChR antagonist (dihydro-β-erythroidine) was infused into the hippocampus of WT mice chronically treated with nicotine (Davis and Gould 2009). The work also implicated β2* nAChRs in the manifestation of memory-associated deficits during nicotine withdrawal. Furthermore, chronic nicotine treatment upregulated a variety of nAChRs in the hippocampus, and the duration of β2 subunit upregulation relative to other nAChR subunits most closely corresponded to the total duration of memory-associated withdrawal symptoms (Gould, Portugal et al. 2012).

5.4. Extended amygdala

The bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), and shell of the nucleus accumbens (NAc-Sh) form the extended amygdala, an anatomically and neurochemically interconnected system located in the basal forebrain (Smith and Aston-Jones 2008). The system has been implicated in stress-related components of drug withdrawal, and is a site of interaction between corticotropin-releasing factor (CRF) and norepinephrine (NE) transmission. Nicotine withdrawal leads to an increase in CRF in the central nucleus of the amygdala (CeA), and blockade of CRF1 receptors diminishes nicotine withdrawal-induced anxiety-like behavior (George, Ghozland et al. 2007). The CRF/CRF1 receptor signaling in the CeA also mediates nicotine withdrawal-induced increases in nociceptive sensitivity in rats that are dependent on nicotine (Baiamonte, Valenza et al. 2014).

Given the polygenic nature of nicotine addiction, many other mechanisms and many other brain areas are likely to influence the manifestations of withdrawal. Preclinical research is increasingly benefitting from the information coming from genetic studies and pharmacogenetic trials and the speed of discovery is destined to increase in the coming years.

6. Summary and Research Moving Forward

Individuals undergoing nicotine withdrawal experience both affective and somatic symptoms beginning between 4 to 24 hours after ceasing intake. The syndrome is most severe in the first week but it can persist for longer periods of time. During this time, relapse is incentivized by the ability of nicotine to alleviate or abolish withdrawal symptoms. In addition, there are cognitive deficits that manifest during nicotine withdrawal, including difficulty concentrating, increased reaction times in tasks requiring sustained attention, and impaired episodic and working memory (Myers, Taylor et al. 2008, Wesnes, Edgar et al. 2013). Knowledge of the molecular mechanisms that govern the emergence and intensity of withdrawal symptoms, and elucidation of the genetic variants associated with successful smoking cessation, will facilitate the development of personalized treatments.

New techniques in neuroscience are enabling researchers to ask previously unapproachable questions regarding which genes, circuits, and neurochemical systems mediate and modulate different aspects of addiction and withdrawal. Using transgenic mouse lines or viral delivery, designer receptors (DREADDs (Rogan and Roth 2011)) and light-driven ion channels (opsins (Yizhar, Fenno et al. 2011)) can be expressed in particular anatomical structures or in particular neuronal types. These genetically

targeted receptors and ion channels can be used to control the activities of specific circuits and populations of neurons in freely behaving mice. When combined with receptor KO mice, or with the delivery of nAChRs derived from specific human SNPs, these techniques enable researchers to manipulate neuronal activity while monitoring the dynamics of withdrawal-related behaviors.

Genetic studies of nicotine dependence and smoking cessation have identified several risk factors using GWAS, candidate gene approaches, and pharmacogenetic analyses. These studies provide targets that can be validated with large population samples and across ethnicities. Once putative genes are identified, hypotheses of the functional roles of such candidate genes can be tested in pre-clinical animal models. The functional consequences of some SNPs can be addressed relatively easily if the SNPs are non-synonymous coding variants. The best example is provided by rs16969968, the SNP that leads to an aspartic acid for an asparagine substitution (D398N) in CHRNA5(Jackson, Marks et al. 2010). The impact of the mutation that defines the risk allele has been validated repeatedly in vitro and in animal models (Kuryatov, Berrettini et al. 2011, Morel, Fattore et al. 2013). For many of the SNPs, however, there is no change in protein sequence, and therefore, it is harder to formulate clear functional hypothesis. Candidate SNPs can have a multitude of biochemical functions, such as altering DNA methylation, histone modification, or accessibility of DNA to transcription factor binding, that can impact when, where, and how much a gene, and its protein, is expressed. Fortunately, analytical tools such as the Encyclopedia of DNA Elements (ENCODE), can help the design of experiments that explore disease-related variants located within non-coding regions (Siggens and Ekwall 2014). Increasing attention is being paid to the functional analysis of rare variants, as common variants can explain only a small

percentage of the variance in smoking-related phenotypes. More work is necessary at the bench and in the clinic, as a collection of genes likely operates collectively to predispose or protect individuals to or from nicotine addiction and withdrawal. Ultimately, this increased capability to comprehensively characterize the etiology of withdrawal symptoms will inform more competent and efficient drug design. The future undoubtedly holds greater development of pharmacological nicotine cessation therapeutics.

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CHAPTER 4: GENERAL CONCLUSIONS & FUTURE DIRECTIONS

The goal of my research was to identify if components of the dorsal diencephalic conduction system (DDC) regulate anxiety-associated behavior independently of chronic exposure to habit-forming drugs. I began with a broad perturbation of one of the primary junctions of signaling within the pathway, the interpeduncular nucleus (IPN), using excitatory DREADDs. Next, I determined whether glutamatergic output from the nucleus was responsible for the modified behaviors I first observed, evaluating whether behavioral responses were sexually dimorphic. I then identified a substantial serotonergic response in the ventral hippocampus correlated with chemogenetic stimulation of the IPN, and then confirmed direct projections from the IPN to the ventral hippocampus for the first time in decades.

While our group and others have established a role played by the MHb-IPN axis in withdrawal symptoms from multiple habit-forming drugs, only paltry evidence has characterized the regulation of affect by this pathway independently of chronic drug exposure. Given the substantial comorbidity between anxiety-associated psychiatric conditions and substance use disorders (SUDs), we set out to investigate whether the DDC might represent a point of shared neurophysiology between the two. Ultimately, we corroborated the placement of the DDC in a broader anatomical context that regulates anxiety, verifying direct projections from the IPN to the ventral hippocampus. Additionally, for the first time, we identified significant increases in serotonin concentrations in the ventral hippocampus following chemogenetic stimulation of IPN neurons.

The IPN Regulates Affect

Based on prior research demonstrating the role of IPN signaling in the manifestation of withdrawal symptoms, we hypothesized that stimulation of the IPN would result in increased anxiety-associated behavior. To our surprise, however, we observed anxiolysis. While some groups have identified specific signaling pathways from subnuclei within the IPN (Zhao-Shea et al., 2015; Zhao-Shea et al., 2013), it remains uncertain which neuronal subpopulations within the structure mediate this behavioral output. Given the distinct efferent projections emerging from IPN subnuclei that some groups have identified, and the limitations of viral interrogation of this structure in my hands, I remain unable to provide more granular insights regarding this response. However, these data demonstrate that stimulation of IPN neurons regulates anxiety-associated behavior.

IPN Stimulation Correlates with Increased Serotonin in the Ventral Hippocampus

The IPN is a junction of signaling arriving principally from the MHb, which sends efferent projections to a variety of structures that span the fore-, mid-, and hind-brain. There are two pathways that may account for the increased serotonin concentrations we observed following chemogenetic stimulation of the IPN. Projections from the IPN to the raphe nuclei were demonstrated many years ago, though the functional characteristics have yet to be determined. Additionally, projections from the IPN to the hippocampus were posited several decades ago, and have been verified by recent studies. However, to date, this work is the first to identify direct projections from the IPN to the ventral hippocampus. Serotonergic neurons have been identified within the IPN (Montone et al.,

1988; Wirtshafter et al., 1986), and a recent study suggests the apical subnucleus may be where the majority can be found (Quina et al., 2017). As there is only a sparse presence of serotonergic neurons within the IPN, future studies may exploit transgenic mouse lines that express Cre recombinase in serotonergic neurons to achieve more granular functional characterization. In particular, studies may explore whether elevated serotonin in the ventral hippocampus, following chemogenetic stimulation of the IPN, is attributable to a direct projection to the ventral hippocampus, or is rather due to an indirect activation of structures like the raphe nuclei (Behzadi, Kalen, Parvopassu, & Wiklund, 1990; Groenewegen et al., 1986; Quina et al., 2017).

Viral Interrogation of the MHb-IPN Axis

While interpreting behavior that results from general stimulation of brain structures, the tropism of adeno-associated viruses (AAV) serotypes (Mason et al., 2010; Srivastava, 2016) is important to consider. This methodological limitation applies to all viral interrogations of neurophysiology using AAVs. As a result, some neuronal populations in the IPN may be excluded by the use of specific AAV serotypes in this series of experiments, though hybrid serotypes may enable broader expression (Y. Mao et al., 2016). However, the selectivity, verifiable by immunofluorescence, afforded by viral methods helps to reveal the role the IPN plays in regulating affect at baseline. As a result, there remains room for more comprehensive interrogation of the structure with the application of more targeted techniques, such as the use of transgenic models expressing transcription factor driven Cre recombinase with superior anatomical selectivity (Bandin, Morona, Lopez, Moreno, & Gonzalez, 2014; Moreno et al., 2014).

Optimizing Viral Tools & Methods

Lessons from Working with New Transgenic Lines & Commercially Derived Viruses

The initial experimental design of the primary dissertation experiments described in earlier sections included studies with the combination of a Cre recombinase-dependent virus and a transgenic mouse line expressing Cre driven by the promoter/enhancer regions of the vesicular inhibitory amino acid transporter (*Viaat*) gene. The *Viaat*, or vesicular GABA transporter (VGAT) (Juge, Omote, & Moriyama, 2013), encodes a protein that transports GABA and glycine into synaptic vesicles (Chaudhry et al., 1998). This would enable the selective expression of viral products in neurons expressing VIAAT, principally GABAergic neurons. The line had recently been utilized by labs focused on developmental biology and Rett syndrome to explore GABAergic components of the condition (Chao et al., 2010). Publicly available *in situ* hybridization studies had demonstrated broad *Viaat* expression in the IPN (Allen Brain Atlas). In addition, studies had identified the involvement of IPN GABAergic signaling in the behavioral effects of nicotine (Hsu et al., 2013). We were fortunate that a lab at the University of Pennsylvania had maintained a colony of the line, and generously contributed founder breeders so that our lab could establish a colony.

Chemogenetics, described in Chapter 1, had become a widely-used platform to selectively interrogate neuronal populations *in vivo* (Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Rogan & Roth, 2011; Roth, 2016). Accordingly, viral vectors to drive the expression of designer receptors exclusively activated by designer drugs (DREADDs) had become available to be purchased from academic institutions. As a result, during the optimization of stereotactic viral infusion procedures, DREADD-expressing viruses

were obtained commercially. I had found that commercially obtained AAV8-hM3Dq-mCherry successfully drove expression when surgical and infusion procedures were successful.

At the beginning of my efforts to achieve expression in VIAAT^{+} IPN neurons using the Cre-dependent virus, AAV8-DIO-hM3Dq-mCherry, I encountered additional difficulties. I had effectively confronted the challenges of achieving expression in the IPN, but had learned that a sufficient proportion of potential failure points were present in surgical and infusion procedures that, essentially without exception, failure to achieve expression was attributable to opportunities to improve tools and techniques.

Variability in the performance of syringe components was often the failure point during infusion procedures, and a single syringe was devoted to each virus to avoid cross-contamination. Optimizing the performance of these tools will be discussed in later sections. However, while typically successful, these maintenance procedures are imperfect and can still produce unpredictable failures during viral infusions.

Furthermore, the IPN is a relatively dense, small, unpaired anatomical structure (Bianco & Wilson, 2009) located in the ventral midbrain. Due to the presence of a fairly large vascular structure – perhaps the superior sagittal sinus (Xiong et al., 2017) that runs along the sagittal suture at the anterior/posterior coordinate of stereotactic entry - approaching the structure at a 20° angle proved to result in a vastly superior survival rate due to minimized bleeding. As a result, anatomical variability between mice relative to stereotactic atlases, and positioning of bregma in particular, can result in missed stereotactic targeting even under optimal circumstances. A higher rate of off-target expression when targeting the IPN was, therefore, anticipated – even despite successful optimization of prior viral infusion groups.

Initial viral infusions of transgenic VIAAT-Cre mice proved unsuccessful. Following multiple infusion attempts, and despite consistent histological verification of successful targeting of the IPN, failure to achieve expression persisted (Fig 1).

Figure 1.

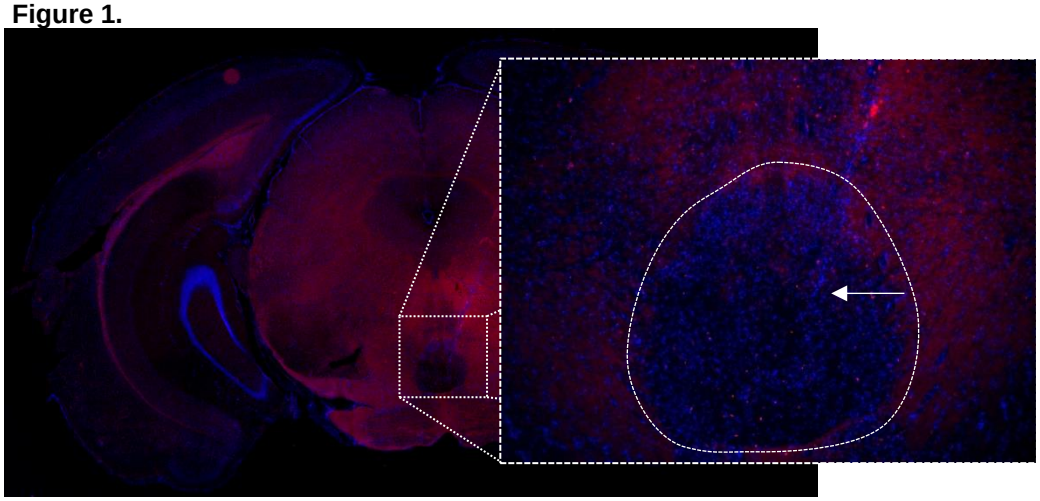


Figure 1. Representative coronal slice from VIAAT-Cre transgenic mouse infused with AAV8-DIO-hM3D-mCherry obtained from a commercial source into the IPN with infusion syringe tract visible in overview (left) and higher magnification image (right). Despite multiple infusions into transgenic mice expressing Cre recombinase driven by the VIAAT promoter, no expression was observed. Arrow indicates syringe tract. Blue indicates A-T rich regions of DNA stained with DAPI.

As experience had instructed that failures of expression were typically attributable to failure points in surgical procedure, the following optimizations were applied: the frequency of syringe needle replacement increased, the stringency of the cleaning protocol was increased to between each mouse infusion, different infusion volumes were evaluated, and different ranges of expression times were assessed. The rationale was the following: while the specific syringe used to infuse the Cre-independent AAV8-hM3Dq-mCherry yielded successful expression, perhaps some unknown feature of mechanical variability was resulting in different rates of blockage for the infusion of the Cre-dependent virus. Accordingly, when a given syringe needle became blocked more frequently than usual, checked both before and after each infusion using a test fluid, it

was replaced. Next, while syringes were initially cleaned between each cohort of viral infusions, more frequent cleaning might more reliably reduce unforeseeable clogging, preventing successful expression. Then, while an optimal infusion volume and rate of infusion had been established with Cre-independent virus, perhaps Cre-dependence resulted in unique expression dynamics. Therefore, increased infusion volumes were attempted. Finally, longer expression time was assessed to account for the possibility that Cre-dependent expression simply took more time in IPN GABAergic neurons than Cre-independent expression.

Despite these optimization measures, successful expression remained evasive. While preparing for another round of viral infusions with further adjustments to stereotactic and infusion protocols, I presented my ongoing progress to a group of colleagues, some of whom were attempting experiments using Cre-dependent DREADD-expressing viruses. Following commiseration over our shared difficulties derived from attempted Cre-dependent DREADD expression, a colleague contacted me regarding a notification that irregularities in virus production had been identified at the institution from which we obtained our viral vectors. Through a personal connection, my colleague conveyed the findings of individuals who were close to the production of the vectors, indicating concerns regarding anomalies in expression patterns reported by other recipients of the vectors.

While the expression products of viruses obtained remained unknown, I assumed that the virus must be either independent or dependent upon Cre recombinase. If Cre-dependent, then - regardless of expression products – I ought to observe some result of infusion. However, no expression was observed.

While continuing to evaluate expression patterns in transgenic VIAAT-Cre mice infused with the commercially obtained Cre-dependent virus, our lab began producing viral

vectors. After obtaining plasmids from a distinct institution, which the individuals identifying anomalies in vector production indicated were free of inconsistent quality, we produced an equivalent, Cre-dependent, DREADD expressing virus. Following *in vitro* confirmation of successful expression in Cre-expressing neurons, I proceeded to infuse the viral constructs produced in the lab into transgenic VIAAT-Cre mice.

A small number of transgenic VIAAT-Cre mice were infused to assess the viability of viruses produced in the lab. Successful expression was observed in the IPN, with an expression pattern indicating sub-structure selectivity (Fig. 2).

Figure 2.

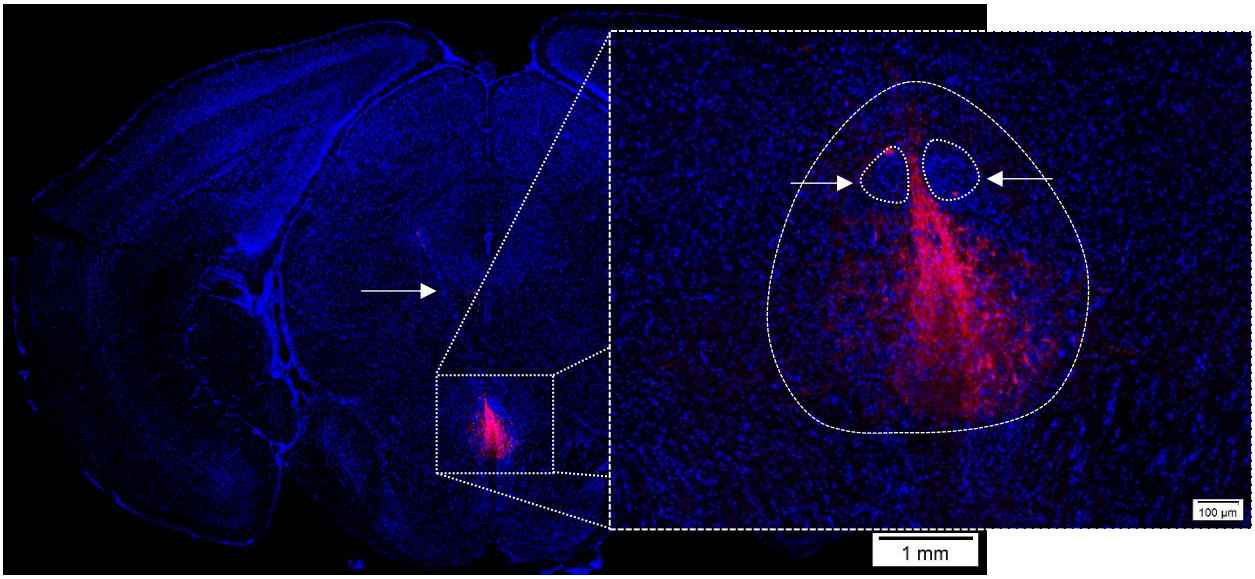


Figure 2. Representative coronal slice from VIAAT-Cre transgenic mouse infused with AAV8-DIO-hM3D-mCherry (red), produced in the MDB lab, into the IPN with infusion syringe tract visible in overview (left, arrow) and higher magnification image (right). Sub-anatomical expression patterns were observed, largely excluding the IPDM. Blue indicates A-T rich regions of DNA stained with DAPI. (IPDM=dorsomedial subnucleus of the interpeduncular nucleus, arrows in right image)

However, members of the lab that contributed breeders of the VIAAT-Cre line indicated that they had identified non-specific off target expression of Cre recombinase. Given the heterogeneous neuronal composition of the IPN, and reports of the synthesis of

monoaminergic neurotransmitters within the typical anatomical delineations of the structure (2017 Quina, Turner 28387937) – as well as the resources and time applied to this component of the project up to this point – I decided to abandon this particular set of experiments to avoid the possibility of sustained unproductivity.

Recommendations for Viral Interrogation of Small Anatomical Structures: *In vitro* validation & serotype evaluation

When using viral vectors to interrogate an anatomical structure that few studies have explored previously, several considerations are warranted prior to beginning infusions. *In vitro* assessments of the viability of any sample of virus is warranted. While commercially-obtained viruses are often reliable, defective lots can result in poor expression, the attribution of which can be laborious to identify otherwise. Ensuring the virus to be used effectively infects target neurons prior to *in vivo* infusion will help to avoid lengthy and wasteful optimization.

The tropisms of adeno-associated virus (AAV) serotypes can result in the unintentional exclusion, or unforeseen ineffective infection, of neuronal populations within the target structure (Mason et al., 2010; Srivastava, 2016). Furthermore, studies have identified different propensities of viral particles to diffuse through tissue following infusion as a function of serotype (McFarland, Lee, Hyman, & McLean, 2009). Accordingly, given equivalent efficiency of neuronal infection, some serotypes may yield preferable diffusion characteristics according to the desired region of infection. Some studies indicate that serotypes confer distinct times to expression, resulting in peak expression occurring at different intervals following infusion, and optimal waiting periods prior to behavioral experiments as a result (McFarland et al., 2009). There is evidence that some serotypes produce an immune response of greater severity than others. For example, when targeting hippocampal neurons, Klein et al. found that AAV8 resulted in broader spread

of expression compared to AAV2, though AAV5 produced the widest expression (R. L. Klein et al., 2006). However, they also corroborated findings of greater levels of expression within neurons initially characterized by Paterna et al. (Paterna, Feldon, & Bueler, 2004). Further, toxicity derived from expression of viral products was mitigated by dilution of viral doses (R. L. Klein et al., 2006).

Additionally, some serotypes are used far more widely than others, and are therefore more thoroughly characterized. When beginning the work described in this dissertation, there had been very few viral interrogations of the IPN in the literature, and members of the De Biasi lab were just beginning to explore the utility of AAV-based vectors in the MHb – as opposed to lentivirus- or rabies-based vectors - with an eye towards the IPN. Accordingly, when beginning my work, I qualitatively evaluated the efficacies of serotypes that were commonly used in the ventral midbrain in experiments using optogenetics – namely, AAV2, AAV5, and AAV8. AAV8 seemed to produce an optimal combination of reliable infection, controllable diffusion, and levels of toxicity.

Given all of the points of variation discussed, unexpected results can arise from the infusion of an AAV packaged in the same serotype used in other brain regions. Accordingly, early evaluation of the viabilities of multiple serotypes is warranted for efficient viral interrogation of specific neuronal populations, followed by subsequent characterization of resulting expression patterns, prior to initiating behavioral experiments.

Stereotactic Targeting of Small Anatomical Structures: Fastidious maintenance of surgical tools

When targeting small anatomical structures in the brain, several non-biological parameters determine whether infection is successfully restricted to the intended structure. The optimal volume of viral fluid infused will vary according to viral serotype,

as well as the anatomical features characterizing the target. In my experience, larger syringe needle gauges tend to develop blockages less frequently than smaller gauges, though smaller gauges resulted in reduced disruptions of tissue when traveling ventrally to the target structure. It seems there is, therefore, a tradeoff between the risk of unsuccessful infusion due to blockage of a small gauge syringe needle and larger lesions due to wider gauge needles. Additionally, I found that the rate of infusion can influence the spread of infection, with lower infusion rates helping to limit the extent of spreading. Once again, however, there seems to be a tradeoff between using slow rates of infusion to minimize unintended diffusion of viral particles beyond the bounds of the anatomical target and the risk of syringe clogging at slower rates.

The syringe system I ended up using for my experiments was a 10 µL Hamilton syringe terminating in a removable needle. The ability to replace syringe needles ended up being a critical capability when performing viral infusions in a sufficient number of mice to perform behavioral experiments. Removable needles are available in a variety of point styles, ranging from blunt to beveled at a variety of angles. Following assessment of initial infusions into the IPN using beveled needle points, I determined that blunt points tended to yield more consistent expression patterns, presumably due to a closer correspondence between the direction of fluid infusion and stereotactic navigation coordinates. While I developed the sense that beveled point styles tended to develop blockages less frequently, I didn't quantitatively evaluate this potential advantage of beveled styles.

During initial cohorts of viral infusions, I cleansed infusion syringes – clearing syringe needles in the process - both before and after groups of infusions. While optimizing my protocol for the infusion of Cre-dependent chemogenetic viruses, I began cleansing syringes between each viral infusion. While this change in protocol did not end up

accounting for poor expression patterns among the Cre-dependent virus infusions in VIAAT-Cre transgenic mice, as described previously, I found that doing so improved rates of success in subsequent viral infusions. This cleansing protocol proceeded as follows: before each cohort of viral infusions, flush the fully assembled syringe with sterilized deionized water multiple times. If fluid does not flow coherently from the syringe, use a cleaning wire dedicated to that particular syringe and virus to purge the lumen of the syringe needle and its connection point with the syringe barrel. The connection point between the syringe needle and barrel is particularly prone to clogging, and so ensuring the syringe cleaning wire can pass through unobstructed is critical to ensuring successful flow. Following flushing with purified water, flush the syringe with sterile saline multiple times – once again ensuring fluid flows coherently upon depression of the syringe plunger.

The syringe plunger consists of a long metal pole with a rather small plunger tip (gasket/stopper) at the end composed of polytetrafluoroethylene. The composition of the plunger tip is worth noting due to potential incompatibilities with solvents that might be otherwise intuitively effective when clensing syringes. Over time, the plunger tip deteriorates – thereby compromising the seal between the plunger and fluid loaded in the chamber. This can be identified during tests of coherent fluid flow of small volumes (~30 nanoliters) from the syringe. The most conspicuous indication of compromised integrity of the plunger tip is, following depression of the syringe using the infusion pump to deliver small volumes (~2-3 nanoliters), if the fluid appears to re-enter the syringe after forming a droplet at the tip of the syringe needle.

Following syringe cleansing and assessment of its ability to infuse fluid, place the syringe in the infusion apparatus such that a small volume of air fills the barrel. As some virus-containing fluids tended to have a propensity to clog syringes after prolonged

periods of time, I found that loading syringes immediately prior to lowering them to stereotactic targets – following the majority of surgical procedures – minimized such clogging. When loading syringes with viral fluid, monitoring the syringe barrel with a stereo microscope for the inclusion of any air bubbles within the volume of loaded fluid ensured only the intended fluid was infused. Following arrival at the anatomical target, an interval of several minutes – 5 minutes, in the case of the IPN – prior to initiating infusion helped to minimize diffusion of viral particles beyond the boundary of the structure. Following completion of infusion, another waiting period of several minutes prior to withdrawal of the syringe was similarly beneficial to limiting off-target expression. Finally, during syringe withdrawal, very slow removal helped to minimize expression along the tract of the syringe.

While perhaps unnecessarily laborious for the targeting of larger brain structures located more dorsally relative to the IPN, this protocol resulted in the highest frequency of successful expression patterns in my experience. However, while necessary for selective targeting of the IPN in my experience, this protocol ought to improve rates of success when targeting any brain region solely at the expense of surgical procedures of longer duration.

Relevance to the Understanding of Anxiety

As discussed in Chapter 1, the DSM-5 describes anxiety as the anticipation of a future threat (Crocq, 2015). This is distinct from fear, elicited in response to the perception of an imminent threat ("Anxiety Disorders,"). While anxiety-associated and depressive disorders are distinct diagnostic categories, elements of both are frequently comorbid. Estimates suggest that approximately 85% of patients diagnosed with depression exhibit anxiety-associated symptoms as well, and a comorbidity of anxiety and depression is

frequently observed (Moller et al., 2016). The consistency of these overlapping symptoms – when those associated with either one is not clearly dominant - has prompted the consideration of a diagnosis of mixed anxiety and depressive disorder (MADD) in the International Statistical Classification of Diseases and Related Health Problems (ICD-10). Early concepts of psychiatric diagnoses framed anxiety as a component of all psychiatric conditions, rather than a distinct category of mental illness (Moller et al., 2016). However, upon the inception of the use of anti-depressants and anxiolytics in psychiatry, as discussed in Chapter 1, a diagnostic distinction between depression and anxiety was established. The merits and value of these distinctions are beyond the purview of this dissertation, but the clinical similarities between both sets of symptoms suggest some shared neurophysiology (Steimer, 2002).

A majority of insights regarding the functional roles of the MHb and IPN characterize the neurobiology of addiction. In particular, as discussed previously, preclinical examinations of the MHb-IPN axis affirm a role played by the pathway in the aversive syndrome arising during withdrawal from a variety of habit-forming drugs – including alcohol, opioids, nicotine, and psychomotor stimulants as well (McLaughlin et al., 2017; Molas et al., 2017). As described in Chapter 1, the anatomy of the habenular complex renders it prohibitively small to resolve in most human neuroimaging efforts (Batalla et al., 2017; Salas et al., 2010; Salas et al., 2009), though some progress has been made in distinguishing the medial from lateral habenulae (Strotmann et al., 2014). Given the reported interaction between the habenular subnuclei (Kim & Chang, 2005), and the reported connectivity between the LHb and IPN (Quina et al., 2017), activity in one of the habenular subnuclei likely influences signaling in the other. Accordingly, reports of the role that LHb activity plays in major depressive disorder (Browne, Hammack, & Lucki, 2018) suggests a further interaction of the MHb-IPN axis with affective states.

As noted in Chapter 1, a high comorbidity between addiction and both depression and anxiety suggest at least some overlapping neurophysiology. A more detailed enumeration of the anatomical context of the MHb-IPN axis was included in Chapter 1, the salience of which here being that the IPN sends efferents to a network of structures implicated in affect regulation, psychiatric illnesses, and anxiety in particular.

The studies described in this dissertation indicate a role played by signaling within the IPN in the regulation of anxiety-associated behavior independently of chronic drug exposure. Until the studies described here, the role played by IPN signaling in baseline anxiety-associated behavior had yet to be demonstrated. Alternatively stated, signaling within the IPN is not only implicated in the pathophysiology of addiction, but perhaps anxiety-associated conditions as well. Accordingly, the IPN may be one component of the broader anatomical bond between addiction, anxiety, and psychiatric illnesses.

Further, efferent projections from the IPN to the raphe nuclei had been established, and evidence of its projections to the hippocampus had been proposed several decades ago – with one recent study confirming them (Quina et al., 2017). However, the studies described in this dissertation confirm that – among its efferents to the hippocampus – the IPN sends projections to the ventral hippocampus in particular. Given the role of the ventral hippocampus in the regulation of affect - and anxiety in particular, as described in Chapter 1 - this may be an important mechanism by which the IPN modulates anxiety-associated behavior.

Similarly, while anatomical connectivity between the IPN and raphe nuclei has been widely appreciated, a correlation between anxiety-associated behavior, IPN signaling, and serotonin levels had yet to be demonstrated. These studies indicate that activation of IPN neurons is anxiolytic and correlates with significant elevations of serotonin concentrations in the ventral hippocampus in particular. Recent tracing experiments I

conducted suggest that at least some of these IPN efferents to the ventral hippocampus are glutamatergic. Furthermore, the IPN sends glutamatergic efferents to other anatomical structures associated with the regulation of affect, including the lateral habenula and lateral hypothalamus (Fig. 1). Additional tracing studies I performed suggest that the IPN sends efferents to components of the basal forebrain – a network associated with the integration of arousal, behavioral inhibition, and sensory information (Cassidy et al., 2019).

Figure 3.

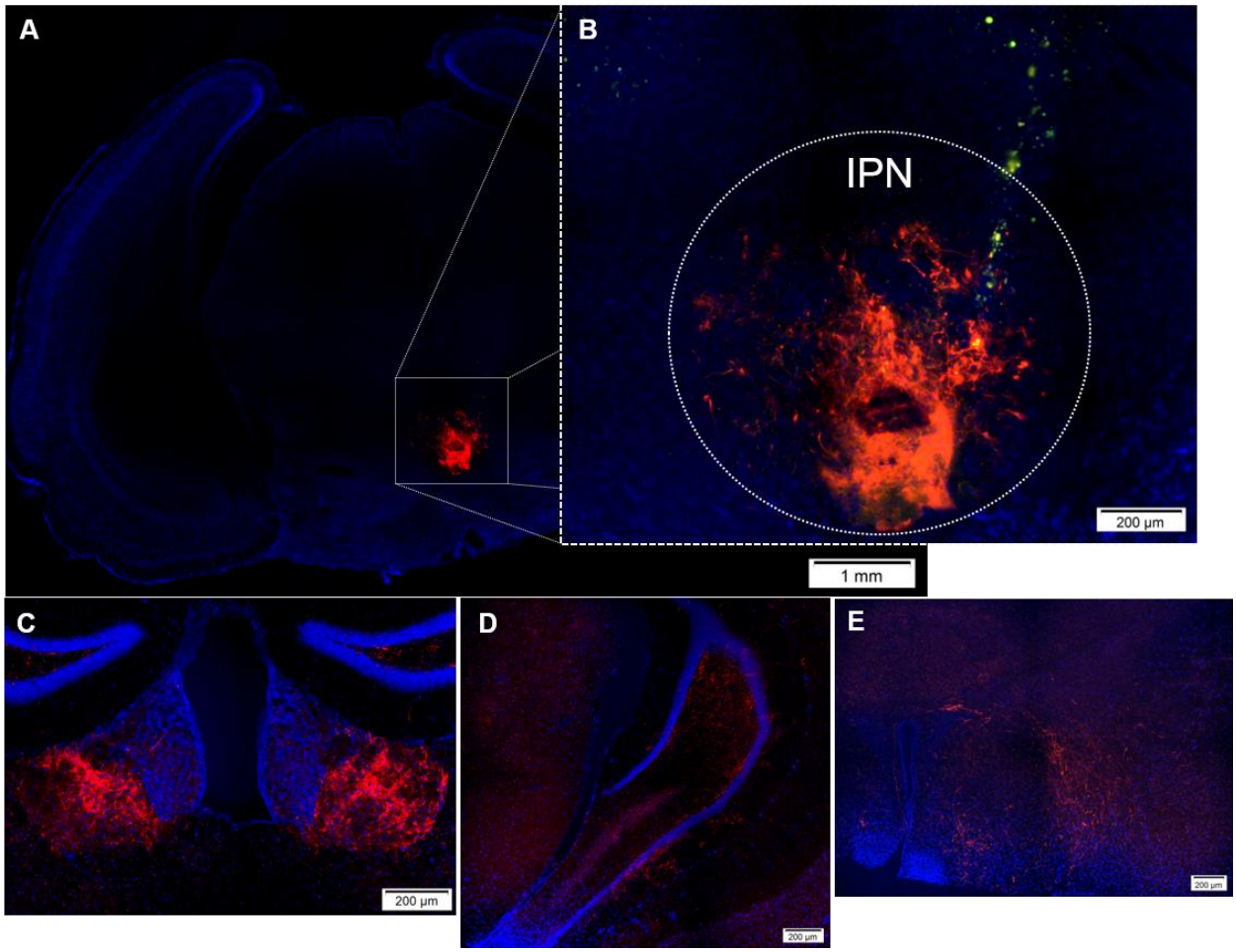


Figure 3. Slices from Vglut3-Cre transgenic mice infused with AAV8-DIO-ArchT-tdTomato (red) into the IPN, stained with DAPI (blue). A low-magnification image of a coronal section (A), complemented by higher magnification image of the IPN, displaying Cre-dependent infection of glutamatergic IPN populations (B). Non-specific fluorescence induced by damage along the syringe infusion tract is identified in green/yellow. tdTomato^{+} projections are observed in the lateral habenula (C), the ventral hippocampus (D), and the lateral hypothalamic area (E).

While other efferent signals involved in the regulation of affect very likely emerge from the IPN, these data help to begin enumerating which among them reach anatomical structures that participate in this activity.

More broadly, these data integrate the principal recipient of DDC signaling, the IPN – which has been implicated in the manifestation of drug withdrawal symptoms - into a

broader anatomical foundation of the regulation of mood and anxiety-associated conditions. Further, the MHb-IPN axis – a junction of signaling by which activity in the forebrain influences the mid- and hind-brain (McLaughlin et al., 2017) – may represent a point of feedback, such that elements of activities in caudal structures are fed back to the forebrain.

Given the anatomy of the DDC and IPN, and evidence linking activity therein with both addiction and psychiatric illnesses, the Hb-IPN axis is likely to continue yielding insights regarding the pathophysiology of both conditions. Further, a pursuit of druggable targets within this pathway in preclinical studies for the treatment of both psychiatric conditions is likely warranted.

Future Directions

While the majority of behavioral analyses of the IPN have been performed in animal models of chronic drug exposure and withdrawal, there are opportunities to evaluate how activity in this pathway regulates context-dependent affective output. The behavioral experiments in these studies were performed in environmental conditions empirically determined to have no significant effect on baseline anxiety-associated behavior. Ethological assays like those used in this series of studies, the open field arena and elevated plus maze, are based upon the tension between predispositions of mice to explore novel environments and an aversion to open and illuminated or elevated environments (K. R. Bailey & Crawley, 2009; M. T. Bailey, Kinsey, Padgett, Sheridan, & Leblebicioglu, 2009). Accordingly, modifying environmental stimuli during chemogenetic perturbations of the MHb-IPN axis may reveal context-dependent regulation of anxiety, such as illumination, aversive olfaction, or adversarial social interaction.

An enticing set of experiments is to interrogate the functional properties of efferent IPN projections to structures established to be associated with the regulation of affect and manifestation of withdrawal symptoms. Principally, while initially proposed decades ago (Groenewegen et al., 1986), discussion of efferent projections from the IPN to the LHb has recently been revived (Quina et al., 2017; Quina et al., 2015). While few studies have confirmed them, recent experiments I performed suggest these projections likely do indeed exist. The behavioral relevance of such a network is likely considerable, given the regulation of dopaminergic signaling by the LHb (Lammel, Lim, & Malenka, 2014; Matsumoto & Hikosaka, 2007; Stamatakis et al., 2013), particularly given that the signaling between the LHb and IPN appears to be rather complex (Beier et al., 2015). Another projection worthy of interrogation is that between the IPN and septum. The MHb receives projections from the medial septum that are thought to be principally GABAergic (Qin & Luo, 2009). Recently published tracing studies suggest there are projections from the IPN to the septal nuclei, as well as the diagonal band nucleus (Quina et al., 2017), and pilot studies I performed comport with these findings. Accordingly, given that these structures regulate anxiety (Henry, Vale, & Markou, 2006; Parfitt et al., 2017), interrogation of projections from the IPN may yield insights that broaden the anatomical regulation of anxiety by the IPN.

An experiment I attempted, that intuitively follows the findings of elevated ventral hippocampal serotonin concentrations correlated with IPN stimulation, is to inhibit the raphe nuclei while stimulating the IPN. In the ventral hippocampus, serotonin has been shown to regulate anxiety-associated behavior (Adams, Kusljic, & van den Buuse, 2008; Barr et al., 2013; Tu et al., 2014). As I am currently unable to attribute the source of this elevation of serotonin levels in the ventral hippocampus, removal of a prime source of serotonin may help to elucidate the functional relationships between these structures.

Accordingly, inhibition of serotonergic neurons within the raphe nuclei while simultaneously stimulating IPN neurons may reveal the source of ventral hippocampal serotonin following IPN stimulation. Finally, while this series of studies used an AAV serotype that appears to infect a significant proportion of the IPN, a systematic comparison of the efficacies of a broader spectrum of serotypes will be very helpful in subsequent viral interrogations of the structure.

CHAPTER 5: SUPPLEMENTARY

ADDITIONAL SCIENTIFIC CONTRIBUTIONS

This chapter section presents two collaborative studies that are in preparation for publication followed by additional publications during the progression of my graduate work.

α5-dependent Modulation of nAChR Function and DA Release during Chronic Nicotine Exposure and Nicotine Withdrawal: Effects of the rs16969968 polymorphism.

Kechun Yang, Ian McLaughlin, John A. Dani, Mariella De Biasi

Contributions:

Electrophysiological studies conducted by Kechun Yang

Virus infusions, immunohistochemistry, and microscopy performed by Ian McLaughlin

Abstract

A D398N (rs16969968) single-nucleotide polymorphism (SNP) in the human CHRNA5 gene has been linked to increased use of tobacco. Accumulating evidence from studies that re-express this α5 SNP suggests that it plays an important role in regulating Ca^{2+} permeability and the function of nicotinic acetylcholine receptors (nAChRs), nicotine consumption, and the treatment outcomes of some neuropsychiatric disorders (Koukouli et al., 2017). It remains challenging, however, to directly measure functional changes in α5-containing nAChRs, especially in adult animals exposed to nicotine treatment for extended periods of time and following nicotine withdrawal. We studied adult α5 null mice and their littermate controls, as well as mice expressing the rs16969968 polymorphism in dopaminergic neurons (α5 SNP). We systematically examined dynamic changes in nAChR function by measuring ACh-induced whole-cell currents in VTA dopamine (DA) neurons using patch-clamp recordings in mice treated with nicotine for 8 weeks, as well as up to 8 weeks after nicotine withdrawal. We also measured the corresponding DA release in the dorsolateral striatum using fast-scan cyclic voltammetry. We demonstrated that deletion of the α5 nAChR subunit leads to a dramatic decrease in nAChR function and single-pulse evoked DA release. Expression of the rs16969968 CHRNA5 polymorphism in dopaminergic neurons of α5 null mice only partially restored nAChR function. Chronic nicotine exposure produced the most profound enhancement of both nAChR function and DA release at single-pulse evoked basal levels, and 20 Hz evoked maximal levels in SNP-expressing mice. Our results provide new insights into the roles of this α5 SNP in chronic smoking and smoking cessation, and reveals new potential treatment strategies for aiding smoking cessation.

Introduction

Tobacco smoking is a major public health concern that leads to millions of preventable deaths every year worldwide. The majority (80%) of smokers have attempted to quit, but only 3% quit successfully (Benowitz, 2010). Nicotine is the principal addictive component of tobacco, which exerts complicated effects on nicotinic acetylcholine receptors (nAChRs) and further modulates the release of a variety of neurotransmitters, including dopamine (DA), in the brain (Dani & De Biasi, 2013). Most functional nAChRs in the brain consist of a combination of five α and/or β subunits (Dani, 2015). The α5 subunit is distinguished by its role as an obligate accessory subunit, and functional α5 containing nAChRs can be formed by different α and β subunits (Berrettini et al., 2008; Ramirez-Latorre et al., 1996; Sciaccaluga et al., 2015). Different lines of evidence from patch-clamp studies show that genetic deletions of the α5 subunit reduces the functionality of nAChRs, reflected by smaller peak amplitudes of whole-cell currents (Chatterjee et al., 2013; Morel et al., 2014; Sciaccaluga et al., 2015).

Genome-wide association studies have found that the α5 subunit containing a nonsynonymous variant, rs16969968 - a single-nucleotide polymorphism (SNP) in which aspartic acid [D] 398 is replaced by asparagine [N] (D398N) - is highly associated with heavy smoking (Berrettini et al., 2008; Bierut et al., 2008; Lips et al., 2010; Sarginson et al., 2011). We and others have shown that mice expressing the α5 SNP, in either dopaminergic cells or the entire brain, self-administer larger doses of nicotine relative to control mice (Morel et al., 2014; O'Neill et al., 2018). However, the neurobiological mechanisms underlying this increased nicotine intake caused by the SNP remain unclear. With the development of SNP re-expression in cell lines, and targeted re-expression of the SNP in ventral midbrain neurons, it has been shown that the

polymorphism results in significant changes in Ca^{2+} permeability and function of both α3β4-nAChRs and α4β2-nAChRs (Sciaccaluga et al., 2015; Tammimaki et al., 2012). Acutely applied nicotine produced significant differences in DA release in striatal synaptosomes from the offspring of nicotine-treated, SNP-expressing mice compared to untreated SNP-expressing mice. It was also shown that chronic nicotine could significantly influence the central nervous system and reverse both neurocognitive and behavioral deficits observed in mice expressing the human SNP (Koukouli et al., 2017). However, the neurobiological mechanisms underlying changes resulting from chronic nicotine exposure remain largely unknown. In this study, we directly examined the dynamic changes in both nAChR function, and the corresponding DA release, induced by chronic nicotine exposure followed by extended nicotine withdrawal. We found that chronic nicotine treatment resulted in altered functionality of nAChRs and DA release probability, and these changes depended upon the α5 subunit. These findings shed light on the pivotal roles played by this α5 SNP in both smoking and smoking cessation.

Materials and Methods

Animals

We studied 4- to 8-month-old mice of both sexes, including α5 null mice and their littermate controls, and α5 nulls crossed with mice expressing Cre recombinase driven by the dopamine transporter (DAT-Cre). We worked with α5 null x DAT-Cre mice to study the effects of α5 WT vs. α5 SNP re-expression in dopaminergic neurons. All mice were maintained in a 12-h light/dark cycle, temperature-controlled room. All behavioral tests were performed during their light cycle, and were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania, according to the guidelines for intramural animal research provided by the National Institutes of Health in an animal care facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Nicotine treatment

Mice received either 2% saccharine (SAC) as control or 200 mg/l nicotine + 2% saccharine in their drinking water for 8 weeks after weaning. After the 8 weeks of nicotine treatment, mice were withdrawn from nicotine to study nAChR responses after short term (1-4 days), intermediate (2 weeks) or long-term (2 months) nicotine withdrawal.

Viral injections

Intracranial viral infusions were performed with mice approximately 2 months old under general isoflurane (1-2%) anesthesia coupled with meloxicam (2 mg/kg). Following fur removal, disinfection, and exposure of the skull, small holes were drilled to enable stereotactic targeting with a 10 µL syringe with removable needle tips (Hamilton, Reno,

Nevada). Virus-containing fluid (AAVDJ-DIO-α5-copGFP or AAVDJ-DIO-α5SNP-copGFP) was infused at 0.01 µL/min with a microinfusion pump (kdScientific, Hollistoin, MA), attached to the stereotactic apparatus (Kopf, Tujunga, CA). 1.70 – 2.0 µL of viral fluid was infused into the VTA (A/P: -3.2 mm - -3.25 mm, M/L: ± 0.05 mm - 0.12 mm, D/V: -4.20 mm - -4.35 mm).

Slice preparation

Mice were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine/xylazine, and transcardial perfusion was performed as previously described (Broussard et al., 2016; Yang et al., 2017) with an ice-cold, N-methyl-D-glucamine (NMDG) based artificial cerebrospinal fluid (ACSF, in mM): 92 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, and 10 $MgSO_4$, pH 7.3-7.4 with concentrated hydrochloric acid (Ting et al., 2014). After decapitation, the brain was quickly removed from the skull and placed in ice-cold NMDG solution, saturated with 95% O_2 and 5% CO_2. Horizontal slices, containing either the ventral tegmental area (VTA, 230 µm) for patch-clamp recordings or the dorsal striatum (300 µm) for fast scan cyclic voltammetric studies, were cut using a vibratome (Leica VT 1200s) in ice-cold NMDG solution. The slices were recovered in the NMDG solution at 32^{o}C for 13 min, and then transferred to HEPEs-based holding ACSF (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2$, and 2 $MgSO_4$ at room temperature for at least 1 hour until recording. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Electrophysiological Recordings

Slices containing the VTA were placed in a home-made recording chamber, and were continuously bathed in well-oxygenated standard recording ACSF (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $CaCl_2$, and 2 $MgSO_4$, maintained at 32–34°C using an inline heater system (TC-324B, Warner Instrument Corp, Hamden, CT). Responses were recorded using glass recording electrodes (~2-3 MΩ), which were pulled from borosilicate glass capillaries (TW 150-4, World Precision Instruments, Inc, Sarasota, FL) using a micropipette puller (Narishige PC-10, Tokyo, Japan), and were filled with a K-gluconate-based intracellular solution (in mM): 140 K-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine, pH 7.3 with KOH. Tight-seal patch-clamp recording configuration was first achieved by applying a brief gentle suction. Under tight-seal patch-clamp mode, spontaneous action potential firing could be observed in most recorded neurons, and the identification of DA neurons was first confirmed by sensitivity to puff-applied quinpirole (2 μM), a selective D2-receptor agonist (Fig 1D). Then, whole-cell mode configuration was achieved by briefly applying strong suction. Only access resistance (Ra) < 10 MΩ was accepted, and the Ra was monitored throughout the experiment. Putative DA neurons were then identified by the presence of hyperpolarization-activated potassium currents (H-current, Fig 1C), a typical electrophysiological property of DA neurons. Some recorded putative DA neurons were labeled with neurobiotin during patch-clamp recording, and were further confirmed as DA neurons with positive tyrosine hydroxylase (TH) immunohistochemistry (Fig 1B). To fully activate nAChRs in the recorded neurons, 1 mM ACh was puff-applied by Picospritzer II (Parker Instrumentation, Fairfield, NJ) every 2 min via a puff electrode (marked as P in Fig 1A), an electrode identical to those used for recording, while the recorded neurons were clamped at -60 mV (VH = -60 mV)

under voltage-clamp mode (sFig 1). Atropine (1 μM) (Yang et al., 2011; Yang et al., 2009a) was added to recording ACSF to block muscarinic receptors.

Fast-scan cyclic voltammetry

Slices containing the dorsal striatum were transferred to the same recording chamber as above and were continuously perfused with the same well oxygenated standard recording ACSF (32-34 °C). Fast-scan cyclic voltammetry was performed using homemade carbon fiber (10 μm diameter, Amoco Polymers, Greenville, SC) electrodes (~ 100 μm of exposed fiber) within the dorsal striatum (Le, Zhang, Xie, Li, & Dani, 2015). The carbon-fiber electrode potential was linearly scanned at a rate of 300 mV/ms every 100 ms from 0 to −400 to 1000 to −400 to 0 mV against a silver chloride reference electrode. DA transients were evoked by electric stimulations via a bipolar tungsten electrode (Stereotrode Tungsten; WPI) with two poles spaced about 150 μm apart, placed within the dorsal striatum. The tip of the carbon-fiber recording electrode was about 150 μm away from each of the two poles of the stimulating electrode. Different stimulating protocols were designed with the aid of the Master-8 (A.M.P. Instruments LTD. Jerusalem, Israel) to mimic physiological DA release, induced by either single pulse stimulation or by tonic or phasic bursting stimulations. Each stimulus pulse (about 0.15 mA) was 1 ms in duration, produced with an A365 Stimulus Isolator (World Precision Instruments, Inc. Sarasota, FL). Single pulses were applied every 120 seconds to allow recovery of the DA release. To measure phasic/tonic ratios ($[DA]_{5p}/([DA]_{1p}$ or $[DA]_{20p}/[DA_{1p}$), single stimulations and the phasic burst stimuli, at an intraburst frequency of 20 Hz, were separated by 120 seconds. Peak DA signals were converted into DA concentrations based on a post-experimental calibration of the carbon-fiber electrode

against fresh DA solutions (from 0.5 to 10 μM). All data were collected using Clampex 10 software via an Axopatch 200B amplifier and a digitizer 1550 (Molecular Devices).

Chemicals

Dihydro-β-erythroidine hydrobromide (DHβE) and quinpirole were purchased from Tocris. Nicotine hydrogen tartrate salt was purchased from Glentham Life Sciences. NEUROBIOTIN™ tracer was purchased from Vector Laboratories. All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analyses

All values were expressed as mean ± SEM, and the number of experiments was indicated by n. Statistical analyses were conducted using either paired/unpaired Student's *t* test, or one-way ANOVA with *post hoc* Tukey's test, and $p<0.05$ was considered to be statistically significant.

Results

The α5-nAChR subunit is critical to nAChR function in VTA DA neurons

Our previous work (Gangitano et al., 2009; Morel et al., 2014), and studies from other groups, found that knock-out of the α5 nAChR subunit influences behavior (Fowler et al., 2011; Koukouli et al., 2017; X. A. Liu & Kenny, 2017). Moreover, the CHRNA5 polymorphism significantly influences nicotine intake, anxiety-associated behavior, DAergic responses to nicotine, and hypofrontality observed in patients with schizophrenia (Koukouli et al., 2017).

To better understand the role of the α5 subunit in the dynamics of nicotine exposure and withdrawal, we first compared nAChR function in DAergic neurons from age-matched drug-naïve (SAC-treated), wild-type (WT), α5 null mice, their littermate controls, and α5 SNP mice. Only neurons with clear H-currents (Fig 1C) were considered to be DAergic. Under voltage-clamp mode, 1 mM ACh was puff-applied every 120 seconds onto recorded putative VTA DA neurons held at -60 mV (VH= -60 mV). ACh-induced inward currents were able to be stably recorded for at least 20-30 minutes (Fig 2), with some lasting up to 1 hour (sFig1). The greatest peak amplitude of the inward currents (99.34 ± 11.59 pA, n=17) induced by 1 mM ACh was recorded in VTA DA neurons of WT mice (Fig 2A, D). In null mice, nAChRs without the α5 subunit showed very limited function, as evidenced by the small peak amplitude of inward-currents (9.66 ± 1.17 pA, n=20) induced by 1 mM ACh in VTA DAergic neurons (Fig 2B, D). These results corroborate those of previous works, showing that the α5 subunit enhances baseline nAChR currents in VTA DA neurons from both young (Chatterjee et al., 2013; Sciaccaluga et al., 2015) and adult mice (Morel et al., 2014). The function of nAChRs was partially recovered by expression of the rs16969968 polymorphism in DAergic neurons (α5 SNP), resulting in larger ACh-induced inward currents (22.51 ± 2.76 pA, n=10, $p<0.001$, SNP vs. null, Fig 2C, D), that were, however, still smaller than those measured from WT neurons ($p<0.001$, SNP vs. WT, Fig 2D). One-way ANOVA showed significant differences in the peak amplitudes of ACh-induced currents among WT, α5 null, and α5 SNP mice ($F_{(2,44)} = 40.5$, $p< 0.0001$, Fig 2D), suggesting that the α5-nAChR subunit plays key roles in modulating nAChR function in VTA DA neurons.

α5-containing nAChRs play an important role in the modulation of DA release in the dorsolateral striatum

At physiologically relevant levels, nicotine can modulate striatal DA release, either via nAChRs expressed in DA neurons/axons, or via nAChRs locally expressed in cholinergic interneurons (L. Wang et al., 2014; T. Zhang et al., 2009; Zhou, Liang, & Dani, 2001). Frequency-dependent modulation of DA release by nicotine has been observed in both the ventral and dorsolateral striatum (H. Zhang & Sulzer, 2004; L. Zhang, Doyon, Clark, Phillips, & Dani, 2009).

To date, the α5 nAChR subunit has proven to be particularly critical to the regulation of DA release in the dorsal striatum, but not the nucleus accumbens (Exley, McIntosh, Marks, Maskos, & Cragg, 2012). In addition, compared with DA terminals in the ventral striatum, those in the dorsal striatum are able to gain a significantly higher proportion of DA release from presynaptic nAChR activation. A single action potential arriving at DA terminals in the dorsolateral striatum is more likely to trigger DA release than one arriving at DA terminals in the NAcc shell (T. Zhang et al., 2009). To better understand the role played by α5-containing (α5*) nAChRs in the regulation of DA release in the dorsal striatum, and the effects of the rs16969968 SNP, we first compared basal DA release in the dorsolateral striatum with a single-pulse stimulus (1p), delivered every 120 seconds, in drug naïve mice. The biggest peak amplitude of DA signals (1.63 ± 0.16 μM, n=29) was recorded in WT mice (Fig 3 A, D), suggesting that WT mice have the greatest DA release probability. The smallest DA signals (0.40 ± 0.04 μM, n=13) were recorded in α5 null mice (Fig 3B, D), indicating that a lack of α5 is associated with the lowest DA release probability. Interestingly, the peak amplitude of DA signals (0.98 ± 0.07 μM, n= 22) is partially recovered in α5 SNP mice, which is greater than that of null mice

(p<0.001, SNP vs. null, Fig 3C, D), but remains smaller than that of WT mice (p<0.001, SNP vs. WT, Fig 3D). One-way ANOVA detected significant differences in the peak amplitudes of single pulse-evoked DA signals among WT, null, and SNP mice ($F_{(2,61)}$ = 7.27, p< 0.001, Fig 3D). These results imply that the probability of DA release in the dorsolateral striatum greatly depends on the functional levels of nAChRs, is determined by the nAChR α5 subunit, and is affected by the presence of the CHRNA5 polymorphism.

To further confirm that striatal DA release is mainly regulated by nAChRs, we examined the effects of 0.1 μM DHβE by applying the β2-nAChR antagonist via 20-minute bath application. As shown in Fig. 4 A&B, inhibition of β2-nAChRs dramatically decreased single-pulse induced DA release to 0.23 ± 0.02 (n=5, p< 0.001 vs. baseline, Fig 4A, E) and 0.25 ± 0.03 (n=7, p< 0.001 vs. baseline Fig 4B, E) of baseline in WT and α5 null mice, respectively. 60 minutes after DHβE washout, the DA signal was partially recovered to 0.64 ± 0.05 (n=5, p<0.001, washout vs. DHβE, Fig 4A,E) and 0.78 ± 0.06 (n=7 p<0.001, washout vs. DHβE, Fig 4 B,E) of the baseline in WT and α5 null mice, respectively. Reversible inhibition of DA release by DHβE is consistent with our previous findings (L. Zhang et al., 2009; Zhou et al., 2001), and corroborates studies from other groups (Threlfell et al., 2012), indicating that dorsolateral striatal DA release largely depends on nAChR function.

We further tested the effects of nicotine (Nic) on striatal DA release, as it is well-known that nicotine desensitizes nAChRs (Dani, 2015; McLaughlin et al., 2015) and inhibits DA signals (L. Wang et al., 2014; T. Zhang et al., 2009; Zhou et al., 2001). Single-pulse evoked DA release was suppressed to 0.22 ± 0.03 (n=5, p<0.001 vs. baseline, Fig 4C, F) and 0.22 ± 0.03 (n=7, p<0.001 vs. baseline, Fig 4D, F) of baseline levels in WT and

α5 null mice, respectively, by 20 min of bath-application of 0.5 μM nicotine. The suppression of DA signals resulting from nicotine-induced desensitization partially recovered to 0.83 ± 0.07 (n=5, p<0.001, washout vs. Nic, Fig 4C, F) of baseline in WT mice, and to 0.49 ± 0.10 (n=6, p<0.05, wash out vs. Nic, Fig 4D, F) of baseline in α5 null mice, 60 min after nicotine wash-out. Neither DHβE nor nicotine completely abolished single-pulse evoked DA release in the dorsolateral striatum, indicating that nAChRs, although major contributing factors (L. Wang et al., 2014), work in concert with a variety of other neurotransmitters and receptors in the regulation of local DA release. The reversible suppression of DA signaling by DHβE and nicotine, however, strongly supports the important roles played by α5* nAChRs in modulating the probability of dorsolateral striatal DA release.

Dramatic and long-lasting changes in nAChR function after chronic nicotine treatment

Chronic nicotine exposure may lead to long-term homeostatic regulation of nAChR function that contributes to the addiction process. Several lines of evidence from studies measuring nAChR binding (Nguyen, Rasmussen, & Perry, 2003; Perry, Davila-Garcia, Stockmeier, & Kellar, 1999) and using electron microscopy (Pakkanen, Jokitalo, & Tuominen, 2005) suggest that long-term exposure to nicotine results in upregulation of nAChRs in WT rodent brains. A recent study, using quantitative ligand-binding autoradiography, reported that chronic nicotine treatment down-regulates α6β2-nAChRs and up-regulates α4β2-nAChRs in DAergic and optic-tract nuclei. Conflicting lines of evidence from the above studies indicate that chronic nicotine, depending on subunit composition, can upregulate, down regulate, or have no effect on nAChR function. Most electrophysiological studies that directly examined nAChR function in VTA DA neurons

were carried out in young animals (Chatterjee et al., 2013; Wooltorton, Pidoplichko, Broide, & Dani, 2003; Wu et al., 2004; Yang et al., 2009b). Given the challenges associated with patch-clamp recording in VTA DA neurons of adult animals chronically treated with, and withdrawn from, nicotine, our understanding of the dynamic changes in the function of VTA DA neurons with nAChRs is limited. No information is available on the effects of chronic nicotine treatment and withdrawal on the function of α5* nAChRs.

In this study, we directly measured nAChR-mediated whole-cell currents in VTA DA neurons of adult WT, α5 null, and α5 SNP mice undergoing either chronic nicotine treatment or nicotine withdrawal. In WT animals, ACh-induced whole-cell currents were comparable between SAC- treated controls (99.34 ± 11.59 pA, n=17) and animals treated for 8 weeks with nicotine (89.47 ± 10.15 pA, n=16, $p>0.05$ vs. SAC) (Fig. 5A, E). This result is consistent with previous findings indicating that chronic nicotine does not change nAChR function in midbrain DA neurons, including both the VTA and the substantia nigra pars compacta (Nashmi et al., 2007; Xiao et al., 2009). ACh-induced whole-cell currents, however, became significantly smaller after nicotine withdrawal (WD 1-4d: 54.40 ± 4.96 pA, n=10, $p<0.05$ vs. SAC/Nic, Fig. 5A), indicating down-regulation of nAChR function resulting from nicotine withdrawal. Under our experimental conditions, the suppressed ACh-mediated currents did not recover after 2-4 weeks of withdrawal (WD 2-4 wk: 50.14 ± 4.06 pA, n=7, $p<0.05$ vs. SAC/Nic), and, surprisingly, remained suppressed even after 8 weeks of withdrawal (WD 8 wk: 48.40 ± 4.97 pA, n=10, $p<0.005$ vs. SAC/Nic). One-way ANOVA revealed significant differences in the peak amplitudes of ACh-induced currents during withdrawal ($F_{(4, 55)} = 5.08$, $p<0.005$;Fig. 5E) in WT mice. The results from WT mice clearly suggest that chronic nicotine did not change nAChR function, but nicotine withdrawal resulted in long-lasting down-regulation of nAChRs. We

next tested the effects of chronic nicotine treatment and nicotine withdrawal on the function of nAChRs in α5 null mice. VTA DA neurons from null mice that were chronically treated with nicotine were more responsive to ACh (26.34 ± 3.39 pA, n=15 p<0.005 vs. SAC, Fig. 5B, E) compared to SAC-treated controls. This indicates that chronic nicotine treatment leads to upregulation of nAChR function in the absence of α5. In contrast to the downregulation observed during nicotine withdrawal in WT mice, the upregulation of nAChR function in null mice persisted, even after 8 weeks of withdrawal from chronic nicotine. As shown in Fig. 5B & E, the peak amplitudes of ACh-induced currents remained larger 1-4 days (42.65 ± 7.88 pA, n=13, p<0.005 vs. SAC), 2-4 weeks (36.91 ± 6.67 pA, n=13, p<0.005 vs. SAC), and 8 weeks (37.08 ± 7.33 pA n=12, p<0.005 vs. SAC) after nicotine withdrawal. One-way ANOVA showed significantly increased amplitudes of ACh-induced currents ($F_{(4,68)}$=7.39, p<0.001) in null mice that underwent chronic nicotine treatment and nicotine withdrawal compared to WT.

Chronic treatment of α5 SNP mice with nicotine for 8 weeks further improved nAChR function, demonstrated by bigger ACh-induced currents (60.03 ± 10.22 pA, n=11, p<0.005 vs SAC, Fig. 5C, E). This indicates that chronic nicotine exposure enhances nAChR function in mice expressing the rs16969968 SNP. Enhancement of nAChR function persisted in the days immediately following cessation of nicotine treatment (days 1-4 of nicotine withdrawal; 72.59 ± 12.22 pA, n= 12, p<0.005 vs. SAC, Fig. 5C, E). However, the peak amplitudes of ACh-induced currents returned to baseline levels beginning at 2 weeks following withdrawal (35.19 ± 9.65 pA, n= 8, p>0.05 vs. SAC, Fig. 5C, E). Normalization of the currents was also confirmed at 8 weeks after nicotine withdrawal (28.15 ± 3.24 pA, n= 14, p>0.05 vs. SAC). Dramatic changes in nAChR function were further revealed by one-way ANOVA ($F_{(4,50)}$ = 6.85, p<0.001). We further

compared the differences in nAChR function across the three different animal groups tested in response to the same treatment (Fig. 5D). As shown in Figs. 2D & 5D, significant differences ($F_{(2,44)}$ = 40.5, p< 0.001) in the peaks of ACh-induced currents were detected across nicotine naïve WT, α5 null, and α5 SNP mice. After 8 weeks of chronic nicotine treatment, significant differences among the three groups were detected ($F_{(2,39)}$ = 15.58, p<0.001, Fig. 5D). There was no difference among nAChR currents in WT, null, and SNP mice after 1-4 days ($F_{(2,32)}$ = 2.86, p=0.07, Fig. 5D), up to 2-4 weeks ($F_{(2, 25)}$ = 0.99, p= 0.38, Fig. 5D) of nicotine withdrawal. After 8 weeks of withdrawal, ACh induced smaller currents in SNP mice compared to WT and SNP mice (*t* test: WT vs. SNP, p< 0.01; one-way ANOVA: $F_{(2,33)}$ = 3.52, p=0.04, Fig. 5D). Accordingly, the results from WT, null, and SNP mice suggest that chronic nicotine and nicotine withdrawal can result in increased, decreased, or unchanged function, and the α5 subunit - and its rs16969968 polymorphism - play a key role in determining nAChR function during different states induced by nicotine.

Dramatic changes in single-pulse evoked DA release in the dorsolateral striatum after chronic nicotine treatment

We next examined whether chronic nicotine and nicotine withdrawal may have the same profound effects observed for nAChR function on single-pulse evoked DA release in the dorsolateral striatum in WT, α5 null, and α5 SNP mice. In WT mice, the peak amplitude of single-pulse-evoked DA release was dramatically suppressed after 8 weeks of nicotine treatment, (p<0.001, Fig. 6 A, E) from 1.63 ± 0.16 µM (n=29) to 0.53 ± 0.06 µM (n=16). The DA signal was partially recovered to 0.72 ± 0.05 µM (p<0.05 vs. Nic, n=26, Fig. 6A, E) after 1-4 days of nicotine withdrawal and remained slightly suppressed (0.74 ± 0.03 µM, p<0.05 vs. Nic, n=16, Fig. 6A, E) after 8 weeks of nicotine withdrawal. The

suppression of DA signals by chronic nicotine was a long-lasting effect, and did not recover to control levels ($F_{(3, 80)}$ =20.91, p<0.001, one-way ANOVA) up to 8 weeks after nicotine withdrawal in WT mice. In α5 null mice, however, the same chronic nicotine treatment significantly increased the amplitudes of DA signals (p<0.05, Fig. 6B, E) from 0.40 ± 0.04 μM (n=13) to 0.57 ± 0.06 μM (n=16). DA signals remained elevated after short-term withdrawal (WD 1-4: 0.63 ± 0.17 μM, n=14, p<0.001 vs. SAC, Fig. 6B, E), and this potentiation was sustained 8 weeks after nicotine withdrawal (0.61 ± 0.05 μM, n=23, p<0.001 vs. SAC, Fig. 6B, E) compared to those in SAC-treated controls. Thus, under our experimental conditions, chronic nicotine and nicotine withdrawal resulted in long-lasting potentiation of DA signals in α5 null mice ($F_{(3,62)}$= 3.79, p<0.05, one-way ANOVA). Similar to the results observed in α5 null mice, chronic nicotine produced significant changes in DA release in α5 SNP mice (F(3,57)=3.10, p<0.05). Peak amplitudes of single-pulse evoked DA release were greatly increased from 0.98 ± 0.07 μM (n=22) to 1.42 ± 0.11 μM (n=14) in α5 SNP mice chronically treated with nicotine (p<0.005, Fig. 6C, E). The potentiated DA signals remained high after short-term withdrawal (1.24 ± 0.10 μM, n=17, p<0.05, WD 1-3d vs. SAC), but DA signals were no longer significantly elevated after 8 weeks of withdrawal compared to control levels (1.13 ± 0.26 μM, n=8, p>0.05, WD 8 wk vs. SAC) (Fig. 6C, E).

When we examined the differences in DA release in response to the same treatments across WT, α5 null, and α5 SNP mice, we confirmed significant differences in single-pulse evoked DA release (Fig. 6D). As shown in Figs. 3 and 6, WT mice showed the greatest levels of DA release, while null mice exhibited the lowest levels of DA release. After 8 weeks of treatment with nicotine, followed by withdrawal, probability of DA release was dramatically suppressed in WT mice while probability of DA release was

greatly potentiated in both α5 null and SNP mice. Nicotine-treated WT and α5 null mice were comparable (Fig. 6D), while α5 SNP mice showed increased DA release ($F_{(2,43)}$ = 39.47, p<0.001) during chronic nicotine treatment and during short-term nicotine withdrawal ($F_{(2,54)}$ = 20.13, p<0.001) up to 8 weeks ($F_{(2,41)}$ = 6.46, p<0.005). Therefore, chronic treatment with nicotine, and subsequent nicotine withdrawal, can lead to either suppression or potentiation of single-pulse evoked striatal DA release. This effect is influenced by nAChR function and is regulated by α5* nAChRs.

Modulation of DA release evoked by 20 Hz phasic stimuli in the dorsolateral striatum during chronic nicotine exposure and nicotine withdrawal

Reward-related signaling is associated with changes in DA levels in the brain in response to transitions between tonic and phasic firing. As shown in Fig. 4 and previous studies (Rice & Cragg, 2004; L. Wang et al., 2014; T. Zhang et al., 2009; Zhou et al., 2001), acute application of smoking-related concentrations of nicotine not only suppresses single-pulse evoked DA release, but also modulates DA release elicited by tonic and phasic stimulation in the striatum. The reinforcing properties of nicotine, however, are at least partially related to nicotinic modulation of peak DA release during phasic, but not tonic, firing (Rice & Cragg, 2004). By comparing DA release evoked by bursts of variable lengths, previous studies (L. Zhang, Dong, Doyon, & Dani, 2012; L. Zhang et al., 2009), and studies from other groups (H. Zhang & Sulzer, 2004), demonstrated that maximal DA signals can be reliably generated by 20 Hz phasic stimuli. Furthermore, longer burst firing evokes the greatest DA release in the NAcc of WT mice, during either acute nicotine application or after one day of withdrawal from chronic nicotine.

Here, we explored the role of α5* nAChRs in regulating potential reserve pools of DA reward. This was evaluated by comparing stimulated DA release, elicited by a 20 Hz phasic stimulus, with longer (20 pulses, 20p) to shorter (5 pulses, 5p) trains in the dorsolateral striatum, of WT, α5 null, and α5 SNP mice during long-term nicotine exposure and after short-term (1-3 days) and long-term (8 weeks) withdrawal. Because baseline DA signals evoked by a single pulse were variable, all values were normalized to baseline as ratios to reveal relative changes in DA peak amplitudes evoked by 5p ($[DA]_{5p}/[DA]_{1p}$) and 20p ($[DA]_{20p}/[DA]_{1p}$). We first examined the dynamic alterations in DA release caused by chronic nicotine and by nicotine withdrawal. As shown in Fig. 7A & E, no significant differences between 5p and 20p were detected in WT mice treated with SAC (20p: 1.32 ± 0.08, n=13; 5p: 1.19 ± 0.05, n=14, $p>0.05$) or nicotine (20p: 1.48 ± 0.11, n=14; 5p: 1.37 ± 0.08, n= 13, $p>0.05$), indicating that only limited reserve pools of DA can be accessed by longer phasic firing under control conditions. In addition, more DA was produced by 20p than by 5p in WT mice undergoing both short-term (20p: 2.13 ±0.17, n=20; 5p: 1.55 ± 0.09, n=21, $p<0.01$) and long-term (20p: 2.19 ± 0.22, n=10; 5p: 1.63 ± 0.16, n=10, $p<0.05$) withdrawal from chronic nicotine. These results suggest that the reserve pools of DA are amplified by longer phasic firing in WT mice undergoing nicotine withdrawal. Conversely, in α5 null mice, 20p stimulation always evoked more DA release than shorter, 5p stimulation (Fig. 7B, E) independently of whether mice were treated with SAC (20p: 4.41 ± 0.58, n=13; 5p: 2.24 ± 0.31, n=11, $p<0.01$), nicotine (20p: 1.93 ± 0.28, n=12; 5p: 1.36 ± 0.05, n=13, $p<0.05$) - or whether they were undergoing short-term (20p: 2.02 ± 0.31, n=12; 5p: 1.45 ± 0.09, n=12, $p<0.05$) or long-term nicotine withdrawal (20p: 2.79 ± 0.16, n=22; 5p: 2.02 ± 0.13, n=22). These findings show that, in the absence of α5, longer phasic firing produces greater enhancement of evoked DA release, suggesting that the reserve pools of DA release may not be altered by chronic

nicotine and subsequent nicotine withdrawal. In α5 SNP mice, longer 20p evoked more DA release than shorter 5p did, in groups receiving SAC (20p: 2.18 ± 0.25, n=12; 5p: 1.47 ± 0.08, n=12, $p<0.05$) and those receiving chronic nicotine (20p: 2.07 ± 0.20, n=13; 5p: 1.43 ± 0.06, n=13, $p<0.01$) (Fig. 7C, E). However, the differences in DA release evoked by 20p vs. 5p became undetectable after SNP mice were withdrawn from nicotine measured in the days immediately following withdrawal (WD 1-3 days; 20p: 1.37 ± 0.39, n=9; 5p: 1.33 ± 0.28, n=9, $p>0.05$), up to 8 weeks post nicotine cessation (20p: 1.40 ± 0.23, n=7; 5p: 1.34 ± 0.13, n=7, $p>0.05$). These observations suggest that, in the presence of the rs16969968 polymorphism, mice withdrawn from chronic nicotine may have reduced DA responses to longer phasic firing.

Figure 7D compares the ratios of DA signals evoked by 5p vs. 20p in WT, α5 null, and α5 SNP mice across various experimental conditions. In drug naïve, SAC-treated controls, 20 Hz phasic stimulation with both 5p and 20p produced the greatest increases in DA release in α5 null mice and the lowest increases in DA release in WT mice (5p: $F_{(2,34)}= 9.81$, $p<0.001$; 20p: $F_{(2,35)}=18.50$, $p<0.001$ one way ANOVA). The degree of increase in DA release elicited by phasic stimulation was inversely correlated with the levels of initial baseline DA release probability. The lowest DA release probability by single-pulse observed in α5 null mice (Fig. 3B, D) was associated with greater facilitation of DA release by phasic stimulation with either 5 or 20 pulses (Fig. 7D). The higher DA release probability at baseline observed in WT mice suggests reduced reserve pools available for DA release after phasic stimulation with either 5 or 20 pulses. Such differences were attenuated by chronic nicotine treatment ($F(2,36)=2.38$, $p>0.05$), and persisted during early nicotine withdrawal ($F(2,38)=2.91$, $p=0.06$), although statistically significant differences were apparent after 8 weeks of withdrawal when the effects of 20p

were considered ($F(2,36)=10.52$, $p<0.001$). Overall these results suggest that nicotine exposure and nicotine withdrawal may affect DA reserve pools, and that α5* nAChRs are important mediators of these effects.

Modulation of tonic vs. phasic DA release during chronic nicotine exposure and withdrawal

The differential DA release observed when switching between tonic and phasic frequencies is directly related to reward prediction and reinforcement learning. Acute interruption of nicotinic signaling using newly developed, light-controllable nAChRs has shown that nAChRs greatly influence the firing patterns of VTA DA neurons (Durand-de Cuttoli et al., 2018). Previous work from our group and others has shown that both acute nicotine, and withdrawal from chronic nicotine, regulate DA signals evoked by tonic and phasic activities (Rice & Cragg, 2004; L. Zhang et al., 2012). Here, we focused on the effects of chronic nicotine, and subsequent nicotine withdrawal, on tonic vs. phasic DA release in WT, α5 null, and α5 SNP mice to explore the specific role played by the α5 subunit in the alterations of DA signals that promote nicotine addiction. Given that tonic pulses at 3-4 Hz produced significantly smaller DA signals than single pulses did (Le et al., 2015; L. Zhang et al., 2012; L. Zhang et al., 2009), DA release evoked by 3-4 Hz tonic pulses was difficult to distinguish from background noise in α5 null mice under our experimental conditions due to the low DA release probability shown in Fig. 3B. Thus, we applied very slow, 0.2 Hz tonic pluses to let each single stimulus produce bigger, detectable DA signals (Fig. 8B). As shown in Fig. 8A-C, greater DA release was produced by a 20 Hz phasic stimulus with 5 pulses, which was observed 5 seconds after relatively small DA signals were evoked by 4 0.2-Hz tonic stimuli which reached a pseudo steady state. To examine differences in DA release, we compared the ratios of

phasic-to-slow-tonic DA signals ($[DA]_{5p}/[DA]_{1p}$). In WT mice (Fig.8 A, E), chronic nicotine treatment dramatically decreased the ratios, from 2.44 ± 0.24 (n=18, SAC controls) to 1.67 ± 0.09 (n=14, NIC; $F_{(3,60)} = 3.80$, $p<0.05$). The ratios returned to control levels after short-term (1-3 days;2.16 ± 0.13, n=11) and 8 weeks-long (2.38 ± 0.15, n=21) nicotine withdrawal. In α5 null mice, however, chronic nicotine did not significantly increase the ratios that went from 1.89 ± 0.09 (n=14) at baseline to 2.11 ± 0.12 (n=16) during nicotine treatment. Ratios remained unchanged (2.05 ± 0.10, n=12) shortly after nicotine withdrawal, but were significantly increased to 2.63 ± 0.28 (n=16) after 8 weeks of withdrawal ($F_{(3,54)} = 3.32$, $p<0.05$, ANOVA, Fig. 8B, E). Similar to the results obtained in α5 null mice, the ratios of phasic-to-slow-tonic DA signals were not altered by chronic nicotine or during short-term withdrawal compared to baseline in α5 SNP mice (control: 1.76 ± 0.06, n=13; chronic nicotine1.66 ± 0.10, n=14; WD 1-3 days: 1.54 ± 0.07, n=17). However, contrary to what was seen in the null mice, ratios were significantly decreased to 1.30 ± 0.07 (n=7) after 8 weeks of nicotine withdrawal ($F_{(3,47)} = 4.48$, $p<0.01$,Fig. 8C, E).

Fig. 8D compares ratios of phasic-to-slow-tonic DA signals in WT, α5 null, and α5 SNP mice across the experimental conditions. In the SAC-treated, drug naïve condition, WT mice had significantly greater ratios ($F_{(2,42)} = 4.39$, $p<0.05$,) relative to α5 null and α5 SNP mice, suggesting that both null and SNP mice may experience lower reward signals, generated by a contrast between phasic and tonic DA signals, compared to WT controls. Following chronic nicotine exposure, however, α5 null mice exhibited higher ratios ($F_{(2,41)} = 6.01$, $p<0.01$) compared to WT and SNP mice, indicating that nicotine exposure might enhance reward signals produced by phasic firing. In α5 SNP mice, ratios were unaffected by chronic nicotine exposure, but decreased significantly during

both short-term withdrawal ($F_{(2,37)}$ = 12.74, p<0.001) and up to 8 weeks of withdrawal ($F_{(2,41)}$ = 6.59, p<0.005). These results imply that mice carrying the rs16969968 polymorphism may experience lower levels of reward for an extended period of time following cessation of nicotine exposure. Overall, these results confirm that both chronic nicotine exposure and withdrawal may differently influence an animal's experience of rewarding signals by modulating the function of nAChRs that contain the α5 subunit via distinct mechanisms.

Discussion

In this study, we systematically examined the roles of the α5 subunit in the regulation of dynamic alterations in the function of nAChRs in VTA DA neurons. Further, we found that the α5 subunit plays pivotal roles in modulating DA release evoked either by single-pulse stimulation or by tonic/phasic stimulus trains in the dorsolateral striatum of WT, null, and SNP mice treated with chronic nicotine and following nicotine withdrawal.

Significant regulatory roles of the α5 subunit in controlling nAChR function and DA release in drug naïve controls

It is well-known that the α5 nAChR subunit is an obligate accessory subunit that cannot form functional receptors by itself and requires additional α and β subunits (Berrettini et al., 2008; Ramirez-Latorre et al., 1996; Sciaccaluga et al., 2015). Studies using α5 null mice found that the absence of α5 subunits caused a dramatic loss of nAChR functionality, identified by smaller peak currents recorded in VTA DA neurons (Chatterjee et al., 2013; Morel et al., 2014; Sciaccaluga et al., 2015). Corroborating previous observations, the data from SAC treated naïve controls, presented in Fig. 2, demonstrate that WT mice exhibited the greatest nAChR functionality, evidenced by the

biggest peak currents, while null mice had very limited nAChR functionality with the smallest currents (Fig. 2). Recent developments in genome-wide association studies for nicotine dependence susceptibility genes revealed a strong association of increased risk of nicotine dependence with a single-nucleotide polymorphism (SNP) in which an aspartic acid residue at position of 398 was replaced with asparagine in the α5 subunit (Bierut et al., 2008; Saccone et al., 2007). We re-expressed the SNP in VTA DA neurons of α5 null DAT-Cre mice (Fig. 9), which resulted in a partial recovery of the nicotinic responses. This corroborates very similar observations from previous studies in VTA DA neurons, HEK cell lines, and GH4C1 cells (Morel et al., 2014; Sciaccaluga et al., 2015; Tammimaki et al., 2012). Thus, these data indicate that the responses of nAChRs to nicotine are directly determined by the characteristics of α5 subunit variants, and null mice require much higher concentrations of nicotine to achieve the same dopaminergic responses relative to WT controls (Fowler et al., 2011; Morel et al., 2014). Our results may partially explain why heavy smoking is highly associated with SNPs in CHRNA5 (Lips et al., 2010; Sarginson et al., 2011). The precise mechanisms underlying how the α5 subunit controls nAChR function in the VTA remain unclear. However, it is reported that α4β2-nAChRs are the most commonly expressed nAChRs in VTA DA neurons (Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001; D. Mao, Gallagher, & McGehee, 2011; Yang et al., 2009b), and the α5 subunit plays critical roles in controlling the expression and function of α4* nAChRs in the VTA (Chatterjee et al., 2013). Recent evidence from studies using immunoprecipitation and ligand-binding techniques suggest that α4β2-nAChRs are also highly expressed on both striatal dopaminergic and nondopaminergic terminals (Gotti et al., 2005; Zoli et al., 2002). Indeed, we confirmed that striatal DA signals are predominantly suppressed in brain slices containing the dorsolateral striatum by blockade of β2* nAChRs in WT mice, and in the same brain

slices, in nAChR β2-subunit null mice (L. Zhang et al., 2009). Interestingly, a recent study using mice with deletions of α4, α5, α6, or β3 subunits showed that the α5 nAChR subunit plays an important role in the modulation of DA release by nAChRs containing α4β2 subunits in the dorsal striatum (Exley et al., 2012). However, these roles played by α5 subunit in controlling DA release, especially under the influences of chronic nicotine and nicotine withdrawal, remain poorly understood. In the present study, for the first time, we further demonstrated that the α5 subunit and α5 SNP also proved to play important roles in regulating baseline DA release evoked by single pulse stimuli applied every 120 s with the aid of voltammetry recordings in brain slices containing the dorsolateral striatum of WT, null, and SNP mice. Under the same recording conditions, WT mice exhibited the greatest DA signals, followed by SNP mice, while null mice exhibited the lowest DA signals (Fig. 3). The following 2 mechanisms may underlie the greatly diminished DA release in null and SNP mice: (1) significantly decreased cholinergic signals, identified by much smaller ACh-induced currents (Fig. 2), and (2) a dramatic reduction of nAChR-mediated Ca^{2+} signals (Sciaccaluga et al., 2015; Tammimaki et al., 2012). Confirmation that the activation of nAChRs participates in the modulation of DA release is shown in Fig. 3. DHβE was bath-applied first to block β2* nAChRs. DA signals were dramatically and reversibly suppressed by 20 min application of DHβE (Fig. 4A, B). Furthermore, desensitization of nAChRs by bath-application of 0.5 μM nicotine resulted in similar inhibition of striatal DA release (Fig. 4C, D). The finding that there is overwhelming inhibition of evoked DA release by both DHβE and nicotine corroborates previous studies (Exley et al., 2012; Threlfell et al., 2012; L. Wang et al., 2014; L. Zhang et al., 2009; Zhou et al., 2001), and suggests that nAChRs govern levels of DA release in the dorsolateral striatum. Thus, we conclude that baseline DA release

probability is mainly determined by nAChR function, with the α5 subunit playing a critical regulatory role.

α5 subunit dependent modulation of nAChR function and DA release by chronic nicotine treatment

Chronic nicotine exposure through tobacco smoking may lead to long-term homeostatic regulation of nAChR function, which is the cellular mechanism underlying nicotine addiction. There is considerable evidence that long-term exposure to nicotine results in upregulation of nAChRs in WT rodent brains, indicated by measurements of nAChR binding (Nguyen et al., 2003; Perry et al., 1999), and by studies using electron microscopy (Pakkanen et al., 2005). However, behavioral and *in vivo* electrophysiological studies by Besson et al. showed that upregulation of nAChR function can only be detected in chronic nicotine-treated $\beta2^{-/-}$, but not WT, mice (Besson et al., 2007). A recent study reported that chronic nicotine treatment down-regulates α6β2-nAChRs and up-regulates α4β2-nAChRs in DAergic and optic-tract nuclei using quantitative ligand-binding autoradiography (Marks et al., 2014). The different lines of conflicting evidence from the above studies suggest that chronic nicotine exposure, subunit-dependently, either up-regulates, down-regulates, or does not significantly change nAChR function. In the cortex, however, all the changes in β2/α4 subunit ratios, $(\alpha4)_2(\beta3)_3$ vs. $(\alpha4)_3(\beta3)_2$, elicited by 2-weeks of chronic nicotine returned to basal levels with an average half-life of 2.8 days after nicotine withdrawal (Fasoli et al., 2016). In VTA DA neurons, increased AMPA/NMDA ratios can last at least 3 days after a single injection of nicotine and can last up to 8 days after a treatment of once-daily nicotine injections for 7 days. (Gao et al., 2010). The potential functional alterations of α5* nAChRs caused by long-term chronic nicotine treatment and withdrawal remain largely

unknown. In this study, we extended both chronic nicotine treatment and subsequent nicotine withdrawal up to 8 weeks, and then investigated the influences of chronic nicotine upon nAChR function in VTA DA neurons. We detected significant increases in the amplitude of ACh-induced whole cell currents in null and SNP mice (Fig. 5). Increased functionality of nAChRs in SNP mice mirrored findings in oral keratinocytes that found nicotine stimulates expression of α5* nAChRs (Arredondo, Chernyavsky, Jolkovsky, Pinkerton, & Grando, 2008). Different from both null and SNP mice, chronic nicotine did not show significant effects on ACh-induced whole-cell currents in WT mice, which corroborates previous findings in 2-3 month-old α4YFP mice after 10 days of treatment with nicotine by measuring levels of α4YFP fluorescence (Nashmi et al., 2007). Thus, chronic nicotine results in an α5 subunit-dependent modulation of the function of nAChRs expressed in VTA DA neurons. Normal ACh-induced responses were only partially recovered in null mice, but were almost completely restored in SNP mice to levels comparable to WT mice after chronic nicotine exposure (Fig. 5). The restoration of nAChR function observed in this study could be a key underlying mechanism that contributes to the altered firing of pyramidal neurons in the hypofrontality of α5 SNP schizophrenia models after 14 days of nicotine exposure via mini-pump implantation (Koukouli et al., 2017). Accordingly, restoring normal cholinergic function may be an internal motivation for carriers of the SNP to smoke cigarettes.

Chronic nicotine exposure also significantly influences DA release in the dorsolateral striatum in an α5 subunit-dependent manner. Substantially suppressed single-pulse evoked striatal DA signals were recorded in WT mice after 8 weeks of chronic nicotine treatment (Fig. 6A), which reflects the findings of previous reports (X. A. Perez, Khroyan, McIntosh, & Quik, 2015; X. A. Perez, Ly, McIntosh, & Quik, 2012), indicating that

decreased DA release was detected in the NAcc of both rats and monkeys treated with nicotine in drinking water for 2-6 months. However, the same chronic nicotine treatment resulted in much bigger single-pulse stimulated DA signals in the dorsolateral striatum of null (Fig. 6B) and SNP (Fig. 6C) mice. In particular, SNP mice had the greatest single-pulse evoked basal DA release compared to WT and null mice (Fig. 6D), suggesting that chronic smoking may be more pleasurable for SNP mice. The increases in DA signals may result from both restored nAChR function, shown in Fig. 5, and the corresponding improvement in Ca^{2+} permeability via α5* nAChRs after nicotine exposure (Arredondo et al., 2008). The data from SNP mice further extended our findings from mice to humans, which may explain reported associations between the polymorphism in CHRNA5 and enhanced pleasurable responses to early smoking experiences among current regular smokers (Sherva et al., 2008). Thus, chronic smoking not only restores deficits in cholinergic function, but can also increase DA release, rendering the effects of nicotine even more rewarding for null and SNP mice, and particularly for SNP mice.

Long lasting impact of chronic nicotine upon nAChR function and phasic DA release during extended nicotine withdrawal

Withdrawal from chronic nicotine is believed to trigger powerful negative reinforcement that drives relapse and compulsive tobacco use. Smokers may continue to consume nicotine primarily to alleviate the aversive symptoms of nicotine withdrawal and craving (Tan, Bishop, Lauzon, Sun, & Laviolette, 2009). Therefore, a better understanding of the effects of long-term nicotine withdrawal on nAChR function, and corresponding changes in DA release, is a critical step towards developing more effective therapeutics for smoking cessation. Smoking cessation is a long-term process that has been characterized by 6 stages of progressive changes (Prochaska & Velicer, 1997).

However, most previous studies (Grieder et al., 2012; Natividad, Tejeda, Torres, & O'Dell, 2010; X. Y. Zhang et al., 2012) have only characterized animals that have undergone nicotine withdrawal for hours to days. In the present study, we extended nicotine withdrawal to 8 weeks and systematically investigated the dynamic changes that arose in both the functionality of nAChRs in VTA DA neurons, as well as DA release in dorsolateral striatum. Significantly smaller ACh-induced whole-cell currents were recorded in VTA DA neurons of WT mice undergoing nicotine withdrawal for 1-4 days, 2-4 weeks, or 8 weeks (Fig. 5A), suggesting a long-lasting depression of nAChR function after cessation of long-term nicotine exposure. Nicotine withdrawal also resulted in the same long-lasting reduction of single-pulse stimulated DA signals in WT mice (Fig. 6A). These findings confirm previous observations from our lab and others that nicotine withdrawal substantially lowers baseline DA release in both mice and rats undergoing nicotine withdrawal for 1-10 days (Grieder et al., 2012; L. Zhang et al., 2012). Interestingly, nicotine withdrawal showed distinct effects on nAChR function and DA release in null and SNP mice. The up-regulated nAChR function was sustained during 8 weeks of nicotine withdrawal in null mice (Fig. 5B), but only 1-4 days - then returning to baseline levels - during the same 8 weeks of nicotine withdrawal in SNP mice (Fig. 5C). Corroborating an alteration in nAChR function, under the same nicotine withdrawal conditions, we found that enhanced single-pulse evoked DA signals were maintained at the same significantly higher levels in null mice (Fig. 6B). Meanwhile, DA signals only remained elevated for 1-3 days, returning to baseline levels in null mice (Fig. 6C). SNP mice have the greatest single-pulse induced DA release relative to both WT and null mice during 1-3 days of withdrawal, while no significant difference was detected afterwards between SNP and WT mice (Fig. 6D).

To fully understand the impact of nicotine withdrawal on DA release, we further investigated maximal DA release by applying 20 Hz phasic stimuli, measuring changes in ratios of phasic vs. tonic DA release. SNP mice exhibited greater maximal DA release compared to WT mice after chronic nicotine treatment. Surprisingly, SNP mice exhibited the lowest maximal DA release throughout nicotine withdrawal compared to both WT and null mice (Fig. 7D). In addition, SNP mice had the lowest phasic vs. tonic ratios relative to both WT and null mice (Fig. 8D), which suggests that phasic stimulation produced the lowest efficiency to boost DA release, and failed to generate comparable reward signaling in SNP mice undergoing nicotine withdrawal. Previous studies using *in vivo* microdialysis demonstrated that acute nicotine dramatically increases peak, but not basal, DA release in mice undergoing nicotine withdrawal (L. Zhang et al., 2012). Indeed, nicotine exerts dual effects on striatal DA release, increasing phasic DA release while decreasing tonic DA release (Rice & Cragg, 2004). Therefore, during nicotine withdrawal, it is possible that animals may consume nicotine to temporarily enhance DA release via phasic firing (Grieder et al., 2012). It is reasonable to speculate that SNP mice undergoing nicotine withdrawal may experience the strongest motivation to seek nicotine in order to restore suppressed reward from phasic DA activation that they previously experienced from chronic nicotine exposure.

Summary

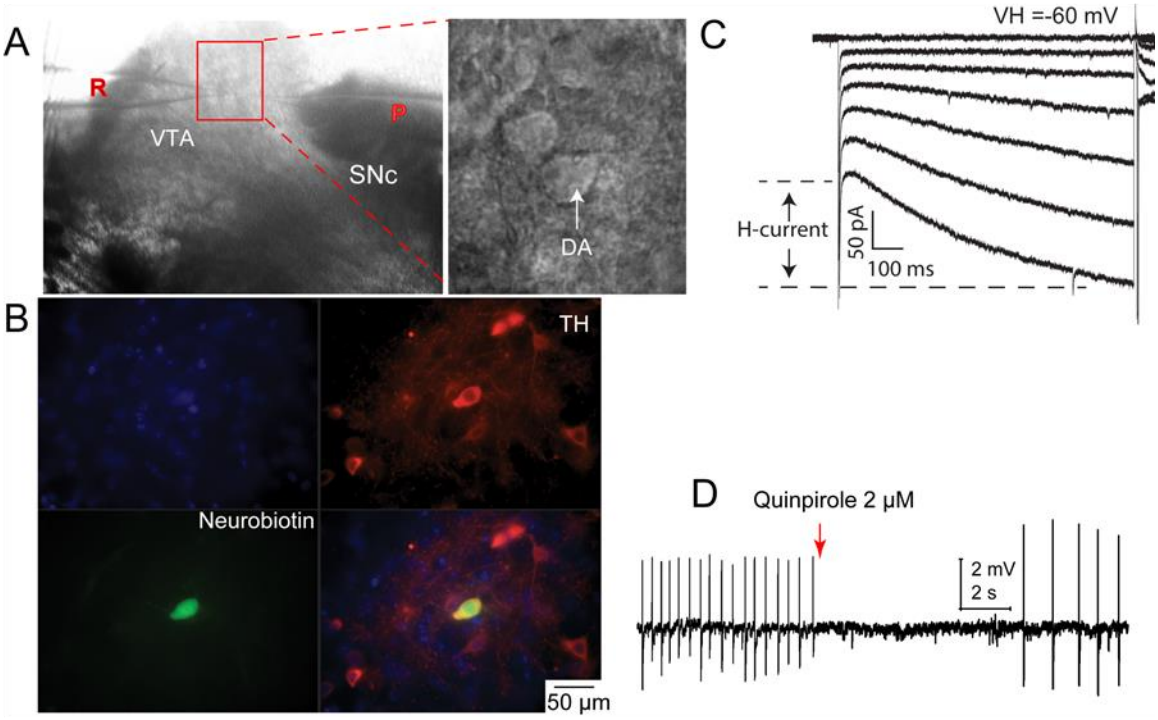
We systematically examined the dynamic functional alterations of nAChRs in VTA DA neurons, and the corresponding DA release in the dorsolateral striatum, induced by 8 weeks of chronic nicotine, as well as 8 weeks of nicotine withdrawal. Overcoming the challenges of performing patch-clamp recordings in 5-7 month-old mice, and employing fast-scan cyclic voltammetry, we worked with α5 null mice, finding that the α5 nAChR

subunit plays important roles in regulating VTA nAChR functionality, as well as striatal DA release. Relative to WT controls, null mice not only displayed limited nAChR function, but exhibited much smaller single-pulse evoked DA release as well. Expression of the human α5 SNP in mice resulted in only a partial recovery of VTA nAChR function and striatal DA release. These data translate our findings from mice to human smokers carrying the α5 SNP, yielding insights regarding the neurobiological mechanisms by which the α5 subunit contributes to both chronic nicotine use and smoking cessation. We further demonstrated that 8 weeks of chronic nicotine exposure significantly enhanced nAChR function and, in turn, dramatically increased single-pulse stimulated DA release in both null and SNP mice. It should be noted that, following chronic nicotine treatment, SNP mice exhibited the greatest single-pulse evoked DA signals compared to both WT and null mice, as well as significantly greater maximal DA release elicited by 20 pulses of 20 Hz phasic stimuli compared to WT controls. However, the same chronic nicotine treatment didn't significantly change nAChR functionality, but substantially suppressed single-pulse induced DA release in WT mice. Accordingly, chronic nicotine can improve nAChR function, enabling carriers of the SNP to experience significantly greater reward-related DAergic responses. These findings suggest that SNP mice respond the most to chronic nicotine exposure, which might elucidate the underlying neurobiological mechanisms that promote heavy smoking in human carriers of the α5 nAChR SNP.

Withdrawal from nicotine, following 8 weeks of chronic nicotine treatment, produced persistent down-regulation of nAChR function in WT mice or up-regulation of nAChR function in null mice. Up-regulated nAChR function in SNP mice persisted for only 1-4 days following withdrawal, however, and returned to basal levels afterwards. Nicotine withdrawal also produced profound effects on DA release. SNP mice exhibited the

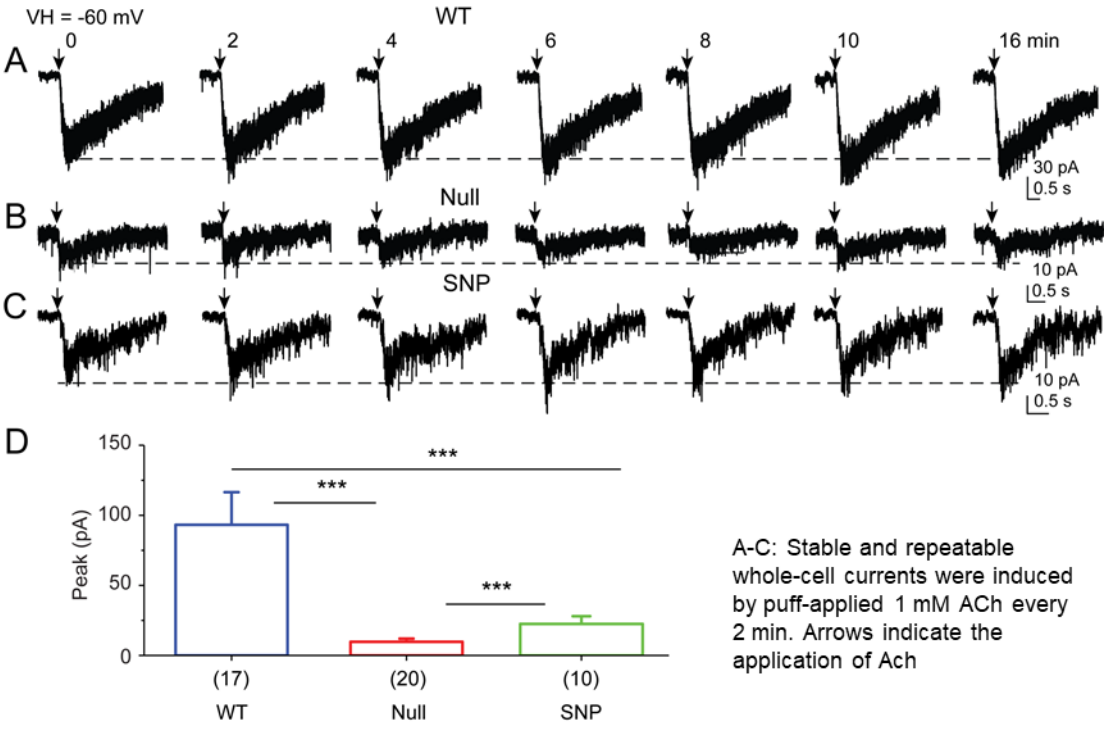
greatest levels of single-pulse evoked DA release for only 1-3 days of nicotine withdrawal, returning to levels comparable to those exhibited by WT. Notably, SNP mice exhibited the lowest maximal DA release, evoked by 20 pulses at 20 Hz, as well as the smallest ratios of phasic/tonic DA signals, during the entire period of 8 weeks of nicotine withdrawal. These results suggest that SNP mice may lose a majority of DA reward signals, and therefore experience withdrawal symptoms of greater severity. These findings elucidate why heavy smokers carrying the SNP may derive greater pleasure from tobacco, and also why they experience stronger cravings during withdrawal.

Fig 1. Identification of VTA DAergic neurons.



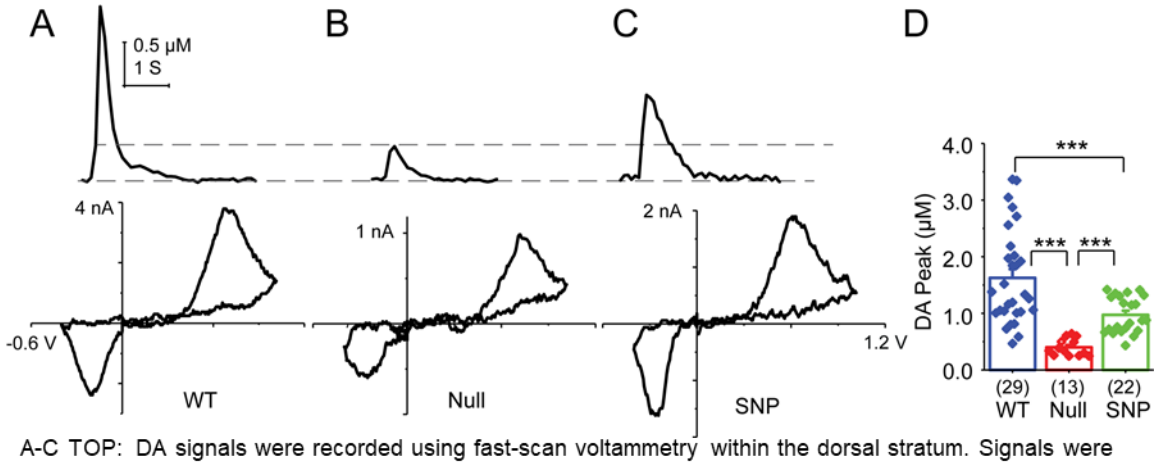
A. R indicates Recording electrode; P electrode used for puff-application.
B. Recorded putative VTA DA neurons was labeled by neurobiotin via recording electrode
C. Cell was clamed at -60 mV, Ih was induced by hyperpolarizing voltage steps (from −60 mV to −120 mV with −10 mV steps)
D. Spontaneous action potential firing recorded via tight-seal patch-clamp recording. Firing was stopped by puff-applied quinpirole (2 μM), a selective D2-receptor agonist.

Fig 2. ACh-induced currents in VTA DA neurons of WT, α5 null, and SNP mice



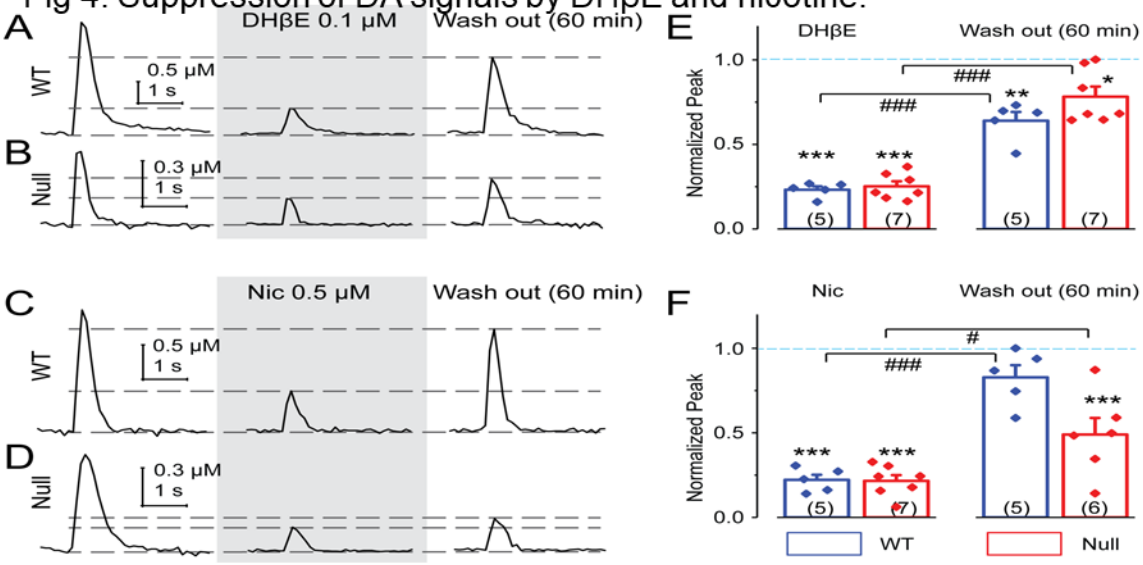
A-C: Stable and repeatable whole-cell currents were induced by puff-applied 1 mM ACh every 2 min. Arrows indicate the application of Ach

Fig 3. DA signals elicited by a single pulse in dorsolateral striatum in WT, null, and SNP mice.



A-C TOP: DA signals were recorded using fast-scan voltammetry within the dorsal stratum. Signals were evoked by electric stimulations via a bipolar tungsten electrode (Stereotrode Tungsten; WPI) with two poles spaced about 150 μm apart. The tip of the carbon-fiber recording electrode was about 150 μm away from each of the two poles of the stimulating electrode.
Bottom: the corresponding voltammograms

Fig 4. Suppression of DA signals by DHβE and nicotine.



DA signals were reversibly suppressed by 15-18 min of bath application of either DHβE (0.1 μM, A-B) or Nic (0.5 μM, C-D) and were recovered after 60 min of wash out.

E: Dashed lines indicate the control (pre-drug application) levels of the DA signals as 100%.

* $p<0.05$, ** $p<0.005$, *** $p<0.001$ *t*-test compared to the corresponding baselines; # $p<0.05$, ### $p<0.001$ *t*-test comparison between indicated groups

Fig 5. Modulation of nAChR function by chronic nicotine (in drinking water) in VTA DA neurons in wild-type (WT), null, and SNP mice.

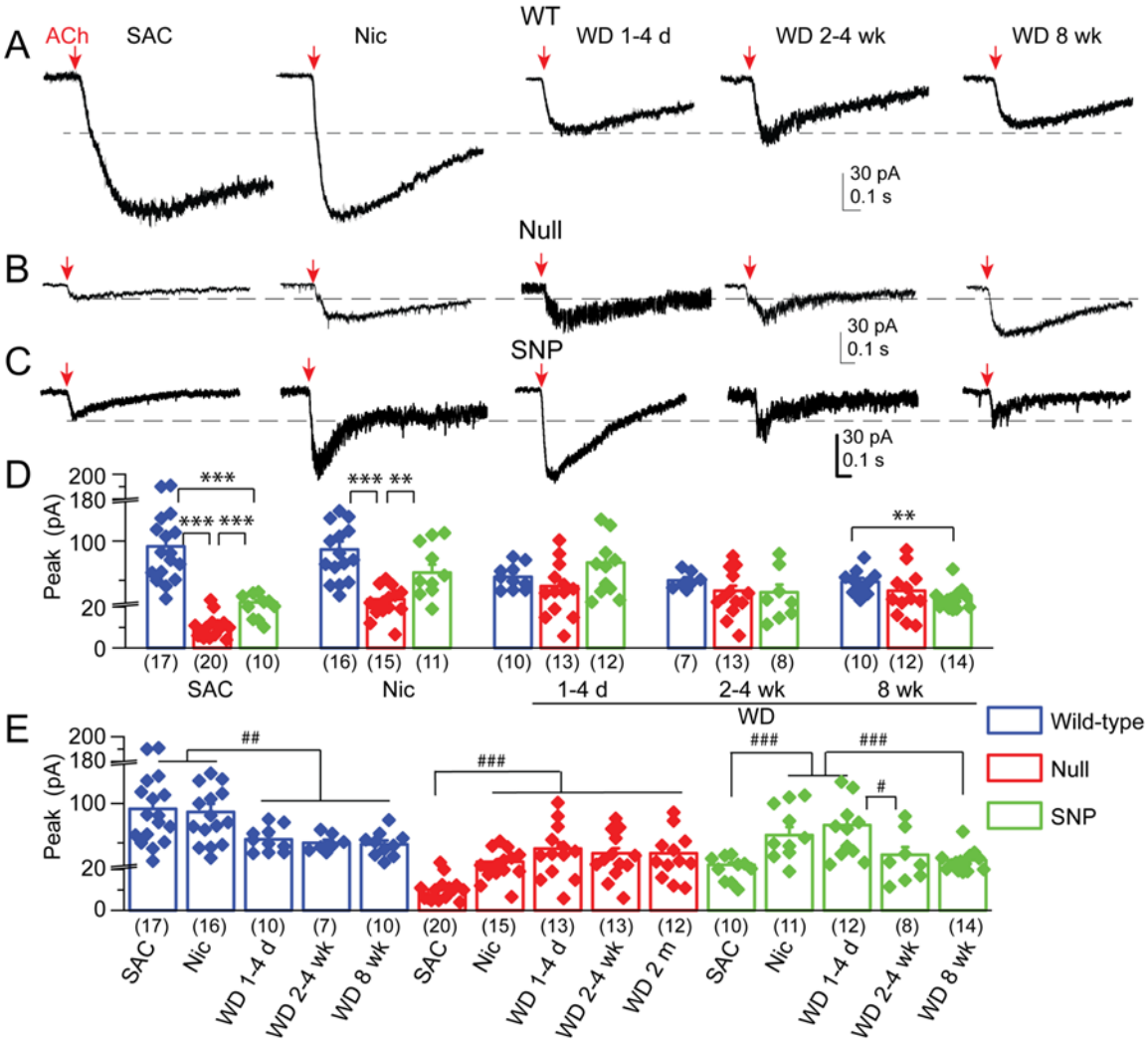


Fig 6. Single-pulse evoked DA release in dorsolateral striatum before (SAC), during (Nic), and after (WD) chronic nicotine in WT, Null, and SNP mice.

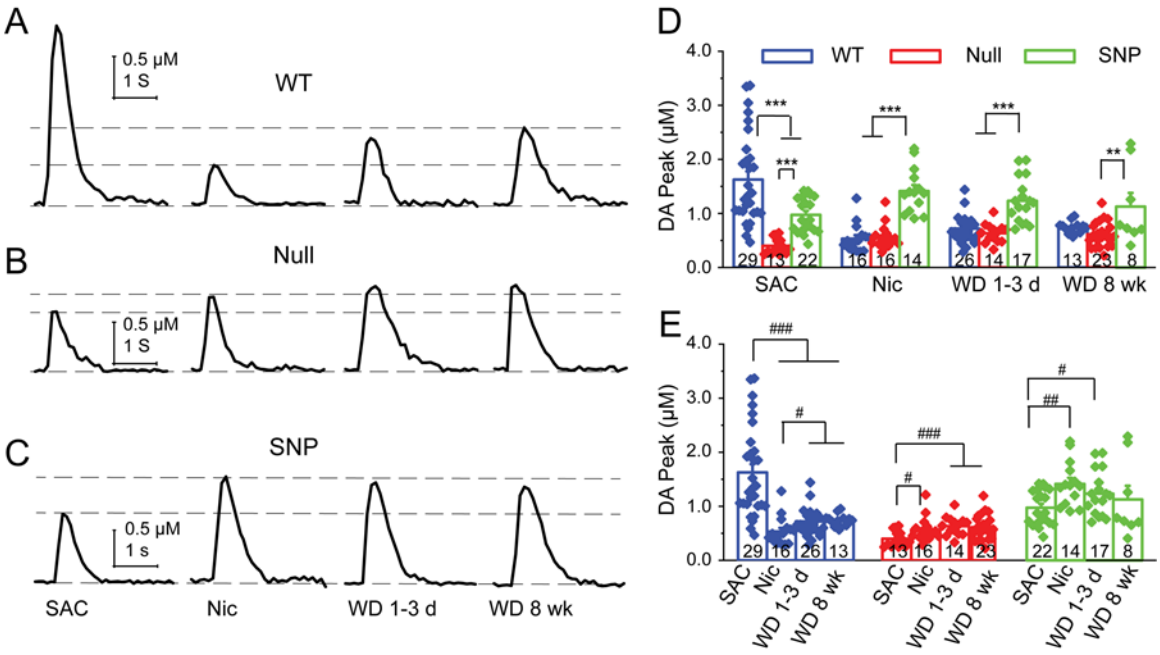
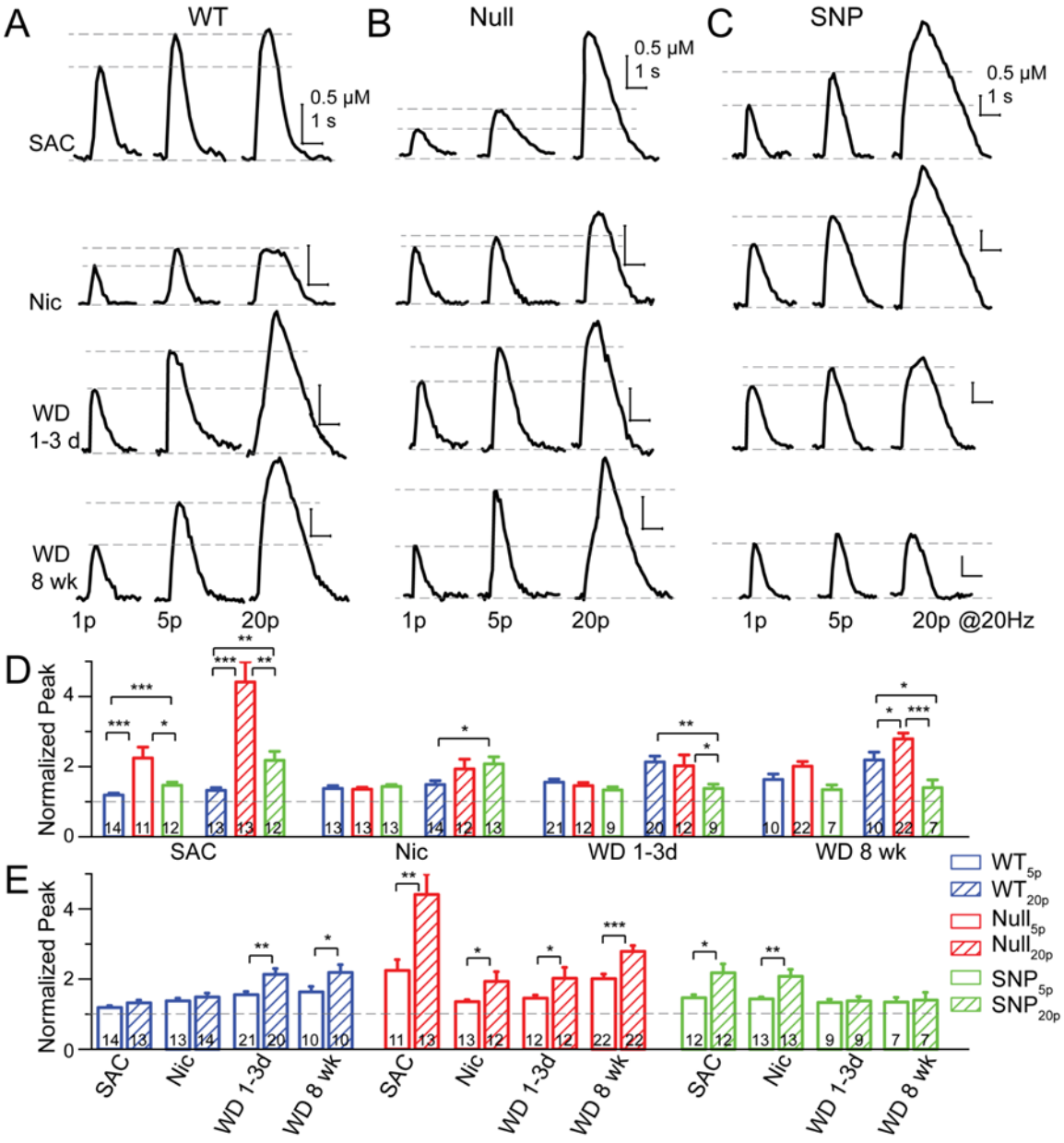


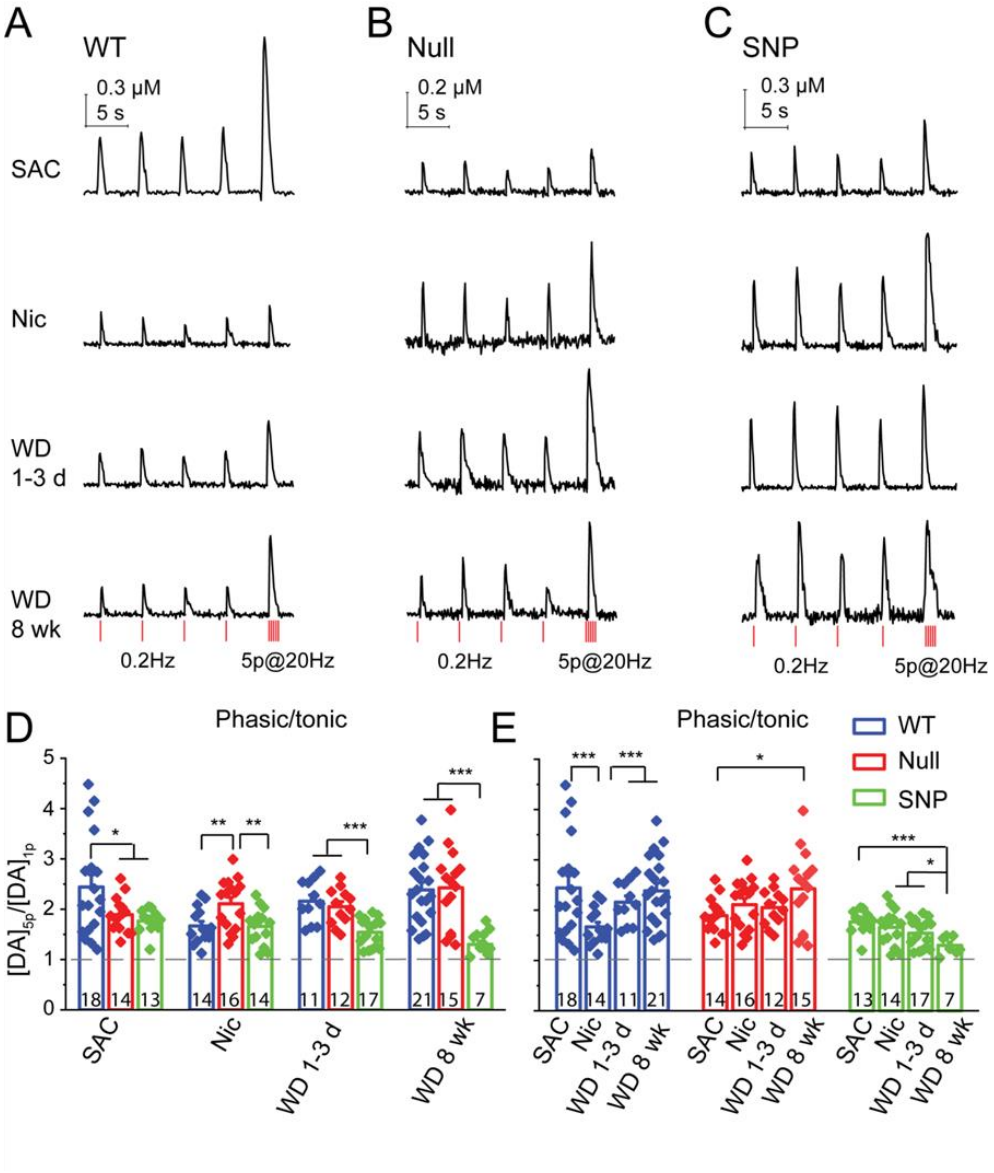
Fig 7. DA signals elicited by a single pulse (1p) or by 5p or 20p @ 20 Hz before (SAC) , during (Nic), and after (WD) chronic nicotine in WT, null, and SNP mice.



A-C: DA signals were evoked by 1p (left), 5p (middle), and 20p (right) @ 20 Hz recorded in the dorsolateral striatum in WT (A), null (B), and SNP (C) mice chronically treated with SAC, Nic, nicotine withdrawal 1-3 d (WD 1-3 d), or 8 weeks (WD 8 wk)

D-E: All values were normalized to the corresponding control levels ($[DA]_{1p}$) as 100% indicated by the dashed line

Fig 8. Ratio of the phasic /slow-tonic DA signals within the dorsolateral striatum before (SAC) , during (Nic), and after (WD) chronic nicotine in WT, null, and SNP mice.



A-C: DA signals were evoked by a slow tonic stimulus (4 pluses @ 0.2 Hz after achieving pseudo steady state) followed by a phasic stimulation (5 pulses @ 20 Hz)

Fig 9. Expression of α5(SNP) nAChR subunit in catecholaminergic neurons of the ventral tegmental area in transgenic mice lacking endogenous expression of the subunit.

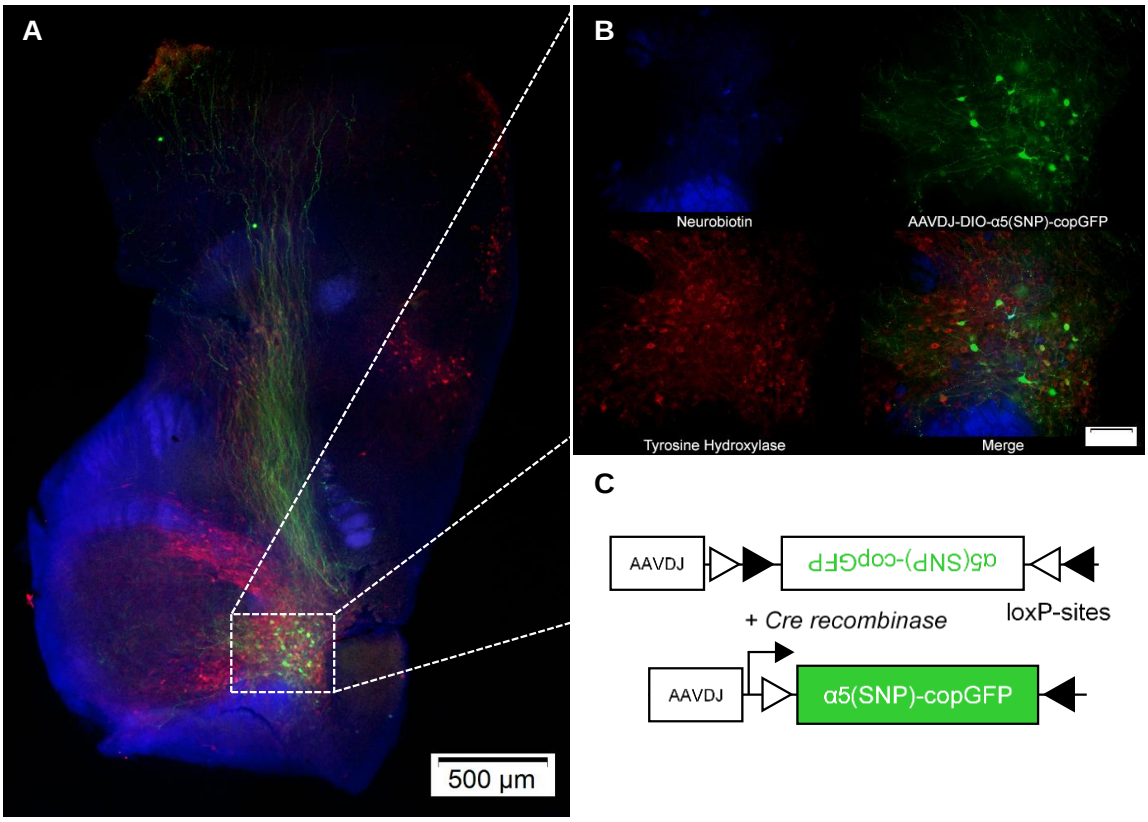


Figure 9. Expression of α5(SNP) nAChR subunit in catecholaminergic neurons of the ventral tegmental area in transgenic mice lacking endogenous expression of the subunit. Transgenic mice lacking expression of the α5 nAChR subunit, expressing Cre recombinase in catecholaminergic neurons (TH-Cre), were infused with a virus (AAVDJ-DIO-α5SNP-copGFP) driving expression of a short nucleotide polymorphism of the receptor subunit into the VTA. Successful expression was observed with some sub-anatomical selectivity.

α5-containing nAChRs Within the Interpeduncular Nucleus Influence the Affective and Physical Symptoms of Alcohol Withdrawal

Erika Perez, Ian McLaughlin, Mariella De Biasi

Contributions:

Erika Perez conducted behavioral experiments

Ian McLaughlin performed virus infusions and collected the immunohistochemistry and microscopy data

Introduction

Alcohol abuse and dependence can be defined as a chronic relapsing disorder (Heilig & Egli, 2006; McLellan, Lewis, O'Brien, & Kleber, 2000). Individuals proceed through repetitive rounds of intoxication followed by cessation, and the resulting emergence of withdrawal symptoms produces cravings and promotes relapse (Heilig & Egli, 2006; McLellan et al., 2000). Understanding the mechanisms responsible for the affective and physical symptoms that emerge after alcohol withdrawal may guide the development of new therapeutics for the treatment of alcoholism. Given the high rate of heavy smoking and nicotine dependence among alcoholics, modulation of the cholinergic system is a possible target for cessation therapies.

Currently, varenicline, a partial nAChR agonist approved by the FDA as a smoking cessation agent, is being evaluated as possible therapeutic for alcohol cessation. Varenicline was initially identified as a possible alcohol cessation aid in laboratory settings by observing smokers who were also heavy drinkers (McKee et al., 2009). Subjects treated with varenicline reported significant reductions in the pleasant subjective effects of alcohol accompanied by decreased cravings and alcohol intake. A more recent study also reported that varenicline can improve smoking abstinence in those with alcohol abuse or dependence while also reducing the number of alcoholic drinks per drinking day in this population of smokers (Hurt et al., 2018). Additional studies focused on alcohol dependent individuals undergoing cessation treatment also observed reduced drinking behavior after varenicline (Fucito et al., 2011; Litten et al., 2013; Mitchell, Teague, Kayser, Bartlett, & Fields, 2012; Plebani et al., 2013). Overall, these studies support the conclusion that varenicline treatment significantly reduces both alcohol cravings and heavy alcohol consumption independently of smoking behavior.

Interestingly, varenicline seemed to have no effect on the total number of alcohol abstinent days. Polymorphisms in CHRNA5, which encodes the α5 nAChR subunit have also been associated with the intensity of alcohol response, age of initiation, and risk of alcohol dependence in humans (Berrettini et al., 2008; Breitling et al., 2009; Choquet et al., 2013; Grucza et al., 2010; Hallfors et al., 2013; Joslyn et al., 2008; J. C. Wang et al., 2009).

We recently reported that deletion of the α5 nAChR subunit in mice enhances ethanol-induced hypothermia, hypnosis, and an anxiolytic-like response in comparison to wild-type controls (Dawson et al., 2018). The α5 KO mice showed reduced conditioned place preference for ethanol, suggesting that the rewarding properties of ethanol are decreased in the mutant mice. Interestingly, Chrna5 gene deletion had no effect on basal ethanol drinking behavior, or ethanol metabolism, but did decrease ethanol intake in the drinking in the dark (DID) paradigm following restraint stress. Another study also showed that rats expressing the non-synonymous CHRNA5 variant rs16969968, which has important consequences on smoking behavior in humans, consume more alcohol, and exhibit increased relapse to alcohol seeking after abstinence (Besson, Forget, Correia, Blanco, & Maskos, 2019).

Several symptoms of alcohol cessation are similar to those reported for nicotine, suggesting shared neurocircuitry (Hughes, Higgins, & Bickel, 1994). Our lab has demonstrated that continued use of nicotine attenuates physical symptoms in mice experiencing alcohol withdrawal after receiving chronic alcohol and nicotine co-treatment, mimicking pharmacological activity in alcoholics who also smoke (E. Perez et al., 2015a). In addition, we were able to precipitate somatic signs of withdrawal by antagonism of nicotinic acetylcholine receptors (nAChRs) in mice treated with either

ethanol or co-treated with both ethanol and nicotine. Pre-clinical research using various nAChR subunit knockout mice has enabled the identification of specific roles for several receptor subunits. For example, β2, α4, and α6 nAChR subunit knockout mice appear insensitive to the rewarding properties of nicotine (Brunzell et al., 2006; Picciotto et al., 1998; Pons et al., 2008). In contrast, β4 and α5 knockout mice exhibit attenuated physical symptoms of nicotine withdrawal and are tolerant to the aversive effects of nicotine (Salas, Cook, Bassetto, & De Biasi, 2004; Salas, Orr-Urtreger, et al., 2003; Salas, Pieri, & De Biasi, 2004; Salas et al., 2009).

Our previous studies have established a role for the cholinergic system in the physical symptoms of alcohol abstinence after either alcohol or alcohol co-administered with nicotine. However, the alcohol withdrawal syndrome is associated with a variety of affective symptoms that are longer-lived and are thought to be critical factors that promote relapse, such as anxiety and depression (K. T. Brady & Lydiard, 1993; R. W. Carlson et al., 2012; Hall & Zador, 1997; Heilig, Egli, Crabbe, & Becker, 2010; Hershon, 1977; McKeon, Frye, & Delanty, 2008; Saitz, 1998). The first objective of this study was to investigate the role of the cholinergic system in the affective manifestations of alcohol withdrawal. Given the potential influence of CHRNA5 polymorphisms on alcohol abuse, a second goal of our experiments was to determine whether and how α5* nAChRs modulate affective and physical symptoms of alcohol withdrawal.

Methods

Animals.

Experiments were conducted with four-month-old, male and female, C57BL/6J mice or α5 null mice and their wild-type littermates. α5 mice were backcrossed into a C57BL/6J background for a minimum of 10 generations (N10). Mice were weaned at postnatal day

21, separated into same sex littermates, and housed in cages containing a maximum of five animals. Genotyping was performed as previously described (Salas, Orr-Urtreger, et al., 2003). Mice had *ad libitum* access to water and food pellets (Labdiet 5001, PMI®, Brentwood, MO). Rooms were maintained on a 12-h light/dark cycle. Investigators were blind to the genotype of each animal until the end of the experiment. All procedures were approved by the Baylor College of Medicine and The University of Pennsylvania Animal Care and Use Committees, and followed the guidelines for animal intramural research from the National Institutes of Health.

Chronic alcohol treatment.

Mice were treated with either daily ethanol injections or a liquid ethanol diet. Both methods have been used to induce withdrawal-associated symptoms 24 hours after ethanol cessation. For the injection delivery method, mice received a daily injection of saline or 2 g/kg ethanol for a minimum of 9 days. 9 mg/kg of 4-methylpyrazole (4MP, Sigma-Aldrich, St. Luis, MO), an alcohol dehydrogenase inhibitor, was added to both the control and ethanol solutions. All testing was performed during the light phase of the cycle, 24 hours after the last injection. Mice treated with the liquid ethanol diet were single housed with a nestlet to mitigate the effects of the stress associated with single housing. During the first week of treatment, mice received the control liquid diet, which is composed of the chocolate flavored, high protein nutritional drink, Boost® (Nestlé Health Science, Lausanne, Switzerland), supplemented with 3 g/L vitamin mixture (MP Biomedicals, LLC, Solon, OH) and 5 g/L mineral mix (ICN Biomedicals, Inc., Aurora, OH) (Gilpin, Misra, & Koob, 2008; Verleye, Heulard, & Gillardin, 2009). Afterwards, mice were randomly assigned to receive a 4% (vol/vol) ethanol liquid diet or control diets containing an isocaloric equivalent amount of sucrose. Diets were prepared and changed daily, two

hours into the dark phase of the light cycle. All testing was performed during the dark phase of the light cycle. Spontaneous withdrawal was triggered by replacing the ethanol diet with control diet 24 hours before testing. To analyze multiple behaviors, the ethanol diet was replaced immediately after testing and mice had free access to the diet for a minimum of 48 hours before a subsequent withdrawal session was induced. Precipitated withdrawal was induced by giving mice a 1 mg/kg intraperitoneal (IP) injection of mecamylamine 3 hours after a fresh ethanol diet was provided.

Open field arena (OFA).

Anxiety-associated behavior was measured during spontaneous and precipitated ethanol withdrawal. Briefly, mice were placed in a clear Plexiglas arena (40 x 40 x 40 cm), and locomotor activity was measured for 30 min. The computer-operated system, ANY-maze (Stoelting, Wood Dale Il) was used to monitor the total distance travelled within the apparatus. The center ratio, i.e. the distance travelled in the center of the arena/total distance traveled, was used as a measure for anxiety (Bouwknecht & Paylor, 2008; Gangitano et al., 2009; Salas, Pieri, Fung, Dani, & De Biasi, 2003).

Marble burying test (MBT).

Ethanol withdrawal-induced compulsive-like behavior was measured using the MBT. Mice were placed in a cage containing a depth of 5 cm of bedding and 20 marbles evenly spaced throughout the cage for 30 min. To be counted as buried, a minimum of 2/3 of each marble was covered in bedding (S. Umathe, Bhutada, Dixit, & Shende, 2008). The total number of marbles buried was used as a measure of compulsive-like behavior.

Physical signs of ethanol withdrawal.

After chronic alcohol treatment, physical signs of withdrawal were measured during either precipitated or spontaneous ethanol withdrawal. Mice were placed into a home cage and observed for 20 minutes. The following signs were recorded and their sums used for comparisons: shaking, scratching, grooming, paw tremors, chewing, ptosis, vocalizations, tail rattling, cage scratching, and writhing behaviors (Salas, Pieri, et al., 2004; Salas et al., 2009).

Ethanol dose-response for locomotor activity.

Mice were tested for changes in locomotor behavior after an acute administration of ethanol in the OFA. On the first day, mice received an IP injection of saline and were placed in the OFA for 30 min. Over the course of five more days, mice received a single injection of ethanol (0.1, 0.5, 1, 2, 3 mg/kg) or saline before being placed into the OFA. Ethanol was administered at increasing doses with a minimum of 24 hours between testing. All injections, including controls, contained 9 mg/kg of the alcohol dehydrogenase inhibitor 4MP. Locomotor behavior was normalized to PBS (day 1) for each dose.

Construction of mouse α5 over-expression plasmids for lentiviral & adeno-associated viral vectors and synthesis of lentiviral particles.

The full-length mouse α5 cDNA (NM_176844.3) was amplified using primers to incorporate the NotI restriction site on either side of the coding region. The cDNA was then inserted into pLL3.7 plasmid (also prepared with NotI digestion) such that it is expressed by the elongation factor 1 alpha (EF-1) AAV-DJ (Grimm et al., 2008) promoter. An internal ribosomal entry site (IRES) is located after the α5 stop codon followed by eGFP/copGFP as a reporter gene. The production of recombinant lentiviral particles was done using the methods of Dr. Kazuhiro Oka (Associate Professor, Department of Molecular and Cellular Biology, Baylor College of Medicine), and AAV

particles by Marion Scott (Department of Psychiatry, University of Pennsylvania). The particles are created through a triple transfection of psPAX2 (a packaging plasmid), pMD2.G (an envelope plasmid, which has the vesiculo-stomatitis virus G-protein), a gift of Dr. Didier Trono (École Polytechnique Fédérale de Lausanne, Switzerland), and α5 over-expression plasmid (or empty vector plasmid consisting of only eGFP/copGFP) into 293T cells using the calcium phosphate method as described previously (Lai et al., 2012). The culture medium was exchanged after an initial 6-hour incubation. Culture medium containing lentiviral particles was collected after 48 h incubation, and was filtered through a 0.45 μm pore size cellulose acetate filter. Lentiviral vectors were concentrated using sucrose gradient ultracentrifugation. Briefly, viral particle containing media is transferred to Polyclear centrifuge tubes and underlaid with 20% sucrose/DMEM. The tubes are then spun at 25000 RPM at 4°C for 2 hours in a Beckman SW 40Ti Rotor. The supernatant is aspirated and the pellet is dissolved into DMEM. Aliquots of 10 – 25 μL are transferred to cryotubes to be stored at -80°C until use. Genomic integration and infectious unit titer of the lentiviral particles was determined. HEK 293T cells were split at a density of$1X10^5$ cells per well with LV particles diluted 1:10. After 48 hours, genomic DNA is harvested (Qiagen DNA Easy Kit) and analyzed with Q-PCR targeting the viral WPRE component

Surgical Procedures.

Mice were anesthetized using isoflurane, then treated with bupivacaine, before being stereotactically injected with lentiviral or AAV vectors expressing the α5 nAChR subunit or GFP controls. The following coordinates (Paxinos & Franklin, 2004) relative to bregma were used for cannula placement: MHb, anterior/posterior (AP) −1.70 mm, medial/lateral (ML) ± 0.25 mm, dorsal/ventral (DV) −2.5 mm; IPN, 20° angle, AP −3.48 mm, ML + 0.17

mm, DV −4.84 mm. A Nanoject or KD Scientific Syringe Pump was used to deliver 0.20 - 0.5 µL of virus at a rate of 0.01 µL - 0.15 µL/min. 2-5 minutes after infusion, the glass pipette or syringe was removed, the hole in the skull was sealed with bone wax, and sutures were placed to seal the incision. Mice recovered for a minimum of 2 weeks before starting ethanol treatment.

Data analysis and statistics.

Data were examined by ANOVA or ANOVA with repeated measures, when appropriate. The Newman-Keuls post-hoc test was used for specific comparisons.

Results

Mecamylamine precipitates ethanol withdrawal-related behaviors.

Our lab has previously shown that nAChRs play a role in the somatic manifestations of withdrawal from ethanol treatment. In that study, wild-type, C57Bl/6J mice were treated with chronic ethanol injections for 9 days before ethanol withdrawal was precipitated using the non-selective nAChR antagonist, mecamylamine. Physical symptoms are one of many behavioral manifestations that occur after ethanol cessation (Kurokawa, Mizuno, & Ohkuma, 2013; Majchrowicz, 1975). Therefore, to test whether mechanisms mediated by nAChRs influence the affective manifestations of withdrawal, we examined withdrawal behaviors in mice exposed to a liquid ethanol diet.

Mice were placed on a 4% ethanol or control liquid diet for 6 weeks. On testing days, 3 hours after the liquid diet was refreshed, mice received an IP injection of 1 mg/kg mecamylamine, and 10 min later mice were monitored for changes in behavior. We previously determined that this mecamylamine dose is sufficient to precipitate withdrawal symptoms and higher mecamylamine doses produce sedation in ethanol-treated mice. Mice were tested in the open field arena (OFA, Fig 6.1A, B), marble burying test (MBT,

Fig 6.1C) or for changes in somatic signs (Fig 6.1D). The total distance traveled in the OFA arena was used as a measure of locomotion and it was unaffected by mecamylamine injections (Fig 6.1A, $F_{injection}[1,23]=0.465$, $p=0.502$; $F_{diet\ x\ injection}[1,23]=0.110$, $p=0.743$). However, there was a significant decrease in the total distance traveled by ethanol treated animals, regardless of acute injection. This is highlighted by a significant main effect of the liquid diet on locomotion ($F_{Diet}[1,23]=10.418$, $p=0.004$). Since the center ratio (measure of anxiety-associated behavior; distance traveled in the center/total distance) is dependent on overall locomotion, we chose to analyze control and ethanol treated groups separately. In control-treated mice, mecamylamine had no effect on the center ratio (Fig 6.1B; Student t test, $p=0.451$). In contrast, mecamylamine produced a decrease in the center ratio of ethanol-treated mice, which did not meet statistical significance (Student t test, 0.058).

Data from our lab and others (S. Umathe et al., 2008) show that ethanol withdrawal induces an increase in compulsive-like behavior when measured in the MBT during spontaneous ethanol withdrawal. Mecamylamine injections in control diet-treated animals had no effect on marble burying behavior. However, in ethanol treated mice, mecamylamine injections led to a significant increase in the number of marbles buried, suggesting that a nAChR mechanism influences the manifestation of compulsive-like behavior during ethanol withdrawal. Statistical analysis revealed a significant interaction between ethanol treatment and mecamylmine injections ($F_{Diet\ x\ injection}[1,24]=12.765$, $p=0.002$).

In our previous study, we were able to precipitate somatic signs in mice treated with ethanol injections. However, we wanted to replicate this result using a more chronic ethanol treatment. Similar to what was previously observed, a 1mg/kg

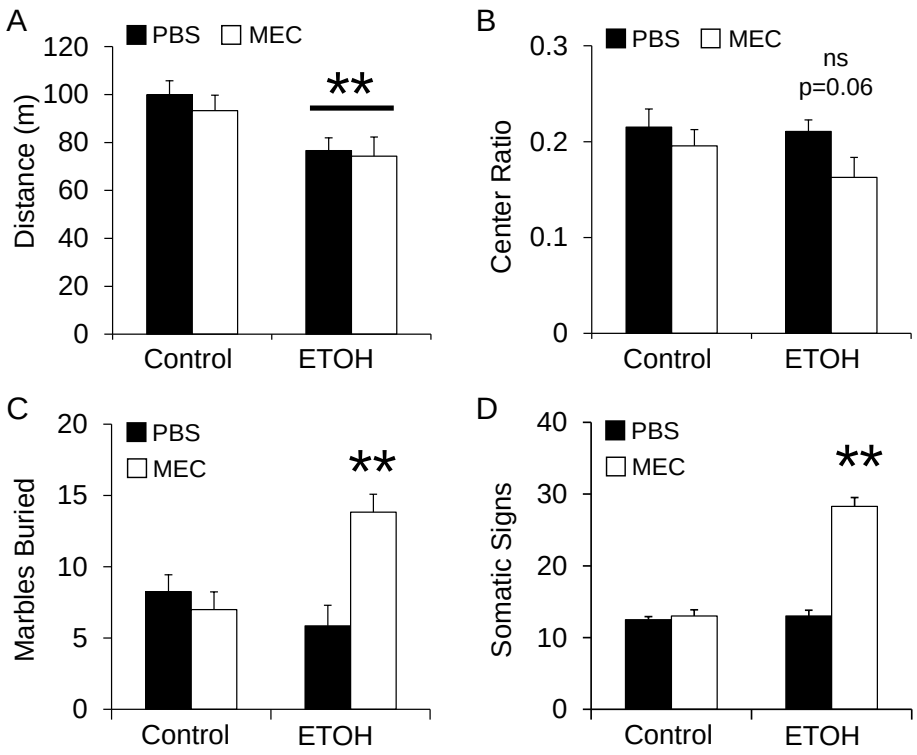


Figure 6.1: Mecamylamine can precipitate affective and physical signs of ethanol withdrawal. C57BL/6J mice were treated with either control or ethanol-containing liquid diet for six weeks. Mice had access to diet until testing. A. Mecamylamine injection (white bars) did not have any effect on the total distance traveled within the open field arena. B. nAChR antagonism did produce a trend towards increased anxiety-like behavior in mice consuming the ethanol diet. C. When tested in the marble burying test, mecamylamine-treated mice buried more marbles than control-injected (black bars) and control diet fed mice. D. Increases in the total number of somatic signs were only observed in ethanol-fed mice receiving mecamylamine injections. Ns for all figures are 8-6 mice for each experimental group. **p<0.01 compared to control-diet and control-injected mice.

mecamylamine injection is sufficient to precipitate somatic signs during withdrawal in mice treated with ethanol liquid diet (Fig 6.1D, $F_{\text{Diet x Injection}}[1,23]=60.202$, $p<0.001$). The number of precipitated signs in liquid ethanol diet-treated mice was similar to what is induced by mecamlyamine in mice treated with ethanol injections or chronic nicotine (Salas, Pieri, et al., 2004; Salas et al., 2009)). Given that nAChR antagonism can precipitate somatic signs in ethanol treated mice, regardless of the type of treatment, these data suggest that nAChRs modulate the physical signs of ethanol withdrawal.

α5 null mice show a normal dose-response to acute ethanol injections.

Since α5 null mice and their wild-type littermates were chronically treated with ethanol injections containing the alcohol dehydrogenase inhibitor, 4MP, it was important to establish that α5 null mice did not differ from their wild-type littermates in their responses to the acute effects of ethanol. A dose response curve was measured by injecting mice with increasing concentrations of ethanol (0, 0.1, 0.5, 1, 2, or 3 mg/kg) + 9 mg/kg 4MP before being placed in the OFA over the course of six days. Low doses of ethanol produced small changes in locomotor behavior in both α5 null and wild-type mice. Larger doses (2, 3 mg/kg) of ethanol significantly reduced locomotor behavior in all mice. Overall, there was no significant interaction between genotype and ethanol dose (Fig 6.2, $F_{geno \times ethanol}[5,55]=0.470$, $p=0.797$) or main effect of genotype ($F_{geno}[1,11]=0.017$, $P=0.900$) on locomotor behavior after acute ethanol injections.

A short-term ethanol treatment suggests a role for α5 nAChRs in ethanol withdrawal.

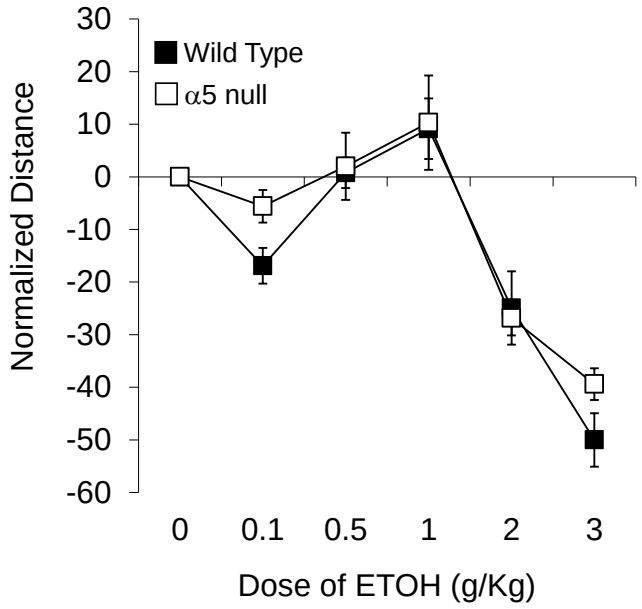


Figure 6.2: α5 null mice do not differ from wild-type littermate in acute effects of ethanol. α5 null mice and their wild-type littermates were treated with various doses of ethanol (0, 0.1, 0.5, 1, 2 and 3 g/kg) + 9mg/kg 4MP before being placed in the open field arena. Distance traveled for each mouse was normalized to the distance traveled after saline control injection. Although ethanol affected locomotor activity, there was no difference overall between the two genotypes. n = 6 for wild-type, 7 for α5 null mice.

Alcohol withdrawal produces a wide range of affective and physical symptoms. Since mecamylamine administration can precipitate various symptoms of ethanol withdrawal, we tested the hypothesis that α5* nAChRs influence the manifestations of ethanol withdrawal-induced behaviors. α5 null mice have previously been shown to be resistant to the physical symptoms of nicotine withdrawal (Salas et al., 2009). We also showed that neuronal structures that express the α5 nAChR subunit (the MHb-IPN axis) are important modulators of somatic signs during nicotine and ethanol cessation (Salas et al., 2009). Therefore, α5 null mice and their wild-type littermates were treated with ethanol injections to evaluate the role of this nAChR subunit in ethanol withdrawal.

Since anxiety-associated behavior is cited as a common symptom of ethanol withdrawal, and mecamylamine injections produced a strong trend towards increased anxiety-associated behavior (Fig 6.1B), mice were tested in the OFA for spontaneous withdrawal 24 hours after the last ethanol injection. No significant differences in the total distance traveled by mice treated with either control or ethanol solutions - of either genotype - was observed, suggesting that locomotor behavior was not affected by ethanol withdrawal (Fig 6.3A; $F_{\text{withdrawal x genotype}}[1,35]= 1.21$, $p=0.65$). Unlike the total distance traveled, the center ratio (distance traveled in the center/total distance traveled) for ethanol withdrawal was significantly different relative to control-treated mice in a genotype-dependent manner (Fig 6.3B; $F_{\text{withdrawal x genotype}}[1,35]= 4.24$, $p=0.047$). Ethanol withdrawal induced a significant increase in anxiety-associated behavior in wild-type

mice only, suggesting that α5* nAChRs play a role in regulating the manifestation of anxiety during ethanol withdrawal.

In addition to the OFA, mice were assessed with the MBT for changes in compulsive-like behavior during ethanol withdrawal. Ethanol-treated wild-type mice buried significantly more marbles than control-treated mice. Unlike their wild-type littermates during ethanol withdrawal, α5 null mice did not bury more marbles (Fig 6.3C, $F_{withdrawal\ x\ genotype}[1,26]=9.688$, $p=0.004$), suggesting that α5*nAChRs are important for compulsive-like behavior after ethanol cessation.

α5* nAChRs have been shown to play a role in somatic signs during nicotine withdrawal. Therefore, we tested the hypothesis that they are also important for physical signs of ethanol withdrawal. Significant increases in somatic signs during ethanol withdrawal were only observed in wild-type mice (Fig 6.3D; $F_{withdrawal\ x\ genotype}[1,38]=38.367$, $p<0.001$). Since there was no effect of ethanol withdrawal in α5 null mice, α5* nAChRs also likely play a role in the manifestation of physical signs during ethanol cessation.

α5 null mice treated with an ethanol liquid diet do not exhibit affective or somatic signs during ethanol withdrawal.

Testing with daily ethanol injections suggests that expression of the α5 nAChR subunit is important for ethanol withdrawal-associated behaviors. However, it was important to show that this relationship remains valid when mice are administered a longer course of ethanol treatment, such as a liquid ethanol diet. Mice were provided with control or a 4% ethanol liquid diet for six weeks. Behavioral testing was conducted 24 hours after the replacement of the ethanol-containing diet with control diet. In the OFA, similar to what

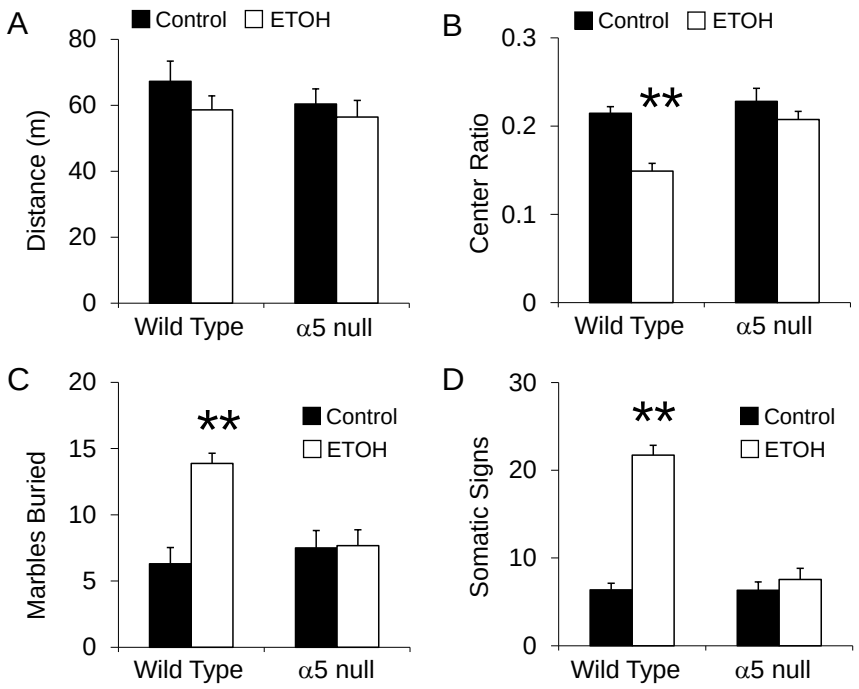


Figure 6.3: α5 null mice treated with ethanol injections do not display affective or physical signs of withdrawal. α5 and their wild-type littermates were treated daily with 2 g/kg ethanol + 9mg/kg 4MP or control IP injections. All testing took place 24 hours after the last ethanol injection. A. Neither ethanol withdrawal nor genotype had an effect on the locomotor behavior in the open field arena. B. The center ratio, a measure for anxiety-like behavior, was significantly decreased in wild-type mice undergoing ethanol withdrawal. No effect on the center ratio was observed in α5 null mice during withdrawal. C. In the marble burying test, upon ethanol withdrawal, wild-type mice undergoing ethanol withdrawal buried significantly more marbles, suggesting that α5* nAChRs play a role in compulsive-like behavior after alcohol cessation. D. Increases in physical signs of withdrawal were only observed in wild-type, ethanol-treated mice. N's are 6-14 mice per experimental group. **p<0.01 compared to control-treated mice and wild-type mice.

was observed with the short-term ethanol treatment, locomotor behavior was not affected by either genotype or ethanol withdrawal. (Fig 6.4A; $F_{\text{withdrawal x genotype}}[1,41]=$ 0.08, p=0.78). The center ratio decreased significantly in wild-type mice undergoing ethanol withdrawal, suggesting an increase in anxiety-associated behavior (Fig. 6.4B). Interestingly, α5 null mice did not exhibit significant changes in the center ratio compared to control treated null mice. Statistical analysis confirmed a significant interaction between withdrawal behavior and genotype ($F_{\text{withdrawal x genotype}}[1,41] =6.14$, p= 0.017).

Mice were also tested in the MBT during spontaneous withdrawal occurring 24 hours after the removal of ethanol diet. During withdrawal, wild-type mice buried significantly more marbles than their control-treated littermates, suggesting that ethanol withdrawal leads to increased compulsive-like behavior (Fig 6.4C; $F_{withdrawal\ x\ genotype}[1,44]=24.644$, $p<0.001$). Similar to the previous result obtained in α5 null mice treated with ethanol injections, withdrawal did not increase the total number of marbles buried.

Lastly, we measured somatic signs during ethanol withdrawal. Consistent with our previous result, somatic signs were significantly elevated in wild-type mice undergoing ethanol withdrawal while α5 null mice did not manifest physical symptoms and behaved like control-treated mice (Fig 6.4D). Statistical analysis revealed a significant interaction between withdrawal and genotype, $F[1,47]=29.022$, $p<0.001$. Overall, we observed that α5* nAChRs affect the manifestations of ethanol withdrawal following both short- and long-term ethanol treatment.

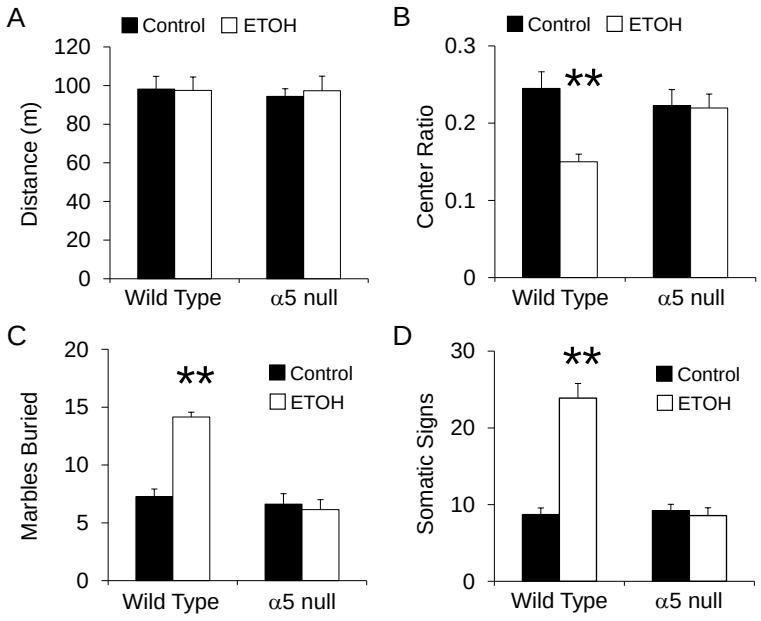


Figure 6.4: α5 null mice treated with a liquid ethanol diet do not manifest ethanol withdrawal symptoms. α5 and their wild-type littermates were provided with either a

control or ethanol-containing liquid diet for six weeks. All testing took place 24 hours after ethanol-diet was replaced with control-diet. A. Total distance traveled was not affected by either ethanol withdrawal or genotype in the open field arena. B. Similar to what was observed in ethanol-injected mice, the center ratio was significantly decreased in wild-type animals only during withdrawal, but anxiety levels did not change in α5 null mice. C. In the marble burying test, only wild-type mice undergoing ethanol withdrawal buried significantly more marbles, suggesting that α5* nAChRs play a role in compulsive-like behavior after cessation of alcohol administration. D. Increases in physical signs of withdrawal were only observed in wild-type, ethanol-treated mice. n = 6-10 mice per experimental group. **p<0.01 compared to control-treated mice and wild-type mice.

Re-expression of the α5 nAChR subunit in the IPN of null mice rescues compulsive-like behavior and somatic signs associated with ethanol cessation.

We have previously shown that the MHb and IPN play roles in the emergence of physical signs associated with nicotine, ethanol, or concurrent nicotine and ethanol cessation (Salas et al., 2009). *In situ* hybridization studies done by our lab and others (Salas, Orr-Urtreger, et al., 2003; Wada, McKinnon, Heinemann, Patrick, & Swanson, 1990) have shown that α5* nAChRs are expressed at low densities within the MHb and at high densities in the IPN. Given that our current results using mecamylamine and α5 null mice suggest that the nicotinic system plays a role in both affective and physical signs of ethanol withdrawal, we hypothesized that α5* nAChRs expressed within the IPN modulate these behaviors. Although the MHb also expresses the α5 subunit, we chose to focus on the IPN due to it being the primary recipient of MHb output.

Viral vectors were produced and packaged by the Baylor College of Medicine Viral Vector Core Facility and in our lab at the University of Pennsylvania. α5 null mice were infused into the IPN with viral particles expressing either a control vector encoding copGFP or a vector encoding α5 subunit and copGFP. Mice recovered from the surgery for one week before being single housed. Control or alcohol-containing liquid diet was introduced two weeks after surgery and was provided daily for a total of 6 weeks before

testing sessions began. Mice infused with viruses expressing only copGFP exhibited no differences in total ethanol consumption compared to those infused with viruses expressing the α5 subunit. All mice were tested in either the OFA, MBT, or for changes in somatic signs 24 hours after ethanol cessation.

In the OFA, viral expression of α5 did not affect the total distance traveled in the maze (Fig 6.5A; $F_{virus}[1,39]=0.217$, $p=0.644$). Similar to our previous results, ethanol withdrawal did not produce changes in locomotion and there was no significant interaction of ethanol treatment with viral treatment ($F_{withdrawal}[1,39]=0.695$, $p=0.410$; $F_{virus\ x\ withdrawal}[1,39]=0.366$, $p=0.549$). In addition, we compared the locomotor behavior of virus-injected mice fed the control liquid diet to what was previously observed in α5 null mice not treated with viral infusions (Fig 6.5A, red line). Overall, mice infused with viral vectors did not differ from non-injected α5 null mice ($F_{virus}[2,31]=0.473$, $p=0.628$). When anxiety-associated behavior was analyzed, we found that there was no significant difference between α5 null mice expressing copGFP or the α5 nAChR subunit in the IPN (Fig 6.5B). Interestingly, ethanol withdrawal did not have a significant effect on the center ratio ($F_{virus\ x\ withdrawal}[1,39]=0.592$, $p=0.446$), suggesting that re-expression of α5* nAChRs within the IPN is not sufficient to restore anxiety-associated behavior during ethanol withdrawal. However, when we plotted the center ratio baseline behavior of α5 null mice that were not infused with virus, we found that all virus-treated animals exhibited significantly lower center ratios regardless of treatment (Fig 6.5B, redline; $F_{virus}[2,31]=3.711$, $p=0.036$), making it harder to confidently conclude that α5* nAChRs within the IPN are having no effect on anxiety-associated behavior during ethanol withdrawal. These experiments are currently being repeated.

Compulsive-like behavior during withdrawal was measured by testing mice in the MBT 24 hours after alcohol removal. The total number of marbles buried by α5 null mice expressing the control vector did not decrease upon ethanol withdrawal (Fig 6.5C). In mice expressing the α5 nAChR subunit, there was a significant increase in the total number of

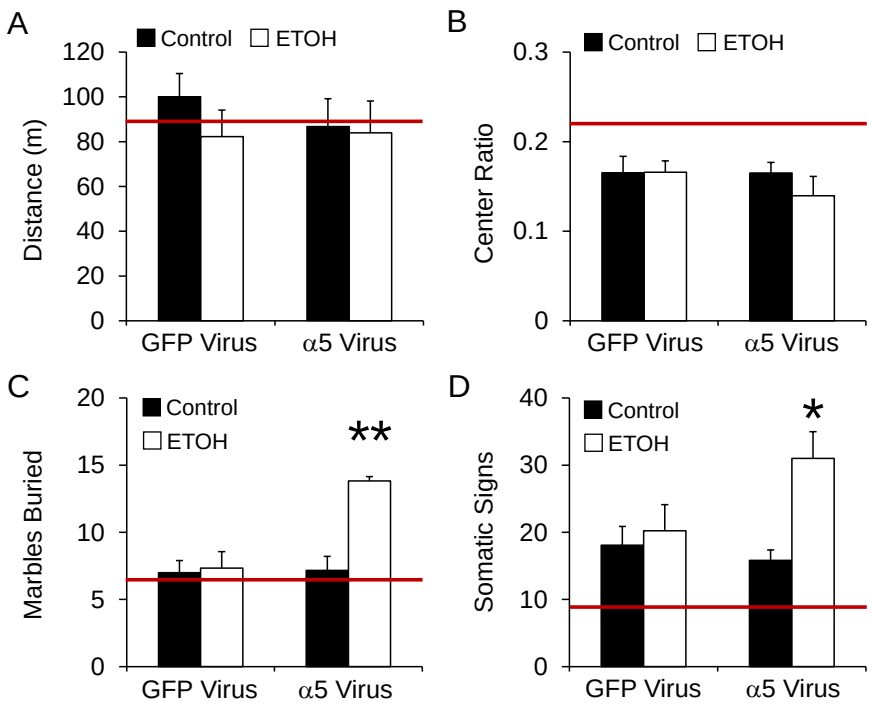


Figure 6.5: Re-expression of the α5 nAChR subunit in the IPN rescues compulsive and physical signs of ethanol withdrawal in α5 null mice. α5 null mice were injected with viral particles expressing either the α5 nAChR subunit + copGFP or solely copGFP. Two weeks after viral infusions, mice were provided with either a control or ethanol-containing liquid diet for six weeks. All testing took place 24 hours after the ethanol-diet was replaced with a control diet. A. Total distance traveled was not affected by either ethanol withdrawal or viral expression in the open field arena. B. No significant difference was observed in the center ratio of mice injected with the α5 viral vector or treated with ethanol. Compared to the baseline behavior of α5 null mice not infused with any viral particles (red line), all control-fed mice displayed a significant decrease in the center ratio. C. In the marble burying test, mice expressing the α5 nAChR subunit undergoing ethanol withdrawal buried significantly more marbles, suggesting that α5* nAChRs within the IPN play a role in compulsive-like behavior after alcohol cessation. D. Increases in physical signs of withdrawal were only observed in ethanol-treated mice expressing the α5 nAChR subunit in the IPN. n = 9-12 mice per experimental group. *p<0.05 and **p<0.01 compared to control-treated mice and cop-GFP injected mice.

marbles buried during ethanol withdrawal, suggesting that α5* nAChR expression within the IPN is important for the manifestation of compulsive-like behavior during ethanol withdrawal. Statistical analysis also reflected a significant interaction between α5 viral expression and ethanol withdrawal ($F[1,39]=11.778$, $p=0.001$). When we compared the

baseline behavior of α5 null mice that were not infused with viral vectors to control-fed viral-treated mice (Fig 6.5C, red line), we found that there was no significant shift in baseline behaviors (Fvirus[2,33]=0.092, p=0.912). Overall, we can confidently conclude that α5* nAChRs within the IPN play a role in the compulsive-like behaviors induced by ethanol withdrawal.

Our lab has previously highlighted the role of nAChRs within the IPN in the modulation of somatic signs of withdrawal after ethanol and nicotine cessation (Salas et al., 2009). In addition, we have explored the role of the IPN in mice co-treated with nicotine and ethanol. Since the α5 nAChR subunit is highly expressed within the IPN, we tested the hypothesis that the nAChRs involved in the physical manifestations of ethanol withdrawal contain the α5 nAChR subunit. Somatic signs were not affected by ethanol withdrawal in α5 null mice expressing solely copGFP, while re-expression of the α5 subunit in the IPN led to the emergence of physical signs of ethanol withdrawal (Fig 6.5D). Statistical analysis confirmed a significant interaction between α5 expression and ethanol withdrawal (F[1,39]=4.392, p=0.043). Interestingly, when control-virus injected mice were compared to non-virus treated α5 null mice (Fig 6.5D, red line), there was a significant increase in baseline somatic sings in virus-infused animals (F_{virus}[2,33]=6.702, p=.004). Despite this shift in baseline, it remained evident that re-expression of α5* nAChRs within the IPN is sufficient to reinstate physical signs of ethanol withdrawal.

Discussion

The symptomology of alcohol and nicotine withdrawal is similar, suggesting a common regulatory neuronal circuit (Hughes et al., 1994). Using techniques previously utilized to understand the mechanisms underlying nicotine withdrawal, we showed that similar nicotinic cholinergic mechanisms and brain areas were implicated in ethanol withdrawal

(Salas et al., 2009). The non-selective nAChR antagonist, mecamylamine, has been shown to precipitate somatic signs of withdrawal in mice treated with chronic nicotine and ethanol injections (Salas, Pieri, et al., 2004; Salas et al., 2009), and we used the same approach to precipitate an array of withdrawal behaviors in mice chronically treated with a liquid ethanol diet. Mecamylamine led to a significant increase in compulsive-like behavior and physical signs in ethanol-treated mice and produced a strong trend towards increased anxiety-associated behavior without affecting total locomotion.

Pre-clinical studies with mice that overexpress the human *CHRNA5/CHRNAα3/CHRNBβ4* gene cluster of α5, α3 and β4 nAChR subunits found that global overexpression results in a significant decrease in alcohol consumption (Gallego et al., 2012). Interestingly, the α5 nAChR subunit has also been shown to influence nicotine self-administration (Fowler et al., 2011; Morel et al., 2013). It has also been established to play a role in the physical manifestations of nicotine withdrawal (Salas et al., 2009). Results from ethanol injections, and a longer treatment using a liquid ethanol diet, suggest that α5* nAChRs influence anxiety-associated behavior, compulsive-like behavior, and physical symptoms during ethanol abstinence. We tested a small group of α5 null mice in the elevated plus maze (EPM), which is considered a behavioral measure of anxiety-like behavior. In the EPM, α5 null mice did not exhibit a reduced entry ratio or time spent in the open arms that were exhibited by their wild-type littermates during ethanol withdrawal (data not shown). It should be noted the α5 null mice display an anxiolytic phenotype in the EPM in basal conditions (Gangitano et al., 2009). We therefore chose to focus on the OFA as a measure of anxiety-like behavior.

Given the number of brain structures in which the α5 nAChR subunit is expressed, and the high density of expression found in the IPN (Salas, Orr-Urtreger, et al., 2003), we decided to re-express the α5 subunit in the IPN. Previous studies using mecamylamine had shown that the MHb and IPN are involved in nicotine and ethanol withdrawal (Salas et al., 2009). We used hippocampus and the ventral tegmental area as control sites, neither of which influenced the modulation of physical signs during withdrawal from either nicotine or ethanol. In the experiments presented here, re-expression of α5* nAChRs in the IPN of α5 null mice resulted in significant increases in compulsive-like behavior and physical signs during withdrawal. Interestingly, re-expression in the IPN did not have an effect on anxiety-associated behavior during withdrawal. However, baseline anxiety-associated behaviors exhibited by control-diet fed mice, infused with either α5- or copGFP-expressing viruses, were altered. Furthermore, the animals appeared more anxious than α5 null mice that did not receive viral infusions. Given that the absence of α5* nAChRs is protective against the manifestations of anxiety-associated behavior induced by ethanol withdrawal, it is possible that the expression of α5* nAChRs in the IPN alone is insufficient to modulate anxiety-associated behavior induced by ethanol withdrawal. The MHb, which also expresses the α5 subunit, is another potential region that has been shown to play a role in drug withdrawal and affective behaviors (P. R. Baldwin, Alanis, & Salas, 2011; Gorlich et al., 2013; Lecourtier & Kelly, 2007; Mathuru & Jesuthasan, 2013; Mirrione et al., 2014; Neugebauer et al., 2013; Panchal et al., 2005; Salas et al., 2009; Viswanath et al., 2013; Yamaguchi et al., 2013).

In conclusion, our study has highlighted α5* nAChRs as important modulators of the behavioral manifestations of alcohol withdrawal, suggesting that this receptor subtype could be targeted to assist alcohol cessation treatments. Given that an absence of α5

nAChR subunit expression reduces withdrawal-induced behaviors, drugs targeting receptors including this subunit may prove to be superior to varenicline, which seems to affect how much a person drinks during a drinking session without affecting overall cessation rates (Litten et al., 2013; McKee et al., 2009; Muller, Geisel, Banas, & Heinz, 2014; Plebani et al., 2013). We also found that α5* nAChRs within the IPN influence the emergence of compulsive behavior and physical signs of alcohol withdrawal. Accordingly, therapeutics that modify the activity of the MHb-IPN axis may be worth evaluation as candidates for the treatment of alcoholism.

Figure 5.6

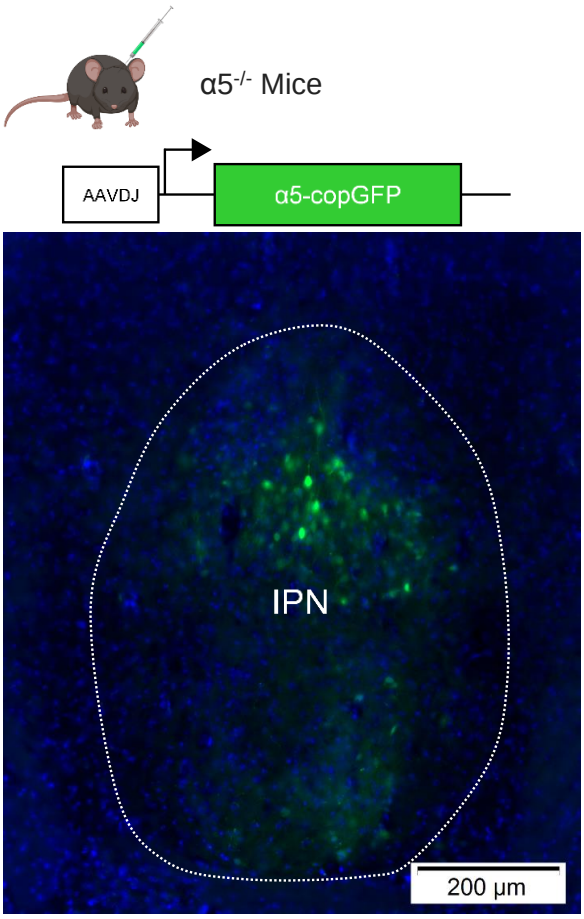


Figure 6.6. Viral rescue of α5 nAChR subunit expression in α5 null mice. Mice lacking expression of the α5 nAChR subunit were infused with a virus (AAVDJ-α5-copGFP) driving expression of the receptor subunit into the IPN. Successful expression was observed with some sub-anatomical selectivity.

The medial habenula & interpeduncular nucleus is critical in addiction, anxiety, and mood regulation

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Abstract

Abstinence from chronic use of addictive drugs triggers an aversive withdrawal syndrome that compels relapse and deters abstinence. Many features of this syndrome are common across multiple drugs, involving both affective and physical symptoms. Some of the network signaling underlying withdrawal symptoms overlaps with activity that is associated with aversive mood states, including anxiety and depression. Given these shared features, it is not surprising that a particular circuit, the dorsal diencephalic conduction system, and the medial habenula and interpeduncular nucleus (MHb-IPN) in particular, have been identified as critical to the emergence of aversive states that arise both as a result, and independently, of drug addiction. As the features of this circuit continue to be characterized, the MHb-IPN axis is emerging as a viable target for therapeutics to aid in the treatment of addiction to multiple drugs of abuse as well as mood-associated disorders.

Introduction

Drug addiction is a substantial public health and societal burden, causing over $700 billion in annual costs associated with crime, lost work productivity, and healthcare (National Center for Chronic Disease et al. 2014, Sacks et al. 2015, Center 2011). Although they have unique pharmacological effects, all addictive drugs share actions on the dopaminergic system that contribute to their rewarding effects (Di Chiara & Imperato 1988). The withdrawal syndrome that follows cessation of chronic use has also been identified as having common substrates. Additionally, epidemiological data have identified Aanxiety and mood disorders, as the first and second most common psychiatric disorders in the United States (Kessler et al. 2005), may also reflect alterations in overlapping circuits. Interestingly, Rresearch characterizing the

mechanisms underlying addiction, withdrawal, and aversive moods states has implicated a common pathway. The medial habenula (MHb) and interpeduncular nucleus (IPN) are two components of the dorsal diencephalic conduction system (DDC), a highly evolutionarily conserved pathway which, along with the medial forebrain bundle, conveys signals from the limbic forebrain to the midbrain and hindbrain (Bianco & Wilson 2009, Okamoto et al. 2012). In higher vertebrates, the DDC has evolved to be a significant pathway by which the forebrain regulates midbrain motivation and reward circuitry (Sutherland 1982). The epithalamic MHb receives inputs from a very variety of structures, including the triangular septal nucleus, septofimbral nucleus, ventral tegmental area, and raphe nuclei (Herkenham & Nauta 1977, Lecourtier & Kelly 2007, Phillipson & Pycock 1982). Studies also indicate that the MHb receives inputs from the nucleus accumbens (Sutherland 1982), locus coeruleus and superior cervical ganglion (Gottesfeld 1983), diagonal band nucleus and medial septum (Qin & Luo 2009), as well as the median raphe nucleus (Sutherland 1982, Conrad et al. 1974). Some studies suggest that the MHb may project to the pineal body (Ronnekleiv & Moller 1979, Guglielmotti & Cristino 2006), as and it may also send sparse efferents to the VTA (Cuello et al. 1978), as well as extend boutons en passant to the LHb (Kim & Chang 2005). However, the MHb It then sends its most dense efferent projections to the mesencephalic IPN through the core of the fasciculus retroflexus (FR) (Herkenham & Nauta 1979), which, in turn, sends efferents to a wide variety of mid- and hindbrain structures implicated in regulating affective states. Those structures, includeing the dorsal tegmental nucleus (Shibata & Suzuki 1984), hippocampus (Shibata & Suzuki 1984, Baisden et al. 1979, Wyss et al. 1979), lateral hypothalamus (Massopust & Thompson 1962, Morley 1986, Kemali & Guglielmotti 1982, Smith et al. 1980), ventral tegmental area (Hayakawa et al. 1981, Smaha & Kaelber 1973), septum, preoptic area,

and nucleus of the diagonal band (Smaha & Kaelber 1973, Morley 1986). Additionally, there are data indicating projections from the IPN to the dorsal and median raphe nuclei (Groenewegen et al. 1986, Behzadi et al. 1990), as well as the lateral habenula (Massopust & Thompson 1962, Morley 1986). Though the major source of innervation in the IPN arrives from the MHb, there is evidence of afferents arriving from structures including the horizontal limbs of the diagonal band nucleus (Contestabile & Flumerfelt 1981), substantia innominata (Vertes & Fass 1988), infralimbic region of the medial prefrontal cortex (Takagishi & Chiba 1991), preoptic nucleus (Shibata et al. 1986), hypothalamic nuclei (Contestabile & Flumerfelt 1981, Hamill & Jacobowitz 1984), supramammillary nucleus (Contestabile & Flumerfelt 1981, Hamill & Jacobowitz 1984), raphe nuclei (Conrad et al. 1974), nucleus incertus (Hamill & Jacobowitz 1984) and dorsal tegmental nucleus (Hamill & Jacobowitz 1984). While some of these anatomical studies were conducted recently, some were conducted many years ago, so corroborative reproducibility experiments are likely warranted, given the availability of more targeted tracer techniques. A summary of these afferent and efferent projections for the MHb and IPN are available in figure 1 and tables 1 and 2.

While both are fairly small anatomical structures, the MHb and IPN host the synthesis and release of a wide variety of neurotransmitters. Studies have identified acetylcholine (McCormick & Prince 1987), substance P (SP) (Burgunder & Young 1989, De Biasi et al. 2016, Jackson et al. 2015), glutamate and GABA (Qin & Luo 2009), norepinephrine (Gottesfeld 1983), serotonin (Kinsey et al. 2001), ATP (Edwards et al. 1992, Sperlagh et al. 1998), interleukin-18 (Sugama et al. 2002), and a host of neuropeptides in the MHb-IPN pathway (McLaughlin et al. 2015, Kopp et al. 2002). Additionally, the MHb-IPN circuit has been implicated in mechanisms that mediate some of the acute and aversive

features of withdrawal from multiple drugs, including alcohol, opiates, nicotine, opiates, and other stimulants. This review will focus on studies that specifically implicate the DDC, and the MHb-IPN pathway in particular, in the neurophysiology associated with both addiction and mood-related psychiatric conditions, with an eye towards the possibility of identifying druggable targets within the pathway that may yield therapeutic benefits for the treatment of both sets of conditions.

Alcohol

The pharmacological activity of ethanol encompasses a broad range of targets in the central nervous system, and studies have indicated that the MHb-IPN circuit represents a significant component of its affective and behavioral effects. Local cerebral glucose utilization rates in alcohol-preferring rats are significantly elevated in both divisions of the habenular complex relative to non-preferring rats (Smith et al. 2001). Data from our lab have identified an interaction between ethanol and nicotine withdrawal, as well as a role played by nAChRs in the MHb or IPN in ethanol withdrawal. Specifically, intraperitoneal injection of a non-selective nAChR antagonist, mecamylamine, is capable of precipitating a withdrawal syndrome in mice chronically treated with ethanol. Withdrawal symptoms are also exhibited when mecamylamine is infused into the MHb or IPN, but not the ventral tegmental area (VTA) or hippocampus (Perez et al. 2015), indicating that nAChR blockade in the MHb-IPN circuit is sufficient to precipitate the ethanol withdrawal syndrome. Another recent finding has identified changes in signaling molecules associated with apoptosis, inflammation, neurodegeneration, and senescence in the habenula, among other structures, following chronic treatment with ethanol (Roux et al. 2015). Receptor knock-out studies have shown that neuropeptide Y (NPY) acts as an important regulator of alcohol intake, with knock-out mice exhibiting increased alcohol

ingestion relative to wild-type mice (Thiele et al. 2002). More recently, it was shown that alcohol-preferring rats exhibit an absence of NPY mRNA in the MHb while non-preferring rats do have a signal of mRNA for the neuropeptide (Hwang et al. 2004). Taken together, the literature suggests that activity in the MHb-IPN circuit likely modulates the effects and ingestive behavior of alcohol, as well as its withdrawal symptoms. Variations between individuals in the signaling within this circuit may underlie predispositions to pathological intake patterns.

Opioids

Drug overdose represents the leading cause of accidental death in the United States (Medicine 2016), and addiction to opioids, including both illicit substances, such as heroin, and prescription analgesics, such as oxycodone and fentanyl, drive this epidemic (Prevention 2015). Interestingly, overdose fatalities, sales, and substance use disorder treatment admissions have increased in parallel from 1999 to 2008 (Paulozzi 2014), indicating a significant need for improved treatments for substance abuse disorder and opioid addiction in particular.

One of the densest regions of opioid receptor expression is the DDC, and the MHb, FR, and IPN are particularly rich with expression (Gackenheimer et al. 2005, Gardon et al. 2014, Zhu et al. 1998, Sim-Selley et al. 1999). Additionally, there is a plethoradiversity of neurotransmitters that the MHb-IPN circuit synthesizes or is sensitive to, including acetylcholine, neurokinins, interleukin-18 (IL-18) (Viswanath et al. 2013, Sugama et al. 2002), and purines (Pankratov et al. 2009, Pankratov et al. 2006, Kanjhan et al. 1999) that may be modulated by the activities of opioids. Given the broad efferent targets of the IPN that are known to regulate affect and substance use, including thethe raphe nuclei, nucleus incertus, lateral septum, lateral dorsal tegmentum (LDTg), and

hypothalamus (Sutherland 1982, Ryan et al. 2011, Bianco & Wilson 2009, Morley 1986, Gardon et al. 2014), the MHb-IPN circuit is likely an anatomical node, centrally involved in the signaling underlying both acute effects of, and withdrawal from, opioids. Evidence over the past several decades has corroborated such a role. For example, lesions of the MHb have been observed to induce hyperalgesia and increase the analgesic efficacy of morphine (Meszaros et al. 1985), and morphine is capable of inducing analgesia when infused directly into the habenular complex (Cohen & Melzack 1985). When evaluating the effects of intracranial self-stimulation of the VTA on opioid release, a unique reduction of endogenous opioid binding was observed in the MHb (Stein 1993). Following chronic morphine administration, altered acetylcholinesterase (AChE) activity is observed in the MHb following chronic morphine administration, and precipitation of withdrawal with the opioid receptor antagonist, naloxone, resulted in altered AChE activity in the IPN (Neugebauer et al. 2013). Additionally, chronic morphine administration induced a trend towards increased nAChR expression in the MHb (Neugebauer et al. 2013). Acute administration of 18-methoxycoronaridine (18-MC), an α3β4 nAChR antagonist, reduced signs of naltrexone-precipitated withdrawal from morphine (Rho & Glick 1998), an effect that appears to be mediated by activity in the MHb and IPN (Panchal et al. 2005, Taraschenko et al. 2007). 18-MC was also observed to reduce morphine self-administration upon intracranial infusion into the MHb or IPN (Glick et al. 2006). In the MHb, RSK2, a component of the ribosomal S6 kinase 90kDa family, which act as substrates of extracellular-regulated kinases 1 & 2 to regulate cytosolic and nuclear targets, has been identified as critical to morphine-induced analgesia (Darcq et al. 2012). Finally, the LDTg, an efferent target of the IPN, has been shown to exhibit significant increases in vesicular ACh transporter markers following chronic morphine administration (Bajic et al. 2015, Gardon et al. 2014). Altogether, these

data implicate the DDC, and MHb-IPN circuit in particular, in the signaling underlying some of the acute effects of opioids, as well as aspects of their addictive properties. Furthermore, adaptations in cholinergic components may represent a significant facet of these changes.

Nicotine and Psychomotor Stimulants

The role of the lateral division of the habenular complex (LHb) in regulating dopaminergic activity in the VTA via the rostromedial tegmental nucleus has been established, and represents an important mechanism by which aversion and addiction are modulated (Barrot et al. 2012, Sanchez-Catalan et al. 2016, Jean-Richard Dit Bressel & McNally 2014, Quina et al. 2015). Both the LHb and MHb send dense efferent projections through the fasciculus retroflexus, and anatomical studies suggest that projections emerging from the MHb course through the core and those from the LHb through the sheath of thise dense fiber bundle (Bianco & Wilson 2009). Degeneration of dopaminergic fibers in the caudate Following chronic exposure to psychomotor stimulants like amphetamines, degeneration of dopaminergic fibers in the caudate washad been observed decades ago (Ellison et al. 1978). In addition to dopaminergic fibers, similar degeneration has been observed in axons populating the sheath of the FR following chronic exposure to cathinone, cocaine, amphetamine, methamphetamine, and MDMA (Ellison 2002, Carlson et al. 2000). In rats treated with cocaine, increased expression of Fos-protein, a marker of neuronal activation, was observed in the MHb associated with cue-induced reinstatement (James et al. 2011). Additionally, similar to its interference with opioid-derived reward, 18-MC administration results in reduced methamphetamine self-administration in rats (Glick et al. 2000). Once again, direct infusions directly into the MHb and/or IPN induced similar reductions of self-

administration, with what appears to be greater efficacy when infused into the IPN, suggesting that activity in the MHb-IPN circuit likely mediates this effect (Glick et al. 2008). Finally, methamphetamine and cocaine have been shown to increase extracellular concentrations of ACh in the IPN, with cocaine inducing a dose-dependent biphasic effect (Hussain et al. 2008).

Probably the best-characterized activity of a drug in the MHb-IPN pathway is that of nicotine, perhaps attributable to the considerable density of a variety of nAChRs that populate the structure (Mugnaini et al. 2002). Studies have suggested that up 90-100% of MHb neurons express nAChRs, with the majority containing the αα3, αα4, αα5, ββ2, and/or ββ4 subunits (Viswanath et al. 2013, Sheffield et al. 2000). Some data suggest that approximately 20% of nAChRs in the MHb expressed by neurons that project to the IPN contain the αα5 subunit (Picciotto & Kenny 2013, Grady et al. 2009). In the IPN, high levels of α2 subunit- containing nAChRs can be found (De Biasi & Salas 2008, Grady et al. 2009), and the distributions of nAChRs composed of specific subunit combinations can help distinguish subnuclei within both the MHb and IPN (Shih et al. 2014).

As many reviews of the effects of nicotine in this circuit have been written over the years (McLaughlin et al. 2015, De Biasi et al. 2014, De Biasi & Dani 2011, Dani & De Biasi 2001, Jackson et al. 2015), this section will focus on recent advances in characterizing the effects of chronic use and withdrawal from nicotine in the MHb-IPN pathway. For example, it was recently shown that, during withdrawal from nicotine, the spontaneous action potential frequencies in MHb cholinergic neurons are doubled after mice are administered nicotine relative to mice in withdrawal treated with saline (Gorlich et al. 2013). Further, these studies demonstrated that the pacemaking activities of MHb

cholinergic neurons are determined by the activities of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, and pharmacological inhibition of these HCN channels resulted in the manifestation of nicotine withdrawal-associated behaviors, including both somatic and anxiety-associated symptoms. A study from our lab showed that nicotine enhances the intrinsic excitability of MHb neurons by activating αα5-containing nAChRs, which results in the facilitation of neurokinin release onto NK1 and NK3 receptors (Dao et al. 2014). Notably, pharmacological blockade of NK1 & NK3 receptors in the MHb of mice chronically treated with nicotine resulted in the precipitation of somatic symptoms of nicotine withdrawal. Further implicating the αα5- containing nAChRs in the MHb in the physiology of nicotine addiction and withdrawal, mice lacking the expression of the subunit have been observed to self-administer doses of nicotine at levels that are aversive to wild-type mice, and virus-mediated re-expression of the subunit in the MHb rescues self-administration to levels resembling those consumed by wild-type mice (Fowler et al. 2011a). Additionally, mice lacking the αα5 nAChR subunit exhibit reduced IPN activation following exposure to nicotine relative to wild-type mice, suggesting a significant role played by this subunit in the MHb-IPN pathway in determining the range of nicotine doses capable of facilitating activity in brain reward circuitry (Fowler et al. 2011b, Fowler et al. 2013). Mice lacking the αα2 nAChR subunit exhibit elevations of both glutamate and GABA in the IPN, suggesting that αα2-containing nAChRs may participate in the signaling underlying the effects of nicotine (Lotfipour et al. 2013). Studies have also demonstrated a significant role played by IPN signaling in somatic symptoms of nicotine withdrawal by the IPN, with GABAergic neuronal activation enhanced by increased glutamate release, perhaps from the MHb (Zhao-Shea et al. 2013). This group also showed that pharmacological inhibition of NMDA receptor activation reduced symptoms of withdrawal, suggesting a glutamatergic

signal from the MHb playing a significant role in the nicotine withdrawal syndrome. Following chronic nicotine exposure, mecamylamine infusion into the MHb or IPN has been observed to induce anxiety-associated behaviors and symptoms of nicotine withdrawal (Zhao-Shea et al. 2015, Salas et al. 2009). Given that mecamylamine infusion into nicotine-naïve mice does not result in significant changes in anxiety-associated behavior, the MHb-IPN axis represents a node of neuroplastic adaptations resulting from chronic nicotine exposure that underlie the affective and somatic symptoms of nicotine withdrawal upon cessation.

Accordingly, while not necessarily a direct pharmacological target of most psychomotor stimulants apart from nicotine, the MHb-IPN circuit likely represents a system that modulates the acute effects of psychomotor stimulants, and contributes to signaling underlying withdrawal and relapse.

Depression-like behavior

In addition to playing a role in the acute effects of multiple drugs and the manifestation of their withdrawal from multiple drugs, as well as their acute effects, the MHb-IPN circuit has been implicated in mood-associated conditions. For example, one group worked to identify brain regions exhibiting activation during uncontrollable stress that then correlated with a subsequent manifestation of helplessness behaviors (Mirrione et al. 2014). Using positron emission tomography (PET) with rats, the habenula and lateral septum emerged as central in a network of brain regions, corroborating a role played by these structures in vulnerability to uncontrollable stress (Mirrione et al. 2014). An evaluation of the effects of chronic mild stress in rats on somatostatin (SST) receptor expression and SST release revealed that this signaling system significantly changes in the MHb (Faron-Gorecka et al. 2016). In fact, SST2 receptor expression in the MHb, and

peripheral SST concentration in plasma, were identified as particularly sensitive biochemical indicators of whether an animal would be stress responsive or non-responsive. Following an effort to characterize changes in the expression of microRNA (miRNA) species in the MHb and LHb of rats that underwent learned helplessness identified, six miRNAs were shown to be significantly altered (Svenningsen et al. 2016). The miRNA species identified are associated with MAPK, neutrophin, and ErbB signaling pathways.

Though the MHb is a relatively small structure, it can be segregated into subnuclei based upon localization of neurotransmitter and transporter protein expression in specific regions. The MHb is glutamatergic, and is often sub-divided into a dorsal sub-region, which expresses SP, and a ventral sub-region, which expresses ACh (Kobayashi et al. 2013). Some studies suggest that it can be further divided based on transporter protein and receptor densities (Aizawa et al. 2012). Moreover, efferent projections from the MHb target the IPN topographically, with efferents from the dorsal MHb targeting the lateral IPN, those from medial regions of the MHb terminating in the ventral IPN, and those from the lateral MHb terminating in the dorsal IPN (Bianco & Wilson 2009). Given the neurochemical differences in these topographic projections, it is not unsurprising that studies suggest these anatomical distinctions correspond to different functional roles (Ichijo & Toyama 2015). As altered physical activity and anhedonia are diagnostic indicators of major depressive disorder according to the Diagnostic and Statistical Manual of Mental Disorders (Association 2013), the MHb has been evaluated as a regulator of analogous behaviors like wheel running activity (WRA) in rodents (Hsu & Wang 2014). Working with a genetic ablation model of the dorsal subnucleus of the MHb, reduced WRA and sucrose preference was observed, both of which are

indicativeons of depressive-like behaviors. Interestingly, despite no known direct synaptic connection with dopaminergic populations, mice exhibited a significant preference for optogenetic intracranial self-stimulation of the dorsal MHb (Hsu & Wang 2014). Conversely, inhibition of the terminals arriving in the IPN from the dorsal MHb yielded place aversion. This corroborates studies that characterized metabolic differences in brain regions of rats bred for susceptibility to helplessness, which exhibited significantly increased markers of metabolic activity in both the lateral and medial habenula (Shumake et al. 2003). Using the same metabolic profiling method in Holtzman rats, a strain that exhibits susceptibility to stress-evoked helplessness, Padilla and colleagues characterized the effects of a two-week treatment with fluoxetine following a learned helplessness paradigm were characterized (Padilla et al. 2011). The group identified a protective effect of fluoxetine against depression-like behaviors in the forced swim test relative to rats treated with vehicle. This protective effect correlated with changes in regional metabolic activity in a variety of brain networks, including the habenular complex, IPN, and dorsal raphe nucleus (Padilla et al. 2011). In particular, fluoxetine treatment was associated with comparatively stronger positive correlations with prefrontal regions, including the dorsal, orbital, and prelimbic cortices. The IPN was positively correlated with the lateral orbital cortex in rats treated with fluoxetine, and this correlation was not present in vehicle-treated rats. Finally, metabolic markers in the dorsal raphe become somewhat positively correlated with the habenula in fluoxetine-treated rats, while strongly negatively correlated among the vehicle-treated group.

Due to challenges derived from the small size and sub-cortical location of the MHb-IPN circuit, the majority of studies characterizing its function have been performed in animal models. However, a few studies with humans have corroborated a role played by the

circuit in depression. Accumulating literature over the past decade indicates that ketamine may represent a novel pharmacological treatment strategy for major depressive disorder (Han et al. 2016, Burger et al. 2016, Zarate et al. 2006, Murrough et al. 2013). When evaluating changes in cerebral glucose metabolism using PET, 20 unmedicated patients with treatment-resistant major depressive disorder were scanned before and after a ketamine infusion. The habenular complex, among several other brain regions, exhibited reduced metabolism in association with a rapid antidepressant effect of ketamine (Carlson et al. 2013). Finally, a post-mortem study of sections from the brains of patients diagnosed with various mood disorders identified significant reductions in the volumes, cell numbers, and mean cell areas in the MHb of depressive patients (Ranft et al. 2010).

Anxiety, fear, and stress

While anxiety and fear have been associated for quite some time with anatomical structures including the amygdala, hippocampus, hypothalamus, and periaqueductal grayfor quite some time, recent research has implicated the DDC in these behaviors as well (Okamoto & Aizawa 2013). A genetic lesion study indicates that selective elimination of MHb afferents arriving from two forebrain structures, the triangular septum and bed nucleus of the anterior commissure, results in disrupted anxiety- and fear-associated behaviors (Yamaguchi et al. 2013). In particular, lesions of the projections from the triangular septum, which terminate in the ventral subnucleus of the MHb, disrupted anxiety-associated behavior. Conversely, lesions of projections arriving from the bed nucleus of the anterior commissure, which terminate in the dorsal subnucleus of the MHb, disrupted fear-associated behavior. Following both acute and chronic restraint stress in rats, significant elevations of the pro-inflammatory cytokine, IL-18, have been

observed in the dorsal MHb (Sugama et al. 2002). Mast cells are another immune system-associated signaling component that appears to be sensitive to environmental stressors and aversive mood states (Georgin-Lavialle et al. 2016, Nautiyal et al. 2008, Silver & Curley 2013, Frenzel & Hermine 2013). Mice treated with a 3-week behavioral subordination paradigm, either via exposure to an aggressor or placement in a clean cage, exhibited increased numbers of mast cells in the habenula, thalamus, and hypothalamus (Cirulli et al. 1998).

When characterizing fear-associated behavior in zebrafish, inhibition or lesion of the dorsal habenula (analogous to MHb in mammals) resulted in elevated freezing behaviors in response to a conditioned fear stimulus (Agetsuma et al. 2010, Lee et al. 2010). Both control and habenula-disrupted fish froze upon first exposure to an electric shock, but as subsequent shocks were administered, control fish exhibited reduced freezing behavior while those with disrupted habenular function exhibited no such behavioral adaptation. Another study identified behavioral signatures of elevated baseline anxiety in zebrafish following dorsal habenula lesions, including responses to novel environments and alarm substance secretion in response to overhead shadows (Mathuru & Jesuthasan 2013). This has led to the suggestion that reciprocal connectivity between thethe IPN, raphe nuclei, and dorsal tegmental region may be critical to behavioral responses to stressors. Furthermore, activity in the habenular complex may be an upstream determining factor in selection of behavioral strategies to cope with stressors (Okamoto et al. 2012, Jesuthasan 2012). A unique characteristic of the habenular complex is its asymmetry, with the left habenula larger than its right counterpart (Ahumada-Galleguillos et al. 2016, Hetu et al. 2016). When this asymmetry is lost in zebrafish, behavioral assays indicated

elevated manifestations of anxiety, as well as elevated cortisol levels in response to stressors (Facchin et al. 2015).

While the size of the habenular complex renders current neuroimaging technology incapable of distinguishing the LHb from the MHb in human studies, some studies indicate a role played by the habenular complex in the pathophysiology of bipolar disorder (BD). In a study using high-resolution magnetic resonance imaging (MRI), it was found that patients diagnosed with BD who had either never been medicated, or had been un-medicated for at least two months, exhibited smaller habenular volumes than healthy controls (Savitz et al. 2011).

Studies have also implicated the IPN in regulating anxiety and fear. Early studies of the IPN identified a role for the structure in the retention of avoidance conditioning. Rats were trained to perform a jumping response following a visual stimulus to avoid a shock. After a learning period, a group of trained rats were treated with electrolytic lesions of the IPN. Following recovery from the procedure, rats re-learned the task and retention of the response was compared to controls. Rats with IPN lesions exhibited comparatively inferior retention of the response, though exhibited other signatures of a fear reaction, implying a role played by IPN signaling in specific components of fear learning (Thompson 1960). Following chronic nicotine administration, increased corticotropin releasing factor (CRF) synthesis is observed in dopaminergic neurons in the VTA, which appear to send efferents to the ventral IPN (Zhao-Shea et al. 2015). This is accompanied by an increase in CRF1 receptor expression in a particular subnucleus of the ventral IPN, and withdrawal induces release of CRF by the VTA onto ventral IPN neurons. Blockade of CRF1 receptor binding in the IPN was shown to reduce anxiety-associated behavior generated during withdrawal from nicotine.

Conclusion

The dorsal diencephalic conduction system is emerging as an important node in the pathophysiology of addiction to multiple drugs, including alcohol, opioids, and nicotinealcohol. In addition to this involvement in substance abuse and addiction, the DDC also appears to regulate affect and mood-associated psychiatric conditions. Given the high comorbidity of substance use disorders and psychiatric illnesses, it makes sense that both conditions engage some overlapping pathways. For example, approximately one-third of individuals diagnosed with major depressive disorder also have a substance use disorder (excluding nicotine dependency), with almost half of all patients with depression having a family history of substance use disorders (Davis et al. 2008). Individuals with both disorders tend to experience earlier onsets of depression, greater persistence of the disorders, and exhibit more suicide attempts. When considering another addictive drug, nicotine, individuals diagnosed with mental illnesses are approximately twice as likely to smoke tobacco (Lasser et al. 2000), and studies have shown that 81.8% of individuals diagnosed with bipolar disorder, and 76.8% diagnosed with generalized anxiety disorder have smoked daily for at least a month (Lasser et al. 2000). Anxiety disorders have been implicated in alcohol, opioid, and stimulant abuse (Vorspan et al. 2015). Among adolescents, the majority of substance use disorders occur among youth with prior psychiatric disorders, with alcohol abuse observed in 17.3% and drug abuse observed in 20% of those diagnosed with prior anxiety disorders (Conway et al. 2016). A relationship between anxiety, stress, depression, and other psychiatric conditions and increased nicotine consumption has been established for many years (Lasser et al. 2000, Feldner et al. 2007, Morissette et al. 2007, Patton et al. 1998, Zvolensky et al. 2005, Kassel et al. 2003, Cougle et al. 2010), and a recent study has indicated that this dynamic has not changed, despite

declining overall usage over the past 6 decades (Prochaska et al. 2016). This relationship even translates to a unique puff volume among those with psychiatric conditions, with smokers who have a history of panic attacks exhibiting increased puff volumes relative to those without histories of panic attacks (Farris et al. 2016). Accordingly, mounting data indicates an involvement of the DDC, and the MHb-IPN pathway in particular, in both addiction to multiple drugs of abuse and mood-associated conditions, and the MHb-IPN circuit may represent a junction at which signaling underlying both sets of conditions occurs. Given the presence of nAChRs composed of unique subunit compositions in the MHb-IPN pathway (Antolin-Fontes et al. 2015), this circuit may yield strategic targets for pharmacological therapeutics to improve treatment outcomes of both sets of conditions.

Table 1.

MHb Afferent & Efferent Connections			
Afferent		**Efferent**	
Structure	**Reference**	**Structure**	**Reference**
Triangular Septum	Herkenham & Nauta, 1977	Interpeduncular Nucleus	Morley, 1986; Herkenham & Nauta, 1979
Medial Septal Nucleus	Qin & Luo, 2009	Pineal body	Ronnekleiv & Moller, 1979; Guglielmotti & Cristino, 2006
Diagonal Band Nucleus	Qin & Luo, 2009	Lateral Habenula	Kim & Chang, 2005
Septofimbral Nucleus	Herkenham & Nauta, 1977	VTA	Cuello, 1978
VTA	Herkenham & Nauta, 1977		
Interfascicular nucleus of the VTA	Phillipson & Pycock, 1982		
Locus coeruleus	Gottesfeld, 1983		
Superior cervical ganglion	Gottesfeld, 1983		
Preoptic area	Groenewegen, 1986; Herkenham & Nauta, 1977		
Median Raphe Nucleus	Conrad, 1974; Sutherland, 1982		
Nucleus basalis	Herkenham & Nauta, 1977		
Nucleus accumbens	Sutherland, 1982		

Table 2.

IPN Afferent & Efferent Connections			
Afferent		**Efferent**	
Structure	**Reference**	**Structure**	**Reference**
Medial Habenula	Morley, 1986	Hippocampus	Shibata & Suzuki, 1984; Baisden, 1979; Wyss, 1979
Diagonal band nucleus	Contestabile & Flumerfelt, 1981	Lateral Hypothalamus	Massopust, Thompson, 1962; Kemali, Gugliemotti, 1982; Smith, 1980
Substantia innominata	Vertes, 1988	Ventral Tegmental Area	Morley, 1986; Hayakawa, 1981; Smaha, 1973
Hypothalamic nuclei	Contestabile & Flumerfelt, 1981; Hamill & Jacobowitz, 1984	Septum	Morley, 1986; Hayakawa, 1981
Supramammillary nucleus	Contestabile & Flumerfelt, 1981; Hamill & Jacobowitz, 1984	Preoptic Area	Morley, 1986; Hayakawa, 1981
Dorsal tegmental nucleus	Hamill & Jacobowitz, 1984	Nucleus of the Diagonal Band	Morley, 1986; Hayakawa, 1981
Preoptic nucleus	Shibata, 1986	Dorsal Raphe Nucleus	Groenewegen, 1986
Infralimbic region of medial prefrontal cortex	Takagishi & Chiba, 1991	Median Raphe Nucleus	Groenewegen, 1986; Behzadi, Wiklund, 1990
Raphe nuclei	Conrad, 1974	Lateral Habenula	Morley, 1986; Massopust, Thompson, 1962
Nucleus Incertus	Hamill & Jacobowitz, 1984	Dorsal Tegmental Nucleus	Hamill & Jacobowitz, 1984; Groenewegen, 1986

Table 3.

Roles in the actions of drugs

MHb			IPN		
Technique	**Finding**	**Reference**	**Technique**	**Finding**	**Reference**
Local cerebral glucose utilization	Higher in MHb of EtOH-preferring rats	9	Naloxone-precipitated opioid withdrawal	Altered AChE activity in the IPN	31
IP nAChR antagonist injection	Precipitates EtOH withdrawal syndrome	10	18-MC IPN infusion	Reduced morphine self-administration	35
NPY Receptor knock-out	Knock-out mice ingest more EtOH	11	18-MC IPN infusion	Reduced methamphetamine self-administration	46
In situ hybridization	EtOH-preferring mice lack NPY mRNA in MHb	12, 13	Methamphetamine & cocaine administration	Increased extracellular [ACh]	48
MHb lesion	Hyperalgesia, increase analgesic efficacy of morphine	28			
Intracranial infusion to MHb	Analgesia	29			
VTA ICSS	Reduced endogenous opioid binding in MHb	30			
Chronic morphine administration	Altered AChE activity in the MHb	31			
Chronic morphine administration	Trend to increased MHb nAChR expression	31			
18-MC MHb infusion	Reduced morphine self-administration	35			
RSK2 knock-out in MHb	Reduced morphine analgesia	36			
Cocaine cue-induced reinstatement	Increased Fos-protein in MHb	45			
18-MC MHb infusion	Reduced methamphetamine self-administration	46			

Table 4. Roles in Mood Disorders

MHb			IPN		
Technique	**Finding**	**Reference**	**Technique**	**Finding**	**Reference**
PET in stress-treated rats	Hb identified as central in network of brain regions	49	Inhibition of afferent terminals in IPN	Place aversion	56
Chronic mild stress in rats	Altered SST release and SST2 receptor expression in MHb distinguish stress-responsive from non-responsive	50	Stress-evoked helplessness treated with fluoxetine in rats	Fluoxetine was protective, correlated with altered metabolic activity in IPN	58
Learned helplessness in rats	Altered miRNA associated with MAPK, neutrophin, and ErbB in MHb & LHb	51	Electrolytic lesion of IPN	Inferior retention of conditioned avoidance response	80
Genetic ablation of dorsal MHb	Reduced wheel running activity	56	Chronic nicotine administration	Increased CRF release to ventral IPN	81
Optogenetic self-stimulation of dorsal MHb	Preference for stimulation	56	Chronic nicotine administration	Increased CRF1 receptor expression in ventral IPN	81
Rats bred for susceptibility to helplessness	Increased markers of metabolic activity in MHb & LHb	57	Blockade of CRF1 receptor in IPN	Reduced anxiety-associated behaviors during nicotine withdrawal	81
Stress-evoked helplessness treated with fluoxetine in rats	Fluoxetine was protective, correlated with altered metabolic activity in Hb	58			
PET in depressed patients treated with ketamine	Reduced metabolism in the Hb	63			
Post-morten study of brain sections of patients with depression	Reduced volume, cell number, and mean cell areas in MHb	64			
Elimination of MHb afferents from TS & BAC	Disrupted anxiety-associated behaviors	66			
Acute & chronic restraint stress	Elevated IL-18 in dorsal MHb	22			
Subordination paradigm	Elevated mast cell numbers in the Hb	71			
Inhibition or lesion of dMHb in zebrafish	Elevated freezing behavior, elevated anxiety, elevated alarm substance secretion	72, 73, 74			
MRI of unmedicated patients with bipolar disorder	Reduced Hb volumes	79			

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Scientific Overview: 2013 BBC Plenary Symposium on Tobacco Addiction

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Abstract

Nicotine dependence plays a critical role in addiction to tobacco products, and thus contributes to a variety of devastating tobacco-related diseases (SGR 2014). Annual costs associated with smoking in the US are estimated to be between $289 and $333 billion. Effective interventions for nicotine dependence, especially in smokers, are a critical barrier to the eradication of tobacco-related diseases. This overview highlights research presented at the Plenary Symposium of Behavior, Biology and Chemistry: Translational Research in Addiction Conference (BBC), hosted by the UT Health Science Center San Antonio, on March 9-10, 2013. The Plenary Symposium focused on tobacco addiction, and covered topics ranging from basic science to national policy. As in previous years, the meeting brought globally-renowned scientists, graduate student recruits, and young scientists from underrepresented populations in Texas and other states together with the goal of fostering interest in drug addiction research in young generations.

General Introduction

Nicotine, the alkaloid primarily responsible for the addictive properties of tobacco products, acts at nicotinic acetylcholine receptors (nAChRs). Found throughout the nervous system, nAChRs comprise numerous combinations of α (α2-9) and β (β2-4) subunits in the form of homo- and heteromeric ion channels (Gotti et al., 2009). nAChRs can mediate either fast synaptic transmission - as they primarily do in the periphery - or modulate the function of other neurotransmitter systems, as is common in the central nervous system (Dani and Bertrand, 2007; De Biasi, 2002).

The majority of smokers desire to quit, but only a small fraction of attempts are ultimately successful (Benowitz, 2010). According to the Center for Disease Control (CDC), approximately 69% of smokers want to quit, and 52% of smokers attempted to quit in 2010 – but only 6.2% were successful (CDCP, 2011).

There are several issues to confront when considering smoking cessation. First, nicotine's ability to interfere with the dopaminergic (DA) reward system is an important factor contributing to both the initiation and maintenance of nicotine use (Picciotto and Corrigall, 2002). Second, smoking is also motivated by the urge to alleviate affective, cognitive, and physical symptoms of withdrawal that emerge during periods of abstinence (De Biasi and Dani, 2011). Therefore, to be successful, smoking cessation strategies must reduce both the motivation to smoke and the symptoms of withdrawal during quit attempts. Nicotine replacement therapy (NRT), bupropion, and varenicline are the most commonly applied pharmacological aids for smoking cessation. Although all three work better than placebo, long-term success rates remain low among smokers attempting to quit (Paolini and De Biasi 2011). The following summarizes progress that contributing authors' labs are making toward understanding the molecular mechanisms of nicotine addiction, as well as the design of pharmacological and non-pharmacological strategies aimed at improving smoking cessation outcomes.

1. Nicotinic Receptor Subunits and Their Influence on Nicotine Addiction and Withdrawal (Mariella De Biasi, Ian McLaughlin, Erika E. Perez)

1.1 Symptoms of nicotine withdrawal. The unpleasant symptoms associated with nicotine withdrawal act as negative reinforcers that promote nicotine dependence (Koob and Volkow, 2010; Piper et al., 2011; Allen et al., 2008). These negative reinforcers include both affective (anxiety, depression, and irritability) and somatic (decreased heart

rate, constipation, general restlessness) symptoms (Malin and Goyarzu, 2009; Salas et al., 2009). Mice chronically exposed to nicotine display withdrawal symptoms that develop spontaneously, peak 24hr following cessation of administration, and can last for several days. Withdrawal can also be precipitated by systemic injection of non-selective nAChR antagonists such as mecamylamine (Paolini and De Biasi, 2011). Several behavioral tests are available to explore both somatic and affective symptoms in rodents. Affective signs of withdrawal can be examined in rodents using behavioral paradigms that test for anhedonia, conditioned place aversion, anxiety, and conditioned fear (De Biasi and Salas, 2008; Damaj et al., 2003; Epping-Jordan et al., 1998; Davis et al., 2005). Physical signs of withdrawal include chewing, teeth-chattering, shakes, tremors, writhing, palpebral ptosis, gasps, and yawns (De Biasi and Salas, 2008; Malin and Goyarzu, 2009).

1.2. A gene cluster on chromosome 15q25 influences nicotine addiction. Ample studies have demonstrated that genetic factors predispose individuals to younger smoking initiation, increased quantities of cigarettes smoked, nicotine dependence, and smoking persistence (Li et al., 2003; Rhee et al., 2003; Schnoll et al., 2007). A cluster of nicotinic receptor genes (CHRNA5/CHRNA3/CHRNB4) located on chromosome 15q25 has been repeatedly associated with nicotine dependence, smoking behaviors, and lung cancer (Amos et al., 2008; Berrettini et al., 2008; Furberg et al., 2010; Greenbaum and Lerer, 2009; Hung et al., 2008; Liu et al., 2010; Rose, 2007; Saccone et al., 2010, 2007; Thorgeirsson et al., 2008). SNPSs rs16969968, rs578776, and rs588765 represent three statistically distinct nicotine dependence loci associated with the CHRNA5/A3/B4 gene cluster (Saccone et al., 2010; Saccone et al., 2009). The α5 risk variant, rs16969968 G/A, causes an Asp398Asn amino acid substitution, and the risk allele (Asn398)

produces hypofunctional α5-containing nAChRs with reduced Ca 2+ permeability and faster desensitization rates than non-risk alleles (Bierut et al, 2008; Kuryatow et al, 2011). Other polymorphisms are associated with different levels of α5 or α3 mRNA (Wang, 2009), and the functional significance of several other gene variants in the CHRNA5/A3/B4 gene cluster is currently being investigated (Flora et al., 2013).

1.3 nAChR mutant mice help reveal mechanisms of nicotine withdrawal symptoms. Pre-clinical rodent models can be used to elucidate the functions of genes and brain pathways involved in nicotine addiction. Our lab took advantage of genetically engineered mice carrying null mutations in nAChR subunit genes to examine the roles of various subunits in the mechanisms of withdrawal. We focused on physical symptoms of nicotine withdrawal - studied either 24 hr after nicotine deprivation, or upon systemic injection of mecamylamine in mice with free access to nicotine in drinking water. We found that mice lacking functional β4 nAChR subunits exhibited reduced somatic signs relative to wild-type mice undergoing nicotine withdrawal (Salas et al., 2004). Further experiments with additional mice lacking nAChR subunits revealed that physical symptoms of withdrawal also depend on α5, α2, and partially on α7 nAChR subunits (Salas et al., 2007; Salas et al., 2009). Interestingly, mice lacking the β2 nAChR subunit displayed symptoms of withdrawal resembling those of wild-type mice (Fig. 1). Mice carrying a null mutation for the α3 nAChR subunit were not studied due to perinatal mortality (Xu et al., 1999).

1.4 Medial Habenula (MHb) and Interpeduncular nucleus (IPN) are key brain areas for physical manifestations of nicotine withdrawal. We focused on the MHb-IPN pathway - which is among the brain areas with the highest co-expression of α5, α3, α2, and β4 – to pursue neuronal circuits associated with physical symptoms of withdrawal (De Biasi and

Dani, 2011). The MHb, together with the lateral habenula (LHb), forms the habenular complex (Hb). The IPN is the main projection target of the MHb, while the LHb sends projections to the rostromedial tengmental nucleus (RMTg) in the midbrain. These brain areas play significant roles in aversion, negative reinforcement, negative prediction errors, and negative motivation (De Biasi and Dani, 2011; Fowler and Kenny, 2014). In mice chronically treated with nicotine, mecamylamine administration was sufficient to induce nicotine withdrawal behaviors only when microinjected into the MHb or the IPN, but not when microinjected into other brain areas, including the ventral tegmental area (VTA) (Salas et al., 2009).

Future studies. One unanswered question is whether the MHb-IPN pathway and the nAChRs contained therein, are important for the affective manifestations of nicotine withdrawal. This is a key question, given the emerging role of the Hb in anxiety-related disorders, and the fact that both β4 and α5 null mice exhibit reduced anxiety-related behavior in the elevated plus maze (Mathuru and Jesuthasan, 2013; Yamaguchi et al., 2013; Gill et al., 2013). In addition, given the role of the MHb-IPN pathway in nicotine aversion and withdrawal, it would be interesting to determine whether the same nAChR subtypes and the MHb-IPN pathway influence withdrawal from other drugs of abuse. Withdrawal from ethanol is a candidate, given similarities between certain manifestations of alcohol and nicotine withdrawal (Hughes et al.,1994). This hypothesis is corroborated by the observation that, although different from those influencing tobacco addiction, polymorphisms in the CHRNA5/A3/B4 gene region are independently associated with alcohol dependence (Wang et al., 2009; Sherva et al., 2010) - some of which are associated with altered levels of α5 mRNA (Wang et al., 2009). Furthermore, the

rs16969968 α5 SNP has been linked to increased risk not only for nicotine, but also for alcohol dependence (Schlaepfer et al., 2008).

1.5 Conclusions. nAChRs, especially those in the CHRNA5/A3/B4 gene cluster, influence aversive manifestations of nicotine withdrawal - and thereby represent novel molecular targets for medications aimed at smoking cessation.

2. Discovery of Nicotinic Receptor Antagonists as Agents for Treating Nicotine Addiction (Peter A. Crooks, Linda P. Dwoskin, Michael T. Bardo)

2.1 Introduction and Rationale. Activation of neuronal nicotinic acetylcholine receptors (nAChRs) evokes dopamine (DA) release within neuronal circuitry associated with reward (Picciotto and Corrigall, 2002). DA release is well known to underlie reinforcing properties of nicotine (Wise and Rompre, 1989). Therefore, tobacco smoking is reinforced and maintained, at least in part, by nicotine activation of nAChRs within DA reward circuitry. Results from a comprehensive molecular genetics study, in which an individual nAChR subunit gene (α4, α5, α6, α7, β2, β3, and β4) was deleted, suggest that nicotine-evoked DA release is mediated by 6 nAChR subtypes (Salminen et al., 2004; Gotti et al., 2005). These include α-conotoxin MII (α-CtxMII)-sensitive (α6β2β3*, α4α6β2β3*, α6β2*, and α4α6β2*) and α-CtxMII-insensitive (α4β2* and α4α5β2*) subtypes, while deletion of α7 and β4 subunits had no effect. Thus, multiple nAChR subtypes likely mediate nicotine-evoked DA release. Based on these data, we set out to develop smoking cessation therapies to identify subtype-selective nAChR antagonists that inhibit nAChRs mediating nicotine-evoked DA release (α6β2-containing nAChRs). We hypothesized that such subtype-selective nAChR antagonists would: i) be efficacious tobacco use cessation agents, ii) have limited side-effects due to their

receptor selectivity, and iii) offer smokers who do not respond well to existing smoking cessation therapies alternative treatment options.

2.2 Target and Drug Discovery. Kulak and colleagues (Kulak et al., 1997) previously reported that the neuropeptide α-CtxMII potently blocks nicotine-stimulated DA release in rat striatal synaptosomes. While relevant to the development of smoking cessation therapies, neurotoxin peptides acting as subtype-selective nAChR antagonists are unlikely to be developed into treatments for tobacco addiction because they are high-molecular weight compounds, and are unlikely to cross the blood–brain barrier (BBB). Thus, our strategy was to develop small, drug-like molecules that are selective antagonists at α6- and β2-containing, α-CtxMII-sensitive nAChR subtypes (i.e., α6β2*, α6β2β3*, α6α4β2β3*, and α4α6β2*). Importantly, since these α-CtxMII-sensitive nAChRs are located on DA neurons that mediate nicotine-evoked DA release (Wickham et al., 2013; Gotti et al., 2010), they represent viable targets for the development of tobacco cessation agents.

We initially focused on a series of N-n-alkylnicotinium analogs and related compounds for their ability to inhibit nicotine-evoked DA release from rat striatal slices and to displace [3H]-nicotine binding to rat striatal membranes (α4β2* nAChRs) (Dwoskin et al., 1992; Crooks et al., 1995). The most potent compound that emerged was S-(-)-N-n-octylnicotinium iodide (NONI, Fig. 2). Though lacking selectivity, NONI exhibited good antagonist potency at α6β2* nAChRs (IC50 = 0.62 mM) (Crooks et al., 2004). NONI was also found to have good affinity for the BBB choline transporter (Crooks et al., 2004; Dwoskin et al., 2004; Lockman et al., 2008), suggesting good brain bioavailability due to its cationic quaternary ammonium structure.

Second generation libraries incorporated a second quaternary ammonium moiety into the scaffold of the N-alkylnicotinium analogs, generating bis-quaternary ammonium analogs containing diverse headgroups separated by variable alkane, alkene, or alkyne linker units. The most potent among them was bPiDDB (N,N'-dodecane-1,12-diyl-bis-3-picolinium dibromide; Fig. 3) (IC50 = 2 nM; Imax = 60% at α6β2*-containing nAChRs) - which had little or no affinity for either α4β2* or α7* nAChRs (Crooks et al., 2004; Dwoskin et al., 2004), and was an excellent substrate for the BBB choline transporter (Lockman et al., 2008). Pharmacokinetic studies carried out in rats treated with 14C-bPiDDB confirmed its brain bioavailability (Albayati et al., 2008). bPiDDB dose-dependently decreased nicotine self-administration in Sprague-Dawley rats with no effect on sucrose-maintained responding (Neugebauer et al., 2006), and attenuated hyperactivity produced by acute and repeated nicotine dosing. Interestingly, in rats repeatedly administered nicotine, the in vitro inhibitory potency of bPiDDB at α6β2* nAChRs increases significantly compared to that exhibited in similar in vitro assays in naïve rats (i.e., IC50 = 5 pM vs. 6 nM, respectively; Smith et al., 2010), demonstrating that repeated nicotine treatment may differentially regulate the stoichiometry and/or conformation of α6β2* nAChRs.

2.3 Exploring Analogs of bPiDDB. As part of an iterative SAR study, the effect of introducing an additional picolinium or other headgroups into the bPiDDB scaffold on inhibition of nicotine-evoked DA release was evaluated. Initially, three lead tris-quaternary ammonium molecules, TRIS-1, TRIS-2, and TRIS-3, emerged that were potent and selective inhibitiors of α6β2* nAChRs (IC50 = 0.2–4 nM; e.g. TRIS-1, Fig. 2; Zheng et al., 2007). However, further development of these molecules was abandoned due to toxicity at higher doses in nicotine self-administration studies in rats, and poor

affinity for the BBB choline transporter. The introduction of two additional picolinium headgroups into the bPiDDB scaffold resulted in tetrakis-analogs (e.g. TETRAKIS-1, Fig. 2) (Zhang et al., 2008), which were selective, lower potency antagonists at α6β2* nAChRs, but were not substrates for the BBB choline transporter, and were thus ineffective in decreasing nicotine self-administration in rats (unpublished results).

Replacement of the above quaternary ammonium head groups in the bis-, tris- and tetrakis-analogs with tertiary amine head groups is predicted to improve oral bioavailability and drug-likeness of such compounds. The resulting analogs should have good water-solubility, and cross the BBB independently of choline transporters due to passive diffusion through lipid membranes. Evaluation of a small library of these tertiary amine analogs as antagonists at α6β2* nAChRs identified several bis-analogs with IC50 values in the low nM or sub-nM range (Zhang et al., 2011).

Two of these bis molecules: r-b3,5L/3PiDDB and r-bPiDDB (Fig. 2) are chemically reduced analogs of bPiDDB, and both compounds potently and selectively inhibit nicotine-evoked [3H]-DA release (IC50 = 0.009–0.058 nM; Imax = 60–74%) at α-CtxMII-sensitive α6β2* nAChRs (Dwoskin et al., 2009; Smith et al., 2010). These analogs were more potent antagonists at α6β2* nAChRs compared to their corresponding quaternary ammonium counterparts (Zhang et al., 2011). Importantly, inhibition produced by a maximally effective concentration of r-b3,5L/3PiDDB and r-bPiDDB was not additive with a maximally effective concentration of α-CtxMII (Smith et al., 2010; Crooks et al., 2014), demonstrating interaction with the same nAChR subtypes with which α-CtxMII interacts. Also, r-b3,5L/3PiDDB and r-bPiDDB both decreased responding for i.v. nicotine in rats at doses that did not produce lethargy, weight loss, or other signs of toxicity, and that had no effect on food responding (Crooks et al., 2014).

r-bPiDI (Fig. 2), a structurally related analog of r-bPiDDB containing a C10 rather than a C12 n-alkane linker, exhibited decreased inhibitory potency (IC50 = 37.4 nM, Imax = 65%) compared to the above two C12 analogs, and did not inhibit [3H]nicotine or [3H]methyllycaconitine binding (Beckmann et al., 2013). Also, r-bPiDI inhibition of nicotine-evoked DA release was not different in the absence or presence of α-conotoxin MII, characterizing it as a potent and selective α6β2* nAChR antagonist. Acute systemic administration of r-bPiDI decreased nicotine self-administration with no effect on food-maintained behavior (Beckmann et al., 2013), indicating that r-bPiDI specifically decreases nicotine reinforcement. Thus, r-bPiDI, is another selective antagonist at α6β2* nAChRs.

The most recent drug-like antagonist identified in this series is r-b3EPDDB (Fig. 2) (pKa = 9.5; Log P = 5.2), a close structural analog of r-bPiDDB in which the two tetrahydro-3-picolino headgroups are chemically reduced to afford a tetrahydro-3-ethylpyridino headgroup. r-b3EPDDB exhibited a remarkable IC50 value of 2 pM in the nicotine-evoked [3H]-DA release assay (Imax = 80%) (Fig. 3). Data in Fig. 3 compare the IC50 and Imax values of r-b3EPDDB and b3EPDDB (the parent quaternary ammonium compound) in the nicotine-evoked [3H]-DA release assay, and demonstrate that chemical reduction of the bis-quaternary ammonium compound, b3EPDDB , affords an analog, r-b3EPDDB, that exhibits 60 times greater inhibitory potency at α6β2* nAChRs with no change in Imax. This highly potent inhibitor is being evaluated in nicotine self-administration studies in rats.

2.4 Summary. Striatal rat brain slices have been used to screen novel analogs that inhibit nicotine-evoked DA release in the search for potential smoking cessation agents. Several libraries of compounds were constructed and SAR generated. Evolution of the

initial series of quaternary ammonium compounds into tertiary amino analogs provided lead candidates with IC50 values in the pM range. r-bPiDDB, r-bPiDB, r-b3,5L/3PiDDB and r-b3EPDDB have been identified as four promising new analogs with drug-like physicochemical properties. These molecules are potent antagonists at α6β2-containing nAChRs. r-bPiDDB, r-bPiDB and r-b3,5L/3PiDDB specifically decrease nicotine self-administration in a rat behavioral model with no effect on food-maintained responding, and are potential preclinical leads for development as smoking cessation agents.

Our future aim is to enhance the drug-likeness of our lead compounds by improving water-solubility through introduction of hydrogen-bond acceptor moieties in the linker, and by decreasing conformational flexibility. We intend to identify orally bioavailable, drug-like preclinical leads for development as smoking cessation and/or relapse prevention pharmacotherapies. Current experiments are focused on determining if these analogues block cue-induced reinstatement of nicotine seeking.

3. Vaccines for the treatment of tobacco addiction (Paul Pentel)

3.1 Rationale. Existing medications used for the treatment of tobacco addiction (nicotine replacement products, buproprion, varenicline) are valuable, but their efficacy is modest. The neuronal pathways they target - which include those mediating reward, cognition and affect - are diverse and essential to many normal functions. Interrupting their function in order to treat tobacco addiction can interfere with normal functions and cause side effects, which limit the usable dose of medication and its efficacy.

3.2 Nicotine vaccines. Vaccination against nicotine provides an alternative medication strategy, targeting the drug rather than the brain (Bevins et al., 2008; LeSage et al., 2006b; Raupach et al., 2012; Shen et al., 2012). The nicotine vaccines most thoroughly

studied consist of nicotine conjugated (covalently attached) through a short linker to a foreign carrier protein. The nicotine binds and stimulates B lymphocytes, which will mature into antibody producing cells, and the carrier protein serves to activate T lymphocytes to provide signals required for B cell maturation (McHeyzer-Williams and McHeyzer-Williams, 2005). This construct is administered with an adjuvant, such as alum, which creates an immune-competent environment at the injection site (Awate et al., 2013).

Nicotine vaccines elicit production of nicotine-specific antibodies that can bind nicotine in serum or extracellular fluid, and reduce or slow its distribution to brain (Maurer et al., 2005; Pentel et al., 2006; Satoskar et al., 2003). The antibodies themselves are excluded from the brain because they are too large to cross the blood-brain barrier. In animals, vaccination blocks or attenuates a wide range of nicotine-related behaviors including the acquisition, maintenance, and reinstatement of nicotine self-administration at clinically relevant nicotine doses (LeSage et al., 2006a; Lindblom et al., 2002). Nicotine-specific antibodies bind nicotine and its minor but active metabolite nornicotine, but essentially nothing else, including acetylcholine (the natural ligand of nicotinic cholinergic receptors), other neurotransmitters, or medications with similar structures. This specificity, and the exclusion of antibodies from the brain, explains the absence of side effects (other than transient minor discomfort at the injection site) of this approach in both animals and humans.

3.3 Vaccine efficacy. As the goal of vaccination is to bind as much drug as possible, efficacy is highly dependent upon the concentration (often estimated as titer) of antibody in serum. High antibody levels can be produced in rodents, but this often requires large vaccine doses, or routes of administration and adjuvants that are unacceptable for

humans. Antibody levels achieved in clinical trials of nicotine vaccines have been considerably lower than in rodents (Keyler et al., 2008; Maurer et al., 2005). In addition, antibody levels in both animals and humans show high individual variability (Cornuz et al., 2008; Hatsukami et al., 2011). Reliably achieving high antibody concentrations has emerged as the principal challenge for translating vaccines into clinical use.

Clinical trials of nicotine vaccines have not shown the efficacy seen in animal studies. A phase II clinical trial of one nicotine vaccine (NicVax) showed a doubling of smoking cessation rates (Hatsukami et al., 2011), but follow-up Phase III trials of the same vaccine showed no treatment effect (Fahim et al., 2013). However, subgroup analysis of the phase II study showed efficacy in the 30% of subjects with the highest serum antibody levels, and a similar subgroup finding emerged from a phase II study of an unrelated nicotine vaccine (NicQb) (Cornuz et al., 2008). These analyses suggest that nicotine vaccines may be effective if sufficient antibody levels can be consistently achieved. Current efforts to improve vaccines involve modifications of conjugate vaccine design, development of nanoparticle vaccines, use of newer adjuvants, combining vaccines with each other or with other types of medications, and passive immunization through gene transfer.

3.4 Improving conjugate vaccines. Nicotine that is modified to allow placement of a linker is referred to as a hapten. Many nicotine haptens, with different linker positions, have proven useful, and none is clearly superior under all conditions (Isomura et al., 2001; Pravetoni et al., 2012). A conformationally constrained hapten that prevents rotation of the pyridine and pyrrolidine rings improved immunogenicity of one nicotine vaccine (Moreno et al., 2012). Fluorination, widely used in medicinal chemistry to improve ligand binding to receptors, also improved a model nicotine immunogen (Cai et al., 2013).

Overall, however, modifications in hapten design have provided only modest benefit. Similarly, many linker lengths and structures have proven effective with particular haptens but no one structure emerges as consistently best.

A variety of carrier proteins have been used in nicotine vaccines, generally selected from proteins known to elicit strong immune responses on their own. Tetanus toxoid, recombinant diphtheria toxin (CRM197), and keyhole limpet hemocyanin are most commonly used, but others are also effective. Intact or disrupted viral capsid can serve the same purpose (Cornuz et al., 2008; De et al., 2013). The choice of carrier protein for a particular hapten remains highly empirical. T cell activation is provided by peptide sequences with the carrier proteins, and these peptides can serve in place of the full protein. To date, this approach has worked but has not been more effective than using the whole protein.

3.5 Nanoparticle vaccines. Nanoparticle vaccines are being developed based on liposomes, synthetic polymers, or novel materials such as DNA (Matyas et al., 2013; Peek et al., 2008). Several are effective in animals and have entered clinical trials, but results are not yet available (Kishimoto et al., 2012). Nanoparticles function as a scaffold to which essential vaccine components can be attached or incorporated with a high degree of control. Liposomes or polymer spheres can have their surfaces modified to allow attachment of nicotine or other vaccine components, enabling their display to immune cell surface receptors. Other components, such as T-cell help peptides, can be encapsulated for delivery to cytoplasmic receptors. Nanoparticles allow greater control of hapten, T cell help peptide, and adjuvant density and spacing than conjugate vaccines. Some, such as those based on DNA, can also achieve precise control of size and shape

that may prove useful to optimize vaccine uptake by phagocytic cells or transport to regional lymph nodes (Liu et al., 2012).

3.5 Multivalent vaccines. It is well established that different vaccines can be combined and administered in the same injection with little or no loss of their individual activities, as is routinely done with common infectious disease vaccines such as MMR (measles, mumps, rubella). Some nicotine vaccines can be combined to address problems of low and variable antibody levels (Keyler et al., 2008). Nicotine haptens that have linkers at different positions can act as distinct immunogens, stimulating different populations of B cells, and eliciting antibodies that do not cross-react (Pravetoni et al., 2012). Each hapten elicits antibodies against nicotine, but these antibodies recognize different features of the nicotine molecule.

In rats, combining 3 nicotine immunogens based on haptens with linkers at the 1' or 3' position of the pyrrolidine ring, or 6 position of the pyridine ring, produced additive antibody titers and greater ability to prevent nicotine distribution to brain than a monovalent vaccine (de Villiers et al., 2013). In addition, some rats with poor responses to one immunogen had brisk responses to one of the others. This independence of response reduced the number of low- or non-responders that had ineffectual antibody titers. An appealing feature of this strategy is that it is quite general, and could be applied to other nicotine vaccines - even those based on very different designs such as nanoparticles, provided that the haptens used incorporate appropriate linker positions.

3.6 Passive immunization and gene transfer. Immunization can be accomplished actively by vaccination or passively through the transfer of pre-formed nicotine-specific monoclonal antibody. Passive immunization can achieve the same effects in animals as

vaccination (Carrera et al., 2004; Keyler et al., 2005), but offers some advantages. The dose of monoclonal antibody, and resulting serum antibody levels, can be controlled to avoid the problem of non-responders, and the amount administered can be increased to provide a higher serum antibody concentration than that achievable with even the best vaccines. Passive immunization also has an immediate onset of effect, whereas vaccination takes weeks to months. The main limitation of passive immunization is the cost of monoclonal antibodies, and the doses needed for this application are high (Roiko et al., 2008). No clinical trials of passive immunization for nicotine have been reported, but if costs are reduced, it will merit further study.

An alternative means of delivering monoclonal antibody is administration of viral vectors containing genes for antibody expression. Vectors based on adeno-associated virus (AAV) have been used to produce very high levels of nicotine-specific antibody expression in mice, resulting in reduced nicotine distribution to brain and attenuated physiologic and behavioral effects (Hicks et al., 2012). These vectors have shown no important toxicity in clinical trials for the transfer of other types of genes (Mingozzi and High, 2011). If safety is confirmed with more extensive use, and high levels of expression can be achieved in humans, gene therapy could prove an effective means of providing therapeutic antibodies for tobacco or other addictions.

3.7 Combining immunotherapy with medications. There are compelling reasons to consider combining vaccines or passive immunization with medications that act via different mechanisms to assist with treating tobacco dependence. Even if very high nicotine-specific antibody levels can be reliably achieved through one or more of the strategies described above, immunotherapy is likely to have limits on both its magnitude and spectrum of therapeutic effects. Reasons include: 1) Immunotherapies reduce, but

do not completely prevent, nicotine distribution to brain, and even low brain concentrations of nicotine can produce substantial nicotinic receptor occupancy (Brody et al., 2009); 2) Immunotherapies can act only when nicotine is present for antibodies to bind. Effects that may occur when nicotine is no longer present, such as withdrawal and craving, cannot be directly blocked using vaccines; 3) Minor alkaloids and other components of tobacco may contribute to tobacco addiction, and are not bound or blocked by nicotine-specific antibodies (Hoffman and Evans, 2013).

As proof-of principle, a combination of the nicotinic receptor antagonist mecamylamine and passive immunization with a nicotine-specific monoclonal antibody (Nic311) was studied and these were found to have strong synergistic effects in rats (LeSage et al., 2012). Mecamylamine can block nicotine actions, but use in humans for this purpose is not feasible because it has side effects due to blocking the actions of endogenous acetylcholine. However, combining a low dose of mecamylamine with a low dose of antibody produced complete blockade of nicotine discrimination even though the individual therapies at these doses were essentially without any effect (Figure 4). These preliminary data support further study of immunotherapy/medication combinations to enhance treatment efficacy.

3.8 Summary. Nicotine vaccines appear quite effective in animals, but have been disappointing in clinical trials for smoking cessation. Many options are under development for improving vaccine efficacy or providing antibody through alternative strategies. These advances are likely to provide the higher serum nicotine-specific antibody levels needed to test the therapeutic potential of immunotherapy.

4. Reducing levels of nicotine in cigarettes (Dorothy Hatsukami)

4.1 Introduction. Rather than targeting the smoker, another area of tobacco addiction intervention is altering the tobacco product itself. Reducing the addictiveness or appeal of cigarettes as a national regulatory measure can potentially lead to significant public health benefits by reducing the prevalence of smoking. Prior reports have stated that prevention of tobacco use, cessation of its use, and reduction of tobacco-caused mortality are greatly impeded by the addictive nature of cigarettes (WHO Study Group on Tobacco Product Regulation, 2012; USDHHS, 1988). As a result, Benowitz and Henningfield (1994) proposed a gradual reduction of nicotine levels in all marketed cigarettes over 10-15 years, with the goal of preventing nicotine addiction in youth. This proposal can also facilitate abstinence among those who are already addicted to cigarettes. For many years, this concept was not fully embraced by the public health community because no governmental agency had regulatory authority to require the reduction of nicotine levels in tobacco products. However, since 2009, when the Family Smoking Prevention and Tobacco Control Act (FSPTCA) was enacted, the U.S. Food and Drug Administration now has jurisdiction over some tobacco products, and the law allows a reduction in levels of nicotine in these products to the point that they are rendered non-addictive, but not to zero.

4.2 Reducing nicotine content. Research in animals (e.g., Donny et al., 2012) and humans (e.g., Hatsukami et al., 2010a) have been conducted that supports the viability of reducing nicotine to non-addictive levels in cigarettes. Most animal studies have shown an inverted U-shaped dose-response curve for nicotine self-administration and that generally doses less than 10 □g/kg are not self-administered more than saline, although in some studies the dose needed to be lower than 3.75 □g/kg (Donny et al., 2012; Smith et al., 2013; Grebenstein et al., 2014). In humans, four clinical trials

examined the effects of reducing nicotine in cigarettes either gradually (Benowitz et al., 2007, 2012) or immediately to very low levels (Hatsukami et al., 2010b, 2012). In general, these studies have shown that cigarette consumption and carbon monoxide (CO) exposure were significantly reduced with very low nicotine content (VLNC) cigarettes (< 0.1 mg nicotine yield), with some compensatory smoking occurring with cigarettes containing > 0.2 mg nicotine yield. There was no evidence of increased exposure to toxicants, nor evidence of adverse effects on cardiovascular biomarkers, even at higher doses of reduced nicotine content (RNC) cigarettes (Benowitz 2007, 2012). Decreased exposure was seen at the lowest nicotine content cigarettes (< 0.05 mg; Hatsukami et al., 2010; 2013). It is important to note that these cigarettes are unlike the “light” and “ultralight” cigarettes, marketed as generating reduced tar and nicotine yield, but resulted in levels of nicotine and toxicant exposure that were similar to “regular” cigarettes (NCI monograph 13). The “light” and “ultralight” cigarettes had the same amount of nicotine content as regular cigarettes, but were reduced in machine-determined nicotine yields, primarily through filter ventilation holes. The RNC cigarettes have reduced nicotine in the tobacco itself, making compensation at very low doses difficult.

These clinical trials also demonstrated no substantial withdrawal symptoms with the switch to RNC cigarettes, and minimal withdrawal when smokers quit using VLNC cigarettes (Benotwitz 2012; Hatsukami et al., 2010b; 2013). Similarly, smokers reported reduced dependence scores with VLNC cigarettes. Although the primary outcome of these studies was not cessation, in the Hatsukami et al. studies, in which participants were motivated to quit smoking, the 7-day point prevalence biochemically verified (cotinine and CO) quit rates with the VLNC was 36% at 6 weeks post-treatment in one

study (Hatsukami et al., 2010b), and 24% at 6 weeks post-product assignment in another study (Hatsukami et al., 2013). For the Benowtiz studies, in which smokers were not motivated to quit, rates ranged from 4% at the end of taper, when nicotine content was changed on a monthly basis (Benowtiz et al., 2012), to 25% when nicotine content was reduced on a weekly basis in a separate study (Benowitz et al., 2007).

4.3 Gradual reduction vs. immediate reduction. To date, minimal studies have examined the best approach to reducing levels of nicotine in cigarettes - that is, gradual reduction over time, or an immediate reduction to non-addictive levels on a specified date. In one study, Smith et al., (2013) showed that whether nicotine doses were reduced immediately or gradually in rats, reduction to 3.75 □g/kg led to significant changes in nicotine self-administration with no significant compensation (increased infusions in response to lowered nicotine dose). Reduction in nicotine doses above 3.75 □g/kg led to limited and temporary compensation. Although, no human clinical trials have been conducted to directly compare these two approaches. Nevertheless, similar to the findings in the animal study, in the Benowitz et al. studies (2007, 2012), with gradual reduction, modest increases in numbers of RNC cigarettes smoked and CO exposure were observed, with no significant change in exposure to toxicants in the initial phases of reduction. No substantial decrease would be seen until nicotine content is reduced significantly. However, no major withdrawal or discomfort may be experienced during the reduction period. On the other hand, with the abrupt reduction to VLNC cigarettes, a more immediate effect is observed in the reduction in numbers of VLNC cigarettes smoked, and consequently more immediate reduction in exposure to toxicants (Hatsukami et al., 2010b, 2013). This approach has the advantage of benefiting public health sooner. Furthermore, the number of individuals likely to engage in significant

compensatory smoking is likely to be limited because the dose of nicotine is so low, whereas with higher RNC cigarettes, greater variability in compensatory smoking is likely to be experienced. However, an immediate reduction in nicotine to very low levels may generate greater discomfort in smokers, particularly in heavily dependent smokers.

4.4 Moderating responses to VLNC cigarettes. Several factors may moderate or mitigate discomfort experienced when switching to a VLNC cigarette, including use of medicinal nicotine products, other medications to reduce withdrawal, or alternative less harmful non-combusted tobacco products (e.g., electronic cigarettes). Few studies have examined this area. One study compared the effects of VLNC (0.05-0.9 mg nicotine yield) cigarettes alone, nicotine patch (NP) alone, and a combination of NP and VLNC cigarettes over the course of 6 weeks (Hatsukami et al., 2013). The combination approach led to fewer VLNC cigarettes smoked and lower CO exposure, than VLNC cigarettes alone, and fewer usual brand cigarettes smoked than the other two conditions. In addition, significantly less severe withdrawal was experienced when smokers were switched from usual brand cigarettes to combination compared to NP condition, and near significant differences when compared to VLNC cigarettes alone. Another study also observed that the combination approach leads to lower consumption of usual brand cigarettes and total number of cigarettes smoked than VLNC alone (Rose et al., 2006). In another short-term 10-day study (Donny and Jones, 2009), smokers were assigned to one of four conditions: 1) placebo patch (PP) plus 0.6 mg nicotine yield cigarette; 2) 7 mg NP plus 0.05 mg nicotine yield (VLNC) cigarette; 3) 21 mg NP plus VLNC cigarette. This study also found that subjects assigned to NP (21 mg and 7 mg) plus VLNC cigarettes, compared to PP plus VLNC cigarettes, showed greater reductions in the numbers of VLNC cigarettes smoked, in total volume of inhaled cigarette smoke, and

fewer withdrawal symptoms. These studies show beneficial effects when combining VLNC with medicinal nicotine, demonstrating less discomfort and need to smoke either usual or experimental cigarettes.

Relatively few studies have examined the effects of a combination approach relative to VLNC cigarettes to facilitate cessation. In the study conducted by Hatsukami (2013), an exploratory analysis of the data showed no differences in cessation rates between the combination vs. VLNC or NP alone. A large clinical trial had been conducted, in smokers who called a telephone-based cessation support system. Abstinence rates were compared in smokers who were randomly assigned to usual care involving both medicinal nicotine products and behavioral treatment plus VLNC (0.05 mg nicotine yield) cigarettes versus usual care alone. The usual care plus VLNC cigarettes had higher 7-day point prevalence abstinence rate at the 6-month follow-up compared to usual care (33% vs. 28%, RR=1.18, 95% CI 1.01, 1.39), and higher continuous abstinence rates (23% vs. 15%, RR=1.50, 95% CI 1.20, 1.87).

4.5 Reducing nicotine in vulnerable populations. One concern is the effect of reducing nicotine content cigarettes in vulnerable populations, such as smokers who have serious mental illnesses. While no clinical trials have been conducted, a laboratory study showed no adverse effects of these cigarettes in smokers with schizophrenia (Tidey et al., 2013). In this study, smokers with schizophrenia and a control group of smokers without psychiatric diagnoses underwent 5 laboratory sessions, during which products were used under controlled conditions for 5 hours: 1) usual brand cigarettes; 2) VLNC cigarettes with 42 mg NP; 3) VLNC cigarettes with PP; 4) no cigarettes with 42 mg NP; and 5) no cigarettes with PP. After the session of product use, participants were allowed to smoke cigarettes ad libitum during a 90-minute period. The results showed that

smoking VLNC cigarettes reduced usual-brand smoking and withdrawal symptoms, did not worsen psychiatric or performance measures, and were reported to be acceptable among smokers with schizophrenia. The addition of an active patch had no effect in this population, which may be a function of the short duration of this study or the fact for smokers with schizophrenia, the act of smoking has greater valence.

4.6 Remaining questions and future: Although studies thus far support a reduction of nicotine content in cigarettes, many issues remain. These include:

- Determining the dose of nicotine that will facilitate cessation and/or minimize the development of addiction among those who experiment with cigarettes.

- Directly comparing a gradual reduction in nicotine content of cigarettes with an immediate reduction to a non-addictive level of nicotine content.

- Determining the effects of RNC cigarettes in vulnerable populations of smokers (e.g., those with co-morbid disorders) in clinical trials.

- Examining other factors that might moderate responses to RNC cigarettes and determining the variability in response.

- Identifying and examining ways to mitigate negative consequences resulting from reducing nicotine levels in cigarettes.

Some of these gaps are already being addressed by NIH grants oriented towards developing tobacco regulatory science. It is important to recognize that reducing level of nicotine in cigarettes alone may not be sufficient. Levels of nicotine in all combustible tobacco products will be required to dramatically reduce death and disease associated with smoking .Should a policy to reduce nicotine to non-addictive levels in all

combustible tobacco products marketed in the U.S. be enacted, it would be critical to establish a surveillance system able to track its impact.

4.7 Summary: The future for tobacco control is bright. We not only have effective existing methods of reducing the prevalence of smoking (e.g., comprehensive smoking bans, increased taxes on cigarettes, anti-smoking media campaigns), we can now regulate the contents of cigarettes under the FSPTCA and Article 9 of the World Health Organization Framework Convention on Tobacco Control. Regulation of nicotine content can reduce or eliminate the use of the deadliest tobacco products sold to consumers (i.e., combusted tobacco products), which would lead to a substantial reduction in tobacco-caused death and disease.

5. Overall Conclusion

The neurophysiological etiology of tobacco addiction is complex, involving both genetic predispositions and environmental influence. Therefore, a broad-based set of interventions ranging from specific neurobiological targets for medications to policies must be considered. The wide range of signaling pathways and circuits that undergo neuroplastic adaptation during prolonged use render quitting very difficult. Effective treatments will need to confront the spectrum of systems involved in dependence and withdrawal. Accordingly, studies of approaches to treating different components of addiction will be critical to revealing comprehensive therapies. Through work with genetically modified mice, we have learned that particular nAChR subunits appear to mediate specific symptoms of nicotine withdrawal, representing potentially druggable targets for more effective pharmacological intervention. The development of highly specific drugs, capable of attenuating or abolishing downstream dopaminergic signaling

in response to nicotine intake, may present the ability to prevent the appeal of cigarettes by blocking their rewarding effects. Innovative approaches to preventing the effects of nicotine by taking advantage of immune system-mediated elimination of the drug may represent another method by which tobacco might be rendered ineffective – and, therefore, unappealing to individuals attempting to quit. As research continues to find new ways to effectively treat smokers, the prospect of reducing nicotine levels in tobacco products through federal regulation may reduce consumption of combusted tobacco products – and, ultimately, reduce the prevalence of tobacco-associated disease.

As this research begins to yield a wider variety of options for smokers attempting to quit, clinicians will be capable of providing treatments with fewer side-effects that are better tailored to the unique characteristics of patients. Additionally, the ability to combine therapies will assist in the treatment of cravings – as well as physical, emotional, and cognitive withdrawal symptoms. Such targeted therapies, or combination of targeted therapies, represent opportunities to more effectively ameliorate the burden of tobacco-associated disease on public health.

Figure legends.

Fig.1 Lack of β4, α5 or α2 nAChR subunits protects against increases in nicotine withdrawal-induced somatic signs. Various nAChR null mice and their wild-type littermates were treated chronically with nicotine (24mg/kg/day free base) or saline, using a mini-osmotic pump, for two weeks. On day 14, each mouse received a 3 mg/kg injection of the nonselective nicotinic antagonist, mecamylamine. Somatic signs were measured for 20 min. Mecamylamine treatment precipitated increases in somatic signs in wild-type, β2 null, and α7 null mice chronically treated with nicotine. Such increases were not observed in β4, α5, or α2 null mice - suggesting that nAChRs containing these subunits participate in the modulation of nicotine withdrawal symptoms. *$p<0.05$. The numbers within the bars indicate the number of animals tested for each strain.

Fig. 2 Chemical structure and pharmacological characteristics of potential smoking cessation agents. IC50 values of for the inhibition of NIC-evoked [3H]DA release from rat striatal slices for NONI, bis-, tris, and tetrakis quaternary ammonium compounds, and the bis-tertiary amino analogues (r-series compounds).

Fig. 3 b3EPDDB derivatives inhibit dopamine release in vitro. b3EPDDB (left) and r-b3EPDDB (right) both inhibit NIC-evoked [3H]DA release from rat striatum in vitro in a concentration-dependent manner. n = 5-8/analog. Data are mean ± SEM total [3H] overflow.

Fig. 4 Synergistic effect of mecamylamine and the nicotine-specific monoclonal antibody Nic311 for blocking the subjective effects of nicotine. Rats were trained in a two-lever nicotine discrimination assay. Data are the mean ± SE % responding on the nicotine lever during consecutive daily sessions with the 0.4 mg/kg s.c. nicotine training dose. Nic311 or control antibody was administered i.v. one day prior to session 1, and mecamylamine or saline was administered s.c. 15 minutes prior to each session. Dashed lines indicate criterion levels of performance for discrimination of the 0.4 mg/kg nicotine training dose. Significantly different from Control antibody+Saline, *p<0.05, ***p<0.001. Significantly different from Nic311+Saline, ##p<0.01, ###p<0.001. Reproduced from (LeSage et al., 2012).

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