THE UNIVERSITY OF CHICAGO

NEURAL SUBSTRATES OF THE AVERSIVE EFFECTS OF NICOTINE

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ABSTRACT

Nicotine addiction remains a major health problem in the US and throughout the world. Nicotine has rewarding effects at relatively low doses and intensely aversive effects at higher doses. The projection from the medial habenula (MHb) to the interpeduncular nucleus (IPN) contributes to these aversive effects. This "aversive" pathway may influence the development and maintenance of nicotine dependence and also contributes to withdrawal effects. Thus, improved understanding of the mechanisms that underlie the aversive effects of nicotine may be important in developing more effective therapies for smoking cessation.

Enhanced activity in the MHb-IPN enhances aversion to nicotine, while decreased activity in this pathway increases appetitive responding for nicotine doses that were previously aversive. Although these results implicate the MHb-IPN circuitry, the downstream post-synaptic targets of the IPN that mediates these effects remain largely uncharacterized. The aversive effects of nicotine may occur through an indirect suppression of the excitability and output of VTA dopamine (DA) neurons. Burst activity in DA neurons is important for reward-associated behaviors, and aversive experiences can suppress DA neuron activity. While the IPN projects to several brain areas, it strongly innervates the lateral dorsal tegmental nucleus (LDTg), a brainstem cholinergic center that controls burst firing of VTA DA neurons. Therefore, we hypothesized that IPN projections to LDTg are inhibitory. We expressed Channelrhodopsin (ChR2) in IPN neurons and stimulated the terminals with light while recording specifically from LDTg neurons that project to the VTA. We found that light-evoked synaptic inputs were blocked by the GABAA receptor antagonist bicuculline. Additionally, optogenetic stimulation of either the IPN directly, or the IPN terminals in the LDTg specifically, results in aversion. We are testing the modulation of these inhibitory inputs by high and low concentrations of nicotine, and

preliminary evidence suggests that high concentrations of nicotine selectively enhance light-evoked GABAergic currents from the IPN onto LDTg neurons that project to the VTA. We have also found evidence that optogenetically inhibiting the IPN terminals in the LDTg not only reduces aversion to a high dose of nicotine, but actually shifts the aversion to reward. These findings highlight the importance of the IPN-LDTg connection in mediating the aversive effects of nicotine and provide further insights into the ways in which reward and aversive circuitries interact to produce an overall affective state.

Chapter 1

Introduction

(1a) Summary.

Nicotine addiction remains a major public health problem in the US and throughout the world. Like many other substances, nicotine has different effects at different doses. At relatively lower doses, nicotine has rewarding effects, but at higher doses, nicotine can be intensely aversive. Despite a substantial body of research investigating the mechanisms by which nicotine results in rewarding effects and the role of those effects in the development and maintenance of nicotine addiction, treatments for smokers who want to quit have been only marginally effective at best. However, understanding the mechanisms by which nicotine mediates aversive effects and the impact of those effects on addiction have been largely neglected. Therefore, the focus of my graduate work has been to elucidate the neural pathways that mediate the aversive effects of nicotine and to determine how these pathways might interact with reward circuitry.

The experimental aims were designed to assess the functional role of a known anatomical connection between a nucleus known to be important for aversion to nicotine and a nucleus known to be important for reward. In addition to understanding the functional role of this connection, I designed experiments to investigate the effects of nicotine at this synapse. First, functional connectivity was assessed using optogenetic tools and whole-cell patch-clamp electrophysiology in acute brain slices. Identification of relevant neurotransmitters was achieved using antagonists of fast transmission. Behavioral relevance of this connection was then assessed

using behavioral assays and optogenetic methods. Second, nicotinic modulation of this connection was assessed using whole-cell patch-clamp electrophysiology in brain slices and application of a range of nicotine concentrations. Optogenetic tools were also used in a variety of experiments. Lastly, in order to assess the role of this connection in mediating the behavioral effects of nicotine, behavioral assays and optogenetics were used in tandem with administration of various doses of nicotine. This introduction provides background and rationale for these experimental aims.

(1b) Nomenclature: Nicotinic Acetylcholine Receptors.

Throughout this document, a number of different terms will be used to describe nicotinic receptors (nAChRs). The term **receptor** will refer to the entire ligand-gated ion channel rather than to a specific site that recognizes acetylcholine or nicotine. nAChRs are ion channels that are made up of five **subunits**, each of which is a discrete protein. There are many types of α and β subunits, and they can combine in a variety of ways to form a variety of different types of functional receptors. The various combinations will be referred to as receptor **subtypes** (i.e. α4β2 is a relatively high affinity nAChR subtype, while the α3β4 subtype is a relatively lower affinity receptor). When a receptor is made up of five identical subunits (e.g. the α7 receptor), the term α7 can refer to either the receptor subunit or the receptor subtype.

An **agonist** is a chemical that binds to and activates a receptor. Acetylcholine is the endogenous agonist for nAChRs. Nicotine is an exogenous agonist at nAChRs. When either of these chemicals binds to the receptor, the ion channel opens. An **antagonist** is a drug that inhibits or blocks the ability of agonists to produce their effects.

(1c) Nomenclature: Optogenetics.

The term **optogenetics** refers to the relatively new set of tools that utilize genetically specific expression mechanisms and light-sensitive proteins to control spatially and genetically defined populations of cells. There are myriad approaches to achieving genetic specificity for targeted protein expression, but the one that I have utilized in my graduate work is viral expression. Briefly, all of the genetic material required for replication is removed, while the genes that allow for incorporation of viral genes into the host organism are conserved. Genes for expression of the desired protein are added, along with a promoter region that targets protein expression to desired cell-types. There are three proteins that will be referred to repeatedly throughout this document that were expressed in neurons using this viral method: **channelrhodopsin** (ChR2), **archaerhodopsin** (ARCH), and **enhanced yellow fluorescent protein** (EYFP). Channelrhodopsin is a non-selective cation channel that is activated by blue (473 nm) light. It is used to enhance activity of neurons by depolarizing the cell. Archaerhodopsin is a proton-pump that is activated by green (532 nm) light. It is used to inhibit activity of neurons by hyperpolarizing the cell. EYFP is a fluorescent protein that emits yellow light when activated by blue light. EYFP is often attached to ChR2 and ARCH as a reporter so that successful expression of proteins can be visually confirmed. It is also often expressed on its own in a separate group of animals as a control for viral infection and expression of foreign proteins. Figure 1.1 shows a schematic of these three proteins and their functions. More details on technical considerations will be provided in the Methodology section.

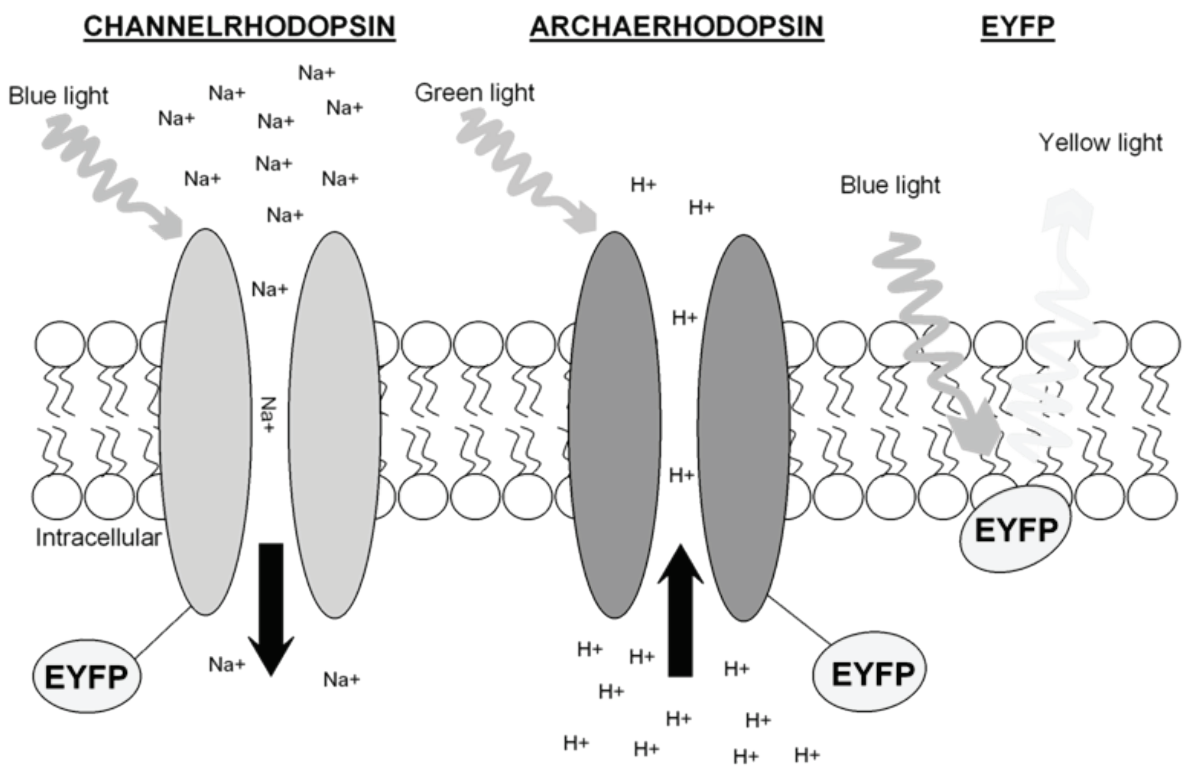

Figure 1.1- Schematic of Optogenetic Proteins. Channelrhodopsin (ChR2) is a non-selective cation channel that is activated by blue light. Exogenous proteins are often tagged with EYFP so that expression can be visualized. When open, there is a large influx of sodium into the cell, resulting in depolarization and enhanced excitability. Archaerhodopsin (ARCH) is a proton pump that is activated by green light. When activated, protons are extruded from the cell, resulting in hyperpolarization and diminished excitability. EYFP is a fluorescent protein that can be visualized under a fluorescent microscope by emitting yellow light in response to exposure to blue light.

(1d) Nicotinic Acetylcholine Receptors.

Acetylcholine binds to two different classes of receptors- nicotinic receptors and muscarinic receptors. Nicotinic receptors are ligand-gated ion channels, and muscarinic receptors are G protein-coupled receptors. Although muscarinic receptors are important for many neural functions, the focus of this thesis will be on nicotinic receptors.

Nicotinic receptors are part of a superfamily of ligand-gated ion channels that includes the $GABA_A$ receptor, the glycine receptor, and the $5HT_3$ serotonin receptor (Papke, 2014). Nicotinic receptors are made up of five subunits, and each subunit consists of four transmembrane domains. The five subunits together form a central aqueous pore, lined by the M2 transmembrane domain from each subunit (Cooper et al., 1991). This pore opens to allow cation flux in the presence of acetylcholine or other agonists. The extracellular domains of the subunits form ligand binding sites, and the intracellular domains allow for modulation of the receptors (Karlin, 1993). This structure seems to be conserved for both nAChRs at the neuromuscular junction (NMJ) and neuronal nAChRs (Martyn et al., 2009).

Nicotinic receptors can be broadly classified as either muscle type or neuronal type. Muscle type nAChRs are highly concentrated at the NMJ endplate, while the neuronal type is found in the brain. These two types of nAChRs have distinct subunit compositions. Muscle type nAChRs are composed of a combination of five different subunits: 2 α, 1 β, 1 γ (or ε), and 1 δ (Mishina et al., 1986). Neuronal nicotinic receptors have more diversity in their subunit compositions. These consist of 9 α subunits (α2-10) and 3 β subunits (β2-4) (Zoli et al., 2015), and have been the focus of my thesis work.

All α subunits share a highly conserved set of six amino acids, including a pair of cysteine residues (Cys 192/193) that share a disulfide bond. These cysteine residues are thought

to be critical to the binding region that exists between adjacent subunits (Karlin, 1993; McGehee and Role, 1995). The β subunits have also been referred to as non-α subunits because they lack these neighboring cysteine residues (Cooper et al., 1991; McGehee and Role, 1995). Therefore, it seems that at least 2 α subunits are required to form a functional receptor such that there cannot be a β subunit homomeric receptor, but there can be α subunit homomeric receptors. Additionally, binding sites will only be present between two α subunits or between an α and a β subunit. Therefore, homomeric receptors will have more binding sites than will heteromeric receptors (Gotti et al., 2006). The numerous nAChR subunits can combine to form a variety of different receptors, and these can vary widely in terms of pharmacological and biophysical characteristics (Zoli et al., 2015). Figure 1.2 shows the structure of nAChR subunits and receptors. Importantly, there are also 2 subunits (α5 and β3) that can only act as accessory subunits (i.e. there can be no α5β2 receptor, but there can be an α5α4β2 receptor) (Ramirez-Latorre et al., 1996; Groot-Kormelink et al., 1998). These accessory subunits can impact functional properties of the receptors and will be discussed more thoroughly in a future section.

Neuronal nicotinic receptors can be further divided into two general classes: α7 and non-α7. Although α7 receptors are not the only subunits that can comprise homomeric receptors, they are by far the most prevalent homomeric receptors in the mammalian brain (Dani, 2001). NAChRs in general are unique in that they are calcium permeable (Fucile, 2004; Shen and Yakel, 2009). Unlike voltage-gated calcium channels or NMDA receptors, nAChRs do not require depolarization for calcium influx to occur. The α7 receptor in particular has high calcium permeability (Fucile, 2004).

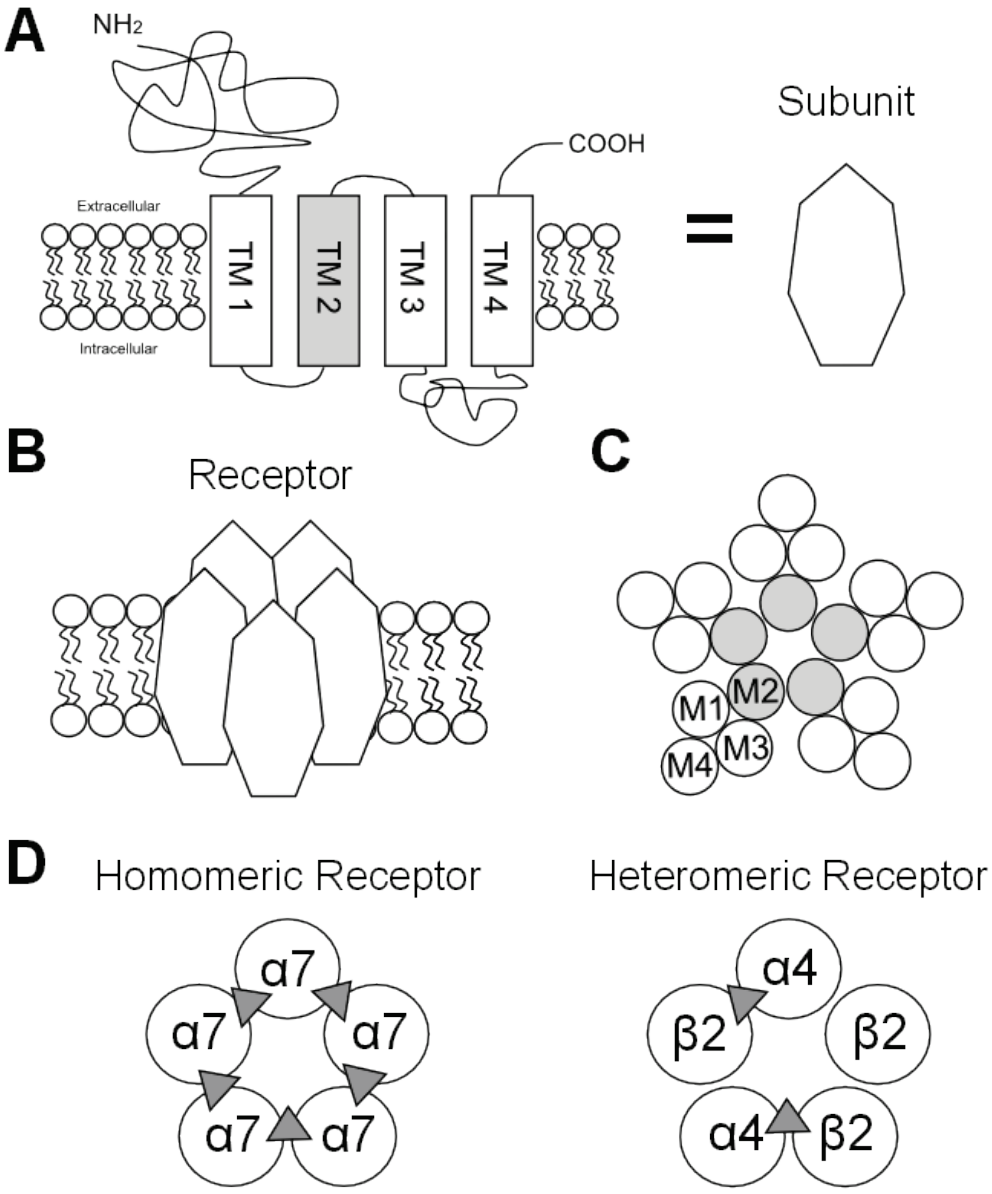

Figure 1.2- Structure of the Nicotinic Receptor. A) The nicotinic receptor subunits are made up of four transmembrane domains, an extracellular N-terminus, and an intracellular C-terminus. The TM2 is thought to line the channel pore. B) Schematic showing that five subunits combine to form a functional nAChR. C) Schematic of a cross-section of the pentameric receptor showing the organization of the transmembrane domains. D) α7 subunits assemble to form homomeric receptors. Because they consist of all α subunits, which contain the vicinal cysteines, an ACh binding site (triangles) is found between each subunit. Heteromeric receptors form from a combination of α and β subunits. Because the β subunit lacks the cysteine residues, ACh binding sites are only found between α and β subunits.

(1e) Localization of nAChRs.

Nicotinic receptors are found in many regions throughout the brain and implicated in many neural processes (Wada et al., 1989; ZM et al., 2003; Gotti et al., 2006; Albuquerque et al., 2009; Fowler et al., 2011; Hsu et al., 2013a; Mineur et al., 2015; Zhou et al., 2015). The receptor subtypes in these various regions often differ, which presents obstacles to fully understanding the role of nAChRs in behavior. Nicotinic receptors are also expressed on axon terminals, axons, dendrites, somata, and intracellular organelles (McGehee et al., 1995; Nashmi and Lester, 2006; Grady et al., 2007; Henderson and Lester, 2015). Figure 1.3 diagrams some of the known locations of nAChRs in the brain and in neurons. The challenges associated with understanding the roles of such a diverse and variously distributed population of receptors are compounded by technical obstacles.

One relatively straightforward approach to identifying the role of a receptor is the use of pharmacological tools. However, small molecule drugs that bind to nAChRs lack specificity, particularly *in vivo* (Gotti et al., 2007; Letchworth and Whiteaker, 2011). Additionally, despite some selectivity of different agonists and antagonists, there are so many combinations of subunits that form functional receptors that identifying the specific receptor complex is nearly impossible (Chavez-Noriega et al., 1997). This is a critical issue because inclusion of various subtypes in the receptor can alter the functional properties of the receptor (Luetje and Patrick, 1991; Kuryatov et al., 2000; Rush et al., 2002; Grady et al., 2010). Furthermore, even the stoichiometry of the receptor subunits (i.e. $(\alpha4)_2(\beta2)_3$ vs. $(\alpha4)_3(\beta2)_2$) can alter the function of the receptor (Moroni et al., 2006; Tapia et al., 2007; d'Incamps and Ascher, 2014). Even if pharmacological approaches alone could conclusively identify which subunits make up a

receptor, it is extremely challenging to distinguish the stoichiometry (d'Incamps and Ascher, 2014).

Another approach that can help to ascertain the role of nAChRs in various processes is to determine their localizations in the brain region or neuronal region of interest. *In situ* hybridization has been used successfully to identify gross structures in the brain that express mRNA for a variety of nAChRs (Wada et al., 1990; Gotti et al., 2007; Grady et al., 2007), but again, there are problems with interpreting these findings. For one, identification of the subunits that are present in a particular brain region does not provide insight into the specific combinations of subunits that form functional receptors in that region. Additionally, although mRNA is required for protein production, levels of mRNA do not necessarily correspond to the amount of protein produced or functionally expressed. Even more challenging is that mRNA has been reported to be present in axon terminals (Giuditta et al., 2015), so these studies cannot definitively provide information about the brain region in which the nicotinic receptors originate nor the part of the neuron in which they might be expressed.

Examining protein levels directly also presents challenges, as radioligands and antibodies for nAChRs subunits lack specificity (Gotti et al., 2007; Letchworth and Whiteaker, 2011; Drenan and Lester, 2012). Electrophysiological assays can be used to determine the location of receptors on either pre- or post-synaptic membranes (McGehee et al., 1995), but these assays rely on pharmacology to determine receptor subunit composition. Many groups are working on overcoming these technical challenges, and better tools are currently being developed (Grady et al., 2010; Drenan and Lester, 2012; Fowler and Kenny, 2012a; Zoli et al., 2015), but for now, identification of exact subunit composition on specific cell-types and at specific subcellular localizations requires immense effort and is rarely conclusive.

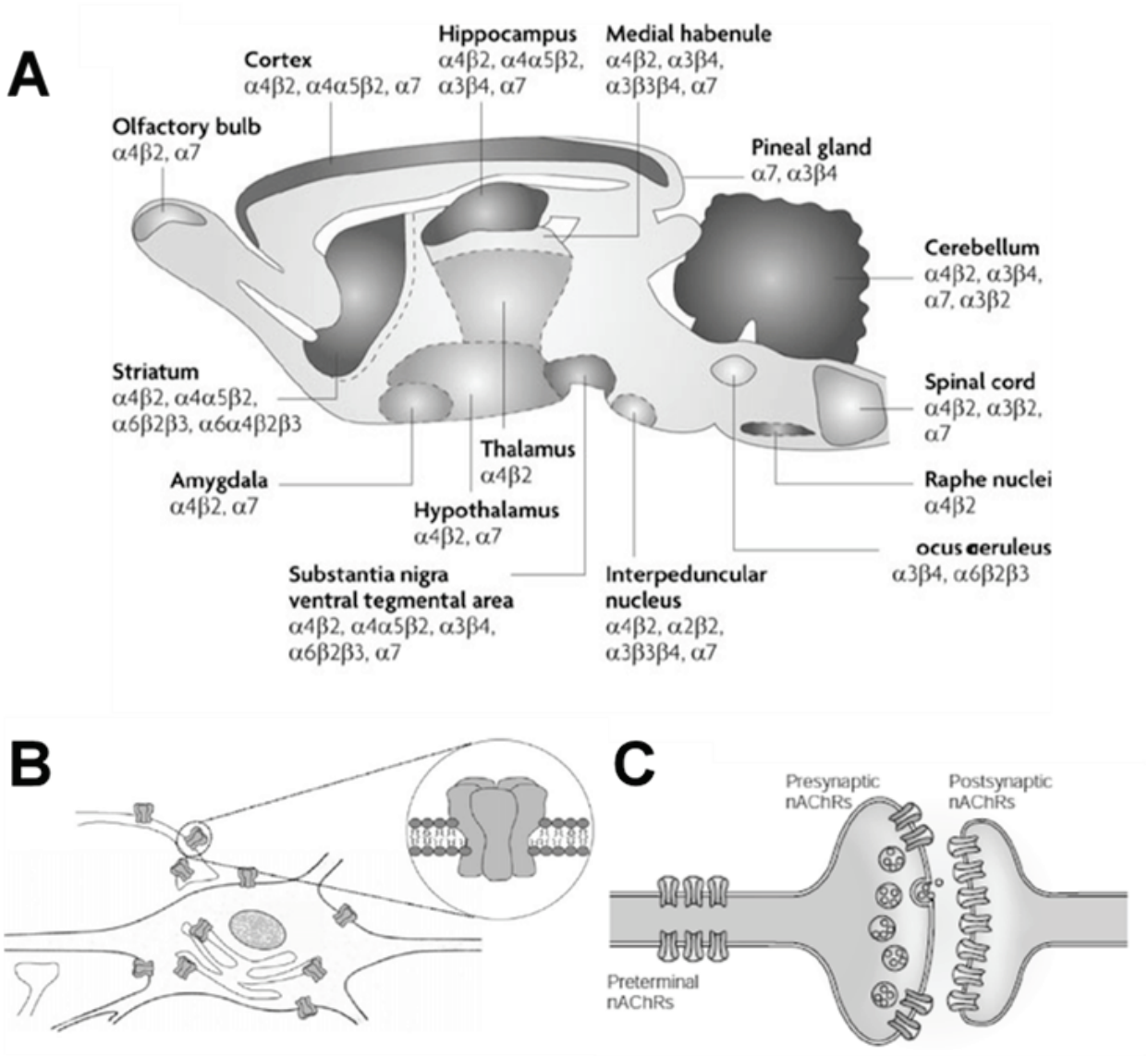

Figure 1.3- Localization of nAChRs. A) Schematic of a sagittal view of a mouse brain. Brain regions and some of the nAChRs that are expressed in those regions are labeled. This is not an exhaustive representation of nAChR expression throughout the brain. Adapted from (Gotti et al., 2006). B) Nicotinic receptors are expressed in a variety of locations throughout the neuron, including on axon terminals, intracellular organelles, and the somatodendritic region. Adapted from (Gotti et al., 2007). C) The various locations of nAChRs at a prototypical synapse. Adapted from (Laviolette and van der Kooy, 2004).

(1f) Nicotine Addiction Overview.

Nicotine addiction remains a major public health problem in the US and throughout the world. It is estimated that 22.5% of adults in the world (~1 billion) smoke tobacco products, and that 16% of male deaths and 6% of female deaths worldwide are due to tobacco use each year (Gowing et al., 2015).

The American Psychiatric Association defines addiction as "a chronic brain disease that causes compulsive substance use despite harmful consequences"(2015). It has also been described as a "progressive, relapsing disease comprised of interlocking stages of disordered motivation" (Edwards and Koob, 2013). The most recent edition of the Diagnostic and Statistical Manual of Mental Disorders from the American Psychiatric Association classifies all addiction-related disorders as substance use disorders that can range in severity. There are also different criteria for each substance that can be abused. Figure 1.4 shows the diagnostic criteria for Tobacco Use Disorder (American Psychiatric Association, 2013a). From these definitions, some main hallmarks of addiction can be summarized as compulsivity or loss of control over drug-taking, craving or extreme wanting of the drug, and propensity to relapse.

While there are many theoretical frameworks through which addiction can be viewed (Robinson and Berridge, 1993; Ahmed and Koob, 2005; Hogarth and Troisi II, 2015), addiction generally has a prototypical progression. An individual uses the drug, escalates drug use over time, loses control over drug taking, and if able to quit, will often relapse. Each of these phases of addiction in general and nicotine addiction more specifically is an active area of investigation. My thesis work has focused on the first stage, investigating how nicotine exerts its effects on the brain during the first exposure.

Diagnostic Criteria

A problematic pattern of tobacco use leading to clinically significant impairment or distress, as manifested by at least two of the following, occurring within a 12-month period:

1. Tobacco is often taken in larger amounts or over a longer period than was intended.

2. There is a persistent desire or unsuccessful efforts to cut down or control tobacco use.

3. A great deal of time is spent in activities necessary to obtain or use tobacco.

4. Craving, or a strong desire or urge to use tobacco.

5. Recurrent tobacco use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., interference with work).

6. Continued tobacco use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of tobacco (e.g., arguments with others about tobacco use).

7. Important social, occupational, or recreational activities are given up or reduced because of tobacco use.

8. Recurrent tobacco use in situations in which it is physically hazardous (e.g., smoking in bed).

9. Tobacco use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by tobacco.

10. Tolerance, as defined by either of the following:

    A need for markedly increased amounts of tobacco to achieve the desired effect.

    A markedly diminished effect with continued use of the same amount of tobacco.

11. Withdrawal, as manifested by either of the following:

    The characteristic withdrawal syndrome for tobacco (refer to Criteria A and B of the criteria set for tobacco withdrawal).

    Tobacco (or a closely related substance, such as nicotine) is taken to relieve or avoid withdrawal symptoms.

Severity: Mild (2-3 symptoms), Moderate (4-5 symptoms), Severe (6 or more symptoms)

Figure 1.4- Diagnostic criteria for Tobacco Use Disorder. The DSM-5 combined its previous addiction-related classifications (substance abuse and substance dependence) into one classification that takes severity into account (American Psychiatric Association, 2013b).

(1g) Dichotomous Effects of Nicotine.

The reinforcing effects of nicotine are well documented in both humans and laboratory animal models (Henningfield and Goldberg, 1983a; Henningfield et al., 1983; Perkins et al., 1997; Jones et al., 1999; Tuesta et al., 2011; Hogarth et al., 2014), but at higher doses, nicotine can have intensely aversive effects (Henningfield and Goldberg, 1983a; Risinger and Oakes, 1995; Rose and Corrigall, 1997; Fowler et al., 2011). Humans and laboratory animals both display inverted-U shaped dose-response curves, with maximal self-administration occurring at intermediate doses. Figure 1.5a shows an example of such a dose-response relationship. In fact, humans and laboratory animals very effectively regulate their levels of nicotine intake when self-administering (Benowitz et al., 1986; Woodward and Tunstall-Pedoe, 1993; Rose and Corrigall, 1997; Lynch and Carroll, 1999) and will perform tasks to avoid the administration of high nicotine doses (Henningfield and Goldberg, 1983a).

Interestingly, acute responses to the same nicotine dose can result in a range of reported subjective effects that vary between participants. For example, current smokers rate nicotine as more pleasurable than non-smokers do (Soria et al., 1996), and women have been found to be more sensitive to nicotine's effects than men (Sofuoglu and Mooney, 2009). Additionally, it has been suggested that some nicotine effects can be reinforcing in some contexts and aversive in others, as indicated by symptoms not directly translating to liking or disliking (Rosecrans, 1979; Henningfield and Goldberg, 1983b; Hogarth and Troisi II, 2015).

There are many factors that can influence the subjective effects of nicotine and/or the development of nicotine dependence (e.g. tolerance, social status, genetic factors, sex, age, affective state, etc.) (Kandel et al., 1997; Blitstein et al., 2003; Patterson et al., 2003; Hu et al., 2006; Buchmann et al., 2011; Haberstick et al., 2011; Hogarth and Troisi II, 2015), but the focus

of this section will be on how initial subjective effects of nicotine might predict propensity for the development of nicotine addiction. Figure 1.5b shows some common subjective effects of nicotine and how they are grouped into "pleasant" and "unpleasant" categories.

It has been hypothesized that initial smoking experiences may predict the likelihood of future dependence (Urban and Sutfin, 2010; de Wit and Phillips, 2012). If true, a pleasurable first experience with nicotine should correlate with more smoking and higher rates of nicotine dependence than would an aversive first experience. More pleasure (or less aversion) could then promote further use, which is a requirement for the transition from use to abuse and dependence (Robinson and Berridge, 1993; Everitt and Robbins, 2005; De Biasi and Dani, 2011). One alternative hypothesis has been that individuals who are most sensitive to the effects of nicotine initially are the most vulnerable for developing dependence (Pomerleau et al., 1993). Figure 1.5c-d illustrates these two conceptual views of individual differences in vulnerability to develop nicotine addiction. Support for both models exists; however, the field seems to have settled on the first model as the one that best fits the data. The remainder of this section will review some of the evidence for this model, along with some questions that remain.

The earliest investigation into whether initial experiences with smoking impact future smoking behaviors was a retrospective study that recruited participants who had wanted to try smoking, or who were tempted to become smokers. They then asked the smokers and non-smokers who had been tempted to smoke to recall their first smoking experience. 80% of the tempted non-smokers reported moderate to severe discomfort during this initial experience, while only 10% of tempted smokers reported these levels of discomfort (Kozlowski and Harford, 1976). This suggests that even in motivated populations, aversive effects of nicotine can deter continued nicotine use.

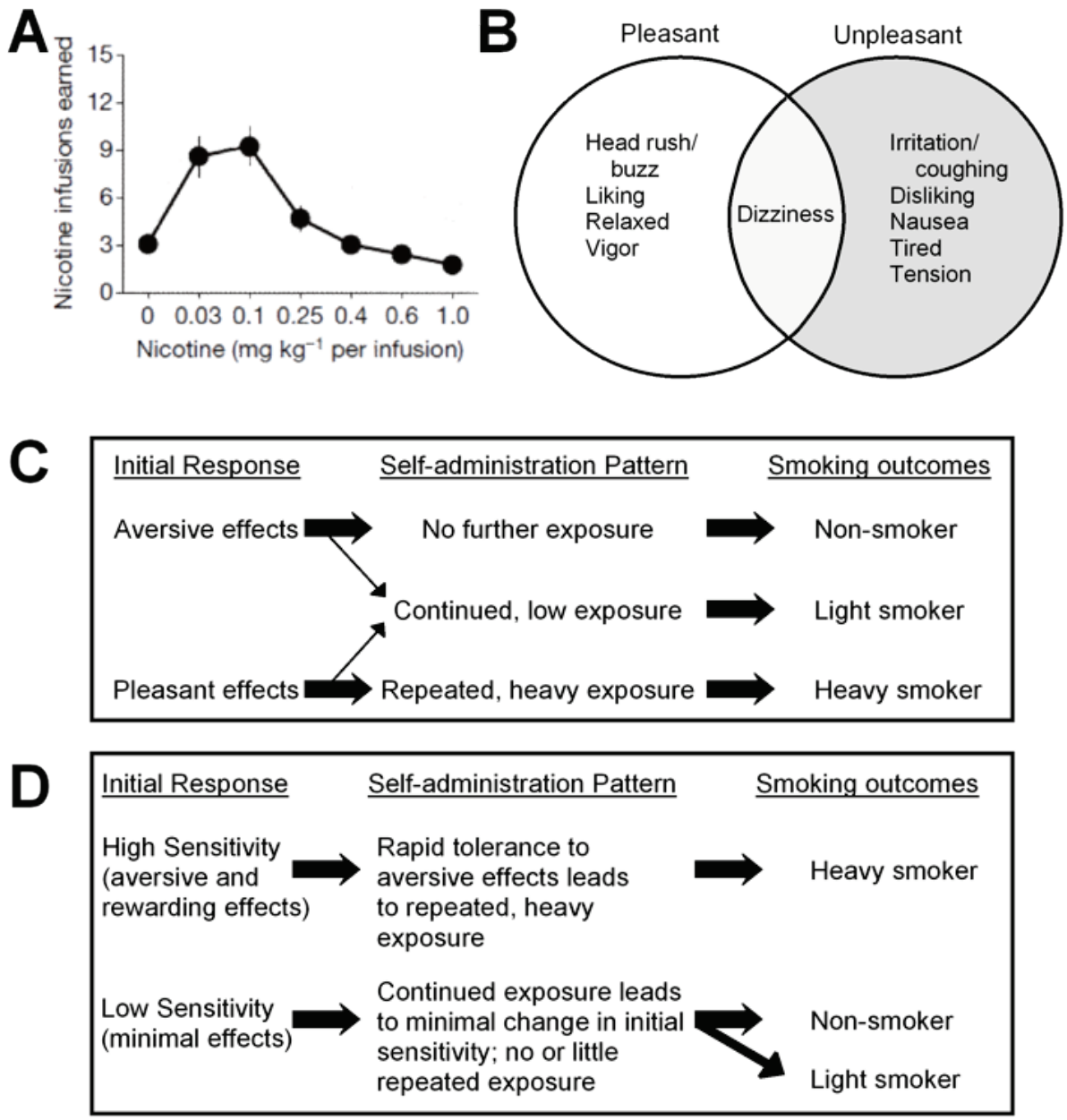

Figure 1.5- Dichotomous Effects of Nicotine. A) Example of a typical dose-response curve for nicotine. Dose is represented on the x-axis, and number of infusions is represented on the y-axis. As the dose increases, self-administration increases, until the maximally reinforcing dose is reached. After that intermediate dose, as the dose increases, self-administration decreases. Adapted from (Fowler et al., 2011). B) Venn diagram showing some commonly reported effects of nicotine. Many studies divide these subjective effects into pleasant or unpleasant. Dizziness, potentially due to the ambiguity in its definition, can be both a pleasant and an unpleasant effect. C) Schematic of one model for how initial subjective effects of nicotine might influence future smoking behaviors. Due to individual differences and a wide variety of factors that also influence smoking behaviors, this schematic only represents theoretically likely scenarios and is not all-encompassing. D) Schematic of an alternative model for how initial subjective effects of nicotine might influence future smoking behaviors. C+D adapted from (Pomerleau et al., 1993).

Since then, many retrospective studies examining reports of initial experiences with smoking have been conducted. For example, in a study of Chinese 10th graders, self-reports of pleasurable buzz, relaxation, and liking the smell during the first smoking experience was associated with increased nicotine dependence (Chen et al., 2003). Similarly, in a study on women smokers, the more dependent women reported higher pleasure ratings in response to their first experience with nicotine than did ex-smokers or never-smokers. They also reported a higher ratio of pleasant to unpleasant effects (Pomerleau et al., 1998). Interestingly, higher ratings of dizziness were also associated with nicotine dependence, and aversive effects such as nausea and coughing did not differ between groups. This finding that aversive effects did not impact nicotine outcomes challenges the model described above; however, there are also numerous reports that aversive effects of nicotine can impact future smoking behavior.

In a study of adolescents asked to recall their first time inhaling a cigarette, irritation and coughing were found to be protective against subsequent dependence (DiFranza et al., 2004). This study also found that reports of relaxation in response to the first smoking experience were the strongest predictor of a substance use disorder. In a similar study, reports of a positive first experience with nicotine were again associated with smoking status, while reports of an unpleasant first experience were shown to make progression to regular smoking less likely, at least during the experimental stage (Urban and Sutfin, 2010). Two additional retrospective studies of adolescents and young adults also found that reports of pleasant initial experiences with smoking were associated with an increased likelihood of regular smoking, while unpleasant experiences with nicotine were associated with inconsistent nicotine use or non-smoking (Urbán, 2010; Haberstick et al., 2011). These studies suggest that aversion can indeed impact the likelihood of continued nicotine use.

Further support for the idea that pleasure from the first nicotine experience can predict future smoking behavior comes from studies of adolescents that look specifically at subjective effects of nicotine and age at which they had their first cigarette. Adolescents are thought to be at higher risk for nicotine dependence than adults, with loss of autonomy over nicotine use sometimes occurring after just one cigarette (DiFranza et al., 2007; Kandel et al., 2007; Scragg et al., 2008). In one such study, although negative sensations in response to the first smoking experience were more frequent than positive sensations and were also more intense, participants who were younger for their first smoking experience were more likely to report positive effects from their initial experiences than participants who were older at the time of first smoking experience (Buchmann et al., 2011). Age at which the first cigarette was experienced and reports of pleasurable effects of nicotine have both been positively associated with smoking status and nicotine dependence (Hu et al., 2006; Ríos-Bedoya et al., 2009; Buchmann et al., 2011).

The findings concerning unpleasant or aversive experiences in these studies are a little less cohesive. In two of the studies, reports of an unpleasant first smoking experience were negatively correlated with smoking (Hu et al., 2006; Ríos-Bedoya et al., 2009). However, in the other study, reports of unpleasant effects were not different according to age at which the first cigarette was experienced (Buchmann et al., 2011). These studies suggest that the level of pleasure derived from smoking is potentially more relevant to continued drug-taking than the level of aversion, but that aversion might still be an important aspect in predicting future smoking behaviors. Buchmann et al. (2011) in particular also suggests that the positive and negative effects of nicotine can occur simultaneously, and that the balance between reward and aversion might determine the likelihood of future use.

One potential problem with all of the studies mentioned so far is that they all rely on self-reported recollections. Not only can memories change over time, but current smokers might be allowing current feelings about nicotine's effects color memories of their first experiences. Indeed, it has been shown that when questioned one year after the initial report of first experiences with nicotine, some participants who had escalated their nicotine intake reported more pleasant effects, when the year prior they had denied experiencing those effects (Riedel et al., 2003). However, other investigations of retrospective data found accounts to be generally reliable over time (Brigham et al., 2008; Urban and Sutfin, 2010). Therefore, these retrospective studies should not be dismissed, but they should be interpreted with caution and in the context of findings based on other methodologies.

One such alternative methodology is to examine the effects of nicotine on non-smoking or never-smoking populations. Two really interesting studies have examined the acute subjective effects of nicotine and how they relate to nicotine self-administration in the laboratory setting. In one study, non-smokers were given the opportunity to self-administer nicotine after reporting on the subjective effects of a test dose of nicotine. Self-administration was correlated with pleasurable effects, such as arousal, pleasant, vigor, and friendly, while self-administration was inversely correlated with aversive effects, such as head rush, feeling the effects, feeling tired, sedation, tension, depression, and fatigue (Perkins et al., 2001). In the other study, participants who had never smoked were given nicotine and placebo capsules to take orally. Once they could reliably discriminate between the two capsules, they were given a choice between the two during daily choice sessions. Half of the participants reliably chose the nicotine capsule, while the other half reliably chose the placebo. The group that chose nicotine had more increases in positive subjective effects after taking nicotine (liking, good effects), while the group that chose placebo

(or avoided nicotine) had more increases in negative subjective effects after taking nicotine (disliking, bad effects)(Duke et al., 2015). These studies suggest that the pleasurable effects of nicotine encourage future drug-taking, while the negative effects discourage drug-taking in drug-naïve or non-smoking populations. Lower likelihood of continued nicotine use should decrease the likelihood of developing tobacco use disorder.

In fact, prospective studies of adolescents support this idea. In a two year study of 6-10th graders, a pleasant initial experience with nicotine predicted both the latency to the first criteria for abuse being met and full dependence (Kandel et al., 2007). In another study of adolescents followed over the course of one year, those who had positive feelings about their first experience with nicotine were more likely to progress to regular smoking, while coughing (a conventionally unpleasant effect) decreased the likelihood that they would progress to regular smoking (Blitstein et al., 2003). The longest prospective study to-date was over the course of 4 years. In that study, relaxation (a conventionally pleasant effect) in response to the first smoking experience predicted loss of autonomy over nicotine taking and dependence (DiFranza et al., 2007). Taken together, these retrospective, acute, and prospective studies suggest that the effects of nicotine during the first smoking experience influence future drug-taking.

Despite this evidence, however, there are some conflicting reports that should be addressed. In one acute study of smokers and non-smokers, non-smokers reported more negative effects in response to nicotine administration than positive effects, while smokers reported more positive effects (Soria et al., 1996). The authors argue that this suggests that repeated exposures to nicotine are required to establish its positive reinforcing effects. This could be interpreted as evidence of the sensitivity model of progression from first cigarette to regular smoking, since initial rewarding effects are not relevant to progression. However, the study only investigated the

subjective effects on average for a few doses of nicotine. In the study mentioned above by Duke et al. (2015), had they simply averaged the results, they would have found that never-smokers had no preference for nicotine or placebo. Therefore, although the idea that nicotine must be repeatedly administered before it can be reinforcing is interesting, there is little support for it, and an abundance of evidence against it.

Another issue that has garnered attention in the literature is the fact that dizziness and/or nausea during the first nicotine experience often positively correlate with increased smoking (DiFranza et al., 2004; Haberstick et al., 2011; de Wit and Phillips, 2012; Agrawal et al., 2014). This seems counter-intuitive, given that dizziness and nausea might generally be considered aversive, and also supports the sensitivity model. However, dizziness has been shown to be both a positive and a negative effect of nicotine (Kandel et al., 2007; Agrawal et al., 2014). This might be useful in explaining an incongruous finding. In a retrospective study of nicotine users and cannabis users, both positive and negative responses to initial smoking experience were positively correlated with future nicotine dependence. The positive symptoms included head rush/buzz, relaxation, and liking the taste, while the negative symptoms included coughing, headache, and nausea. Dizziness was identified as both a positive and a negative symptom (Agrawal et al., 2014). Some of the confusion here may be due to a lack of precise definitions for symptoms given to participants. Dizziness can mean vertigo, giddiness, confusion, silliness, etc. Had the distinction been made between these disparate definitions, the results from Agrawal et al. (2014) and others might be more coherent and pronounced. Furthermore, the inclusion of nausea as a negative symptom intuitively makes sense; however, it has been reported that although other aversive nicotine effects are sufficient to stop self-administration of nicotine, nausea will not alter nicotine-taking (Henningfield et al., 1983). This suggests that nausea might

be a relatively mild negative effect of nicotine in terms of its effects on drug-taking. Self-administration of other drugs of abuse may be similarly unaffected by nausea (de Wit and Phillips, 2012).

A relatively recent study found that initial responses to nicotine can be divided into 4 distinct categories that may be able to help clarify some of the discrepancies in the literature concerning opposing nicotine effects. The categories are: 1) high physiological responding and high pleasure reports, 2) low physiological responding and low pleasure reports, 3) high physiological response and low pleasure reports, and 4) low physiological responding and high pleasure reports. Regardless of physiological responses, high pleasure reports were associated with a more rapid progression from the first cigarette to regular smoking. This suggests that the initial experience can be enjoyable even when coupled with physiological effects that are generally considered to be unpleasant (Sartor et al., 2010). Taken together, the evidence suggests that initial pleasant effects of nicotine (and potentially fewer aversive effects) can predict an individual's future smoking behavior.

If positive and negative drug effects can be considered to summate to produce a gestalt value judgment, then a scenario can be imagined in which high negative reports are counterbalanced by high positive reports. If aversion is mediated by a neural circuit that inhibits reward or positive reinforcement, then perhaps the aversive effects of nicotine limit the extent to which the reinforcing effects of nicotine can be felt. Indeed, preclinical work has implicated specific nAChR subtypes and brain areas that limit the upper limits of nicotine intake without affecting the ascending limb of the dose-response curve (Fowler et al., 2011). Additionally, the relevance of the aversive effects of nicotine may be obscured in many of these clinical studies by other factors that influence smoking. Many of these findings can also be interpreted as

supportive of the sensitivity model of acquisition of nicotine-taking if the initial dose of nicotine were able to be controlled for. Perhaps a very nicotine-sensitive individual might take one puff of a cigarette and enjoy it and then be likely to continue using, while a similarly nicotine-sensitive individual might smoke an entire cigarette and find it extremely unpleasant and then be less likely to continue using. Although further examination is required, and the initial subjective effects of nicotine cannot explain nicotine addiction completely, it does seem that they impact future drug-taking behavior in critical ways.

(1h) Genetic Factors Mediating Nicotine Effects.

Data from genetic studies also suggests that initial subjective effects may be important for future nicotine use. Genetic differences likely account for some of the individual differences in initial responses to and continued use of nicotine. Although there are a variety of genes that can influence tobacco use (Loukola et al., 2014; O'Loughlin et al., 2014; Balfour and Munafò, 2015; Bühler et al., 2015; Pan et al., 2015), this section will focus specifically on genetic variants of nAChR genes.

Many genome-wide association studies (GWAS) have been conducted to determine the genetic underpinnings of smoking behaviors. A recent meta-analysis of GWAS for smoking behaviors reports that the vast majority of the most significant single nucleotide polymorphisms (SNP) associated with nicotine phenotypes belong to nAChR genes, mainly the *CHRNA5-A3-B4* gene cluster on chromosome 15, which encodes the α5, α3, and β4 nicotinic acetylcholine receptor subunits (Bühler et al., 2015). A variety of SNPs in this gene cluster have been linked to smoking behavior, but one in particular has garnered the most attention: rs16969968. This is the only SNP to be implicated in nicotine behaviors thus far that results in a non-synonymous amino

acid change in the resulting protein. The major allele produces α5 subunits with an aspartate (D) in position 398, which is swapped for an asparagine (N) in the minor allele. The minor allele (N398) has been repeatedly confirmed to be highly associated with heavy smoking, intense craving for nicotine, and nicotine dependence (Bierut et al., 2008; Sherva et al., 2008; Stevens et al., 2008; Liu et al., 2010; Breetvelt et al., 2012; Chen et al., 2012; Buczkowski et al., 2015; Olfson et al., 2015).

This and other SNPs in the same gene cluster have also been linked to increased risk of lung cancer and chronic obstructive pulmonary disorder, presumably due to increased smoking and dependence (Berrettini et al., 2008; Stevens et al., 2008; Bierut, 2010; Wang et al., 2013; Chen et al., 2015); however, these SNPs have also been reported to be predictors of lung cancer even in never-smokers, although this risk increases with smoking status (Ji et al., 2015). It has been reported that individuals with one copy of the risk allele are 1.3-fold more likely to develop nicotine dependence, and individuals with two copies are up to 2-fold more likely to develop dependence (Bierut, 2010). There have also been some alleles that have been identified as protective against nicotine dependence in this gene cluster; however, they are all intronic, and the mechanisms by which they might affect protein expression or nicotine-related behaviors remain unclear (Ware et al., 2012; Bühler et al., 2015). Additionally, variants in the *CHRNA5-A3-B4* gene cluster that regulate transcription of *CHRNA5* have also been linked to smoking behaviors (Liu et al., 2010; Smith et al., 2011; Wang et al., 2013; Hancock et al., 2015b). These data suggest that the α5 subunit of the nAChR is critically important for smoking behaviors. This subunit will be discussed further in the next section.

Because of the substantial evidence that risk alleles in this gene cluster predict smoking status and severity, a few studies have looked into the effects of genotype on acute subjective

effects of nicotine. One study looked specifically at the relationship between smokers with the N398 risk allele and those with the D398 allele. They administered nicotine to these smokers in the lab after 8 hours abstinence and found that those with the N398 allele reported significantly fewer aversive responses than those with the D398 allele. There was also a trend toward those with the N398 allele reporting more pleasurable effects than those with the N398 allele (Jensen et al., 2015). Another study compared smokers with non-smokers (fewer than 100 lifetime cigarettes smoked). The relationship between the N398 risk allele and heavier smoking was confirmed, but they also found a link between reported pleasurable experiences during their first cigarette and the N398 risk allele. The study also found that non-smokers were more likely than smokers to report unpleasant experiences and less likely to report dizziness from their first cigarettes, but the sample size used was too small to decipher a potential relationship between genotype and unpleasant experiences or dizziness (Sherva et al., 2008). A study that looked more broadly at SNPs in the *CHRNA5-A3-B4* gene cluster found that a SNP associated with risk for nicotine dependence was also associated with ratings of dizziness during their first smoking experience (Saccone et al., 2009; Pedneault et al., 2014).

Other nAChR genes have been implicated in risk for nicotine dependence as well. SNPs in the *CHRNA4* gene have been found that confer either risk or protection, and two have been found to be related to the effects of nicotine (Feng et al., 2004; Breitling et al., 2009; Han et al., 2011; Hoft et al., 2011; Pedneault et al., 2014; Hancock et al., 2015a). As the α4 nAChR is important for the rewarding effects of nicotine (Picciotto and Kenny, 2013), it is not surprising that genetic variants of this subunit might influence nicotine-related behaviors. Interestingly, variants in the *CHRNB4* gene have also been associated with decreased risk of nicotine dependence (Haller et al., 2012, 2014). Variants in many nAChR subunit genes have also been

implicated in nicotine-related phenotypes, though most have not been found to be as robust or reliable as those in the *CHRNA5-A3-B4* gene cluster (Greenbaum et al., 2006; Ehringer et al., 2010; Hoft et al., 2011; Cannon et al., 2013; Culverhouse et al., 2014; O'Loughlin et al., 2014; Pedneault et al., 2014; Wang et al., 2014b).

These reports suggest that a complex array of genetic and environmental factors influence nicotine dependence and smoking behaviors; however, the importance of SNPs in the *CHRNA5-A3-B4* gene cluster have been shown repeatedly to be important for predicting nicotine phenotypes. Additionally, these risk alleles have been linked to the acute subjective effects of nicotine, supporting the hypothesis that enhanced aversion to nicotine limits intake and prevents dependence while enhanced reward encourages intake and dependence. The neuronal distributions of nAChRs that have been reported to be critical for nicotine-related phenotypes have encouraged the exploration of novel brain circuits that are important for modulating nicotine-taking and dependence. The findings from these investigations have been critical in the development of my thesis work.

(1i) α5 Nicotinic Receptors.

The α5 subunit of the nAChR is an accessory or auxiliary subunit, meaning that it cannot form a functional receptor in the absence of additional α and β subunits and that it is not thought to participate in forming a ligand-binding site (Ramirez-Latorre et al., 1996; Groot-Kormelink et al., 1998; Marotta et al., 2013; Zoli et al., 2015). The functional contribution of the α5 subunit to the nAChR likely depends on the other subunits present (Ramirez-Latorre et al., 1996; Nelson and Lindstrom, 1999) and the expression system in which this contribution is being assessed (Krashia et al., 2010). The most commonly studied receptors that include the α5 subunit are the

α3β4 and the α4β2 nAChRs. This section will discuss the functional contribution of α5 to these receptor types and the distributions of these receptor complexes in the brain.

Because of the problems associated with identifying specific nAChR subunits with certainty (section 1e of this chapter), most of the investigations concerning the contribution of α5 to the properties of nAChRs in which it participates have been conducted in heterologous expression systems. Reports of the functional role of α5 in the α3β4 nAChR, which has a very low affinity for nicotine (Kuryatov et al., 2011), have largely been contradictory. One study reported that incorporation of α5 increased single channel conductance, channel open time, and burst duration (Nelson and Lindstrom, 1999). Another study found similar results for channel open time and burst duration, but found no change in single channel conductance between α5-containing and non α5-containing α3β4 nAChRs (Ciuraszkiewicz et al., 2013). Yet another study reported that inclusion of the α5 subunit in the α3β4 nAChR decreased single channel open time (Li et al., 2011a). Further conflicting reports come from studies of macroscopic currents. One study reported that reduced desensitization was the only observable contribution of α5 to α3β4 currents (Li et al., 2011a), while others reported that the presence of α5 increased desensitization and reduced current amplitudes in response to various agonists (Wang et al., 1996; Gerzanich et al., 1998; Tammimäki et al., 2012). However, Gerzanich et al. also found that inclusion of α5 markedly increased calcium permeability of the α3β4 receptor so that its permeability was similar to that of α7 receptors (1998). Tammimäki et al. instead reported no change in calcium permeability (2012).

It has also been suggested that α5 subunits might compete with β4 subunits, which would result in a "dead end" receptor that cannot bind ligand (Frahm et al., 2011; George et al., 2012). If this were true, then the inclusion of α5 in a functional receptor might enhance the receptor

function but ultimately result in fewer of them. This has been proposed as a mechanism by which the amount of functionally active α3β4 is regulated (Frahm et al., 2011; George et al., 2012). To avoid this possibility, George et al. developed concatamers in order to ensure uniformity of the subunit composition of receptors in each oocyte (2012). They found that inclusion of α5 enhanced efficacy of nicotine and increased peak responses to agonist (George et al., 2012).

Many of these contradictory findings might also be explained my methodological differences. For example, Krashia et al. found that α3β4 subunits expressed in oocytes tended to have a different stoichiometry than those expressed in HEK cells and that these differences result in different single-channel properties (2010). Therefore, the addition of α5 might be more or less likely to alter function, depending on which stoichiometry was predominant initially. Additionally, discerning the proportion of receptors that integrate the α5 subunit is extremely challenging, so the materials required to make functional α5α3β4 receptors might be present, but the receptors expressed on the cell surface might predominantly lack the α5 subunit.

Compounding this problem, more complex subunit compositions that form native receptors have been identified in particular brain regions (e.g. α6α4β2β3) (Gotti et al., 2006), and α5 inclusion in receptors these types of receptors might result in different functional effects compared to its inclusion in stereotypical heteromeric receptors. Furthermore, there is evidence to suggest that subunit compositions and/or functional properties can vary based on location in the cell (Fischer et al., 2005), which might mean that heterologous expression misses critical information. Heterologous expression also does not necessarily translate to neuronal expression either (Albuquerque et al., 2009; Zoli et al., 2015). These caveats hold true for the bulk of the data that will be discussed throughout this section.

Because a genetic variant causing a non-synonymous amino acid switch in the *CHRNA5* gene is highly associated with increased nicotine dependence, a few groups have examined the effects of this risk variant (N398) compared to the normal allele (D398) in heterologous expression systems. Although two studies reported that the risk variant had no relevant effect on the functional receptor compared with the normal subunit (Kuryatov et al., 2011; Li et al., 2011a), multiple studies have found that the risk variant does alter function of the receptor compared to the normal subunit. One found that macroscopic current amplitudes were decreased in cells expressing the α5N398 variant compared to the wild-type variant (Frahm et al., 2011), and another found that nicotine-induced rises in intracellular calcium were reduced in cells expressing the α5N398 variant compared to the wild-type variant (Tammimäki et al., 2012). Using the concatamer approach, George et al. showed that maximal agonist effects were reduced when the risk variant was expressed compared with the wild-type variant (2012). Thus, it seems that the SNP that confers vulnerability to nicotine dependence, heavy smoking, and increased pleasurable/decreased aversive effects of nicotine results in α5 subunits that attenuate function of the α3β4α5 receptor.

The α3β4 receptor is heavily expressed in the periphery but has a very restricted expression profile in the brain (Albuquerque et al., 2009). The majority of neuronal α3β4 receptors are expressed in the medial habenula (MHb) and interpeduncular nucleus (IPN) (Marks and Pauly, 1992; Salas et al., 2004; Grady et al., 2009), suggesting that α3β4α5 receptor would be higher in these brain regions than others. Activity in these two regions have been found to be important for the aversive effects of nicotine (De Biasi and Dani, 2011; Fowler et al., 2011; Frahm et al., 2011), and this will be discussed further in a future section as these findings have strongly informed my thesis work. The question of whether α5 is expressed in the MHb is a

contentious one because of contradictory findings (Marks and Pauly, 1992; Sheffield et al., 2000; Hsu et al., 2013b); however, there seems to be a consensus that α5 subunits are expressed at high levels in the IPN (Wada et al., 1990; Marks and Pauly, 1992; Hsu et al., 2013b).

As mentioned, the α5 subunit can also occupy the accessory position in α4β2 receptors (Marotta et al., 2013), which have high affinity for nicotine (Kuryatov et al., 2011). It has previously been reported that the stoichiometry of α4β2 receptors can impact functional properties of the receptors such that a configuration of $(\alpha4)_2(\beta2)_3$ has high sensitivity to agonists while a configuration of $(\alpha4)_3(\beta2)_2$ has lower sensitivity to agonists (Briggs et al., 2006; Moroni et al., 2006). Inclusion of the α5 subunit in the α4β2 receptor increases sensitivity of the receptor to nicotine and acetylcholine compared to the lower sensitivity receptor, reduces the inward rectification indexes compared to both forms of the α-5 lacking receptors, reduces nicotine-induced desensitization, speeds recovery from nicotine-induced desensitization, and increases current amplitudes and calcium permeability two-fold compared to the lower sensitivity receptor (Grady et al., 2012; Marotta et al., 2013; Sciaccaluga et al., 2015). A5 expression is thought to be critical for nicotine-induced calcium influx in both somata and processes, potentially because the α5 subunit regulates expression of the α4 subunit (Chatterjee et al., 2013; Sciaccaluga et al., 2015). More work is still required to determine the changes in receptor function induced by inclusion of α5 compared to the high sensitivity α4β2 receptor, but these findings indicate that α5 enhances the function in many ways of α4β2 nAChRs.

As for the α3β4 receptor, a couple of studies have investigated the differential contributions of the normal α5 subunit and the risk variant α5 subunit. These studies have shown that the risk variant reduces activation-induced rises in intracellular calcium levels, reduces current amplitudes, and increases desensitization rates relative to the normal α5 subunit

(Kuryatov et al., 2011; Sciaccaluga et al., 2015). Similar to the risk variant's effects on α5α3β4 receptors, it seems that the risk variant reduces function of the α5α4β2 receptor.

The α4β2 receptors are the predominant type of nAChR in the mammalian brain (Albuquerque et al., 2009; Picciotto and Kenny, 2013), so expression of the α5α4β2 receptor is likely to be restricted by expression of the α5 subunit. As mentioned previously, the MHb-IPN pathway has substantial α5 expression as well as expression of both α4 and β2 subunits (Wada et al., 1989, 1990; Marks and Pauly, 1992; Sheffield et al., 2000). All 3 of these subunits are also densely expressed in the ventral tegmental area (VTA), a region that has been repeatedly shown to be important for reward-related and drug-taking behaviors (Wada et al., 1989, 1990; Marks and Pauly, 1992; Lammel et al., 2012; Steinberg et al., 2013; Pignatelli and Bonci, 2015; Volkow and Morales, 2015). Figure 1.6 shows photomicrographs of mRNA expression for various nAChRs in the rodent brain. My thesis work has focused on how the MHb-IPN connection might indirectly modulate VTA activity, which will be discussed further in future sections.

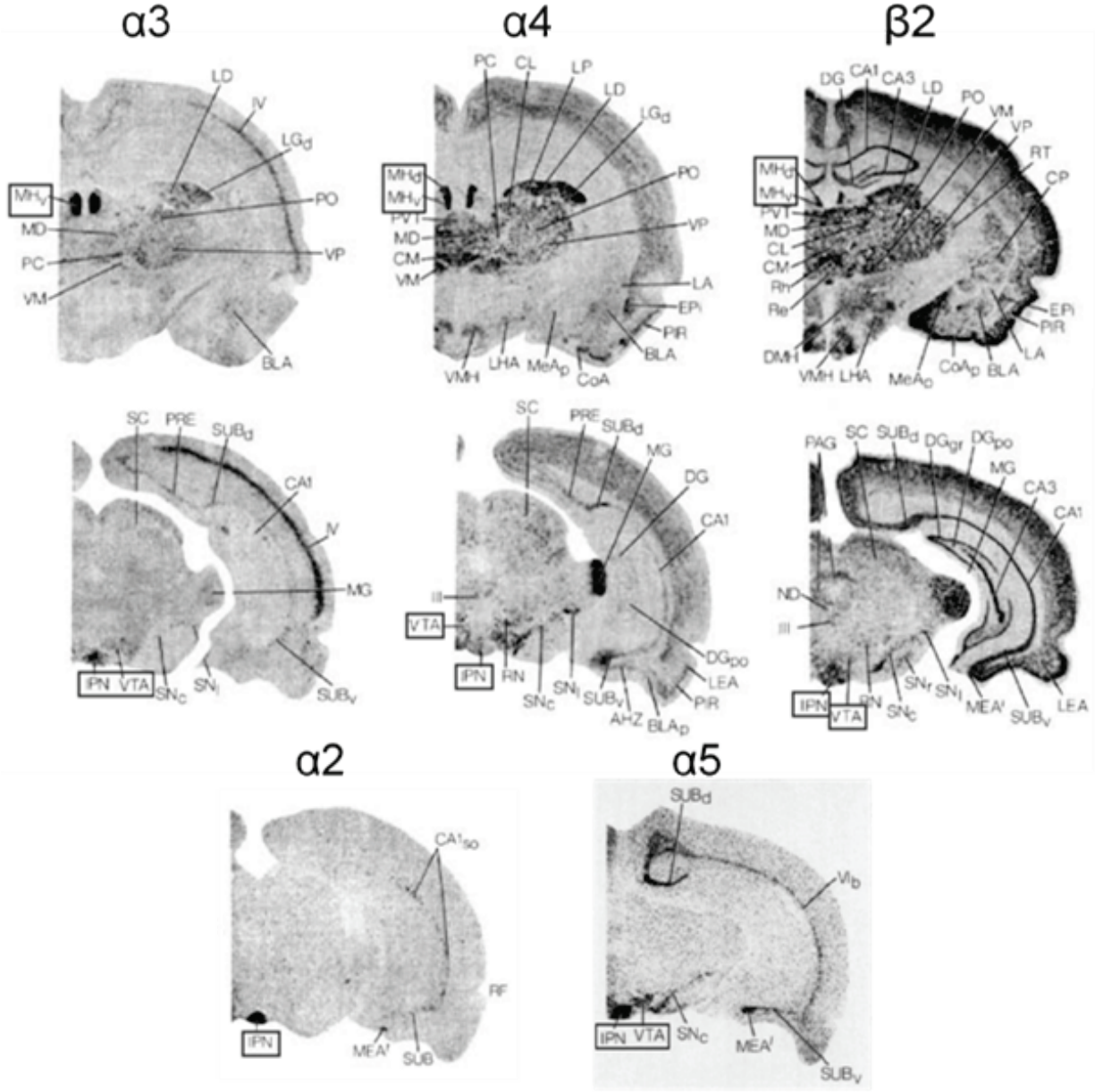

Figure 1.6- nAChR mRNA Expression. Photomicrographs of coronal brain sections showing nAChR subunit expression in various brain regions. The most names of the most relevant regions (MHb, IPN, VTA) are boxed. Adapted from (Wada et al., 1989, 1990).

(1j) Neural Circuits That Underlie Nicotine Reward.

Beneficial actions are more likely to be repeated than those that aren't, and the neural mechanisms that mediate positive reinforcement have been extensively studied. The mesolimbic dopamine system, which includes dense dopaminergic innervation of the NAcc by VTA neurons, has been repeatedly found to mediate positive reinforcement and reward learning (Robinson and Berridge, 1993; Wise, 2004; Fields et al., 2007; Wise and Koob, 2014; Keiflin and Janak, 2015; Pistillo et al., 2015; Volkow and Morales, 2015). This pathway is illustrated in figure 1.7a. Natural rewards and all classes of addictive drugs result in enhanced dopamine release in the NAcc (Di Chiara and Imperato, 1988; Lüscher and Ungless, 2006; Mark et al., 2011; Aitken et al., 2015; Cone et al., 2015).

Dopamine (DA) neurons have two classical modes of firing- tonic and phasic. Tonic firing results in basal levels of dopamine in the NAcc, while phasic, or bursting, activity results in transient, high amplitude spikes in dopamine levels in the NAcc (Goto and Grace, 2005; Goto et al., 2007). Pauses in tonic firing and phasic burst firing of these VTA DA neurons that result in changes in accumbal DA levels are thought to be behaviorally relevant signals that mediate reward learning (Cromwell and Schultz, 2003; Goto et al., 2007; Steinberg et al., 2013). Some of these characteristics of dopamine neurons are illustrated in Figure 1.7b-c.

Recent studies have causally linked activation of the VTA DA neurons and DA release in the NAcc to positive reinforcement and reward learning related to drug addiction. Animals will self-administer specific stimulation of the VTA DA neurons as well as VTA DA terminals in the NAcc (Steinberg et al., 2014; Pascoli et al., 2015). Additionally, phasic-like, specific stimulation of the VTA DA neurons that results in substantial DA efflux in the NAcc is sufficient to condition a place preference in mice (Tsai et al., 2009). These findings suggest that increased

activity of the VTA DA neurons results in positive reinforcement and promotes reward learning. Addiction to drugs of abuse is thought to develop after the initial drug-taking experience is reinforcing, which promotes subsequent drug taking.

Inhibition of VTA DA neurons, pauses in tonic firing, and decreased DA efflux in the NAcc however, have been linked to omission of an expected reward and to aversive outcomes (Schultz, 2007; Chang et al., 2015; Eshel et al., 2015; Fortin et al., 2015). GABAergic neurons in the VTA synapse locally on DA neurons (Omelchenko and Sesack, 2009), are activated by foot-shock, and have been shown to inhibit VTA DA neurons. Selective stimulation of these GABAergic neurons conditions a place aversion (Tan et al., 2012). Additionally, direct, optogenetic inhibition of VTA DA neurons reduces NAcc DA levels and results in aversion (Danjo et al., 2014). However, the VTA DA neurons cannot be discussed as a homogenous group as there are various brain regions innervated by these neurons. Activation of DA neurons that project to the NAcc do appear to mediate reward; however, DA neurons that project to the prefrontal cortex (PFC) mediate aversion (Lammel et al., 2011, 2012). Therefore, reduced tonic or phasic activity of VTA DA neurons that project to the NAcc seems to result in aversion and aversive learning.

The laterodorsal tegmental nucleus (LDTg) is a heterogenous pontine nucleus defined by the presence of cholinergic neurons. The LDTg sends cholinergic, glutamatergic, and GABAergic projections to the VTA and influences DA neuron activity. Lesions of the LDTg result in the inability of VTA DA neurons to transition to phasic firing patterns, even in response to iontophoretically applied glutamate (Lodge and Grace, 2006) and dramatically reduce evoked DA efflux in the NAcc (Blaha et al., 1996). Additionally, selective activation of the LDTg neurons that project to the VTA result in a conditioned place preference (Lammel et al., 2012).

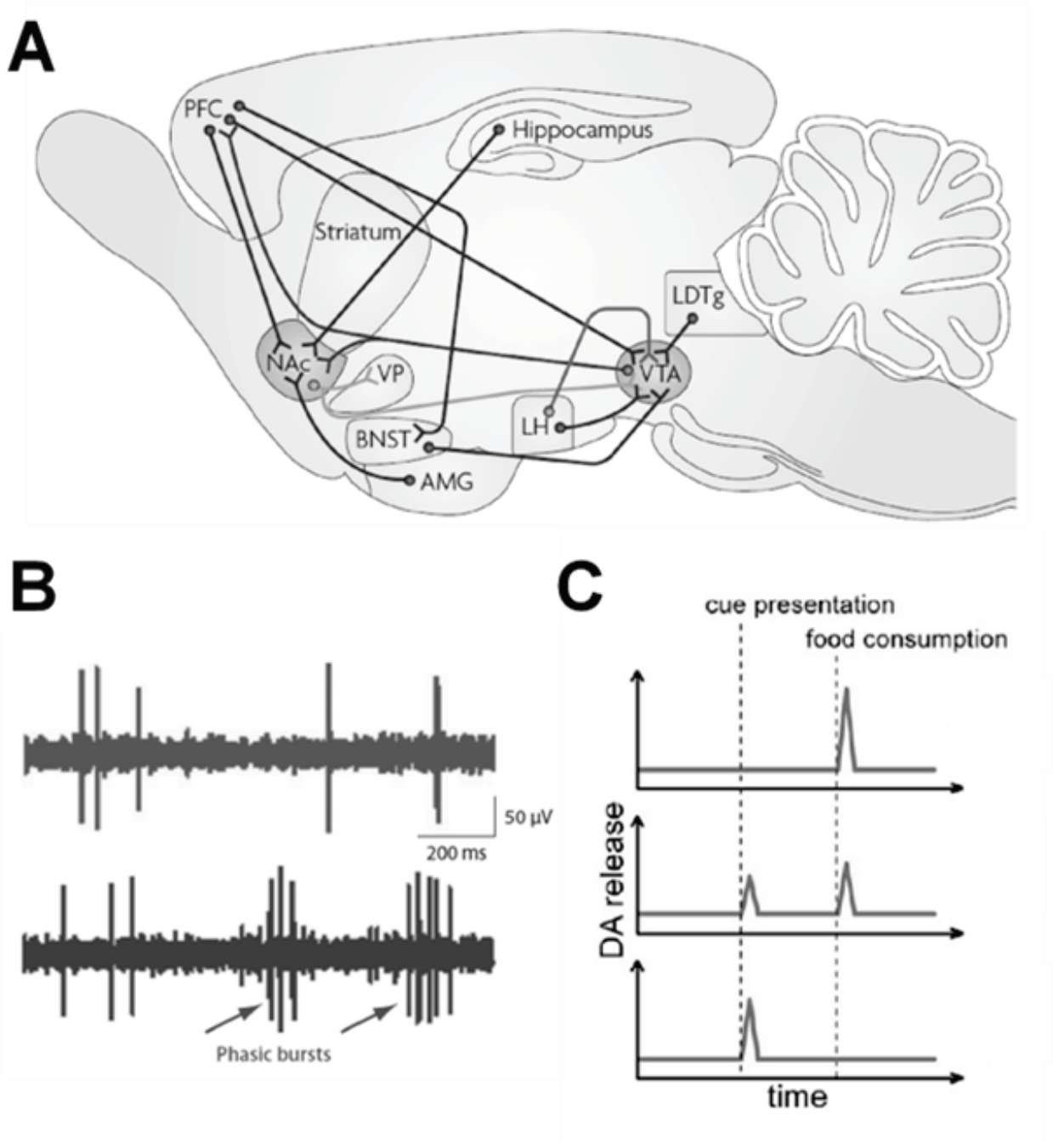

Figure 1.7- Mesolimbic Dopamine System. A) Simplified cartoon of the brain regions and projections that make up the mesolimbic/mesoaccumbens dopamine system. Adapted from (Kauer and Malenka, 2007). B) Single unit recordings of VTA dopamine neurons. Top panel: tonic firing. Bottom panel: phasic bursting. Adapted from (Zhang et al., 2009). C) Dopamine as a reward learning signal. Top panel: no prior training, dopamine efflux in the NAcc occurs when the reward is consumed. Middle: moderate training, dopamine efflux begins to shift to cue that predicts reward. Bottom: well-trained, dopamine efflux occurs upon presentation of the reward-predicting cue rather than the reward itself. Adapted from (Keiflin and Janak, 2015).

These findings may be due to the distinct projection patterns of LDTg inputs to the VTA. Dopamine neurons in the VTA that project to the NAcc primarily receive glutamatergic inputs from the LDTg, while GABA neurons in the VTA (interneurons or projection neurons to NAcc) primarily receive GABAergic input from the LDTg (Omelchenko and Sesack, 2005). Additionally, most of the cholinergic inputs to the VTA synapse onto DA neurons that project to the NAcc (Omelchenko and Sesack, 2006). Taken together, the LDTg seems to be a critical mediator of VTA DA activity and reward-related behaviors. A circuit diagram of the brain regions just discussed is presented in Figure 1.8.

It would be prudent to note here that this explanation of mesoaccumbens circuitry is simplified and incomplete. The dopamine and GABA neurons of the VTA have many projection targets in addition to the NAcc and PFC and receive inputs from many regions other than the LDTg that have also been shown to mediate reward learning (Fields et al., 2007; Ji and Shepard, 2007; Ambroggi et al., 2008; Brown et al., 2012; Stamatakis and Stuber, 2012; Stamatakis et al., 2013). There are also many theoretical frameworks in which dopamine neuron activity is proposed to mediate the transition from drug use to compulsive drug-taking, but these ideas are outside the scope of my thesis work. For comprehensive review, see (Robinson and Berridge, 2003; Everitt and Robbins, 2005; Steketee and Kalivas, 2011; Berridge, 2012; Wise and Koob, 2014; Keiflin and Janak, 2015; Volkow and Morales, 2015) .

Nicotine is the main addictive component of tobacco and, like other drugs of abuse, has been shown to result in prolonged enhancement of VTA DA neuron burst firing and elevation of NAcc DA levels (Di Chiara and Imperato, 1988; Pidoplichko et al., 2004; Zhang et al., 2009; Liu et al., 2012). Nicotine also acts in unique ways to maximize its impact on NAcc dopamine levels. VTA DA neurons express nAChRs on terminals that synapse in the NAcc, and nicotine inhibits

tonic DA release. This seems paradoxical, but nicotine significantly enhances phasic dopamine release. This combination of inhibition of background DA and enhancement of phasic DA results in enhanced contrast in NAcc DA levels when VTA DA neuron firing switches from tonic to phasic activity (Rice and Cragg, 2004; Zhang et al., 2009). Nicotine also tends to synchronize the firing of VTA DA neurons (Li et al., 2011b), which enhances the dopamine signal in the NAcc even further. Nicotine-induced activation of VTA DA neurons is critical in mediating the reinforcing and DA-releasing effects of nicotine, and over the last 20 years, insights into the mechanisms by which nicotine results in enhanced firing of VTA DA neurons have emerged.

Dopamine neurons in the VTA express high affinity α4β2 receptors (Picciotto et al., 1998; Mansvelder and McGehee, 2002) that are activated by physiologicaly relevant nicotine concentrations (Matta et al., 2007); however, these receptors rapidly desensitize. Therefore, nicotine transiently activates VTA DA neurons directly by binding somatodendritic α4β2 receptors, but this mechanism is not sufficient to explain the prolonged increases in VTA DA neuron burst firing and NAcc DA efflux (Zhang et al., 2009).

GABAergic and glutamatergic neurons that synapse on DA neurons also express nAChRs, and it seems that nicotine modulates the balance of inhibitory and excitatory drive to the VTA DA neurons via presynaptic and somatic receptors on these cells (D'Souza and Markou, 2013). The nAChRs expressed on GABAergic interneuron somata are likely also α4β2 (Mansvelder and McGehee, 2002), so GABAergic drive is transiently enhanced to VTA DA neurons at the same time that they are directly activated by nicotine. The nAChRs present on the GABA neurons then also rapidly desensitize (Mansvelder et al., 2002). The glutamatergic inputs, on the other hand, express pre-synaptic α7 receptors, which are highly calcium permeable, facilitating glutamate release at these synapses (Mansvelder and McGehee, 2000). The α7

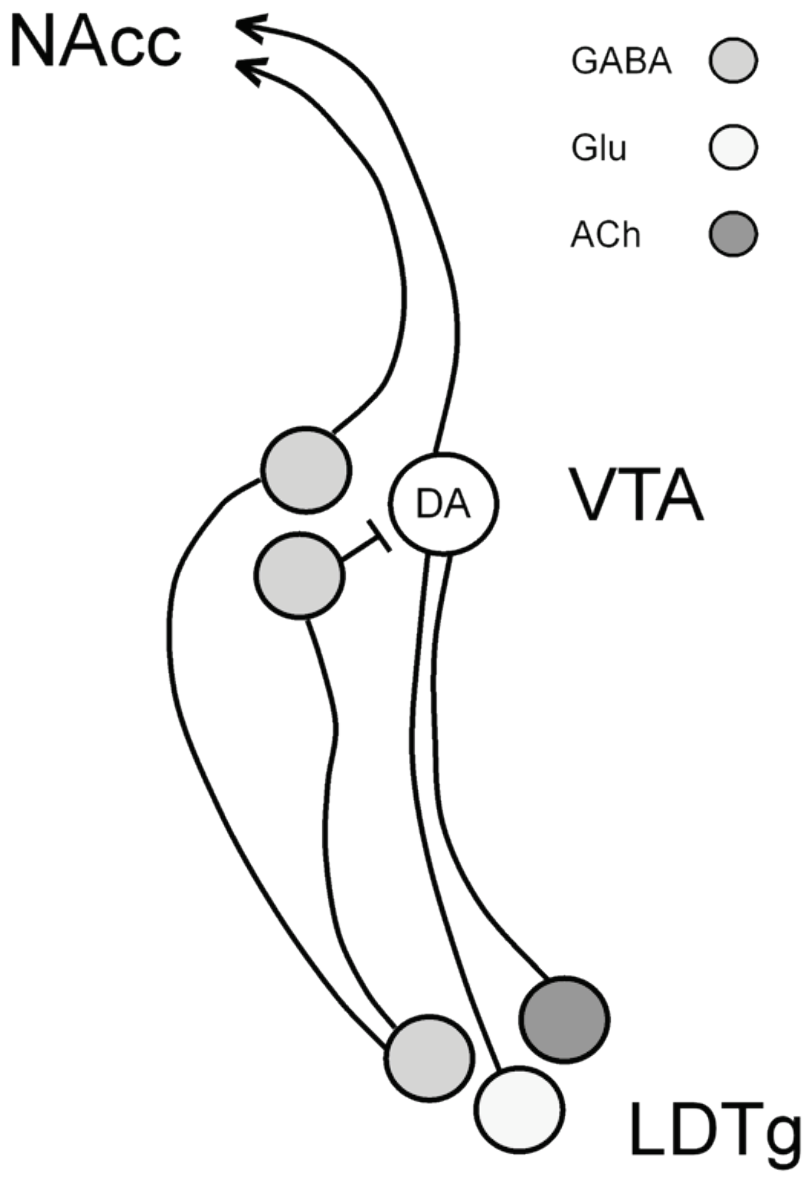

Figure 1.8- Different types of LDTg neurons target specific VTA neurons. Cholinergic neurons from the LDTg preferentially project to VTA DA neurons that project to the NAcc. Acetylcholine often modulates synapses through volume transmission, though, so it is likely to impact other cell types as well. GABAergic neurons from the LDTg preferentially inhibit GABA interneurons or projection neurons in the VTA. Glutamatergic neurons from the LDTg preferentially excite VTA DA neurons that project to the NAcc.

receptors desensitize more slowly, allowing enhancement of glutamatergic drive onto VTA DA neurons to persist for more prolonged periods. This shifts the balance, so to speak, to activation of the DA neurons (Mansvelder et al., 2002). Further contributing to this excitatory balance is the the finding that VTA DA neurons also express somatic α7 receptors (Wooltorton et al., 2003; Drenan and Lester, 2012).

The β2 subunit appears to be critically important for the ability of these mechanisms to transition DA neurons from tonic to phasic firing and thus mediate the rewarding effects of nicotine (Picciotto et al., 1998; Maskos et al., 2005; Subramaniyan and Dani, 2015). It seems that activation of α4β2 receptors switches VTA DA neurons from a resting state to an excited state, and then α7 activation fine-tunes or maintains this state (Mameli-Engvall et al., 2006). The locations of these nAChRs and their effects on DA neuron activity are illustrated in Figure 1.9.

As mentioned above, the LDTg projects to the VTA and gates the ability of VTA DA neurons to transition to burst firing. The LDTg has also been implicated in nicotine-specific reward-related behaviors. For example, lesions of the LDTg block nicotine-induced locomotor sensitization, a process that enhances propensity to self-administer drugs (Vezina, 2004). Lesions of the related pedunculopontine tegmental nucleus (PPTg) or GABAergic agonists administered into the LDTg result in reduced nicotine self-administration (Lança et al., 2000a; Maskos, 2008). LDTg neurons also express somatodendritic nAChRs, and excitatory and inhibitory inputs to the LDTg express presynaptic nAChRs as well (Ishibashi et al., 2009). Nicotine, then, can impact reward-related circuitry at multiple levels, and identifying and integrating all of these effects into a cohesive understanding of nicotine reinforcement still requires more investigation.

The mechanisms described above relate to the acute transition from tonic to phasic firing of DA neurons that occurs during the first experience with nicotine. Those mechanisms,

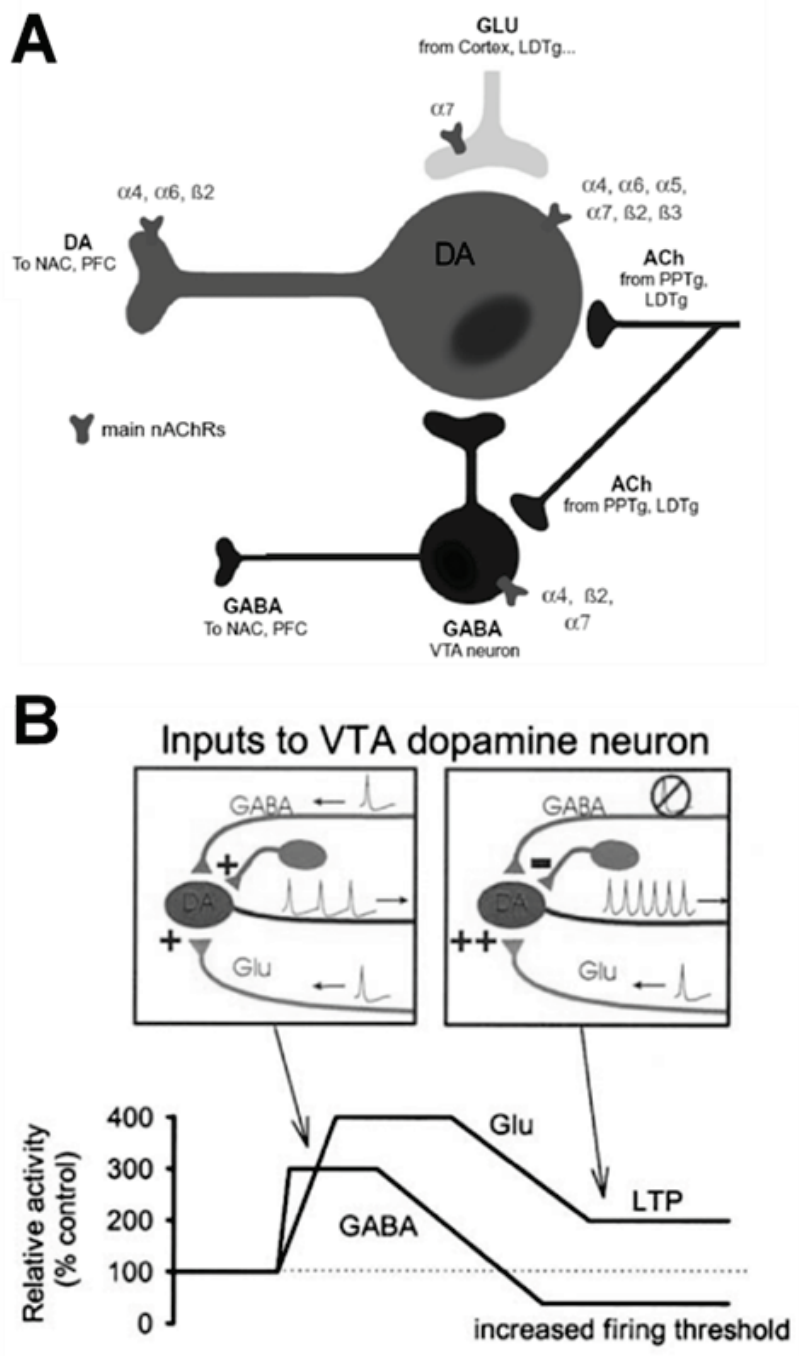

Figure 1.9- Nicotinic receptors modulate the balance between inhibitory and excitatory inputs to VTA DA neurons. A) Diagram of the various nAChRs in the VTA. Adapted from (Pistillo et al., 2015). B) Schematic representing the effects of nAChR activation in the VTA on DA neurons. Nicotinic receptors on GABAergic inputs to dopamine neurons rapidly desensitize such that glutamatergic drive is stronger to dopamine neurons relative to baseline. Adapted from (Mansvelder et al., 2002).

however, also facilitate long term potentiation (LTP) of glutamatergic synapses on VTA DA neurons (Mansvelder and McGehee, 2000; Mao et al., 2011; Pistillo et al., 2015). A single, passively administered dose of nicotine is sufficient to induce LTP at these synapses (Placzek et al., 2009). Addiction is has been characterized as a disorder of aberrant plasticity (Edwards and Koob, 2013), and this initial neural response to nicotine might prime the system to be even more responsive to the next exposure to nicotine. Indeed, enhanced VTA sensitivity to nicotine has been correlated with increased locomotor responses to novelty, a phenotype that predicts propensity to self-administer nicotine (Suto et al., 2001; Fagen et al., 2007).

Alternatively, human genetic findings suggest that decreased sensitivity of α5-containing nicotinic receptors predicts propensity to be a heavy smoker and nicotine dependent (section 1h). As discussed in section 1i, the VTA expresses α5α4β2 receptors, and the SNP that confers risk for nicotine dependence reduces function of this receptor. A recent study investigated the role of α5 VTA expression on nicotine self-administration and found that while wild-type mice self-administered more infusions of low-dose nicotine, α5-/- mice self-administered more infusions of high-dose nicotine and consumed more nicotine per day at these higher doses (Morel et al., 2013). When they restored α5 expression selectively in the VTA, wild-type nicotine self-administration was restored; however, when they restored α5 expression in the VTA using the risk variant, the dose-response curve remained right-shifted (Morel et al., 2013). These results suggest that decreased VTA sensitivity to nicotine confers risk for increased nicotine-taking, specifically that individuals with the risk variant expressed in the VTA are at higher risk for problemmatic nicotine use. The explanation for the right shifted dose-response curve could be similar to why mice don't self-administer many infusions of very low doses of nicotine.

However, other studies have found no differences between α5-/- mice and wild-type mice when investigating lower doses of nicotine (Jackson et al., 2010; Fowler et al., 2011). This could suggest that the α5 variant might confer its risk via an alternative receptor or circuit. Alternatively, perhaps reduced sensitivity of this specific receptor in the VTA allows for α7-mediated LTP to have a relatively larger impact or facilitates LTP in some way. There are a variety of possibilities for how these findings might be reconciled, and more work is required to fully understand the role of α5 in nicotine effects.

In summary, it seems that nicotine reward is mediated by increased VTA DA activity in neurons that project to the NAcc. This includes a transition from tonic to phasic firing, resulting in elevated levels of DA in the NAcc. Therefore, for simplicity, we can conclude that enhanced VTA DA activity corresponds to enhanced reward, while diminished VTA DA activity (pauses in tonic firing or disruptions of phasic firing) corresponds to diminished reward (or enhanced aversion).

(1k) Neural Circuits that Underlie Nicotine Aversion.

The habenula is a bilateral epithalamic structure which consists of two distinct nuclei, the medial habenula (MHb) and the lateral habenula (LHb). These nuclei receive different afferent projections and send efferents to different targets (Herkenham, 1977; Herkenham & Nauta, 1979), though both have been implicated in aversion (Frahm et al., 2011; Lecca et al., 2011; Salas et al., 2010; Stamatakis & Stuber, 2012). More is known about the network functions of the LHb than about the MHb, but the role of the LHb in nicotine-related behaviors specifically remains largely unknown. The LHb sends its glutamatergic afferents largely to the rostromedial tegmentum (RMTg), which in turn sends its GABAergic afferents to the VTA. In this way, LHb

activation indirectly inhibits VTA DA neurons and elicits aversion (Stamatakis and Stuber, 2012). The MHb, however, has been strongly implicated in nicotine-specific behaviors (see sections 1h, 1i)(Fowler et al., 2011; Frahm et al., 2011; Zhao-Shea et al., 2013). Therefore, my thesis work has focused on MHb circuitry, and that will be the focus of this section. Figure 1.10 shows a connectivity diagram of the habenula.

The MHb is a predominantly cholinergic and glutamatergic region that sends all of its projections to the interpeduncular nucleus (IPN) via the fasciculus retroflexus (Marks and Pauly, 1992; Ren et al., 2011). Human genetic studies prompted basic researchers to investigate the habenulo-interpeduncular pathway because it expresses high levels of the α5 nAChR subunit, which has been implicated in vulnerability to nicotine addiction (see sections 1h, 1i). Recall that the risk variant of the α5 subunit results in decreased function of the receptor compared to the wild-type variant.

Recent work has shown that knocking out the α5 nAChR subunit in mice promotes self-administration of high, aversive nicotine doses that wild type (wt) mice will not self-administer (Fowler et al. 2011). This difference was eliminated by rescuing α5 expression in the knock-outs specifically in the MHb (Fowler et al. 2011), suggesting that α5 nAChRs in the MHb mediate the aversive effects of high doses of nicotine. Interestingly, the reinforcing effects of low doses of nicotine that wt mice do readily self-administer were not altered in α5-/- mice. Knock-out of the α5 subunit was intended to mimic the human variant that confers risk for heavy smoking and nicotine as that variant decreases function of nAChRs. Indeed, α5-/- did diminish nicotinic receptor function in synaptosomes taken from the IPN. Also, in wt mice, high, aversive doses of nicotine activate the IPN, but in α5-/- mice, the same dose of nicotine did not significantly activate the IPN (Fowler et al., 2011).

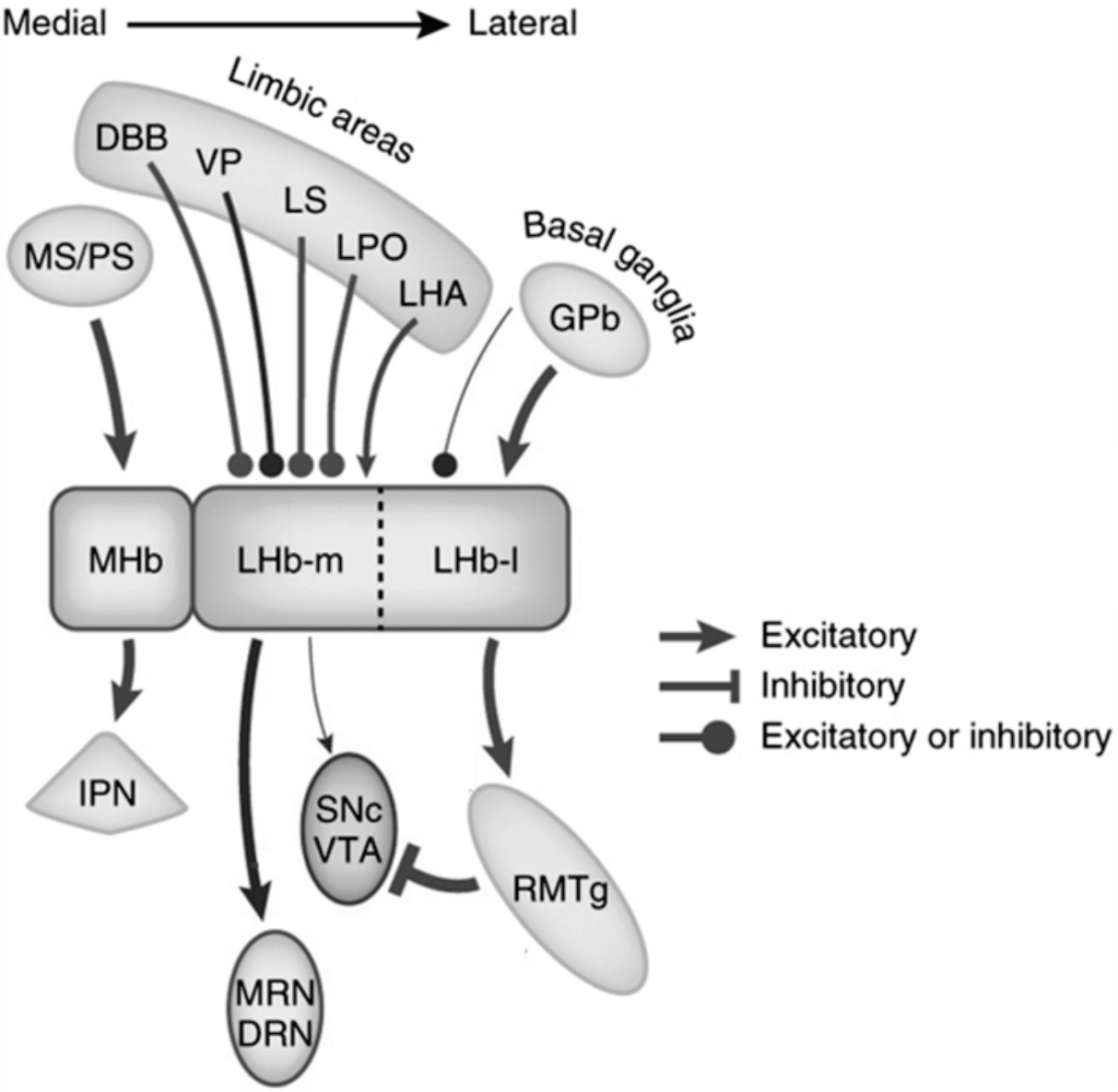

Figure 1.10- Habenular Circuitry. Simplified schematic of inputs and outputs of the medial and lateral habenulae. Substantially more is known about the connectivity, and the nature of those connections, of the LHb than for the MHb. Although there is anatomical evidence of some IPN projections (most notably to the LDTg and the dorsal and median raphe nuclei), the nature of those projections remains largely unclear. Understanding how the MHb-IPN connection mediates aversion to nicotine will require identification of relevant synaptic targets of the IPN. Adapted from (Proulx et al., 2014).

α5-/- mice also develop a conditioned place preference (CPP) to extremely high doses of nicotine that wt mice do not (Jackson et al. 2010). As mentioned, high, aversive doses of nicotine activate the IPN in wt mice but fail to do so in α5-/- mice that find high, aversive doses of nicotine to be reinforcing. Low doses, on the other hand, fail to significantly activate the IPN in wt or α5-/- mice, as shown by c-fos expression (Fowler et al. 2011). These findings suggest that aversive doses of nicotine preferrentially activate the MHb-IPN pathway and that attentuated activity in this pathway removes the upper limits on nicotine intake. Therefore, enhanced activation of this pathway might reduce nicotine intake compared to wt mice.

There has been controversy about whether or not the MHb expresses α5 subunits organically *in vivo* (see section 1i). If the MHb does not normally express α5 subunits, then perhaps deletion of *CHRNA5* impacts the expression of functional nicotinic receptors in the MHb and IPN some other way. It has been shown that α5 expression can affect the expression of α4 in the VTA, and genes that are closely linked can affect transcription of each other (Albuquerque et al., 2009; Wang et al., 2009; Chatterjee et al., 2013). Additionally, the effects seen in α5-/- mice may be driven in part by reduced VTA DA activity in response to nicotine (see section 1j). The rescue of the normal nicotine phenotype may then be explained by atypical α5 subunit expression inhibiting function of MHb nAChRs. These explanations are not as parsimonious as the explanation offered in Fowler et al., but more work is required to decisively determine whether MHb neurons do indeed express α5 subunits (2011).

Another group tested the hypothesis that enhanced MHb-IPN activity mediates aversion by using a genetic manipulation that results in enhanced nicotine-induced currents at α3β4* receptors. Recall from section 1i that the MHb and IPN have the highest density of α3β4 receptors in the brain. The mice that expressed this hyper-functional receptor are called “Tabac”

mice (Frahm et al., 2011). Increased surface expression of the α5α3β4 receptor were observed in the MHb and pre-synaptically in the IPN of Tabac mice, and nicotine application elicited larger currents and increased action potential firing in the MHb neurons of Tabac mice compared to wt mice. This enhanced responding of MHb neurons to nicotine was accompanied by conditioned place aversion (CPA) to a dose of nicotine that was found to be neutral in control animals and decreased consumption of nicotine in a bottle-choice self-administration paradigm compared with controls (Frahm et al., 2011). These results suggest that MHb hyper-sensitivity to nicotine confers hyper-sensitivity to the aversive effects of nicotine. When a mouse homolog of the human α5 risk variant was expressed selectively in the MHb, enhanced aversive responses to nicotine were abolished (Frahm et al., 2011). In oocytes, this variant was found to decrease nicotine-evoked currents compared with receptors containing the endogenous variant of the α5 subunit; however, nicotine's effects on MHb neurons expressing this receptor were not directly tested (Frahm et al., 2011). Nonetheless, these results suggest that enhanced activation of the MHb-IPN pathway results in enhanced aversive responses to nicotine and vice versa.

Recent reports have also found that a variety of SNPs in *CHRNB4* can be either protective variants or risk variants for nicotine dependence, depending on whether they are loss- or gain-of-function (Haller et al., 2012, 2014). Variants that were associated with reduced risk of nicotine dependence resulted in significantly larger nicotine-evoked current amplitudes compared to the wt variant (Slimak et al., 2014). When this gain-of-function variant was virally expressed specifically in the MHb, mice showed aversion to nicotine relative to controls. Over-expression of the normal β4 receptor also showed pronounced aversion to nicotine, but those expressed with a loss-of-function variant showed no aversion to nicotine (Slimak et al., 2014).

These findings lend support to the idea that enhanced responsiveness of the MHb-IPN pathway to nicotine results in aversion that confers protection against nicotine dependence.

It has also been found that direct optogenetic activation of the GABA neurons of the IPN results in withdrawl like-behaviors (Zhao-Shea et al., 2013) and that corticotropin releasing factor (CRF) release into the IPN enhanced glutamatergic drive from the MHb and produces an anxiety-like response (Zhao-Shea et al., 2015). These findings further support the idea that activity in the MHb-IPN pathway mediates aversion.

Interestingly, over-expression of the human *CHRNA5-A3-B4* gene in mice resulted in increased sensitivity to nicotine in many behavioral assays, most notably they acquired nicotine self-administration more rapidly than did their wt counterparts and had higher break-points on a progressive ratio task (Gallego et al., 2011). Considering that α3β4* receptors are most densely expressed in the MHb-IPN pathway, this findings contradicts the idea that enhanced activity in this pathway results in enhanced aversion. However, α5 subunits also form receptors with α4β2 receptors, so the direct enhancement of VTA DA sensitivity due to this global genetic alteration may have offset potential enhancement of aversive behaviors. Indeed, the transgenic mouse exhibited higher c-fos activation in response to nicotine in the MHb than did controls and lower c-fos activation in the VTA after a rewarding dose of nicotine (Gallego et al., 2011).

Additionally, the MHb and IPN express a multitude of various nAChR subtypes, and the factors that influence which cell-types express which receptors remains largely unclear (Grady et al., 2009). However, α3β4 receptors have been shown to be expressed on somatostatin-positive cells IPN, and these are thought to be IPN interneurons that modulate excitatory release from the MHb (Zhao-Shea et al., 2013). In this circumstance, enhanced expression of this receptor type might lead to diminished output from the IPN. Unlike the VTA, the microcircuitries involved in

the effects of nicotine in the MHb and IPN have remained largely unexplored. Figure 1.11 diagrams one of the few known microcircuits within the IPN.

There have been a few studies with similarly contradictory results that might be explained by unknown roles of different receptor types in the net output of these regions MHb or IPN (Jackson et al., 2013; Harrington et al., 2015). Despite interesting findings that implicate various receptors in the regulation of nicotine-taking, these studies fail to examine the activity levels in either the MHb or the IPN. For example, one study used a β4-/- mouse to study the potential role of the MHb-IPN in nicotine-taking. The expectation, given the studies summarized above, would be that β4-/- should result in decreased MHb-IPN function, similar to the α-/-, and that this should result in increased nicotine self-administration. However, the study instead finds that β4-/- mice self-administer less nicotine and show decreased break points on a progressive ratio task, and self-administration can be restored to wt levels by rescue of β4 selectively in the IPN. These findings are similar in nature to those of Gallego et al. (2011); however, unlike Gallego et al., this study did not examine activation of the MHb or the IPN, leaving the exact role of this pathway unclear. These and other studies that use pharmacological tools in various brain regions contribute to the literature by implicating receptor classes in these brain regions that modulate function; however, the conclusions often drawn about the relationship between activity in the MHb and IPN and nicotine reward are drawn prematurely in the absence of examination of activity.

Some insights into potential mechanisms for nicotinic actions in the MHb-IPN circuit have been made, though. For example, infusion of an NMDA receptor antagonist into the IPN dose-dependently increased nicotine self-administration, suggesting that the α5 subunits might participate in pre-synaptic nAChRs that potentiate glutamate release at MHb-IPN synapses,

resulting in an inhibitory motivational signal (Fowler et al., 2011). If true, then α5-/- mice and rats with these receptors antagonized would lack this facilitation of glutamatergic transmission and thus have decreased excitatory drive at this synapse, resulting in a diminished inhibitory motivational signal and increased responding for even high doses of nicotine.

There is some evidence to support this hypothesis, as presynaptic nicotinic receptors have been shown to facilitate glutamatergic transmission at the MHb-IPN synapse (McGehee and Role, 1995; Girod and Role, 2001; Nayak et al., 2001), and it has been proposed that glutamatergic transmission is the main excitatory component of MHb input to the IPN (Brown et al., 1983; Ren et al., 2011). Further work is required to determine if this is in fact a mechanism that contributes to aversion-related behavior. Although the MHb sends almost all of its projections to the IPN, the IPN receives inputs from a variety of other brain regions, and the types of nAChRs expressed on those, if any, remains unclear.

Post-synaptic nAChRs are also present in the IPN and are likely activated by volume transmission of intense activity in the MHb (Ren et al., 2011), as well as by nicotine. The role for these receptors in the effects of nicotine remains unclear. As mentioned, it has been shown that post-synaptic α3β4 receptors are expressed on GABAergic interneurons in the IPN, which may serve to inhibit MHb glutamate release (Zhao-Shea et al., 2013). The differences between endogenous ACh signaling in this region and nicotinic activation also remain unclear. For example, rewarding doses of nicotine decrease ACh levels in the IPN as measured by in vivo microdialysis (Hussain et al., 2008). The mechanism by which a low dose of nicotine might inhibit ACh release in the IPN, or differentially affect function of the MHb-IPN pathway more generally, remains as mysterious as most aspects of this relatively newly popular brain region.

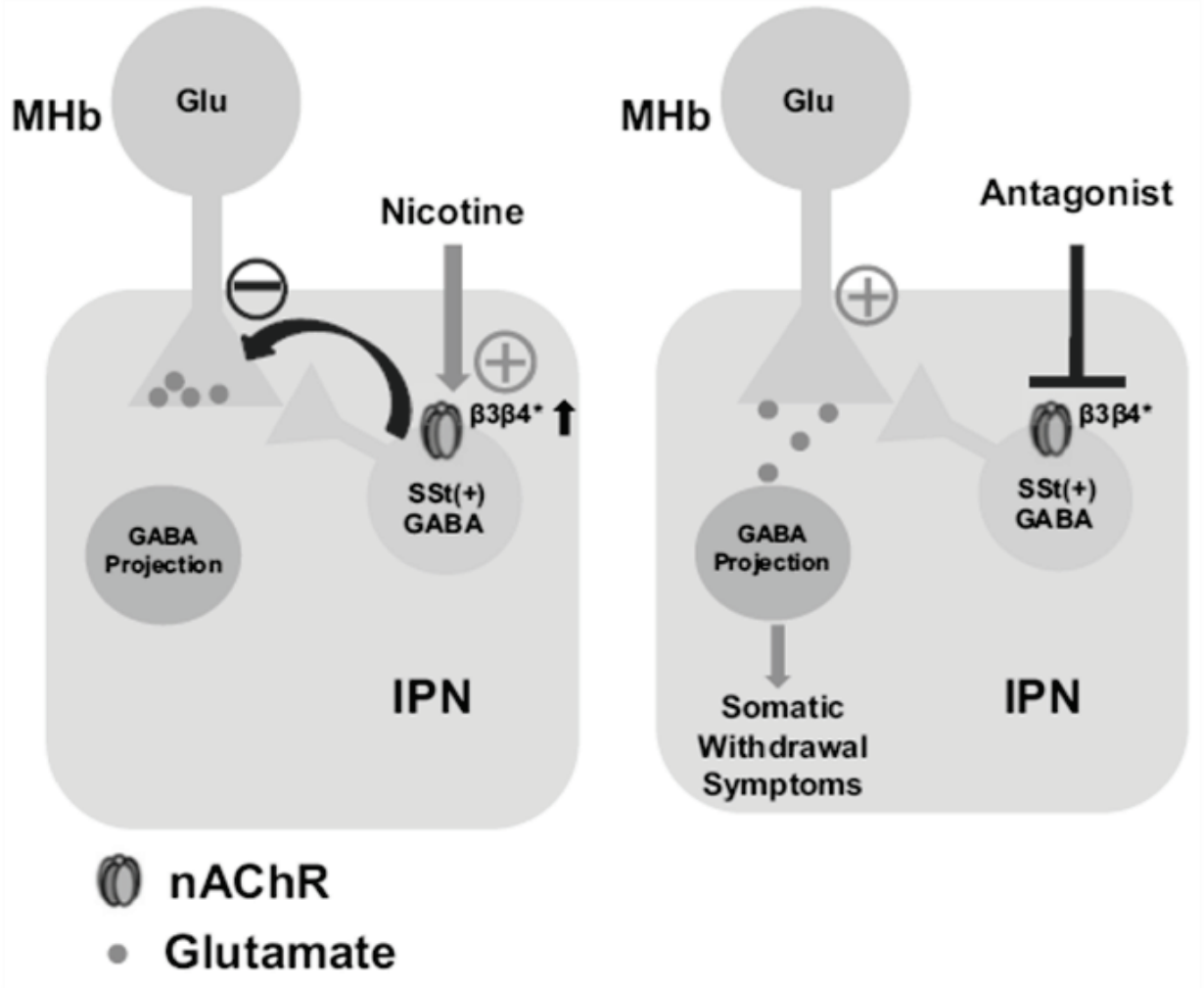

Figure 1.11- Diagram of a proposed IPN microcircuit. Left: In the circumstance of β4 overexpression or upregulation, activation of this receptor enhances the activity of somatostatin-positive (SSt) GABAergic interneurons. This serves to inhibit glutamate release from the MHb. Right: Antagonizing these receptors would then disinhibit glutamate release from the MHb, enhancing the activity of IPN projection neurons, and enhancing aversion. Mechanisms such as this can help to explain contradictory findings that have been reported. Adapted from (Zhao-Shea et al., 2013).

Further complicating the understanding of nicotinic signaling in these regions, MHb and IPN are not homogenous structures that can definitively be thought of as monolithic. Both regions have been divided into a variety of subregions based on anatomical markers, cell-type, and projection targets (Groenewegen et al., 1986; Hsu et al., 2013b). Investigations of the potentially variable roles of these regions are underway (Drenan and Lester, 2012; Hsu et al., 2013b; Zhao-Shea et al., 2015), and hopefully will yield a more complete picture of the functional roles of the MHb and IPN in behavior.

Although the MHb and LHb both seem to be important for aversion, the level at which each is understood differs substantially. Given the similarities in function and the fact that the MHb sends projections to the LHb, the LHb and MHb might interact with each other directly (Kim & Chang, 2005) or indirectly to work toward a common endpoint.

The LHb and its DA neuron inhibiting circuitry have been well characterized, but its role in the effects of nicotine has received very little attention. This circuitry also fits nicely into a reward-prediction error or an incentive motivation theory on reinforcement learning, but even this theoretical framework has been studied only rarely in relation to nicotine. Conversely, the MHb has been studied more extensively in relation to nicotine, but the mechanism by which the MHb might exert its control over nicotine's effects remains largely unknown. Some evidence suggests that similarly to the LHb, the MHb via the IPN might ultimately influence VTA DA neurons to exert its effects (Nishikawa et al., 1986; McCallum et al., 2012).

Interestingly, the IPN sends a strong projection to the LDTg (Groenewegen et al., 1986), making this a candidate pathway by which the MHb-IPN circuit might mediate nicotine aversion (see section 1j). Although it has been reported that α5-expressing IPN neurons largely project to the LDTg only from IPN subregions that do not receive input from the MHb (Hsu et al., 2013b),

non- α5-expressing IPN neurons might both receive input from the MHb and project to the LDTg. Additionally, IPN microcircuitry may reveal that α5-expressing neurons modulate other IPN projection neurons. Figure 1.12 shows anatomical evidence that relevant IPN neurons project to the LDTg. The IPN projection neurons are largely GABAergic (Hsu et al., 2013b; Zhao-Shea et al., 2013), suggesting that IPN inputs to LDTg are inhibitory. Importance of this connection would fit the model that enhanced VTA activity results in enhanced reward, while enhanced IPN activity results in enhanced aversion.

There is also evidence to indicate that the mechanisms mediating reward and aversion to nicotine are dissociable, and that the balance in activity of the two systems mediates the ultimate experiential outcome (Laviolette et al., 2002; Pastor et al., 2011; Grieder et al., 2012a). Supporting this idea, α5 KO mice experience no alteration in nicotine's rewarding effects, but they are resistant to the aversive effects of nicotine at higher doses. However, there does appear to be interaction between the two systems, since DA transmission has been implicated in both CPP and CPA to nicotine (Acquas et al., 1989), and differential DA signaling can alter the rewarding and aversive effects of nicotine administration (Grieder et al., 2012). Also, antagonism of β2-containing nAChRs in the VTA blocked both the rewarding and aversive affects of intra-VTA nicotine administration (Laviolette and van der Kooy, 2003). This might reflect the heterogeneity of cell-types and projection targets in the VTA, as some DA and GABA neurons are known to mediate aversive signaling (Brischoux et al., 2009; Lammel et al., 2012; Tan et al., 2012).

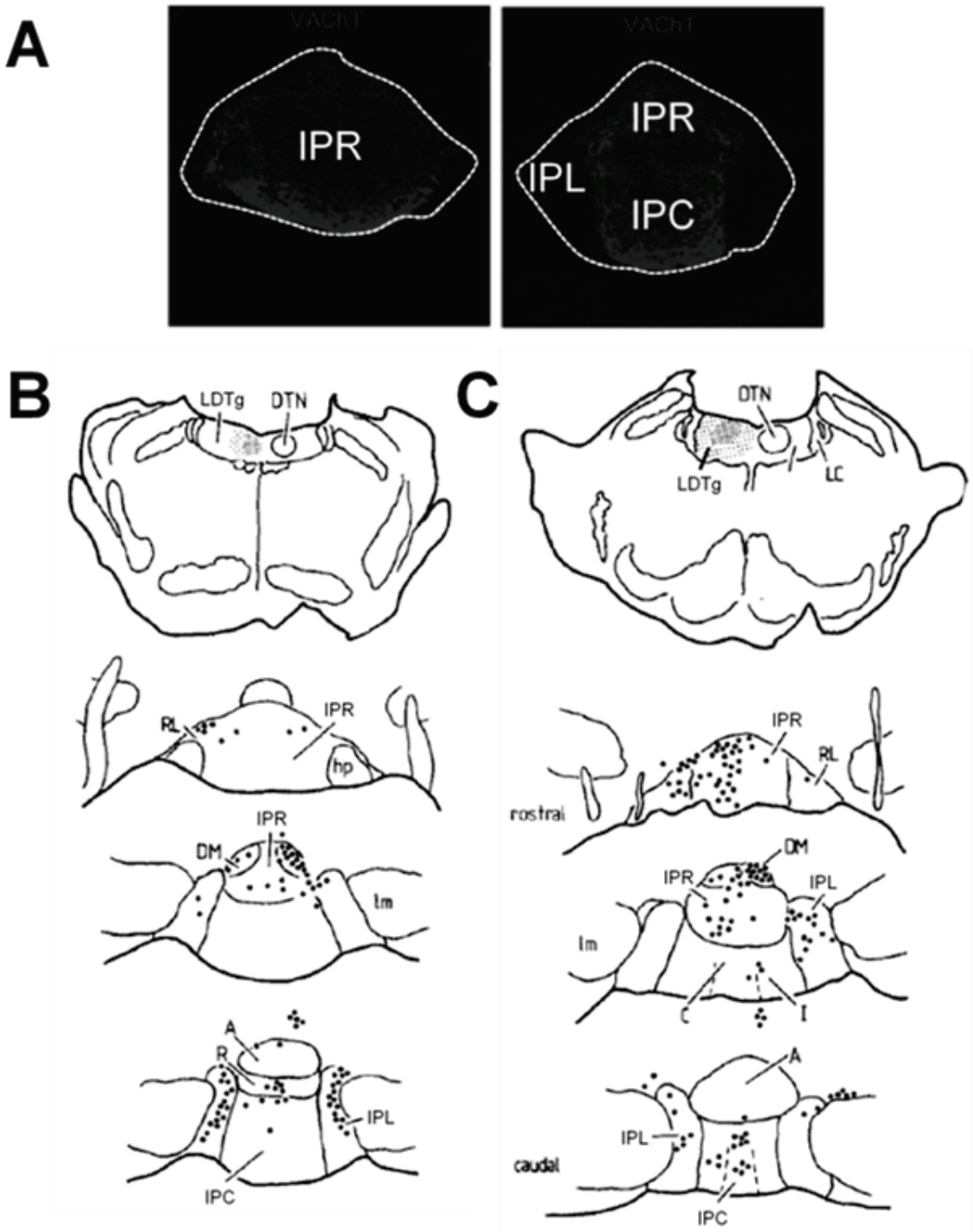

Figure 1.12- IPN Neurons that Receive Input from the MHb may Project to the LDTg. A) Immunohistochemical labeling of MHb cholinergic axons in the IPN (gray). These axons co-release glutamate. Dense innervation is observed in the IPR and IPC (the rostral and caudal subnuclei of the IPN, respectively). Adapted from (Ren et al., 2011). B+C) Drawings of the LDTg (top) showing the locations of retrograde labeling injections and drawings of the IPN (bottom 3) showing locations of IPN neurons that were labeled. Adapted from (Groenewegen et al., 1986). Labeling adapted from (Groenewegen et al., 1986; Hsu et al., 2013b).

In summary, the majority of findings that have manipulated and tested the activity in the MHb or IPN indicate that enhanced activity in this pathway mediates enhanced aversion, and that reduced activity in this pathway reduces aversion. It seems that these processes compete with reward circuitry, with the balance of activity between these systems dictating the ultimate behavioral state. Taken together, these results also suggest that human risk variants for nAChRs might increase risk for nicotine addiction by reducing overall nicotine-induced excitation of the MHb-IPN pathway compared to variants that do not confer this risk for addiction. That possibility, together with previous findings regarding the impact that an initial nicotine experience can have on future use and dependence (see section 1g), highlights the importance of understanding the acute effects of nicotine on these competing neural structures.

(1l) Outline of Experimental Aims.

The research presented in this thesis represents one of the first attempts to examine an important IPN projection target. The following chapters demonstrate that the IPN projection to the LDTg is a behaviorally relevant connection that mediates nicotine reward and is differentially modulated by high and low nicotine concentrations. *Chapter 2- Methodology* outlines experimental protocols, reagents, tissue preparations, behavioral assays, and equipment used for these experiments. *Chapter 3- Characterization and Relevance of IPN projections to LDTg* provides information concerning the neurotransmitters released from IPN axons into the LDTg and evidence concerning the behavioral relevance of this connection. *Chapter 4- Nicotine Dose-Dependently Modulates IPN Projections to LDTg* demonstrates that IPN terminals in the LDTg express low affinity nAChRs, providing a mechanistic explanation for nicotine's dichotomous effects. *Chapter 5-IPN Projections to the LDTg Mediate Aversion to Nicotine*

demonstrates that manipulating this connection is sufficient to alter nicotine behaviors. *Chapter 6- Discussion.*

Chapter 2

Methodology

(2a) Summary.

The experiments outlined in this and the following chapters involve the use of multiple preparations and techniques, each of which has its own experimental advantages and disadvantages. This chapter describes the rationale for using each technique and provides a detailed outline of methods for each experimental procedure.

(2b) Animals.

Adult male (>8 weeks old) C57/Bl6 mice from Jackson Laboratory or that were bred in-house were group housed in a colony room on a standard light-dark cycle (6 AM – 6 PM). Upon arrival (in the case of ordered mice), mice were undisturbed for at least 72 hours to allow acclimatization to the colony room. Water and standard chow were available ad libitum, and cages were changed once/week.

Adult, male mice were chosen so as to avoid confounds associated with different developmental stages and hormonal changes. Differential nicotine responses have been reported between young and adult animals and between male and female animals (Placzek et al., 2009; Levin et al., 2011; Burton and Fletcher, 2012). The studies that informed my thesis work also used adult mice (Fowler et al., 2011; Frahm et al., 2011), so using adult male animals maximized the extent to which I could compare my findings to the literature.

Although a reversed light-dark cycle would have allowed for testing of animals during the times that they are most active, constraints based on shared facilities with other labs prevented the preferable light-dark cycle from being used.

C57/B6 mice were chosen because most genetic manipulations are done in this strain. When I began my thesis work, I did not want to rule out having the choice to utilize more complex genetic manipulations should experiments warrant it. Had I used an alternate mouse strain and decided to transition experiments to a cre-line or a knock-out, for example, I would have had to repeat all of the experiments in a new strain.

(2c) Drugs.

All chemicals were obtained from Sigma Aldrich unless otherwise indicated. Nicotine (Nicotine hydrogen tartrate salt), DNQX (6,7-dinitroquinoxaline-2,3-dione, ABCAM), Bicuculline (Tocris), TTX (Tetrodotoxin citrate, ABCAM).

(2d) Slice Preparation.

Mice were rapidly decapitated following anesthesia with isoflurane (Baxter, Deerfield, IL). The brains were dissected in a solution of ice-cold protective artificial cerebrospinal fluid (aCSF) of the following composition: 92 mM N-methyl-D-glucamine (NMDG), 2.5 mM KCl, 1.25 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM CaCl2.4H2O, and 10 mM $MgSO_4.7H_2O$. The pH of the solution was titrated to 7.3–7.4 with concentrated HCl (which provides Cl^- counter-ions for NMDG). The protective aCSF was bubbled continuously with 95% O_2/5% CO_2, and 250-µm-thick sagittal or coronal slices containing IPN or LDTg were cut with a vibratome (VT100S,

Leica) in this solution. Slices were then incubated in a holding chamber for initial recovery at 32–34 °C for ≤ 15–20 min in the same protective aCSF (saturated with 95% O_2 and 5% CO_2). After this initial recovery period the slices were transferred into a holding chamber containing room temperature carbogenated aCSF of the following composition: 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 12.5 mM glucose, 2 mM CaCl2.4H2O, 2 mM MgSO4.7H2O, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 20 mM HEPES, perfused at a rate of 20 ml/min for at least 30 min before recording.

This neuroprotective recovery method was adapted from (Zhao et al., 2011; Ting et al., 2014; Ting, 2016), and I found this slicing method to more reliably maintain cell and slice health over the course of a recording day compared to slicing in an ice-cold sucrose solution, as in (Mansvelder et al., 2002; Fagen et al., 2007; Campioni et al., 2009; Baker et al., 2013). One concern of using this protective-recovery slicing method rather than the sucrose slicing method is that the protective solution contains N-acetyl-cysteine (NAC). NAC is the precursor for the anti-oxidant glutathione (GSH). Adult brain slices experience oxidative stress during slicing, which can result in rigidity of membranes, lipid peroxidation, and tissue deterioration (Ting et al., 2014), all of which make recording from neurons very challenging. Restoration of depeleted GSH can prevent these effects of slicing and optimize cell and slice health.

The problem with adding NAC to the slicing solution is that multiple studies have linked NAC to glutamate transmission and the reduction of addictive behaviors (Brown et al., 2013; Deepmala et al., 2015)(Bowers et al., 2015), including nicotine behaviors. If the effect of NAC in the slicing solution simply restores GSH levels to what they were prior to slicing, then this method is an improvement on the protective cutting (sucrose) method of slicing. However, if GSH levels are increased in excess and contribute to some synaptic change during slicing and

incubation that could attenuate addiction-relevant synaptic mechanisms, then this method may confound interpretation of data. Because slices are only transiently exposed to NAC and do undergo considerable oxidative stress during slicing, it seems likely that this method would only restore GSH levels not in excess of those prior to exposure.

(2e) Slice Electrophysiology.

For recordings, an individual 250 μm slice was transferred to a recording chamber superfused (~2 ml/min) with room temperature aCSF (in mM, 125 NaCl, 25 NaHCO3, 20 glucose, 2.5 KCl, 2.5 CaCl2, 1 MgCl2, 1 NaH2PO4, and pH 7.4, saturated with 95% O_2 and 5% CO_2). All physiology experiments were performed at room temperature on backlabeled neurons in the laterodorsal tegmental nucleus or on neurons of the interpeduncular nucleus which expressed either channelrhodopsin (ChR2) or archaerhodopsin (ARCH). Neurons were visualized under infrared illumination using an up-right microscope (Axioskop, Zeiss). Data were acquired with a Multiclamp 700A/Axopatch 200B amplifier and pCLAMP 9 software (Molecular Devices). Retrogradely-labeled or virally infected neurons were visualized under fluorescence and their fluorescence was bright enough that the electrode could be guided using combined fluorescence and bright field illumination.

Whole cell patch-clamp recordings were achieved with microelectrodes (3–6 MΩ) pulled on a Flaming/Brown micropipette puller (model P-97, Sutter Instrument, Novato, CA). Recording electrodes were filled with either normal potassium gluconate internal solution (154 mM K-gluconate, 1 mM KCl, 1 mM EGTA, 10 mM HEPES, 10 mM glucose, 5 mM ATP, 0.1 mM GTP, pH 7.4 with KOH) or an intermediate chloride concentration potassium gluconate internal solution (K-Gluconate 70 mM, KCl 70 mM, EGTA 1 mM, HEPES 10 mM, Glucose 10

mM, ATP 5 mM, GTP 0.25 mM, sucrose 15 mM, pH 7.4 with KOH). Light (473 nm or 532 nm) was delivered through the objective at maximal power (>40 mW) for activation of light-sensitive proteins. To record GABAergic currents, DNQX (100 μM) was added to the aCSF. To block GABAergic currents, 20 μM bicuculline was included in the aCSF and bath applied. To record miniature inhibitory post-synaptic currents (mIPSCs), TTX (1 μM) was included in the aCSF. Photocurrents were evoked using light delivered through the objective from fluorescent mercury lamp. Data were only included from recordings with series resistance <30 MΩ, and where input resistance or series resistance varied <25%.

For some experiments, nicotine was bath-applied to slices. Concentrations of nicotine were chosen that correspond to serum levels in a smoker's blood (100 nM) (Matta et al., 2007) or concentrations that were well beyond levels that would be voluntarily administered. Very high (10 μM) concentrations were used to pharmacologically determine whether relevant nAChRs were low or high affinity. Intermediate doses (1 μM and 500 nM) were also chosen in an effort to generate a dose-response curve and determine the relationship between the pathway of interest and dose of nicotine.

(2f) Methodology Rationale – Use of Optogenetic Tools.

Traditionally, the behavioral relevance of a particular brain region has been assessed either by local infusion of pharmacological agents, lesion studies, or electrical stimulation. While useful, these methods cannot target specific inputs to a particular brain region. For example, electrical stimulation, even in brain slices, activates all axon terminals in the vicinity of the stimulating electrode, regardless of their origin. My thesis work has focused on the IPN inputs to the LDTg. Infusion of drugs into the IPN, lesion of the IPN, or electrical stimulation of the IPN

would modulate the neurons (and glia) of the IPN regardless of terminal regions. Similar manipulations in the LDTg would modulate LDTg neurons (and glia) regardless of which inputs they receive. Optogenetic manipulation allows for the targeting of light-activated ion channels to specific cell-types in a specific brain region. Light can then be shined in terminal regions, specifically activating IPN neurons that project to the LDTg. Figure 2.1 diagrams the advantages of using optogenetic approaches in slice electrophysiology and *in vivo*.

One potential problem with activating terminals specifically in a particular brain region is that evoking an action potential in the axon terminal is likely to result in an antidromic action potential. If the neuron that receives stimulation collateralizes to a different brain region, then specificity of activation is compromised. Infusion of GABA agonists or other inhibitory agents into the cell body region that expresses photosensitive proteins can limit these effects. Alternatively, inhibitory opsins can be used in complementary experiments that suggest that stimulation is region-specific because inhibition at the terminal is unlikely to reach the soma or collaterals. This is the method used in the experiments outlined in the following chapters.

(2g) Surgical Procedures.

Anesthesia was induced and maintained with isofluorane at 4% and 1% respectively. Mice were placed in a stereotaxic frame and craniotomies were performed for brain injections. AAV2-hSyn- hChR2(H134R)-EYFP, AAV2-hSyn-EYFP, and AAV-hSyn-eArch3.0-EYFP were obtained from the University of North Carolina vector core and were injected directly into the IPN at a volume of 300 nL (AP: -3.5 mm, ML: -1.0 mm, DV: -4.92 mm from bregma at a 10° angle). These coordinates target the ventral IPN to avoid infection of the nearby VTA. For behavioral experiments, fiber optic ferrules were permanently implanted either above the IPN

(AP: -3.5 mm, ML: -1.0 mm, DV: -4.36 mm from bregma at a 10° angle) or unilaterally above the LDTg (AP: -5.25 mm, ML: + or -0.4 mm, DV: -3.4 mm from bregma). These coordinates target the caudal LDTg to avoid IPN terminals in the nearby dorsal raphe nucleus (DRN).

Dental acrylic (Lang Dental, Wheeling, IL) was used to secure the fiber optic implants (Sparta et al., 2012). For electrophysiology experiments, retrograde labeling was accomplished by injecting fluorescent microspheres (FluoSpheres, Life Technologies) bilaterally into the VTA (AP: -3.0 mm, ML: +/- 0.5 mm, DV: -3.0 mm from bregma). For all experiments requiring direct light stimulation of the IPN, animals were allowed to recover for at least 3 weeks before experiments were conducted. For experiments requiring light stimulation of IPN terminals, animals were allowed to recover for at least 6 weeks before experiments were conducted. Mice with incorrect viral, dye, or fiber optic placements were excluded from analysis.

(2h) Methodology Rationale – Stereotaxic Coordinates for Fiber Optic Implants.

Light spread through brain tissue has been calculated (Yizhar et al., 2011), and this was taken into account when choosing coordinates for implantation of permanent fiber optics. Shorter wavelengths penetrate tissue less than longer wavelengths, and my experiments used only 473 nm and 532 nm light. It has been reported that ~1-5mW of light is required to generate an action potential in neuron expressing ChR2 (Yizhar et al., 2011), but the light power required to depolarize terminals is unknown. Therefore, it is always possible in my *in vivo* optogenetic studies that some terminals are activated that are not in the LDTg. However, the relative effect of that non-specific activation should be relatively small, given the rapid decay of light through the brain and my efforts to avoid confounding structures being targeted by the fiber optic implant. Figure 2.2 shows the transmission of light through tissue relative to my fiber optic placements.

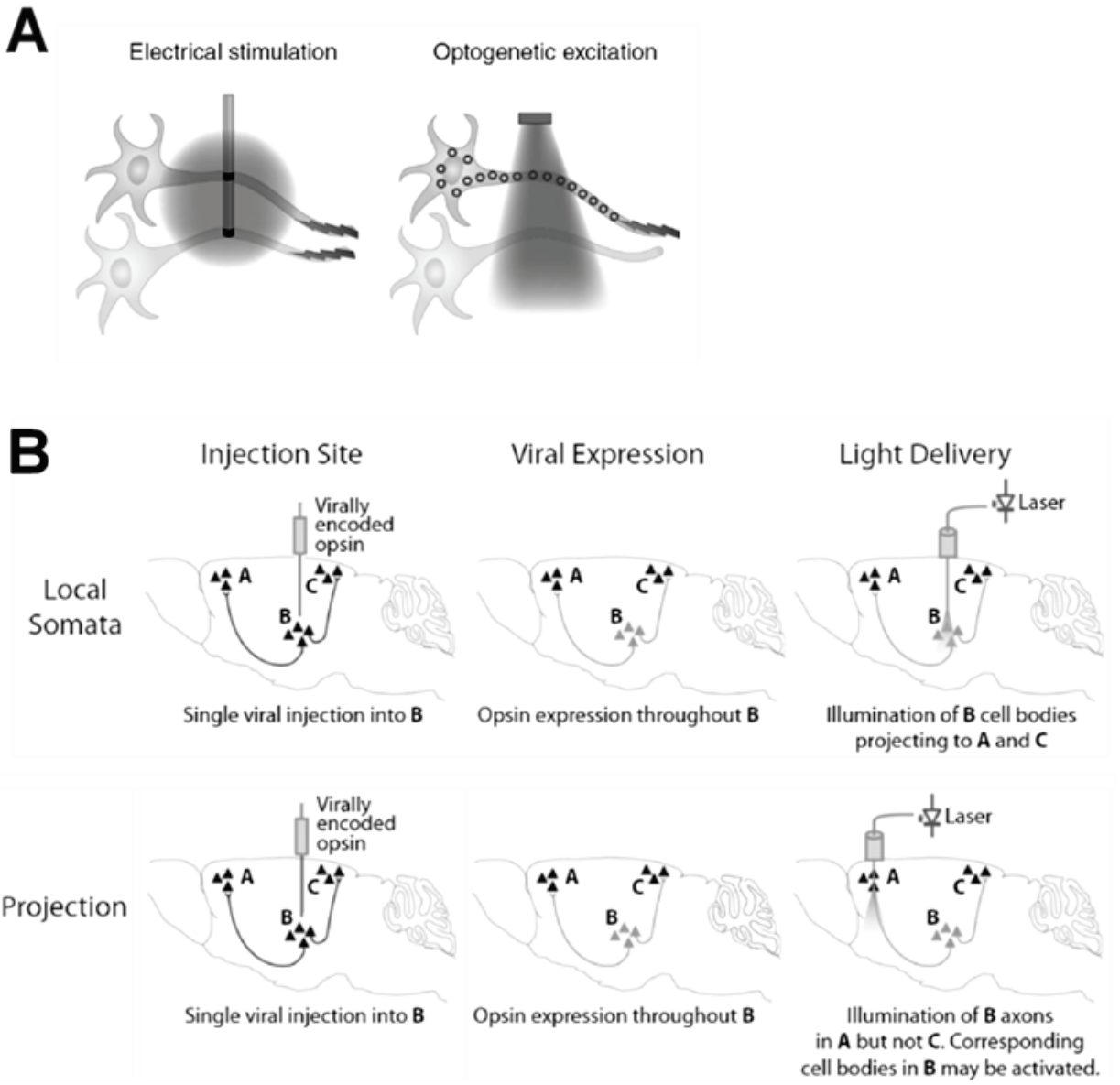

Figure 2.1- Advantages of Optogenetic Methods. A) Cartoon representing that traditional electrical stimulation activates any nearby cells with no specificity (left), while optogenetic stimulation can selectively activate opsin-expressing neurons without affecting nearby cells that do not express light-sensitive proteins (right). B) Top: Illustrates an experiment in which somata are directly activated by light. Left- virus containing the opsin is infused into brain region B. Middle- neurons in B express the opsin, including along their axons. Right- light is delivered directly to somata in B regardless of projection targets. Bottom: illustrates an experiment in which terminal projections of brain region B to brain region A are activated. Top- virus containing the opsin is infused into region B. Middle: neurons in B express the opsin, including along their axons. Bottom: light is delivered to region A only, selectively activating terminals from B that project to A. Adapted from (Deisseroth, 2011; Yizhar et al., 2011).

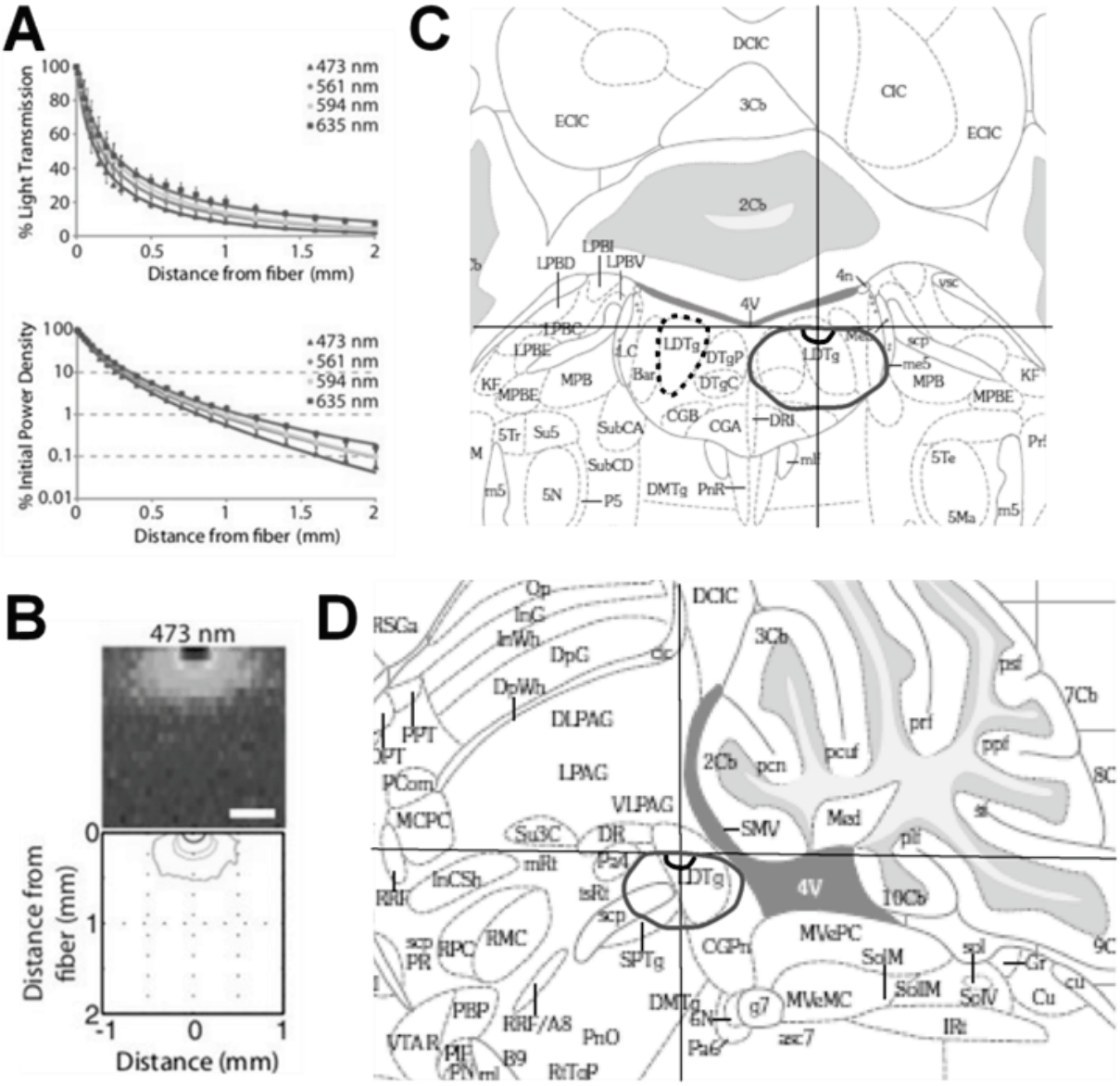

Figure 2.2- Light transmission through brain tissue. A) Relationships between light transmission and distance from the fiber and between power density and distance from the fiber. B) Dorsal-ventral and medial-lateral light transmission through brain tissue for blue light. Top panel: Darkest circular shape near the top represents the most light-intensity. Botton panel: Concentric rings demonstrate the distances at which light intensity is 50%, 10%, 5%, and 1% of maximum (innermost circle is max). C) Coronal slice at approximate AP coordinate for implantation of fiber optic implants above the LDTg. Intersection of the perpendicular lines indicates the point at which the fiber optic implant tip is placed. Contralateral LDTg is demarcated with dashed line. Black circle beneath fiber optic point corresponds to the maximal light intensity. The gray, larger circle corresponds to the distance at which light intensity is 1% of maximum. While the light will shine on regions outside of the LDTg, the caudal placement makes activation of terminals within the DRN less likely. D) Saggital slice at the approximate ML coordinate for fiber optic implant placement. Intersection and concentric circles represent the same as in C. Adapted from (Paxinos and Franklin, 2008; Yizhar et al., 2011).

(2i) Methodology Rationale – Viral Constructs and Opsins.

Adeno-associated virus (AAV) was chosen because it has low immunogenicity, high expression levels over long periods of time, does not infect fibers of passage, and has high multiplicity of infection. Additionally, AAV viral vectors were easily obtainable from the University of North Carolina vector core, and AAV is safer than lentivirus alternatives. AAV2 specifically was chosen because it has a more restricted expression pattern than the also commonly used AAV5 (Yizhar et al., 2011).

The human synapsin (hSyn) promoter was chosen because it specifically targets neurons and does not infect glia (Yizhar et al., 2011). The aim of my thesis work was in part to determine the nature of the projection from the IPN to the LDTg; therefore, a pan-neuronal approach was most appropriate.

The variant of channelrhodopsin chosen was hChR2(H134R) over ChR2 wild-type sequence because it is optimized for expression in mammalian systems (Deisseroth, n.d.). For optical inhibition, we chose archaerhodopsin (eArch3.0). We chose ARCH for photoinhibition rather than halorhodopsin (a light-activated Cl^- channel) because ARCH elicits larger inhibitory photocurrents than even third generation halorhodopsins and it does not result in rebound excitation upon inactivation (Mattis et al., 2011). Also, ARCH is responsive to green light, and green lasers are more cost-effective than yellow (which halorhodopsin is responsive to).

(2j) Histology.

For slice electrophysiology experiments: After recordings, slices were transferred to 4% paraformaldehyde for at least 24 hours. Correct localization of dye was confirmed by conducting immunohistochemistry. A rabbit antibody to tyrosine hydroxylase (TH) (1:500) was used to

identify dopamine neurons and used as an indicator of the boundaries of the VTA. If the majority of the dye was found to be outside of the VTA, the data recorded from that animal was excluded from analysis. Correct localization of viral infection was confirmed by immunohistochemistry as well. A chicken antibody to GFP (which also detects EYFP) (1:5000) was used to enhance the fluorescence for visualization. If the expression of EYFP was not confined to the IPN and segregated from the proximal VTA, data recorded from that animal was excluded. In a subset of slices, immunohistochemistry to visualize acetylcholine-expressing neurons was performed in an effort to confirm that recordings from back-labeled neurons were indeed conducted in the LDTg. A goat antibody to choline acetyltransferase (ChAT) (1:1000) was used to this end.

For behavioral experiments: Animals were anesthetized with isofluorane and transcardially perfused with 4% paraformaldehyde. Brains were kept in paraformaldehyde for >24 hours and then transferred to 30% sucrose in PBS for >24 hours. Brains were frozen in embedding medium (OCT Compund, Tissue-Tek, Sakura Finetechnical) and 50 μm slices were then taken using a cryostat (Leica CS3050 S). Fiber optic and viral expression were confirmed using anatomical markers (Paxinos and Franklin, 2008). Animals with either incorrect fiber optic placements or viral injections were excluded from analysis.

(2k) Methodology Rationale – Real-Time Place Preference Test.

As the name implies, the real-time preference test (RTPT) can be used to test the aversive or rewarding effects of a manipulation in real time. This behavior assay is largely used in optogenetic studies because of the temporal control required. The real-time apparatus consists of two sides of an arena that importantly lack contextual cues (see section 2m.1). When the mouse is on one side of the apparatus, no light stimulation/inhibition is delivered; when the mouse

crosses to the other side, light is delivered. This serves as an operant-like task in which the mouse can decide to ignore, seek, or avoid light delivery. Seeking light delivery suggests that the manipulation is reinforcing, while avoiding light delivery suggests that the manipulation is aversive. Results from the RTPT have been reported to correspond to those from the more traditional conditioned place preference paradigm (Stamatakis and Stuber, 2012; McCall et al., 2015) and are similarly reported by comparing times spent in each compartment.

The lack of contextual cues ensures that mice will not prefer one side of the apparatus over the other in the absence of light delivery and ensures that the only factor driving behavior is the immediate experimental manipulation. This allows for the same mice to be tested using different light stimulation frequencies (order of frequencies used is randomized and balanced between groups). Not only does this reduce the number of animals required to test a hypothesis, but it also allows for within group and individual comparisons based on intensity of stimulation. One potential limitation of this assay is its reliance on unchanged locomotor activity. Optogenetic stimulation or inhibition of neural pathways could potentially result in changes in locomotor behavior, so distance traveled and velocity measurements were taken during each RTPT. No gross changes in locomotion were observed during any RTPT conducted (see Chapter 3), and mice that exhibited abnormal locomotion were excluded from analysis. Given the reliance on this test of mobility, however, experiments in which nicotine was administered instead used conditioned place preference.

(2l) Methodology Rationale – Conditioned Place Preference Test.

The conditioned place preference (or aversion) test (CPP/CPA) has long been used to examine the reinforcing effects of a variety of stimuli as well as to investigate the potential abuse

liability of drugs (Tzschentke, 2007; Napier et al., 2013). Place preferences can also be conditioned to natural rewards and drugs of abuse in humans, supporting CPP as a valid model of measuring positive reinforcement (Childs and de Wit, 2009, 2013; Napier et al., 2013).

Although there are limitations to the interpretations of results from CPP tests, this test is thought to measure incentive-driven behavior mediated by Pavlovian conditioning (Huston et al., 2013). If an unconditioned stimulus (UCS) elicits a positively reinforcing unconditioned response (UCR), then the UCR is associated with the stimulus properties of the place, and the place becomes a conditioned stimulus (CS). The CS may then acquire positive incentive value of its own, and animals may seek the CS, which results in more time spent in that place. Figure 2.3 illustrates this process. Alternatively, if the UCS elicits a negatively reinforcing UCR, then the place becomes associated with a negative UCR and becomes a CS with negative incentive value.

Because nicotine at high doses substantially inhibits locomotor activity in mice (Jackson et al., 2010), assessing the effects of optogenetic manipulation on the aversive effects of nicotine was not feasible in the RTPT. CPP is an advantageous behavior test for these experiments because it tests animals in a drug-free state, so the acute locomotor effects of nicotine will not interfere with the expression of preference or aversion. Additionally, this procedure is sensitive to both reward and aversion, allowing the use of the same behavioral task to measure the effects of nicotine at either high or low doses. Although the interpretation of results from CPP is not necessarily more straightforward than self-administration, the procedure is much less technically challenging. The challenges associated with conducting nicotine experiments in rodents are well documented even amongst experienced behaviorists (Tzschentke, 2007; O'Dell and Khroyan, 2009), so the smaller technical challenge of establishing CPP was attractive as our lab primarily uses electrophysiological techniques.

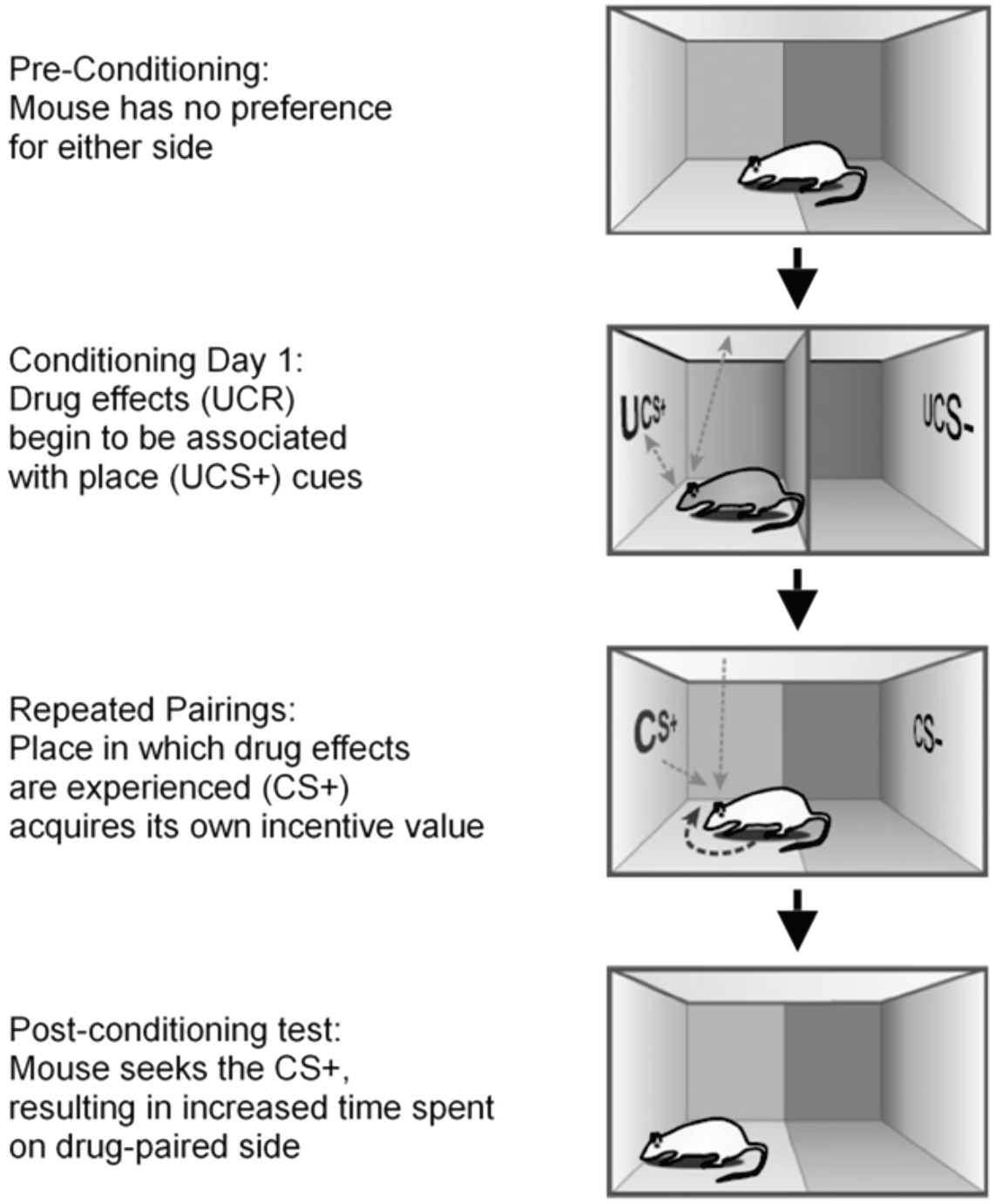

Figure 2.3- Pavlovian conditioning underlying conditioned place preference. Before conditioning, the mouse (presumably) has no preference for either side. During conditioning, the mouse experiences drug effects (indicated by his color change) that he begins to associate with the cues from the environment. After repeated pairings, the UCR to the drug becomes associated with the place in which it was experienced, and the place acquires its own incentive value. During the test for preference, the mouse seeks the place, resulting in increased time spent in the drug-paired compartment. Adapted from (Cunningham et al., 2006).

Although CPP is relatively easier to establish than self-administration, there are limitations to interpreting CPP experiments in general and specifically in the context of the experiments described in future chapters. One limitation is that there are multiple possible explanations for why a mouse might spend more or less time on the drug-paired side. For example, the drug (or other experimental condition) may cause an UCR that includes a behavioral change. The coincidence of the behavioral change and the rewarding effect of the drug may result in superstitious-like behavior that can be triggered by the drug-paired context during testing (Cunningham et al., 2006).

While the distinction between this process and Pavlovian processes is important to the interpretation of many studies, for the specific questions that my thesis aims to investigate, this distinction is not so critical. Both processes describe the animal performing behaviors as a result of a rewarding experience. More troubling is the possibility that CPP results might be a byproduct of conditioned treatment effects. For example, locomotor effects of a drug can be conditioned to a place, and then exposure to that CS can elicit drug-like locomotor behaviors. This might result in increased likelihood that the animal will leave the conditioned compartment, potentially masking a preference. The reverse is also theoretically possible- that a conditioned drug effect might prevent an animal from leaving a compartment regardless of an aversive experience (Cunningham et al., 2006). This alternate interpretation is challenging to rule out, but locomotion was measured during CPP tests in the experiments in the following chapters.

As mentioned, CPP has the advantage of testing mice in a drug-free state, which eliminates confounds associated with acute drug effects. However, as outlined in the paragraphs above, this does not ensure that the CS does not elicit drug-like effects, and recording locomotor or other potential conditioned treatment effects may help in interpreting results. CPP similarly

has the advantage of testing mice that are not immediately receiving photostimulation or inhibition, so outcomes should be independent of any transient effects of light delivery on behavior. However, just as with drugs, it is possible that repeated light treatments may cause a more permanent change or conditioned treatment effects. Again, locomotion was monitored in all CPP tests.

Additional potential issues with using CPP are that the expression of a preference or aversion relies on sensory processes, memory, and motivational states. These issues are more challenging and time-consuming to control for, and my thesis work was unable to encompass controls for all of these potential sources of confounds. All CPP results, therefore, should be interpreted with these caveats in mind.

(2m) Behavioral Assays.

Animals were habituated to the experimenter, the behavior room, handling, and having the fiber optic cable attached to their implants for 3-5 days prior to the start of the experiment. Mice were connected to an optical fiber connected to a laser. For ChR2 experiments, blue light (473 nm) was delivered through the fiber optic implants at a frequency of either 1 Hz or 20 Hz. For ArchT experiments, green light (532) was delivered continuously. All light was delivered at 7-12 mW power.

(2m.1) Real-Time Place Preference Test.

Mice were placed into a custom-made black acrylic two-chambered box (52.5 × 25.5 × 25.5 cm) and allowed to explore each of two chambers for 20 min (Stamatakis and Stuber, 2012; McCall et al., 2015; Siuda et al., 2015). Figure 2.4a shows a cartoon of the real-time preference apparatus. Using Noldus Ethovision hardware controller connected to a master 9 functional generator, light stimulation was delivered through fiber optic implants during the duration of

their time spent in the light-paired side of the chamber, and mice received no stimulation on the side paired with no light. The experimental animals were counterbalanced for both group and conditioning side. Preference or aversion in each experiment were determined by comparing the amount of time spent in the conditioned versus unconditioned sides during this real-time testing. All behavioral data were analyzed using Noldus Ethovision (v11).

(2m.2) Conditioned Place Preference Test.

Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described in (Bruchas et al., 2011; Al-Hasani et al., 2013). The compartmentalized box is divided into two equal-sized outer sections joined by a small center compartment accessed through a single doorway on each side. The compartments differed in wall striping (vertical vs horizontal alternating black and white lines). Figure 2.4b shows a cartoon of the conditioned place preference apparatus. On pre-conditioning day (day 1), mice were allowed free access to all the three chambers for 20 min. Time spent in each compartment was recorded with a video camera and analyzed using Ethovision 11 (Noldus). Mice were randomly assigned vehicle and drug compartments (unbiased design) and received a vehicle injection in the morning (10 ml/kg, i.p.) that was paired with no light, and a nicotine injection (1.5 mg/kg, i.p. as base) in the afternoon that was paired with light, at least 4 h after the morning training on 3 consecutive days. To test for nicotine place aversion, the mice were allowed free access to the three compartments again for 20 min on the 5^{th} consecutive day. For vehicle control experiments, animals received vehicle injections during both light and no light conditioning sessions. Any time that the animals were exposed to the conditioning apparatus, they were connected to the fiber optic cables. Filtered PBS was used as the vehicle for all experiments.

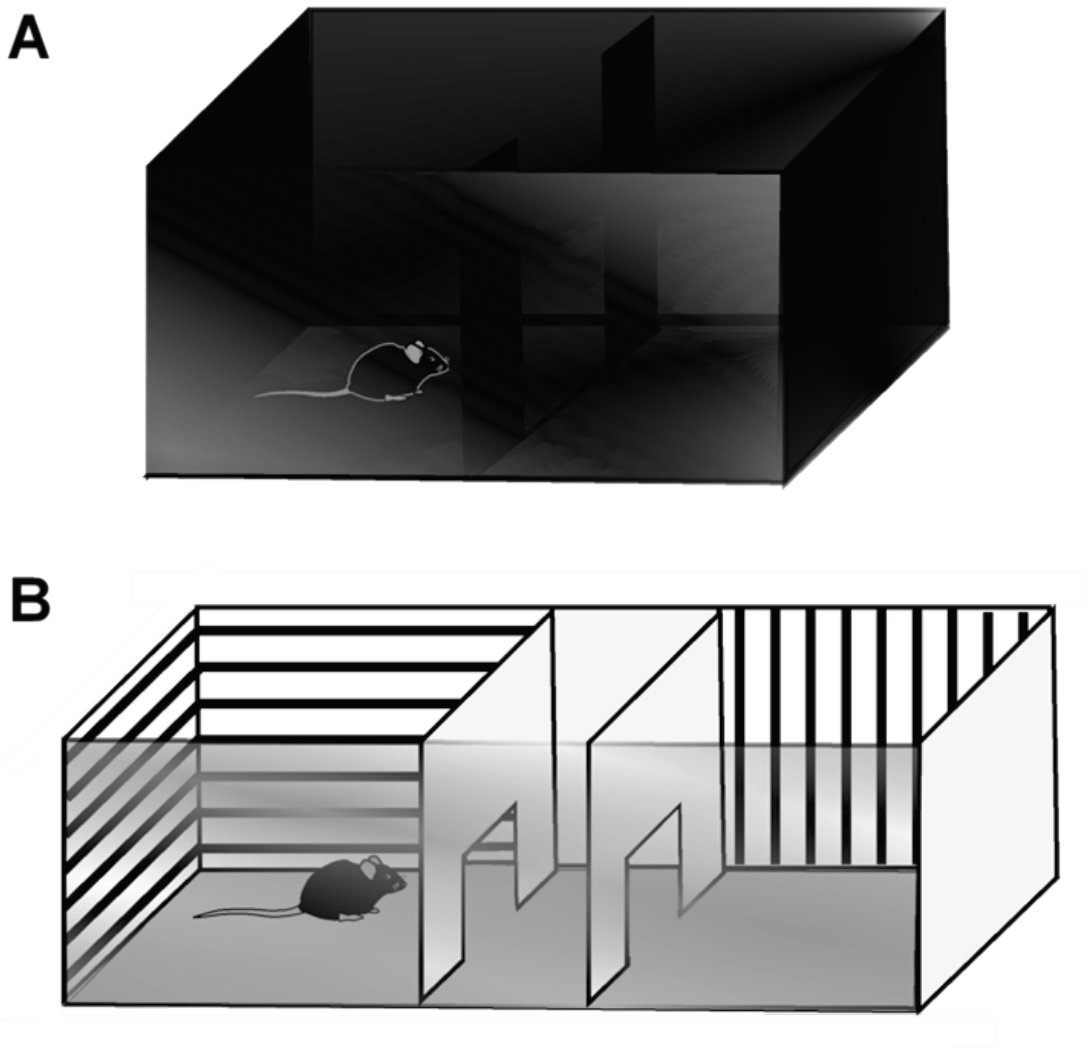

Figure 2.4- Behavioral Apparatuses. A) RTPT apparatus consists of two compartments separated by a corridor. Neither side has contextual cues. B) CPP apparatus consists of three compartments. The middle compartment is neutral and smaller than the other two. One side is horizontally striped and the other is vertically striped.

Although it has been suggested that a biased design is more effective for conditioning place preference or aversion in rodents (Le Foll and Goldberg, 2005), an unbiased design was used for these experiments. The effect of high doses of nicotine on control mice was expected to be expression of CPA; however, the effect of this dose of nicotine on the experimental group of mice was unknown. Therefore, an unbiased design was chosen so as not to artificially obscure or enhance a behavioral effect.

It has been reported that more conditioning sessions generally result in stronger conditioning, and many studies use between 4 and 6 conditioning sessions per treatment (Cunningham et al., 2011). However, in many studies conducted on nicotine CPP/CPA in mice, 3-day conditioning protocols are standard (Tammimäki et al., 2008; Jackson et al., 2009, 2010; Frahm et al., 2011). Our collaborators, who generously helped us to establish behavioral assays and optogenetic tools in our lab, also favor a 3-day conditioning paradigm (Al-Hasani et al., 2013; Al-hasani et al., 2015; McCall et al., 2015). By keeping the experimental design consistent with much of the literature, the results will be easier to interpret in the context of existing data.

Also consistent with much of the literature and with our collaborators, we decided to administer vehicle during the morning conditioning session and nicotine during the afternoon in all experiments. The rationale is that if given first, nicotine, which metabolizes very quickly in mice (Matta et al., 2007), might result in so-called "hangover" effects that could impact the afternoon conditioning session (Cunningham et al., 2006). However, this confound was avoided ultimately by accepting another – that vehicle and nicotine treatments were administered at different points in the sleep-wake cycle.

(2n) Statistical Analyses.

Miniature IPSCs were analyzed with Mini-Analysis (Synaptosoft, Decatur, GA). Amplitude and area thresholds were used to acquire events and each event was visually inspected to protect against software errors. The number of events per 10 sec time bin was assessed by the selection criteria. Unpaired t-test was used to identify significant frequency differences between baseline (100 seconds prior to nicotine application) and nicotine application periods (at least a 30 second duration) ($p<0.05$). All results are expressed as mean ± SEM. Amplitude changes in mIPSCs were tested using the Kolmogorov–Smirnov test on cumulative amplitude probability histograms. Chi square test was used to determine prevalence of nicotine effects between groups. Evoked IPSCs were differentiated from failures by the same amplitude criteria used in the analysis of spontaneous transmission: deflections from baseline >5 x RMS noise, with appropriate rise and decay characteristics, were considered to be successful transmission. Current fluctuation less than threshold were failures. The amplitude, rise time, and decay time of the evoked IPSC were determined in real time by the pCLAMP 9 software (Axon Instruments). Care was taken in all electrophysiological studies that the holding current and series resistance were stable through the entire experiment. Every IPSC was visually inspected to ensure that the software determined the parameters correctly. Unpaired t-test was used to identify significant light-evoked amplitude differences between baseline (100 seconds prior to nicotine application) and nicotine application periods (at least 180 second duration). A new slice was used for each experiment so that each slice was exposed to nicotine only once.

Behavioral data was analyzed using unpaired t-tests and ANOVA as appropriate.

Chapter 3

Characterization and Behavioral Relevance of IPN Projections to LDTg

(3a) Summary.

Although both the IPN and LDTg are thought to be important for regulating nicotine behaviors and a strong connection between these regions has been known for decades, the physiological and behavioral effects of IPN inputs to the LDTg remain unknown. Optogenetic tools were utilized in conjunction with electrophysiological and behavioral techniques to identify relevant neurotransmitters at this connection and assess its behavioral impact. Figure 3.1 shows a schematic representation of what was known prior to the completion of the following experiments.

ChR2 was expressed in the IPN of mice, and fiber optic implants were placed directly above the IPN. In line with previous reports, direct photoactivation of IPN somata resulted in real-time place avoidance in the real-time preference test (RTPT). Having confirmed the ability of IPN activation to drive behavioral avoidance, electrophysiological experiments were done in brain slices to determine the nature of IPN inputs to LDTg. LDTg neurons that project to the VTA were of particular interest, and the use of retrograde labeling dye injected into the VTA facilitated the identification of this population of LDTg neurons. ChR2 was again expressed in the IPN so that neurotransmitter could be released specifically from IPN terminals. Whole-cell

patch-clamp recordings and bath applications of antagonists revealed that the IPN sends GABAergic inputs to the LDTg. To examine the impact of activating these inhibitory projections from IPN to LDTg on behavior, ChR2 was again expressed in the IPN, and fiber optic implants were placed directly above the LDTg. Optogenetic activation of IPN terminals in the LDTg was sufficient to drive a real-time place aversion in the RTPT.

(3b) Background.

There is compelling evidence that enhanced MHb and IPN activity corresponds to enhanced aversion. High, aversive nicotine doses have been shown to activate the IPN more strongly than lower doses (Risinger and Oakes, 1995; Fowler et al., 2011), and decreased activation in the MHb-IPN pathway in response to nicotine results in increased self-administration of high doses of nicotine and diminished aversion to nicotine (Jackson et al., 2010; Fowler et al., 2011; Slimak et al., 2014). Enhanced responsiveness to nicotine in the MHb has been shown to enhance aversion to nicotine, and direct activation of GABAergic neurons in the IPN results in a nicotine withdrawal-like phenotype (Frahm et al., 2011; Zhao-Shea et al., 2013; Slimak et al., 2014). Despite these findings, experiments that explicitly test the relationship between activity in the MHb or IPN and aversion have not been done. There are also studies that report findings that may be contradictory to this hypothesis (Gallego et al., 2011; Glick et al., 2011; Jackson et al., 2013). For this reason, before investigating the impact of IPN activation on LDTg, the role of IPN activation in aversion was directly tested.

The IPN is a largely GABAergic nucleus (Kawaja et al., 1989; Hsu et al., 2013b; Zhao-Shea et al., 2013), but there are many sub-regions of the IPN and a variety of neurotransmitters are expressed (Groenewegen et al., 1986). Although strong inputs to the LDTg from the IPN

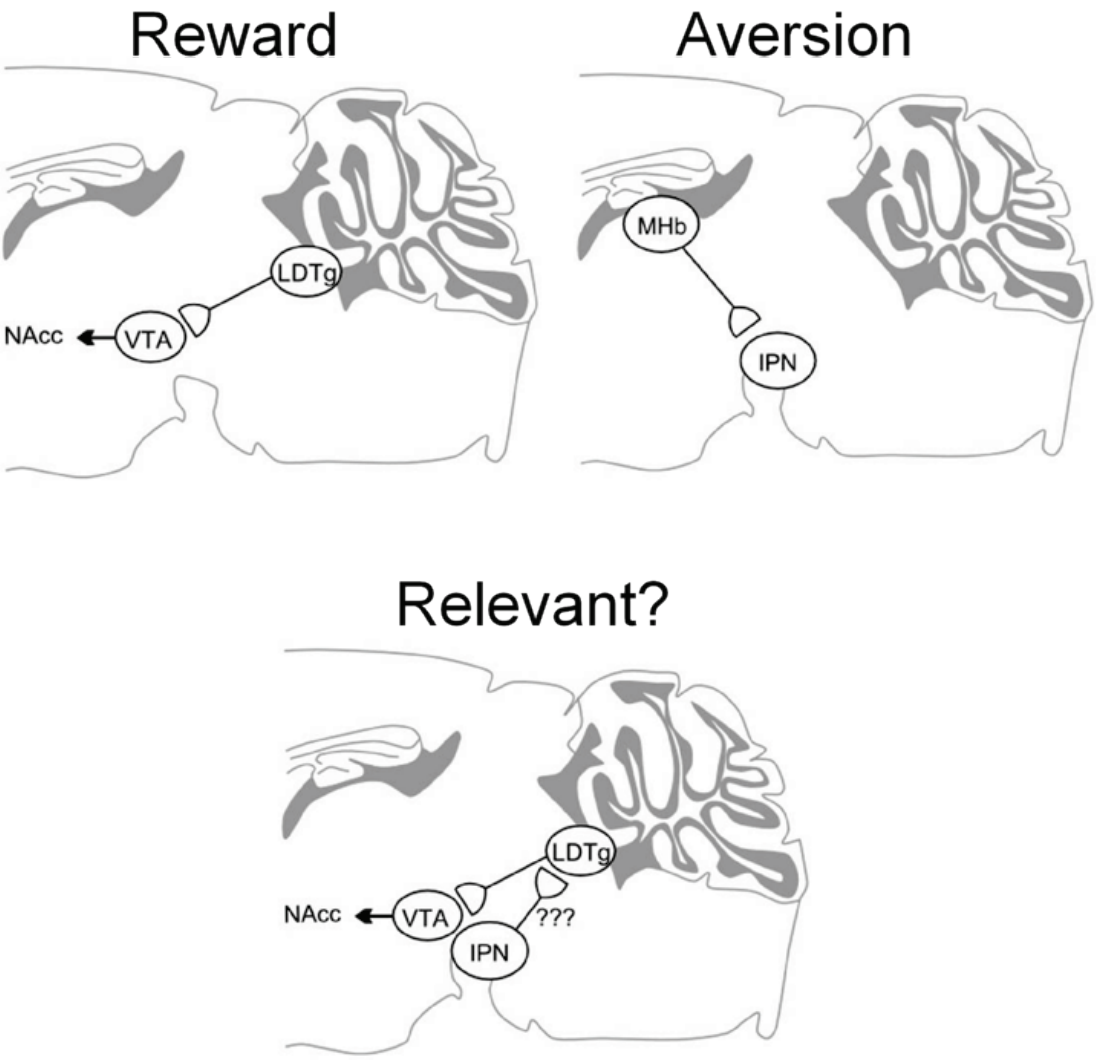

Figure 3.1- Existing knowledge of Reward and Aversive Circuits. Simplified schematic of the existing knowledge concerning the pathways involved in nicotine reward and aversion. Top left: LDTg sends excitatory projections to the VTA DA neurons that project to the NAcc. DA efflux in the NAcc is associated with positive reinforcement and reward learning. Top right: the MHb projection to the IPN seems to be more active in response to aversive doses of nicotine and less active in response to lower, rewarding doses. Altering excitation in the MHb-IPN pathway alters nicotine behaviors. Bottom: IPN is known to project to the LDTg, but the transmitters released at this synapse and its functional relevance was unknown.

have long been known (Groenewegen et al., 1986), investigation into the impact of these inputs on LDTg activity has been lacking. One study has begun to interrogate this connection using anatomical methods, but they only investigated the α5-expressing IPN neurons present in this pathway (Hsu et al., 2013b). While the α5 subunit is thought to be important for mediating nicotine effects in the MHb-IPN pathway (Fowler et al., 2011), its role in either brain region remains unclear.

Despite evidence that diminished function of α5-containing nAChRs contributes to propensity for nicotine dependence and heavy smoking (Fowler et al., 2011; Frahm et al., 2011; Ware et al., 2012; Jensen et al., 2015), understanding of the precise mechanism by which this occurs remains elusive. There is controversy about the expression profile of α5 subunits in the MHb (Hsu et al., 2013b), but even if that were resolved, the locations of the nAChRs that contain these subunits are unclear. Understanding whether α5 is expressed somatically, presynaptically, on projection neurons, on interneurons, or some combination of all of these in both the MHb and IPN is required before strong hypotheses can be generated. Therefore, despite evidence that α5-expressing IPN neurons (which are GABAergic) that project to the LDTg seem to lack input from the MHb (Hsu et al., 2013b), other cell-types in the IPN that do receive MHb inputs may project to the LDTg and thus mediate nicotine effects (Groenewegen et al., 1986). Therefore, the neurotransmitters released by the IPN in the LDTg were examined. Given the evidence that activation of IPN neurons and activation of VTA-projecting LDTg neurons mediate opposing effects of nicotine (aversion and reward, respectively) (Lodge and Grace, 2006; Lammel et al., 2012), and the large population of GABA neurons present in the IPN, it was hypothesized that the IPN would send inhibitory projections to the VTA-projecting LDTg neurons.

Following assessment of neurotransmitters present at the IPN-LDTg connection, the behavioral relevance of the IPN inputs to the LDTg was investigated using the RTPT. Because of the known role for the LDTg in gating VTA DA activity and the evidence to suggest that the IPN mediates aversion to nicotine, it was hypothesized that activating IPN inputs to the LDTg would elicit place avoidance. Indeed, unilateral activation of IPN terminals in the LDTg was sufficient to drive real-time place aversion.

(3c) Methods. – see *Chapter 2* for detailed methodology.

(3d) Confirmation of Functional ChR2 Expression.

Before testing the behavioral impact of IPN activation, assessment of restricted and functional expression of ChR2 in the IPN was required. Fluorescent microscopy and brain slice electrophysiology were used in this endeavor. Channelrhodopsin with an EYFP tag (ChR2) under the control of a pan-neuronal synapsin promoter was expressed by infusion of adeno-associated virus (AAV2-hSyn- hChR2(H134R)-EYFP) directly into the IPN of wild-type mice. After >3 weeks, whole-cell recordings were done in EYFP-labeled IPN neurons. Slices were then fixed for immunohistochemistry. Slices were immunolabeled for tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine production (to define the boundaries of the vicinal VTA) and GFP (to enhance the EYFP signal). Slices were then mounted and imaged on the fluorescent microscope. ChR2 expression was visually confirmed by fluorescence microscopy, and animals with EYFP expression outside the boundaries of the IPN were excluded from the study. Figure 3.2 shows a schematic of virus injection and representative images of ChR2 expression.

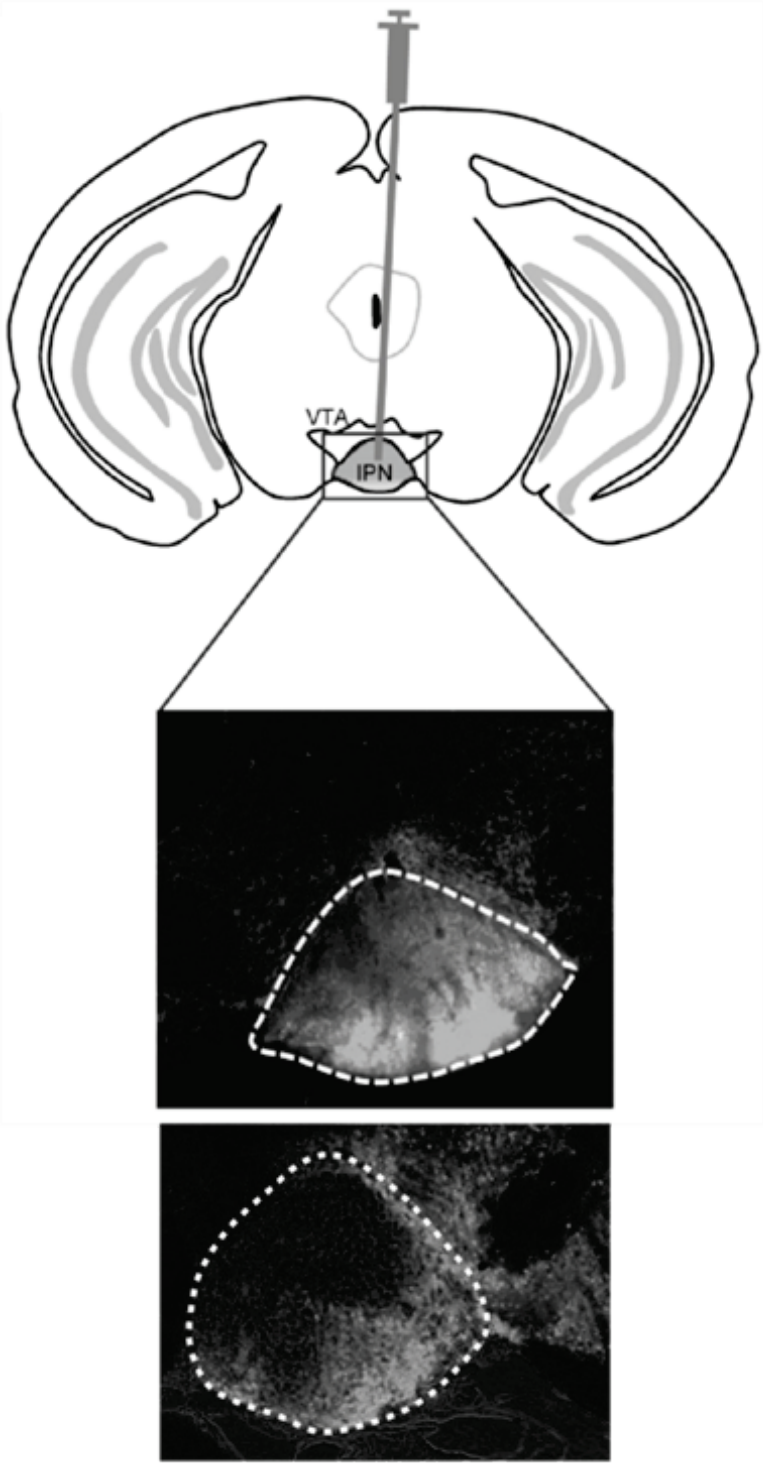

Figure 3.2- Confirmation of restricted ChR2 expression. Top: schematic showing location of virus injection in a coronal slice. IPN and vicinal VTA are indicated. Middle: dashed line demarcates the IPN. Brightness inside the IPN is EYFP labeling (originally green). Labeling outside the IPN is TH expression in the VTA (originally red). Bottom: example of unrestricted ChR2 expression. Dashed line again demarcates the IPN. All labeling in this image is EYFP. Note the EYFP labeling in the VTA (to the right of the IPN).

Functional expression of ChR2 in IPN neurons was confirmed using whole-cell patch-clamp electrophysiology *in vitro*. EYFP-labeled neurons were recorded from, and blue light (473 nm) was delivered through the objective. In voltage clamp recordings, blue light delivery resulted in inward currents that were sustained for the duration of the light pulse. In current clamp recordings, 5 ms light pulses delivered at 10 Hz reliably evoked action potentials, as did 10 ms light pulses delivered at 20 Hz. Figure 3.3 shows experimental set-up and representative traces from these recordings.

(3e) Optogenetic Activation of IPN Neurons Elicits Place Avoidance.

To determine the behavioral effects of IPN stimulation, either channelrhodopsin with an EYFP tag (ChR2) or EYFP alone under the control of a pan-neuronal synapsin promoter was expressed. To this end, Adeno-associated virus (AAV2-hSyn- hChR2(H134R)-EYFP or AAV2-hSyn-EYFP) was infused directly into the IPN of wild-type mice. Blue light (473 nm) was delivered to the IPN neurons through a permanently implanted fiber optic during behavioral testing. Real-time place preference was then assessed in response to photostimulation. Figure 3.5 illustrates the experimental design and shows fiber optic implant placements.

As discussed, a real-time place preference test was used to examine the behavioral effects of IPN activation (McCall et al., 2015; Siuda et al., 2015). When an experimental mouse crossed into the light-paired, contextually similar side of an open field, light stimulation was constantly pulsed (10 ms at 20 Hz) until the mouse crossed back into the non-light paired side. A strong stimulation frequency was chosen to mimic the hypothesized effects of high doses of nicotine on IPN neurons (Brown et al., 1983). Mice expressing ChR2 spent significantly less time on the

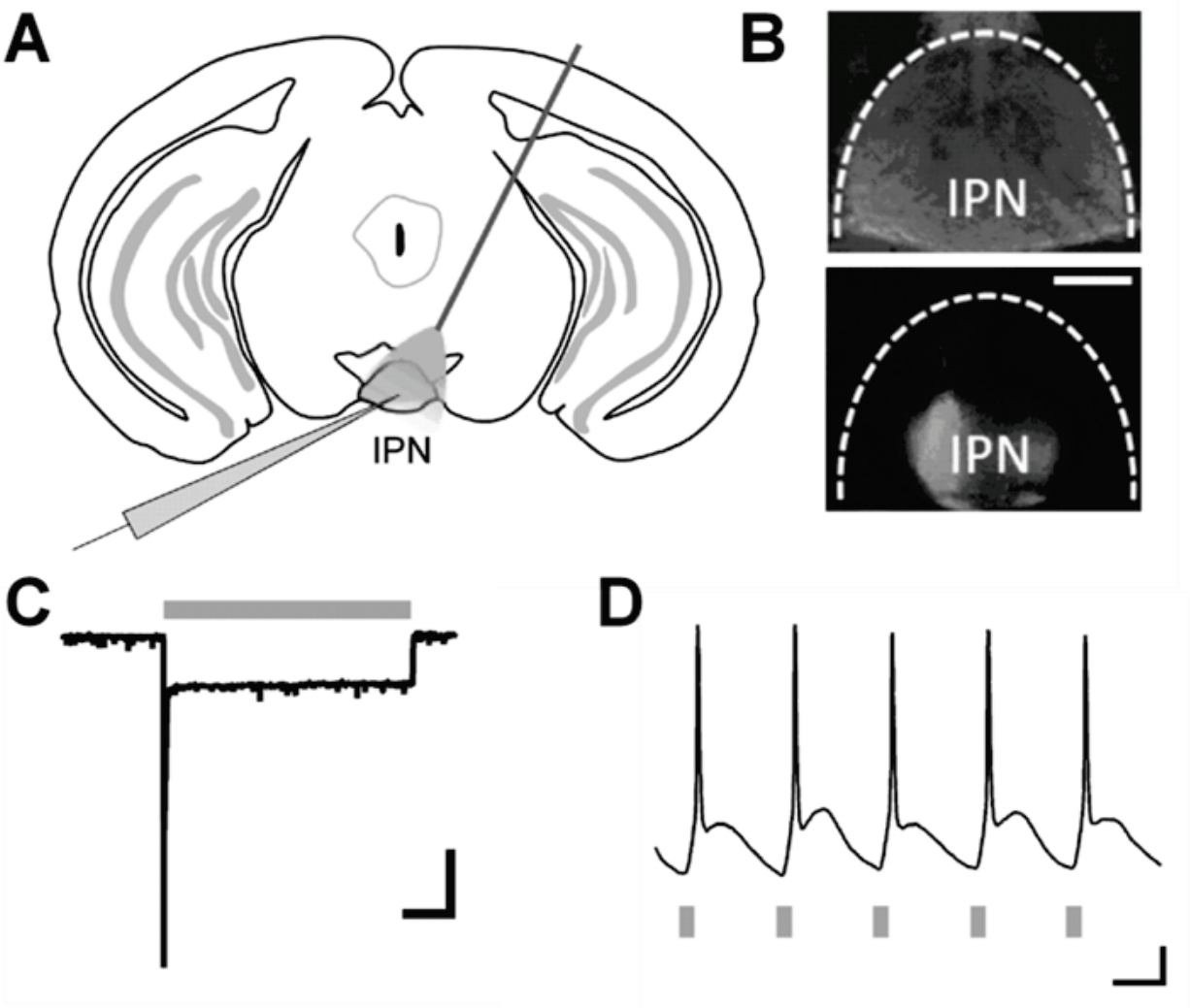

Figure 3.3- Functional ChR2 Expression in the IPN. A) Schematic illustrating that ChR2 was expressed in the IPN, and that those neurons were directly stimulated with blue light during whole-cell recordings. B) Transmitted light (top) and fluorescent (bottom) images of the IPN showing that ChR2 expression was confined to the IPN (250 μm scale). C) Blue light delivered to the slice for 5 seconds during whole-cell patch-clamp recordings results in a sustained inward current in IPN neurons expressing ChR2 (1 second, 100 pA scale). D) Blue light delivered in 5 ms pulses at 20 Hz reliably evokes action potentials in IPN neurons expressing ChR2 (50 ms, 10 mV scale).

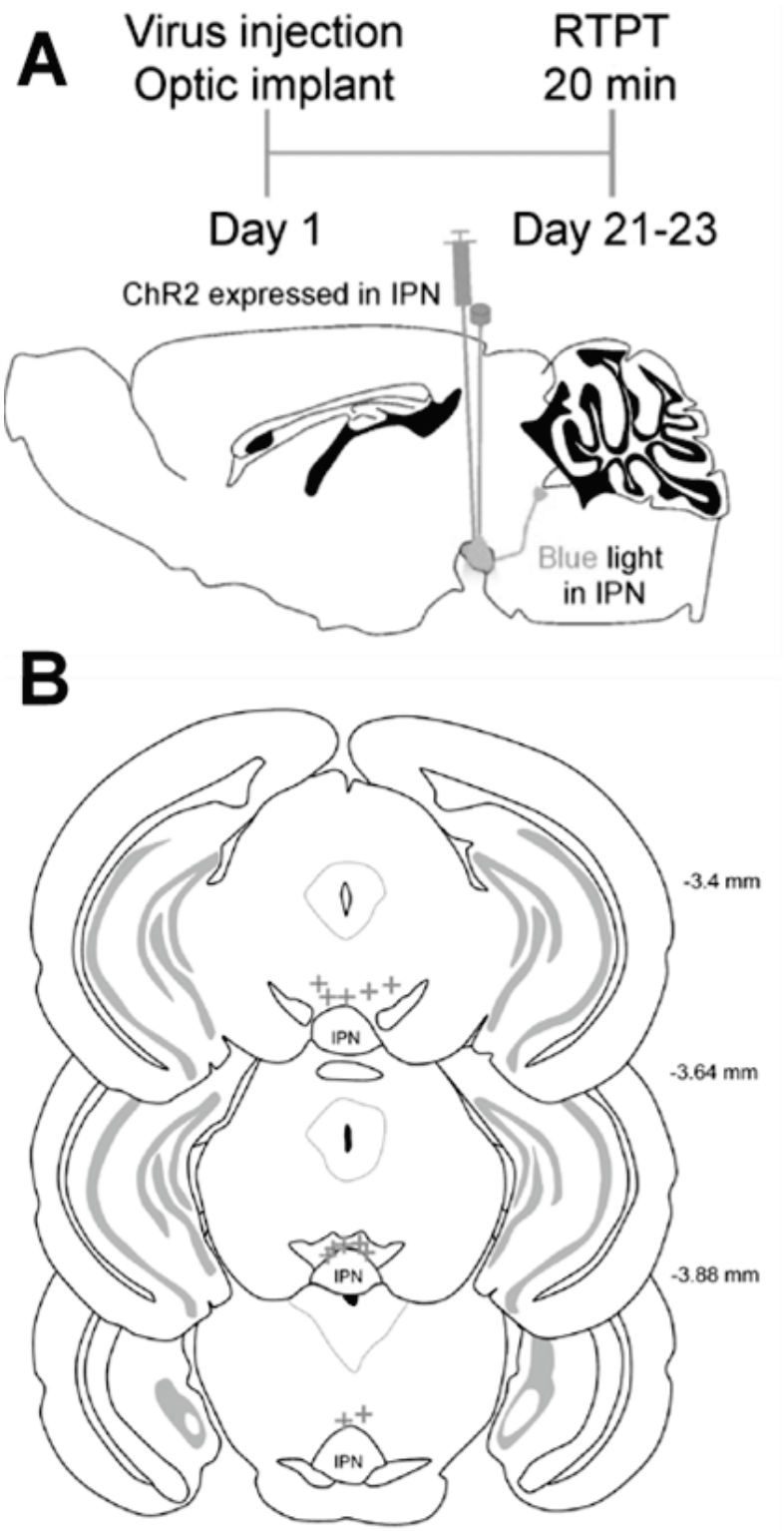

Figure 3.4- Experimental Set-up and Fiber Optic Implant Placements. A) Timeline of experiment from surgery to RTPT. ChR2 was expressed in IPN neurons; blue light was delivered to the IPN. B) Coronal sections showing the confirmed locations (+) of implants above the IPN for both ChR2 and EYFP groups. Numbers indicate distance from bregma.

light-paired side of the real-time apparatus, while mice expressing only EYFP spent equal times on both sides.

To compare these results with more tonic activation of IPN neurons, the same experiment was performed with a weaker stimulation protocol (10 ms at 1 Hz) (Brown et al., 1983). Under these conditions, both groups of mice spent equal times on both sides of the chambers. These different stimulation parameters did not result in differences in total distance traveled, suggesting that the behavioral output was not mediated by gross changes in locomotion. These data suggest that direct stimulation of IPN neurons produces avoidance behavior, which is consistent with previous studies showing that enhanced IPN activity corresponds to enhanced aversion (Fowler et al., 2011; Frahm et al., 2011; Zhao-Shea et al., 2013). Figure 3.5 shows these results.

(3f) IPN terminals make GABAergic synapses onto LDTg neurons that project to the VTA.

The laterodorsal tegmental nucleus (LDTg) contributes to reward through its excitatory projections to the mesoaccumbens dopamine neurons of the VTA (Lodge and Grace, 2006; Lammel et al., 2012). Recent studies suggest that the GABAergic neurons of the IPN are particularly important for nicotine-related behaviors (Zhao-Shea et al., 2013), and the LDTg receives a strong afferent connection from the IPN(Groenewegen et al., 1986). Thus, it was hypothesized that the IPN projections to the LDTg could be an important control point for aversion, and that this might be mediated by IPN inhibition of the LDTg.

To address this hypothesis, ChR2 was again expressed in the IPN of adult male mice. For synaptic studies, the focus was specifically on LDTg neurons that are a part of the LDTg-VTA-NAcc circuit (Lammel et al., 2012). This required injection of fluorescent microspheres bilaterally into the VTA. LDTg neurons that synapse in the VTA take up the dye and label their

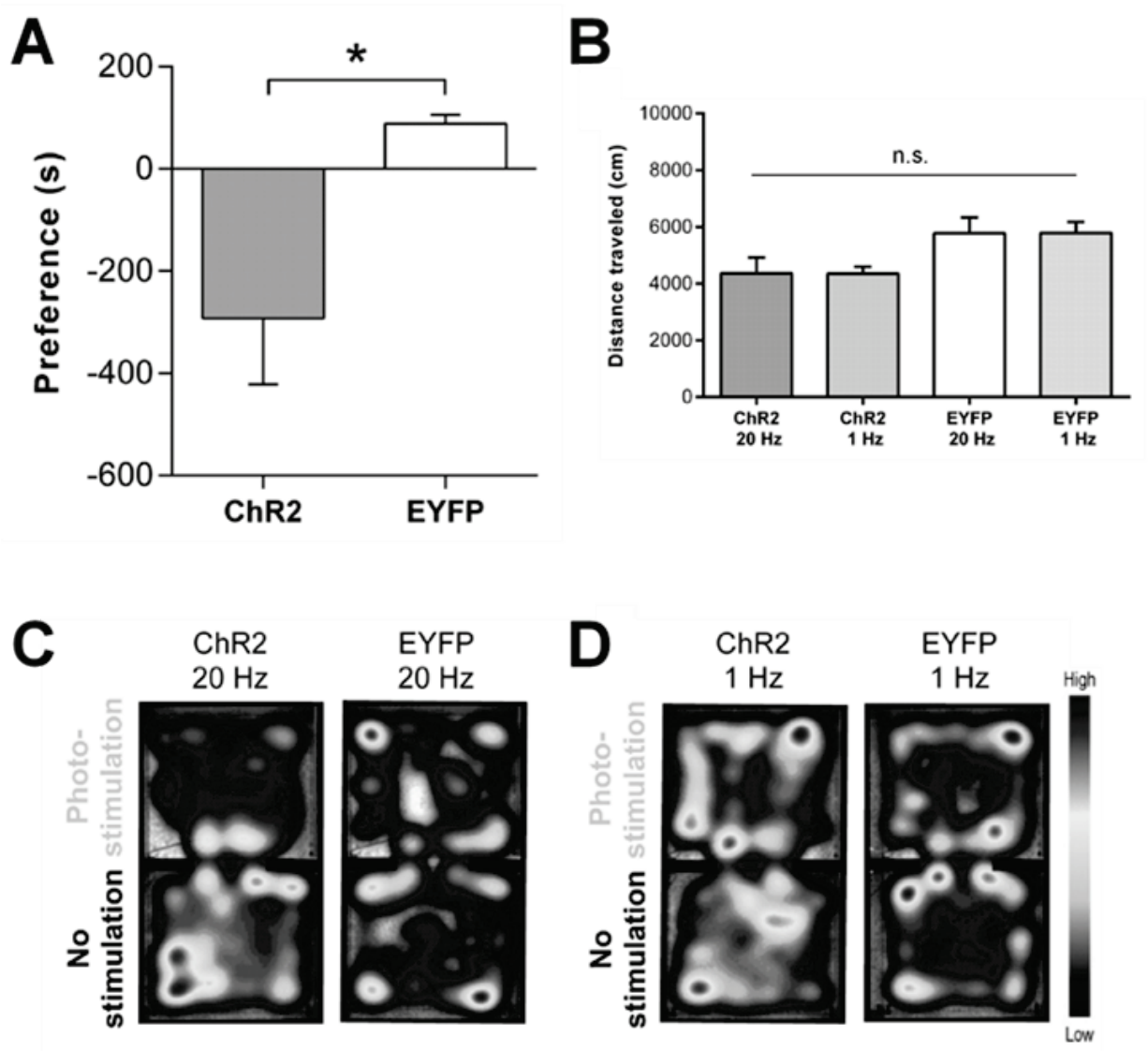

Figure 3.5- Behavioral Effects of Direct IPN activation. A) Direct stimulation in vivo (10 ms pulses at 20 Hz) of the IPN with blue light through permanent fiber optic implants results in a real-time place aversion. Mice expressing ChR2 spend significantly less time in the light-paired compartment compared to mice expressing EYFP (unpaired t-test, P=0.0266). Preference calculated as time in no-light paired side subtracted from time in light-paired side. B) Direct stimulation (10 ms pulses at 20 or 1 Hz) of the IPN does not result in locomotor effects. C) Representative heat maps for ChR2- and EYFP-expressing mice during 20 Hz stimulation. Brighter color indicates more time spent. D) Representative heat maps for ChR2- and EYFP-expressing mice during 1 Hz stimulation. Data are represented as mean ± SEM.

somata, allowing visual identification during slice electrophysiology. After >6 weeks (to ensure ChR2 expression extended fully to the axon terminals), whole-cell voltage clamp recordings were done in back-labeled LDTg neurons. IPN terminals were stimulated with blue light through the objective. Figure 3.6 illustrates the experimental design.

Using an internal solution with a Cl^- reversal potential of -125 mV, light stimulation evoked outward currents. These currents were not affected by bath application of the glutamate receptor antagonist DNQX, but were completely blocked by the $GABA_A$ antagonist, bicuculline (20 μM). Additionally, the current-voltage relationships of the light-evoked synaptic responses were determined using two internal solutions with different Cl^- reversal potentials (-125 mV and -17 mV). The light evoked currents reversed at -114.2 mV when using the internal solution with a Cl^- reversal potential of -125 mV and at -15.971 mV when using the internal solution with a Cl^- reversal potential of -17 mV (after adjusting for junction potentials). Together, these findings indicate that GABA release from IPN neurons activates $GABA_A$ receptors on LDTg neurons that project to the VTA. Figure 3.7 shows these data.

(3g) Optogenetic Activation of IPN Terminals in the LDTg Elicits Place Avoidance.

To examine the behavioral relevance of the IPN inputs to the LDTg, the RTPT was used. Because the IPN projection to the LDTg is GABAergic, activating the IPN terminals in the LDTg should inhibit LDTg neurons. It was hypothesized that this inhibition of LDTg output would induce aversive behavioral effects via indirect suppression of VTA DA neurons.

Either ChR2 or EYFP was expressed in the IPN neurons of adult wild-type mice, and fiber optic implants were placed unilaterally above the LDTg. In this way, selective activation of

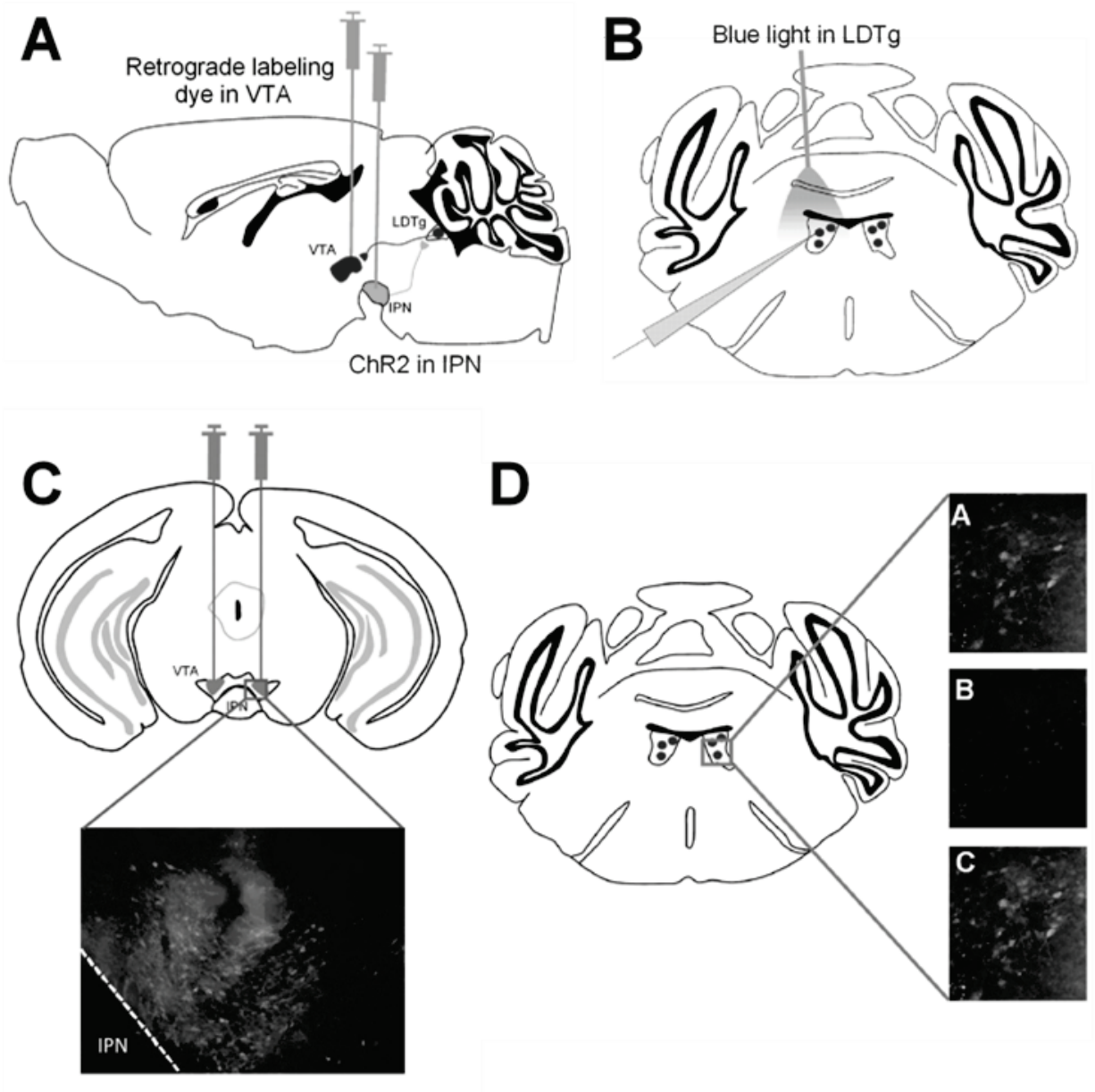

Figure 3.6- Experimental Design for Electrophysiology Experiments. A) Schematic of a saggital slice representing the locations of retrograde microspheres and virus injection. B) Schematic of the recording configuration depicted in a coronal slice. Black dots indicate back-labeled neurons within the outlined LDTg. Recording electrode and blue light delivery are shown. VTA-projecting (back-labeled) neurons in the LDTg were recorded from. Blue light was delivered to stimulate IPN terminals in the LDTg. C) Coronal section depicting retrograde labeling dye infusion sites. Dye was infused bilaterally in the VTA. Representative image below shows hazy (originally red) dye overlaid with TH immunostaining (somata, originally green). D) Coronal slice showing back-labeled neurons in the LDTg. DA: ChAT immunostaining to delineate the boundaries of the LDTg (originally green). DB: back-labeled neurons in the LDTg (originally red). DC: overlay.

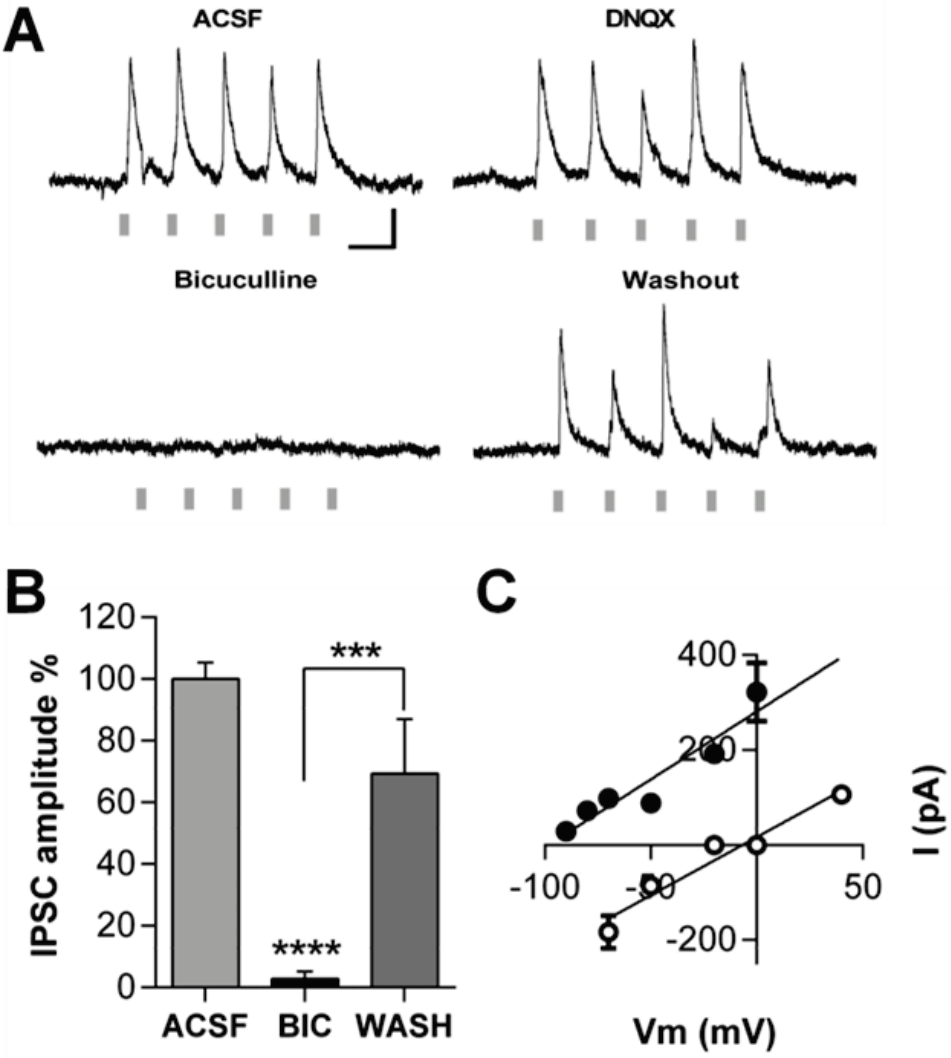

Figure 3.7- LDTg neurons which project to the VTA receive GABAergic inputs from the IPN. A) Stimulation of IPN terminals results in inhibitory currents in VTA-projecting LDTg neurons. These currents are unaffected by bath application of DNQX (100 μM), but are completely blocked by bicuculline (20 μM). The currents recovered after washout of bicuculline (100 ms, 20 pA scale). C) Summary graph showing normalized current amplitudes prior to bicuculline application, in the presence of bicuculline, and after washout of bicuculline (one-way ANOVA, $P<0.0001$, Holm-Sidak test for multiple comparisons). D) Current-voltage relationship of the light-evoked synaptic response was examined using two internal solutions that have differing Cl^- reversal potentials (closed circles: -125 mV, open circles: -17 mV). The light evoked currents reversed at -98.30 mV and -6.671 mV (linear regression, 95% CI: -144.6 to -78.51; 95% CI: -23.78 to 16.94; slopes not significantly different $P=0.4098$; Adjusted for junction potentials, light evoked currents reverse at -114.2 mV and -15.971 mV). Data are represented as mean ± SEM.

IPN terminals in the LDTg could be achieved. Figure 3.8 shows the experimental set-up and fiber optic placements.

Blue light stimulation was delivered through the fiber optic implant and constantly pulsed (10 ms at 20 Hz) when the mouse was in the light-paired side of the apparatus. Again, this strong stimulation frequency was chosen to mimic the strong activation of this pathway that might occur in response to aversive nicotine doses. Mice expressing ChR2 spent significantly less time on the light-paired side under these conditions, while mice expressing EYFP spent equal times on both sides of the chamber.

To compare these results with more tonic activation of IPN neurons, the same experiment was performed with a weaker stimulation protocol (10 ms at 1 Hz). Under these conditions, both groups of mice spent equal times on both sides of the chambers. These different stimulation parameters did not result in differences in total distance traveled, suggesting that the behavioral output was not mediated by gross changes in locomotion. These data support the idea that the LDTg is an important downstream target of the IPN in mediating aversion. Figure 3.9 shows these results.

(3h) Discussion.

Though the MHb projection to the IPN has been implicated in the regulation of reward and aversion to nicotine, the downstream targets of this connection and the mechanisms by which this pathway mediates aversion have remained unknown. These investigations into the IPN projections to the LDTg provide new insights into how aversion-related circuitry interacts with reward-related circuitry. These studies show that direct, optogenetic stimulation of IPN neurons results in behavioral avoidance and that the IPN sends GABAergic projections to LDTg

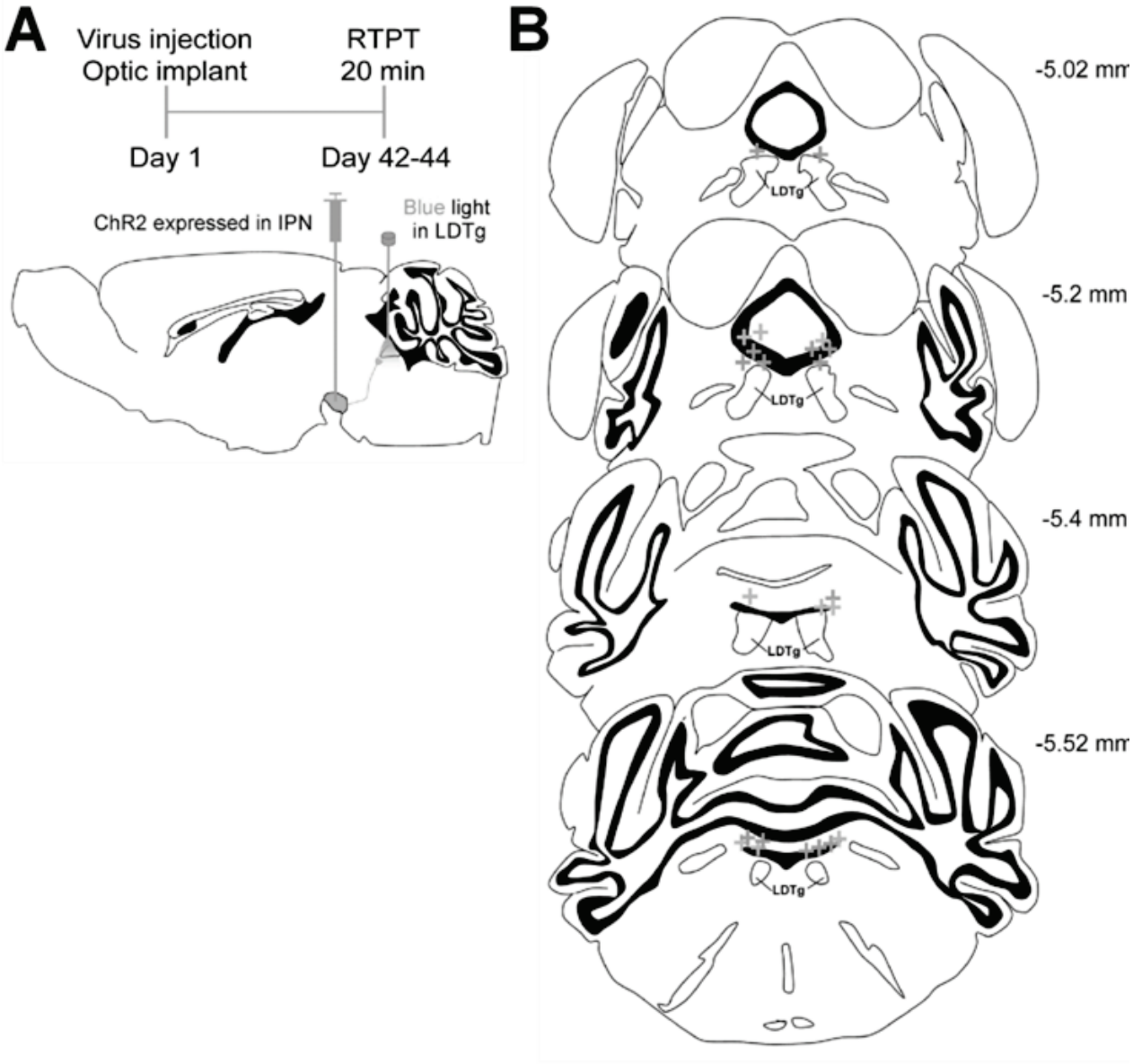

Figure 3.8- Experimental Set-up and Fiber Optic Implant Placements for Stimulation of IPN Terminals in the LDTg. A) Timeline from surgery to RTPT. ChR2 was expressed in the IPN, and fiber optic implants were placed unilaterally above the LDTg. Blue light was delivered to IPN terminals in the LDTg. B) Coronal slices showing fiber optic placements for both ChR2 and EYFP groups. Numbers indicate distance from bregma.

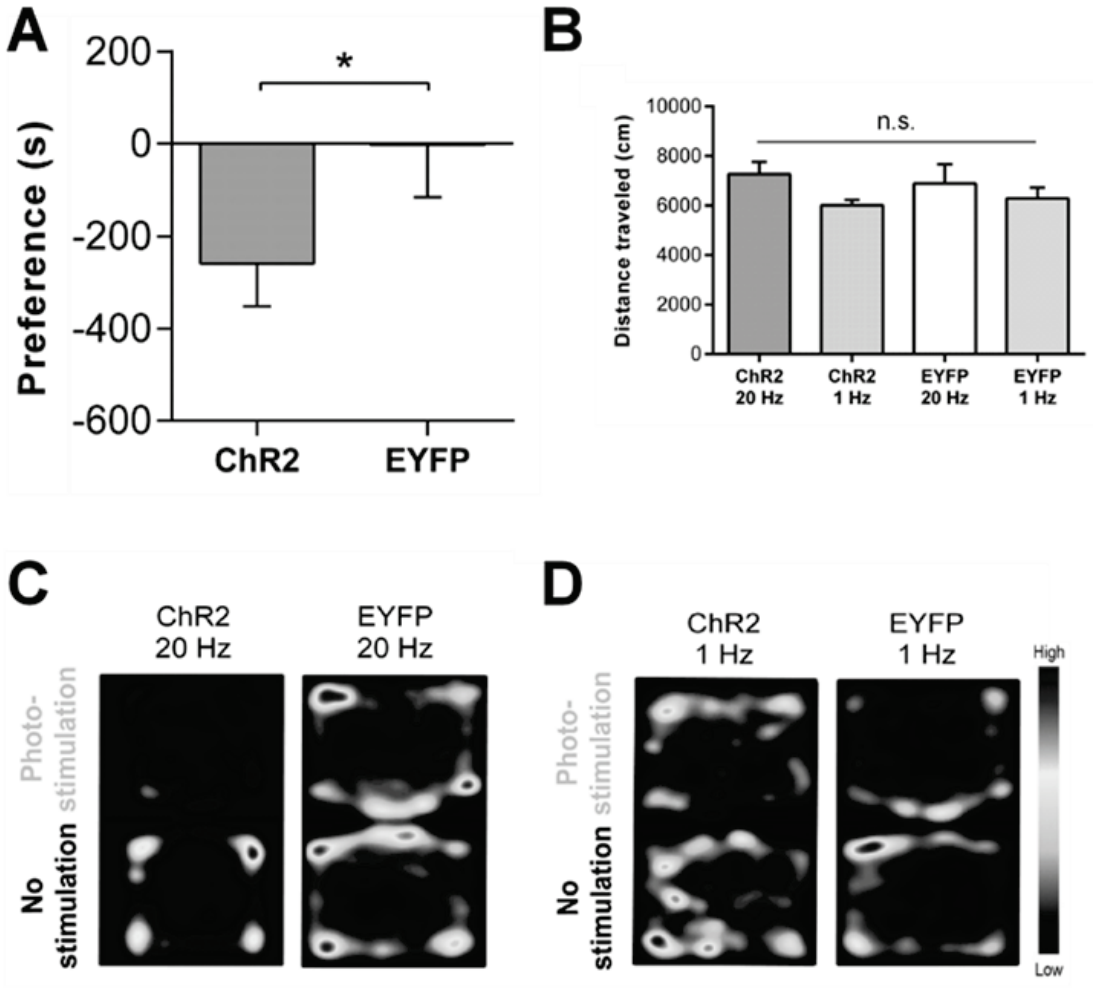

Figure 3.9- Optogenetic activation of IPN terminals in the LDTg elicits place avoidance. A) Stimulation of IPN terminals in the LDTg (10 ms pulses at 20 Hz) results in real-time place aversion. Mice expressing ChR2 spend significantly less time in the light-paired compartment compared to mice expressing EYFP (Two-way ANOVA $P=0.0044$; group x time interaction $P< 0.0001$; between group differences $P<0.01$, Holm-Sidak test for multiple comparisons). B) Neither stimulation resulted in locomotor changes in either group. C+D) Representative heat maps for ChR2- and EYFP-expressing mice during 20 Hz and 1 Hz stimulation of IPN terminals in the LDTg in vivo through permanent fiber optic implants in the real-time place preference assay.

neurons which in turn project to the VTA. These results also show that photostimulation of the IPN-LDTg connection results in behavioral avoidance. Taken together, the data indicate a causal role for the IPN, and more specifically IPN inputs to the LDTg, in aversive behaviors. That the IPN inputs to the LDTg are GABAergic suggests that IPN activity mediates aversion by inhibiting the LDTg, which may in turn inhibit VTA DA neuron firing rates.

The finding that direct activation of IPN somata elicits behavioral avoidance is in line with existing evidence. Selective activation of GABAergic neurons in the IPN produces a nicotine withdrawal-like phenotype even in nicotine-naïve mice, and nicotine withdrawal corresponded to more activity in the IPN, as shown by c-fos expression (Zhao-Shea et al., 2013). Nicotine withdrawal has been repeatedly shown to be aversive in both humans and animals (Kenny and Markou, 2001). Additionally, enhancing the sensitivity of the MHb to nicotine results in augmented MHb activity in response to low concentrations of nicotine, and this results in aversion to doses of nicotine that were previously neutral or rewarding (Risinger and Oakes, 1995; Frahm et al., 2011). The IPN is also preferentially activated by high, aversive doses of nicotine and not by lower doses, and functional inhibition of the MHb and IPN in response to nicotine result in increased self-administration of high doses of nicotine (Fowler et al., 2011). Because the MHb sends the vast majority of its excitatory projections to the IPN, enhanced activity in the MHb also leads to enhanced activity in the IPN (Brown et al., 1983), and diminished MHb activity might decrease activity in the IPN (Zhao-Shea et al., 2013).

These findings also call into question interpretations of data that contradict the idea that enhanced MHb-IPN activity corresponds to enhanced aversion (Glick et al., 2011; Gallego et al., 2012; McCallum et al., 2012; Harrington et al., 2015). As mentioned in *Chapter 1*, many of these studies used local infusions of antagonists or global genetic strategies, and assessment of MHb or

IPN activity in response to nicotine was largely absent. The strategy used here was a targeted approach to specifically stimulate only the somata of IPN neurons. Viral injections were targeted to the ventral portion of the IPN, and ChR2 was largely expressed in regions that receive inputs from the MHb. As suggested by Zhao-Shea et al., microcircuits in the IPN are likely also activated by this stimulation (2013); however, the predominant effect of activating neurons in this region is to elicit aversion.

The results indicating that the inputs to VTA-projecting LDTg neurons from the IPN are GABAergic is also in line with the existing literature. Selective activation of GABAergic neurons in the IPN results in a nicotine withdrawal-like phenotype, and the IPN has an abundance of GABAergic neurons (Kawaja et al., 1989; Zhao-Shea et al., 2013). Additionally, an anatomical study tracing the projections of α5 nAChR-expressing IPN neurons found that these neurons, including those that project to the LDTg, are largely GABAergic (Hsu et al., 2013b). However, as discussed in section 1b, the IPN is a heterogeneous nucleus which remains relatively poorly understood (Klemm, 2004), and the α5 nAChR expression in the MHb, rather than in the IPN, has been the focus of nicotine-related behavioral inquiry, despite controversy concerning the existence of native α5-containing nAChRs in the MHb (Fowler et al., 2011; Frahm et al., 2011; Hsu et al., 2013b; Dao et al., 2014).

As mentioned, viral expression of ChR2 was targeted to the ventral portion of the IPN, which receives dense innervations from MHb neurons (Ren et al., 2011). Hsu et al., however, reported that α5-expressing IPN neurons that project to the LDTg originate instead from the lateral portions of the IPN (2013). There are a few ways to explain the findings reported here and those from Hsu et al. (2013). It is possible that although the most dense ChR2 expression is observed in the ventromedial portion of the IPN, some neurons in the lateral regions of the IPN

also express ChR2. This has in fact often been observed (Figure 3.2). This is not surprising, given that although viral infection was targeted to the ventromedial portion of the IPN, this was largely done to avoid infection of VTA neurons. The MHb also projects to more dorsal parts of the IPN, and the lateral sub-regions are relatively small compared to the central and rostral regions. Therefore, in an effort to express ChR2 in as many IPN neurons that might receive habenular input as possible, the only guideline for infusion volume was restricted expression in the IPN that did not breach the VTA boundary. Maximal ChR2 expression was desirable in these experiments to enhance the likelihood that synaptic and behavioral effects would be observable. It is therefore possible that the bulk of the effects seen in these studies do not reflect MHb-IPN circuitry, but rather IPN circuitry that receives input from elsewhere.

However, α5-expressing neurons are likely not the only important cell-type present in the IPN. IPN neurons from various sub-regions have been reported to project to the LDTg, and it may be through these neurons that the MHb-IPN pathway modulates LDTg activity. Furthermore, even if our results are largely a result of IPN projections from lateral sub-regions, it is possible that cholinergic drive from the MHb is modulating these neurons, even though they do not directly receive dense innervations, as acetylcholine in the IPN is thought to function through volume transmission (Ren et al., 2011). Even more compelling is the fact that the MHb has also been divided into a variety of sub-regions (Qin and Luo, 2009; Shih et al., 2015). The dorsal region of the MHb has been shown to contain substance P and glutamatergic afferents that project to the IPN, specifically including the lateral sub-regions of the IPN (Qin and Luo, 2009). Substance p in the MHb has also been implicated in nicotine-induced increases in excitability of MHb neurons and in the aversive effects of nicotine withdrawal, suggesting that cholinergic neurons in the MHb may not be the only critical mediators of nicotine aversion (Dao et al.,

2014). Another interesting possibility is that microcircuitry of the IPN might result in these lateral sub-regions being indirectly impacted by MHb signaling.

Although these experiments were not designed to clarify the IPN-LDTg pathway with sub-regional specificity, these data do clarify the nature of IPN projections to the LDTg. While it has been reported that the α5-containing IPN projection neurons tend to be GABAergic, those experiments were conducted using histological methods exclusively (Hsu et al., 2013b). Those experiments also investigated the IPN projection to various brain regions without identifying the projection targets downstream. The data presented here show that the functional effect of IPN neuron activation on VTA-projecting LDTg neurons is inhibitory and also specifically identify the nature of this connection in the context of reward-related circuitry.

Another potential confound that was considered was the possibility that the light-evoked GABAergic currents observed were not the product of a monosynaptic connection. Theoretically, IPN neurons could release glutamate onto GABAergic interneurons in the LDTg, which then result in GABAergic currents. This is unlikely, for a couple of reasons. One is that DNQX, the AMPA receptor antagonist, was bath applied to the slices and had no impact on the light-evoked GABA currents. In fact, when using the intermediate Cl^- internal solution (E_{Cl^-} -17mV), the use of DNQX was required to ensure that the inward currents observed were not mediated by glutamate. As discussed in *Chapter 2*, use of DNQX and this intermediate Cl^- internal solution was found to be the optimal strategy for discerning light-evoked currents. Light-evoked current latencies were also consistent with a monosynaptic connection, indicating that the IPN neurons synapse directly on LDTg neurons.

The LDTg is known to control firing patterns of VTA DA neurons (Lodge and Grace, 2006), and enhanced GABAergic drive to the LDTg may be reducing the firing rate or

prohibiting phasic firing of these neurons. Pauses in VTA DA neuron firing correspond to aversive events (Cohen et al., 2012), and most of the LDTg inputs to the VTA ultimately enhance excitation of VTA DA neurons that project to the NAcc and are known to participate in reward-related behaviors (Omelchenko and Sesack, 2005; Lammel et al., 2011). This suggests that IPN-mediated inhibition of LDTg neurons is indeed inhibiting VTA DA neuron firing rates. However, IPN-mediated inhibition of LDTg neurons may be modulating behavior in more complex ways. IPN inhibition of LDTg neurons may instead serve to alter the timing or pattern of VTA DA neuron firing in more subtle ways. Additionally, as alluded to above, the LDTg is a heterogenous nucleus with a unique projection pattern in the VTA (see *Chapter* 1).

While excitatory inputs to the VTA favor the DA neurons that project to the NAcc, the inhibitory inputs favor the GABAergic interneurons that presumably inhibit the VTA DA neurons. However, LDTg projections to VTA DA neurons that project to the PFC are more mingled (i.e. excitatory and inhibitory drive from the LDTg target both DA and GABA neurons in the VTA that project to the PFC). Given the bright and extensive fluorophore expression that was already present in the slices that were recorded from, identification of cell-types that received input from the IPN was not feasible for these experiments. Therefore, it is possible that the LDTg neurons that receive IPN inhibitory inputs are those that would excite VTA DA neurons that project to the PFC and have been shown to mediate aversion. In that case, IPN activation would inhibit an aversive circuit and reduce aversive behavior. However, this possibility is not in line with the behavioral data presented here.

Finally, although targeting of VTA-projecting LDTg neurons was possible during slice electrophysiology experiments, this level of specificity was not possible for the *in vivo* optogenetic studies. It is therefore always possible that there are IPN terminals that synapse onto

LDTg neurons that project elsewhere than the VTA, and that this unknown pathway is contributing to behavioral outcomes. However, given what is known about the VTA in relation to reward and aversion, and given what we know about the impact of the LDTg on VTA DA neuron function, this seems less likely than the proposed hypothesis. The simplest explanation of the data at this stage is that by inhibiting the LDTg, the IPN indirectly inhibits reward-facilitating VTA DA neurons. Studies that directly assess VTA DA neurons that project to the NAcc are required to truly address this hypothesis, and those are currently underway.

Chapter 4

Nicotine Dose-Dependently Modulates IPN Projections to LDTg

(2a) Summary.

In *Chapter 3*, experiments revealed that IPN inputs to VTA-projecting LDTg neurons is GABAergic, and that optogenetic activation of either IPN cell bodies or IPN terminals in the LDTg results in behavioral avoidance. Although these findings suggest that the IPN mediates aversion via indirect inhibition of VTA DA neurons, the effects of nicotine on this pathway had yet to be examined.

It has been well established that lower doses of nicotine tend to be rewarding, while higher doses tend to be aversive. However, the mechanisms that underlie these opposing, dose-dependent effects of nicotine remain unclear. The studies discussed in this chapter were aimed at understanding the how high vs. low doses of nicotine might differentially modulate IPN synapses onto VTA-projecting LDTg neurons.

Optogenetic techniques and slice electrophysiology were used in these experiments. ChR2 was expressed in the IPN, and retrograde labeling dye was infusion bilaterally into the VTA of experimental animals. Whole-cell patch-clamp recordings were done in back-labeled LDTg neurons, and different concentrations of nicotine were applied.

In these experiments, high concentrations of nicotine were found to enhance GABAergic drive from IPN to VTA-projecting LDTg neurons, while low concentrations did not. Further investigations suggest that the relevant, apparently low affinity nAChRs reside on IPN terminals in the LDTg. These results suggest that presynaptic modulation of GABAergic inputs from the IPN to VTA-projecting LDTg neurons is one way in which nicotine may exert its dichotomous dose-dependent effects.

(4b) Background.

Nicotine has repeatedly been shown to result in more rewarding experiences at lower doses and more aversive experience at higher doses (Henningfield and Goldberg, 1983b; Risinger and Oakes, 1995; Soria et al., 1996; Rose and Corrigall, 1997; Picciotto, 2003; Matta et al., 2007; Fowler et al., 2011). Humans and laboratory animals both display inverted-U shaped dose-response curves, with maximal self-administration occurring at intermediate doses. They also very effectively regulate their levels of nicotine intake when self-administering (Benowitz et al., 1986; Woodward and Tunstall-Pedoe, 1993; Rose and Corrigall, 1997; Lynch and Carroll, 1999) and will perform tasks to avoid the administration of high nicotine doses (Henningfield and Goldberg, 1983a).

There is also substantial evidence to suggest that initial smoking experiences may predict the likelihood of future dependence (Urban and Sutfin, 2010; de Wit and Phillips, 2012)(see *Chapter 1*). A pleasurable first experience with nicotine seems to be associated with more smoking and higher rates of nicotine dependence than does an aversive first experience (Kozlowski and Harford, 1976; DiFranza et al., 2004). More pleasure (or less aversion) may then

promote further use, which is a requirement for the transition from use to abuse and dependence (Robinson and Berridge, 1993; Everitt and Robbins, 2005; De Biasi and Dani, 2011).

It was proposed in *Chapter 1* that the positive and negative effects of nicotine occur simultaneously and produce a gestalt value judgment. If aversion is mediated by a neural circuit that inhibits reward or positive reinforcement, then the aversive effects of nicotine may limit the extent to which the reinforcing effects of nicotine can be achieved. Activity in the MHb-IPN pathway has been shown to mediate aversion to nicotine (Fowler et al., 2011; Frahm et al., 2011; Slimak et al., 2014), and results presented in *Chapter 3* indicate that IPN activity controls aversion by inhibiting LDTg neurons that project to the VTA. However, the mechanism by which high and low doses of nicotine result in differential activity in aversive- and reward-related circuitries remains unclear.

Given that a variety of nAChRs exist with a variety of sensitivities for nicotine, and that these receptors are variously expressed throughout the brain (Albuquerque et al., 2009), it was hypothesized that different types of nAChRs might predominate in the MHb-IPN pathway than in the LDTg-VTA pathway. Differential expression of nAChRs with different sensitivities to nicotine could explain the dose-dependent activation of the MHb-IPN pathway as well as the dose-dependent aversive effects of nicotine.

Indeed, clinical and preclinical work has implicated several nAChR subtypes and brain areas that are important for the rewarding and aversive effects of nicotine (Picciotto et al., 1998; Tapper et al., 2004; Fowler et al., 2011; Frahm et al., 2011; Haller et al., 2012; Ware et al., 2012; Slimak et al., 2014). Genetic studies in humans have revealed SNPs in various genes for nAChRs that confer vulnerability to nicotine dependence, and some of these SNPs result in nAChRs with different responses to nicotine than the normal variants (Wang et al., 1996; Gerzanich et al.,

1998; Nelson and Lindstrom, 1999; Frahm et al., 2011; Kuryatov et al., 2011; Li et al., 2011a; George et al., 2012; Tammimäki et al., 2012; Ciuraszkiewicz et al., 2013; Sciaccaluga et al., 2015). It has also been shown that the MHb-IPN is highly enriched in low affinity α3β4 receptors, while the VTA is enriched in high affinity α4β2 receptors that have been shown to be critical for nicotine reward (Marks and Pauly, 1992; Quick et al., 1999; Tapper et al., 2004; Nashmi and Lester, 2006; Marotta et al., 2013).

However, the MHb-IPN pathway expresses many different types of nAChRs, and although α3β4 receptors have been shown to dominate function in parts of the MHb (Quick et al., 1999), the relevance of this dominance to nicotine behaviors has not been assessed. The relevance of nAChRs in IPN downstream signaling has also not been assessed. Therefore, the experiments outlined in this chapter sought to determine if nicotinic modulation of IPN synapses on LDTg neurons that project to the VTA might be one mechanism by which dose-dependent effects of nicotine are mediated. Figure 4.1 illustrates what was known about nAChRs in relevant brain regions prior to these experiments.

(4c) Methods. See *Chapter 2* for detailed methodology.

(5c) Nicotine concentration-dependently modulates light-evoked GABAergic currents from the IPN to VTA-projecting LDTg neurons.

Since the output from the MHb-IPN pathway influences nicotine-related behaviors, we tested whether nicotine could modulate the light-evoked inhibitory postsynaptic currents (IPSC) from the IPN to LDTg neurons that project to the VTA. ChR2 was expressed in the IPN of adult

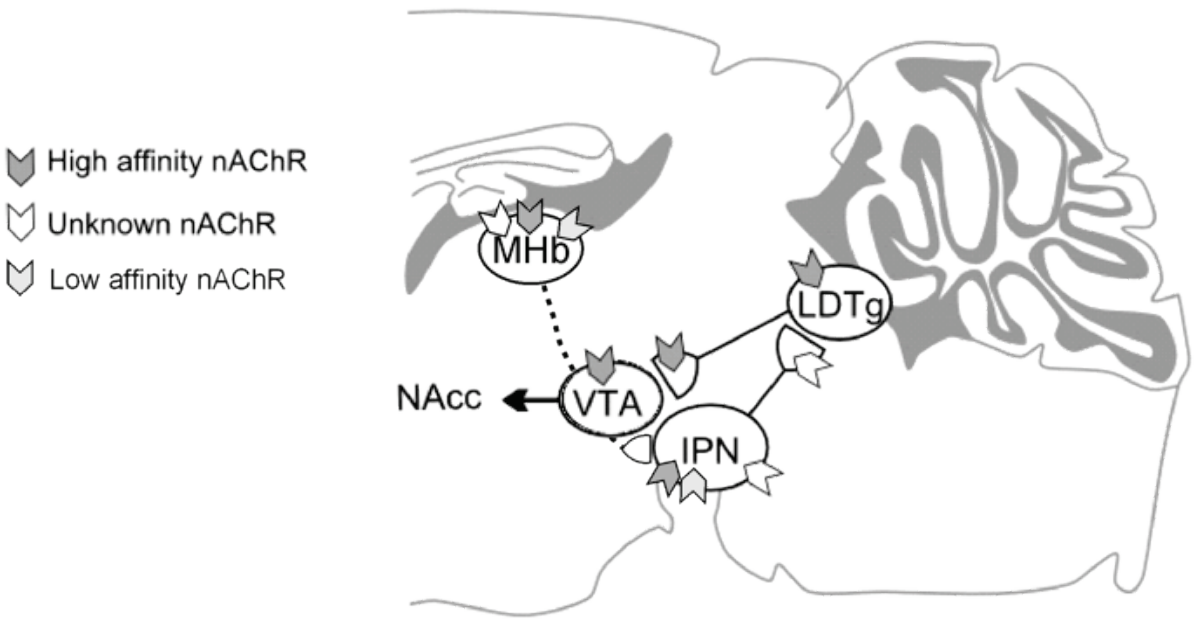

Figure 4.1- Existing Knowledge of the nAChRs Present in Relevant Brain Regions. The MHb and IPN express a variety of nAChRs, but those that are most relevant for the acute aversive effects of nicotine are still largely unknown, especially for the IPN. The regions related to reward are known to be important for the rewarding effects of low doses of nicotine, indicating that they predominantly express high affinity nAChRs.

mice, and fluorescent microbeads were injected bilaterally into the VTA to back-label VTA-projecting LDTg neurons. This is shown in *Chapter 3,* Figure 3.6.

Whole-cell voltage-clamp recordings were then done in back-labeled LDTg neurons. Blue light was delivered through the objective to excite ChR2 expressed on IPN terminals in the LDTg. An intermediate Cl^- concentration was used in the internal solution (E_{Cl^-} -17 mV), so light-evoked GABA currents were observed as inward deflections at a holding potential of -70 mV. DNQX was present throughout these experiments, and blue light was delivered in 5 ms pulses every 30 seconds.

As nicotine has dose-dependent effects on behavior (Risinger and Oakes, 1995), and the MHb-IPN pathway is especially responsive to high doses of nicotine (Fowler et al., 2011), two different nicotine concentrations (10 μM and 100 nM) were tested to start. 10 μM nicotine would be an excessively aversive concentration *in vivo,* while 100 nM is well within the range of serum concentrations in smokers after a cigarette (Patterson et al., 2003; Williams et al., 2010).

Bath application of 10 μM nicotine for 5 minutes resulted in significantly increased amplitudes of light-evoked IPSCs, while 100 nM nicotine resulted in no significant impact on average current amplitudes. Additionally, nicotine-induced increases in light-evoked IPSC amplitudes were significantly more prevalent when a high concentration was used compared to a lower concentration. These data are shown in Figure 4.2A-D. While these data suggest that modulation of inhibitory drive from IPN to LDTg with higher doses of nicotine could contribute to aversion, 10 μM is an extremely high concentration of nicotine. Therefore, more physiologically relevant high doses of nicotine were tested.

In unrestricted smokers, nicotine levels in the blood plateau around 200 nM (Hukkanen et al., 2005). Mice are less sensitive to the effects of nicotine, but doses that have been shown to

condition place preference in mice yield plasma levels >300 nM (Risinger and Oakes, 1995; Matta et al., 2007; Tzschentke, 2007). Doses that condition place aversion in mice yield plasma levels between 600 nM and 1.2 μM, depending on the dose used (Risinger and Oakes, 1995; Matta et al., 2007; Tzschentke, 2007). Therefore, concentrations of 500 nM and 1 μM were chosen for additional experiments. Tests of the effects of 1 μM nicotine on modulation of this synaptic connection are still underway, but 500 nM was able to significantly enhance the average light-evoked IPSC amplitudes. These results are shown in Figure 4.2E. Together, these data indicate that physiologically relevant, aversive concentrations of nicotine facilitate IPN-mediated inhibition of VTA-projecting LDTg neurons. This suggests that nicotinic modulation of this synapse contributes to the ability of high and low nicotine doses to generate opposing behavioral outcomes.

(4e) Nicotine modulates mIPSC frequency in VTA-projecting LDTg neurons in a concentration-dependent manner.

To better understand the mechanism by which nicotine modulates this synaptic connection, studies were undertaken to assess the pre- or post-synaptic locations of relevant nAChRs. To this end, mice received bilateral infusions of retrobeads into the VTA. Whole-cell voltage-clamp recordings were then done in back-labeled LDTg neurons in the presence of DNQX and TTX. The same internal solution and holing potential used in the prior experiments were also used for these studies. Miniature IPSCs (mIPSC) were recorded, and nicotine was again bath applied.

Bath application of 10 μM nicotine significantly enhanced the frequency of mIPSCs, while application of 100 nM had no significant effect. In addition, the prevalence of a nicotine-

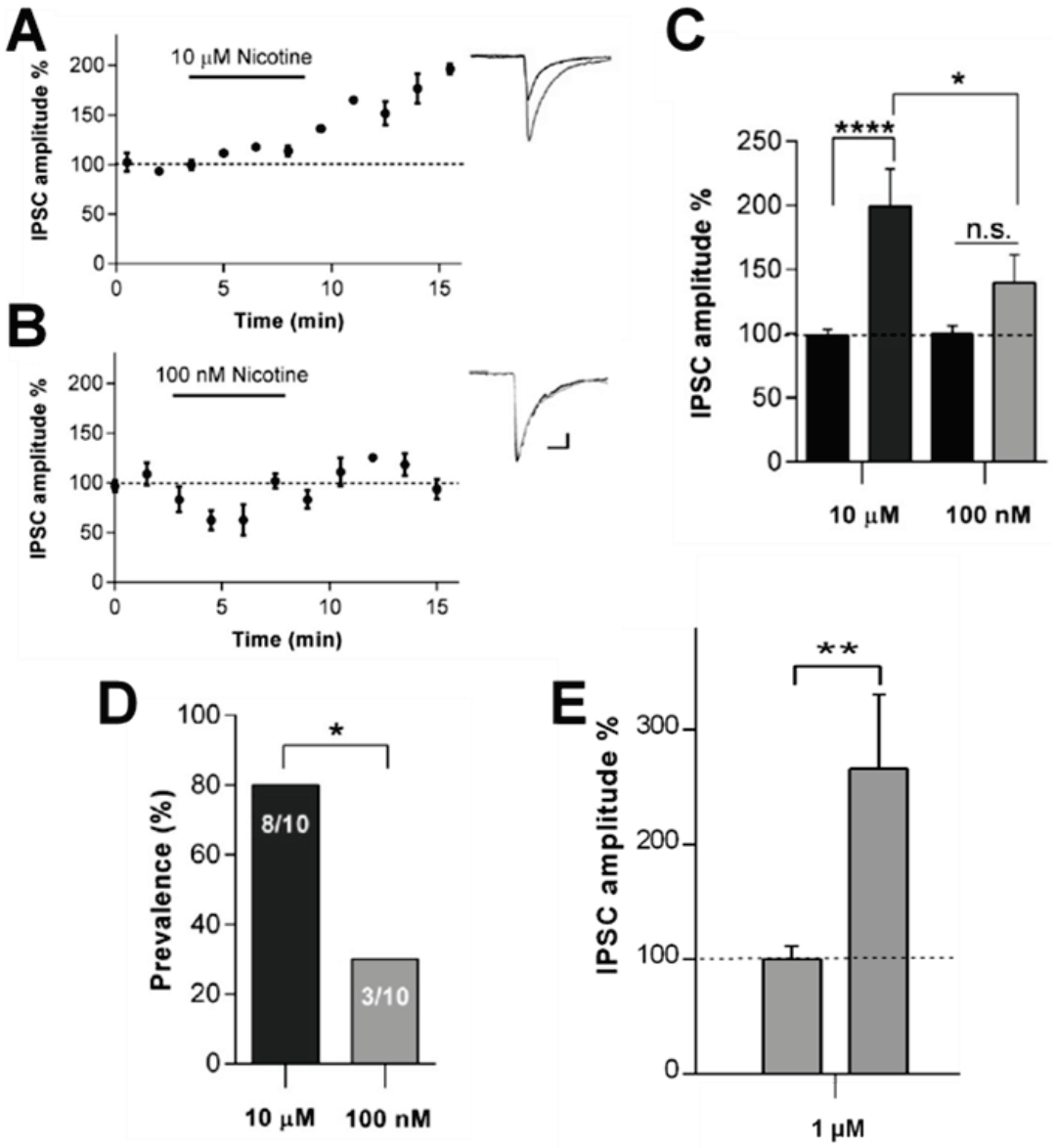

Figure 4.2- Nicotine concentration-dependently modulates light-evoked GABAergic currents from the IPN to VTA-projecting LDTg neurons. A) Representative voltage-clamp recording of the effects of 10 μM nicotine on light-evoked currents from the IPN to VTA-projecting LDTg neurons. IPSCs are normalized to baseline. Inset: representative current traces (black- baseline, gray- 10 μM nicotine). B) Representative voltage-clamp recording of the effects of 100 nM nicotine on light-evoked currents from the IPN to VTA-projecting LDTg neurons. Inset: representative current traces (black- baseline, gray- 100 nM nicotine). C) Averaged effects of the two concentrations of nicotine on light-evoked IPSCs. 10 μM nicotine significantly increased the amplitudes of light-evoked IPSCs compared to baseline and compared to 100 nM nicotine (one-way ANOVA, $P<0.0001$, Holm-Sidak test for multiple comparisons). D) Prevalence of a nicotine-induced increase in IPSC amplitude for the two concentrations of nicotine. 10 μM nicotine increased the amplitudes of light-evoked IPSCs in a significantly higher proportion of neurons than did 100 nM (chi-square test, $P= 0.0246$; n=10 in each group). E) Averaged effect of 1 μM nicotine on IPSC amplitude compared to baseline (t-test, $P=0.0051$).

induced increase in mIPSC frequency was significantly higher after bath application of 10 μM nicotine relative to100 nM nicotine. Importantly, neither concentration of nicotine had a significant impact on mIPSC amplitude. The change in mIPSC frequency without an associated change in mIPSC amplitude suggests that high concentrations of nicotine act pre-synaptically to enhance GABAergic drive onto LDTg neurons that project to the VTA and that the nicotinic receptors underlying these effects are likely to be lower-affinity receptors. These data are shown in Figure 4.3.

As for the experiments assessing nicotinic modulation of light-evoked IPSCs, it seemed prudent to test the effects of more physiologically relevant aversive concentrations. 500 nM and 1 μM were chosen for continuity with the prior experiments. The effects of 500 nM nicotine on mIPSCs are currently still underway, but 1 μM nicotine was found to significantly increase mIPSC frequency. These data suggest that the low-affinity nAChRs mediating GABAergic drive to VTA-projecting LDTg neurons are localized pre-synaptically. However, to rule out the possibility of alternate explanations for an increase in mIPSC frequency (e.g. post-synaptic release of a retrograde signal or other calcium-mediated post-synaptic), the effects of 10 μM nicotine were assessed with BAPTA present in the internal solution.

Because nAChRs are ionotropic and are calcium permeable, any post-synaptic changes caused by nicotine are likely to be calcium-mediated. BAPTA is a calcium chelator, so inclusion of BAPTA in the internal solution should inhibit the ability of 10 μM nicotine to increase the frequency of mIPSCs if this change is due to a post-synaptic nAChR. BAPTA had no impact on the ability of 10 μM nicotine to enhance mIPSC frequency, and these data are shown in Figure 4.4. Therefore, the low affinity nAChRs that enhance GABAergic drive from the IPN to VTA-projecting LDTg neurons are likely expressed pre-synaptically.

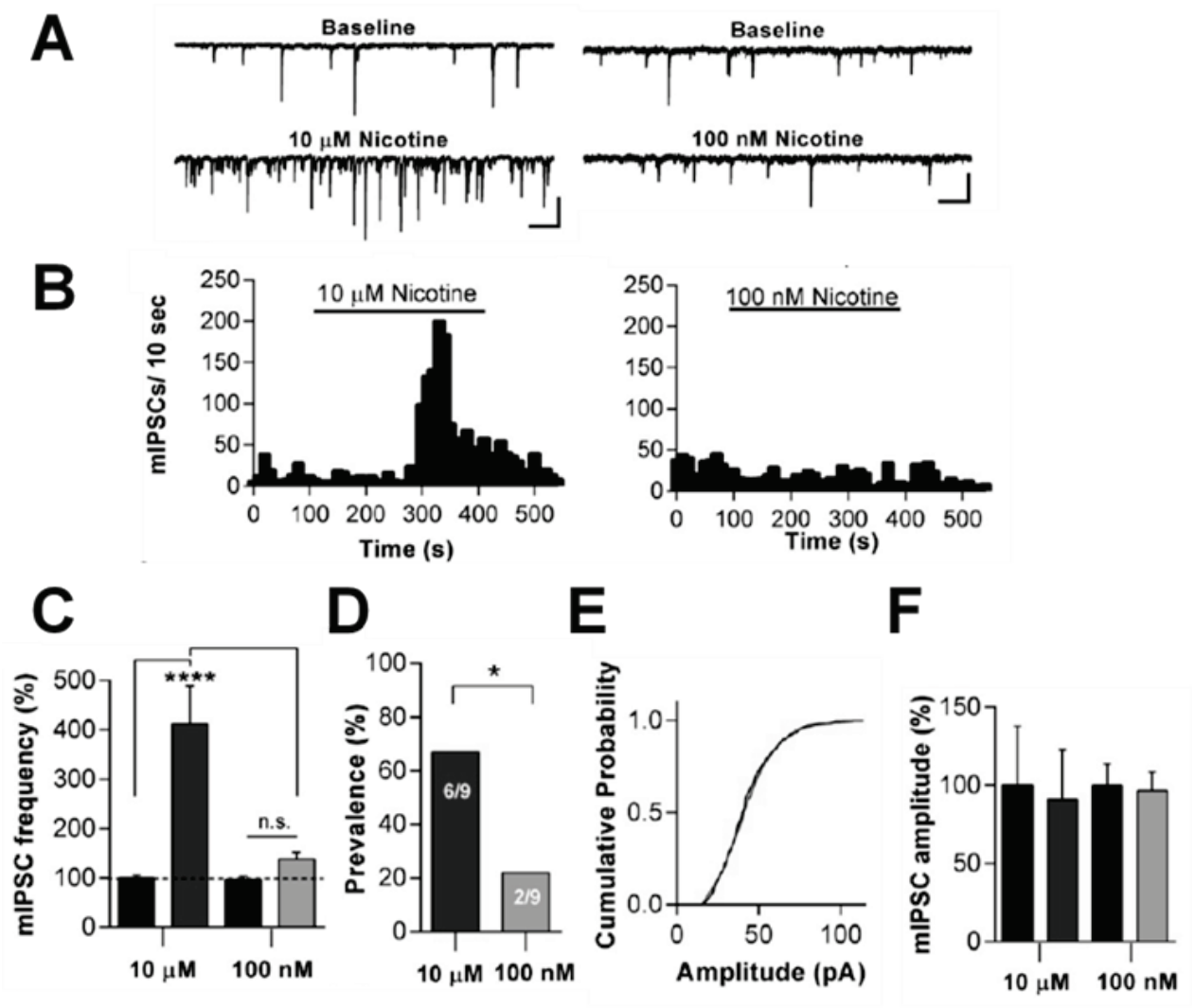

Figure 4.3- Nicotine modulates mIPSC frequency in a concentration-dependent manner. A) Representative mIPSC traces before and after bath application of 10 μM nicotine. Whole-cell voltage clamp recordings were done in vitro in VTA-projecting LDTg neurons in the presence of TTX (1 μM). B) Representative histogram of mIPSC frequency during bath application of 10 μM nicotine. C) Representative mIPSC traces before and after bath application of 100 nM nicotine. D) Representative histogram of mIPSC frequency during bath application of 100 nM nicotine. E) Averaged effects of the two concentrations of nicotine on mIPSC frequency. 10 μM nicotine significantly increased the frequency of mIPSCs compared to baseline and compared to 100 nM nicotine (one-way ANOVA, $P<0.0001$, Holm-Sidak test for multiple comparisons). F) Prevalence of a nicotine-induced increase in mIPSC frequency for the two concentrations of nicotine. 10 μM nicotine increased the frequency of mIPSCs in a significantly higher proportion of neurons than did 100 nM (chi-square test, $P<0.05$; n= 9 for each group). G) Representative cumulative amplitude histogram showing that 10 μM nicotine does not significantly increase mIPSC amplitude (Kolmogorov-smirnov test). H) Averaged effects of both concentrations of nicotine on mIPSC amplitude normalized to baseline.

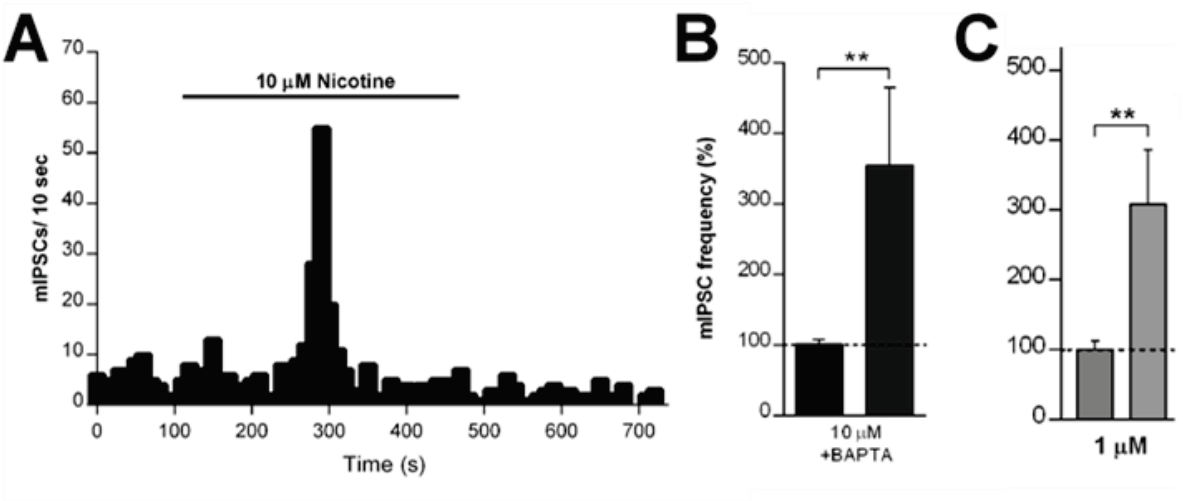

Figure 4.4- NAChRs that Modulate GABAergic drive to VTA-projecting LDTg Neurons are Pre-synaptic and Low Affinity. A) Representative mIPSC trace before and after bath application of 10 μM nicotine. Whole-cell voltage clamp recordings were done in vitro in VTA-projecting LDTg neurons in the presence of TTX (1 μM) and with BAPTA in the recording electrode. B) Averaged effects of 10 μM nicotine on mIPSC frequency with BAPTA in the recording electrode. 10 μM nicotine significantly increased the frequency of mIPSCs compared to baseline. C) Averaged effects of 1 μM nicotine on mIPSC frequency. This physiologically relevant, aversive concentration of nicotine significantly increased the frequency of mIPSC compared to baseline.

(4f) Discussion.

These findings demonstrate that the GABAergic drive from the IPN to LDTg-projecting VTA neurons is selectively augmented by a high concentration of nicotine due to the expression of presumably low affinity nAChRs on the presynaptic terminals of these IPN neurons. They also identified a potentially important mechanism by which different concentrations of nicotine can differentially result in reward or aversion. A high concentration of nicotine enhances the amplitude of light-evoked currents from the IPN in VTA-projecting LDTg neurons, while a lower concentration of nicotine fails to do so.

This is consistent with reports that high doses of nicotine result in aversion while lower doses result in reward (Risinger and Oakes, 1995; Rose and Corrigall, 1997). It has also been reported that higher doses of nicotine activate the IPN, while lower doses fail to do so. Furthermore, higher doses of nicotine fail to activate the IPN in α5 nAChR KO mice which self-administer much higher doses of nicotine than their wild-type counterparts (Fowler et al., 2011). These effects of nicotine on the IPN have been studied in the context of nicotine-sensitivity in the MHb (Fowler and Kenny, 2012b), but taken together, these previous findings and our results point to multiple potential sites of nAChR expression that may be separately and in conjunction mediating the aversive effects of high concentrations of nicotine. Perhaps enhanced MHb activity in response to a high dose of nicotine leads to enhanced IPN activity, and the inhibitory effects of this IPN excitation on VTA-projecting LDTg neurons are maximized by nAChRs expressed pre-synaptically on IPN terminals in the LDTg.

Similar to the electrophysiology experiments discussed in *Chapter 3*, a limitation of these studies is that cell-type specificity was not determined for the back-labeled neurons that were recorded from in the LDTg. Most of the LDTg inputs to the VTA seem to ultimately result in

increased activity of VTA DA neurons. GABAergic neurons from the LDTg preferentially target GABAergic interneurons that would presumably inhibit VTA DA neurons, suggesting that LDTg activity would disinhibit VTA DA neurons. Excitatory inputs from the LDTg preferentially target VTA DA neurons that project to the NAcc directly. However, the projections from the LDTg to VTA neurons that project to the PFC and mediate aversion are mixed (Omelchenko and Sesack, 2005; Lammel et al., 2011).

Therefore, as in the *Chapter 3* experiments, it is possible that some or all of the LDTg neurons that were recorded from targeted VTA neurons that project to the PFC. Because cell-type identification in these studies was not feasible, it is even theoretically possible that IPN inputs to LDTg inhibit GABAergic neurons in that region that project to VTA DA neurons that project to the PFC. This would functionally result in disinhibition of these aversion-mediating neurons. However, this possibility is not supported by the behavioral data reported in *Chapter 3* or the prior studies that have implicated enhanced MHb-IPN activity with enhanced aversion to nicotine (Fowler et al., 2011; Frahm et al., 2011).

Another limitation of these experiments is that mIPSCs could not be recorded selectively from IPN inputs to VTA-projecting LDTg neurons. Investigations into the pre- or post-synaptic localization of the nAChRs that modulate IPN inputs to LDTg neurons may have been assessing pre-synaptic nicotinic modulation of other GABAergic inputs to VTA-projecting neurons in the LDTg in addition to or instead of the IPN inputs specifically. Because it is unlikely that the LDTg receives GABAergic input solely from the IPN, it is very likely that additional inputs were assessed in addition to IPN inputs. It seems unlikely that IPN inputs were not measured at all, though, given the similarities in concentration-dependent effects of nicotine observed in experiments measuring light-evoked and miniature IPSCs. However, future experiments could

utilize an optogenetic approach combined with bath application of strontium. While technically challenging, this method allows for the measurement of light-evoked miniature events.

The final limitation that will be discussed here concerns the heterogeneity of the IPN. While the only fast transmission observed at this synapse was GABAergic, the IPN contains a variety of neuropeptides (Groenewegen et al., 1986). Further experiments are required to determine if any neuropeptides or other modulatory neurotransmitters are present at this synaptic connection and if they have any relevance to nicotine-related behaviors. The possibility that additional neurotransmitters may be important for signaling at this connection complicates the interpretations of some of the results. BAPTA was used in the recording pipette to eliminate the possibility that nicotine might cause some change post-synaptically that is observed as a traditionally pre-synaptic effect. However, it is possible that some neuromodulator is affected by nAChRs and causes post-synaptic changes through a calcium-independent mechanism. For example, substance P is expressed in some neurons of the IPN (Groenewegen et al., 1986), and signaling through neurokinin receptors can take many forms, some of which may result in intracellular changes independent of calcium influx (Steinhoff et al., 2014).

However, it seems that GABAergic signaling is transiently increased by high but not low concentrations of nicotine at the IPN synaptic connections with LDTg neurons that project to the VTA. Although more research is required to fully understand this connection and its modulation by nicotine, these results have revealed a potential mechanism by which nicotine can exert dose-dependently opposing behavioral effects. Together, the studies presented thus far suggest that IPN activity mediates aversion to nicotine by indirectly inhibiting VTA DA neurons, and that this inhibitory drive is enhanced selectively by high, aversive concentrations of nicotine.

Chapter 5

IPN Projections to the LDTg Mediate Aversion to Nicotine

(5a) Summary.

In *Chapters 3 and 4*, experiments revealed that the IPN connection to the LDTg mediates aversion, consists predominantly of inhibitory inputs, and is modulated preferentially by high vs. low concentrations of nicotine. These experiments suggest that the MHb-IPN pathway mediates aversion to high doses of nicotine by inhibiting LDTg neurons that excite VTA DA neurons, and that this function is due at least partially to pre-synaptic, low affinity nAChRs expressed on IPN terminals on the LDTg. However, the behavioral relevance of IPN inputs to the LDTg has not yet been addressed in a nicotine-specific context.

The MHb-IPN pathway has been implicated in mediating the aversive effects of nicotine and restricting the upper bounds of nicotine intake. The LDTg-VTA pathway has been implicated in mediating reward and reward-related learning, and function of this pathway has been shown to be critical for the reinforcing effects of abused drugs. Experiments outlined in the prior chapters have shown that IPN activity can result in inhibition of the LDTg and behavioral avoidance, but these experiments do not conclusively determine that the connection between the IPN and LDTg is a mechanism by which aversion to nicotine is mediated.

The experiments described in this chapter utilize optogenetic techniques and behavioral assays to assess the contribution of the IPN-LDTg connection in controlling the motivational valence of nicotine. Archaerhodospin was expressed in the IPN, and green light was delivered to IPN terminals in the LDTg during a nicotine conditioned place aversion test. Selective inhibition of IPN inputs to the LDTg was not only found to reduce nicotine-induced CPA, but was found to shift nicotine-induced CPA to a strong nicotine-induced CPP. These results indicate that the IPN-LDTg connection is a critical mediator of the aversive effects of high-dose nicotine, and suggest that the IPN gates reward-related signaling to establish the upper limits of nicotine-taking.

(5b) Background.

The reinforcing effects of nicotine are well documented in both humans and laboratory animal models (Henningfield and Goldberg, 1983a), but at higher doses, nicotine also has intensely aversive effects (Henningfield and Goldberg, 1983a; Risinger and Oakes, 1995). In fact, humans and laboratory animals regulate their levels of nicotine intake when self-administering (Rose and Corrigall, 1997; Lynch and Carroll, 1999) and will perform tasks to avoid the administration of high nicotine doses (Henningfield and Goldberg, 1983a). Initial responses to nicotine may impact future dependence. A pleasurable first experience correlates with heavier smoking and higher rates of nicotine dependence than does an aversive first experience (Hu et al., 2006; Kandel et al., 2007; de Wit and Phillips, 2012). Less aversion may promote further use, supporting the transition from use to abuse and dependence (De Biasi and Dani, 2011; de Wit and Phillips, 2012). See *Chapter 1* for extensive literature review. Therefore, the balance between the rewarding and aversive effects of nicotine likely contributes to the development and maintenance of nicotine addiction, and requires further study.

The principal targets for both the rewarding and aversive aspects of nicotine are nicotinic acetylcholine receptors (nAChRs), which are widely expressed throughout the central and peripheral nervous system (Albuquerque et al., 2009). High nicotine doses, particularly with initial exposures, have aversive effects that likely are mediated by nAChRs with various sensitivities to nicotine.

Genetic differences can account for some of the individual differences in initial responses to and continued use of nicotine. A single nucleotide polymorphism (SNP) in the *CHRNA5* gene of the *CHRNA5-A3-B4* gene cluster, which encodes the α5, α3, and β4 nicotinic acetylcholine receptor subunits, has been associated with more pleasurable early responses to nicotine, heavier smoking, more nicotine dependence, and delayed smoking cessation compared with people without this variant (Ware et al., 2012). The addition of the α5 subunit to the α3β4 nAChR enhances function of the receptor (Ciuraszkiewicz et al., 2013), and this disadvantageous SNP decreases function of the receptor compared to the normal variant (Bierut et al., 2008; Kuryatov et al., 2011). The highest density of α3β4α5 expression is found in the medial habenula (MHb) and the interpeduncular nucleus (IPN), to which the MHb sends almost all of its excitatory projections (Marks and Pauly, 1992; Ren et al., 2011).

Recent work has shown that knocking out the α5 nAChR subunit (Ramirez-Latorre et al., 1996; Ciuraszkiewicz et al., 2013) in mice promotes self-administration of high, aversive nicotine doses that wild type (wt) mice will not self-administer (Fowler et al., 2011). This behavioral difference was eliminated by rescuing α5 expression specifically in the MHb (Fowler et al., 2011). α5 KO mice also develop a conditioned place preference (CPP) to high doses of nicotine that wt mice do not (Jackson et al., 2010), and high, aversive doses of nicotine activate the IPN while lower doses fail to do so, as shown by *c-fos* expression (Fowler et al., 2011).

Furthermore, a genetic manipulation that results in enhanced nicotine-induced currents and excitation in MHb neurons results in conditioned place aversion (CPA) to a dose of nicotine that was neutral in control animals (Frahm et al., 2011), while manipulations that decrease this sensitivity reduce aversion to nicotine (Slimak et al., 2014). These genetic and pre-clinical findings suggest that the MHb-IPN pathway mediates aversion to high doses of nicotine and that decreasing its responsiveness can diminish aversion to nicotine and enhance vulnerability to nicotine dependence (see *Chapter 1* for more detailed review).

One of the projections from the IPN is to the laterodorsal tegmental nucleus (LDTg) (Groenewegen et al., 1986). The LDTg is a brainstem cholinergic center that controls many autonomic and limbic functions, including a strong excitatory projection to ventral tegmental area (VTA) dopamine (DA) neurons (Lodge and Grace, 2006; Grace et al., 2007). Because DA transmission contributes to both nicotine-induced CPP and CPA (Acquas et al., 1989; Grieder et al., 2012a), the MHb-IPN pathway might mediate aversion to nicotine by affecting VTA DA neurons. Nicotine activates both the LDTg and the related pedunculopontine tegmentum (PPTg) (Lança et al., 2000b), and activation of LDTg inputs to the VTA results in CPP (Lammel et al., 2012). Conversely, lesions of the PPTg that diminish VTA burst firing also decrease nicotine self-administration (Lança et al., 2000a).

As MHb-IPN activity and LDTg-VTA activity result in opposing behavioral outcomes, and experiments outline in *Chapters 3 and 4* indicate that activation of the IPN inputs to the LDTg mediates aversion and is concentration-dependently modulated by nicotine, it was hypothesized that this pathway might underlie the MHb-IPN pathway's role in nicotine aversion. However, it was also possible that although the IPN could inhibit LDTg neurons and result in behavioral avoidance, the aversive effects of nicotine might be mediated predominantly by an

alternative IPN output circuit. Therefore, the role of the IPN-LDTg connection in the aversive effects of nicotine was directly assessed.

(5c) Methods.- see *Chapter 2* for detailed methodology.

(5d) Optogenetic Inhibition of IPN Terminals in the LDTg Elicits CPP to a High Dose of Nicotine.

Because experiments described in previous chapters revealed that the IPN projection to the LDTg is important in mediating aversion, and that high concentrations of nicotine preferentially modulate this connection, the effects of manipulation this connection on nicotine-related behavior were assessed. To this end, either archaerhodopsin (ARCH), which is a light-driven proton pump, or EYFP only were expressed in the IPN of adult mice (AAV2-hSyn-eArch3.0-EYFP or AAV2-hSyn-EYFP). Permanent fiber optic implants were placed unilaterally above the LDTg. Figure 5.1A shows the experimental set-up and timeline. Figure 5.2 shows the locations of fiber optic placements for both the ARCH and EYFP mice.

To confirm functional expression of ARCH, whole-cell patch-clamp recordings from IPN neurons were done in brain slices. Green light (532 nm) was delivered through the objective. In voltage-clamp, brief pulses of green light evoked hyper-polarizing currents. In current-clamp, green light halts tonic firing for the duration of the light pulse. Representative traces from these recordings are shown in Figure 5.1B. These results confirm that ARCH is functionally expressed in IPN neurons and that it can powerfully inhibit these cells.

An unbiased conditioned place preference/aversion (CPP) assay was used to assess the behavioral effects of nicotine. On day 1 of CPP, mice were allowed to explore all three

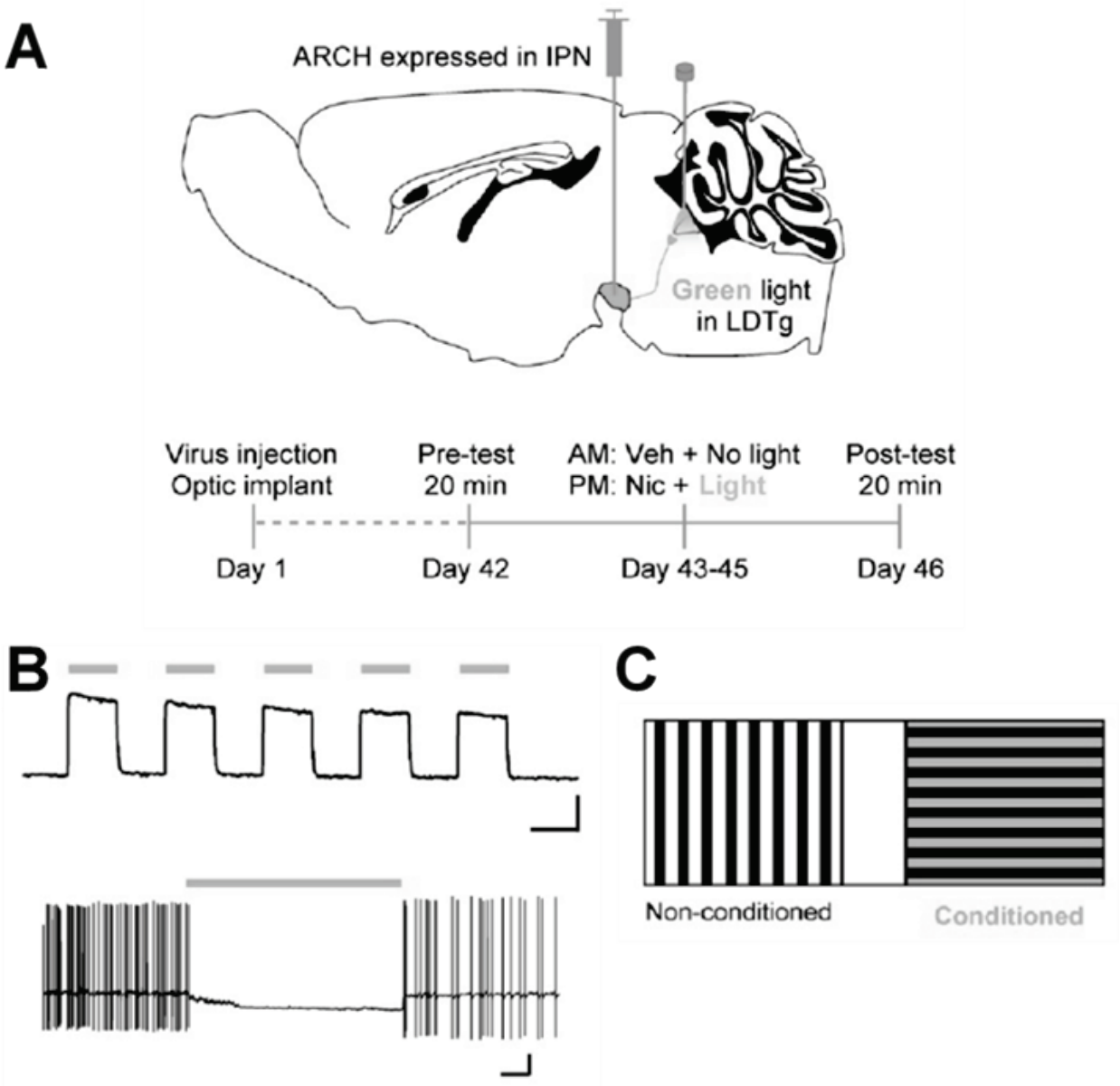

Figure 5.1- Experimental Set-up and Functional ARCH Expression. A) Top: Schematic showing that ARCH was expressed in the IPN and permanent fiber optic implants were placed above the LDTg unilaterally. Green light was delivered to the LDTg to inhibit IPN terminals in that region. Bottom: Timeline of experiment from surgery to behavioral testing. Traces from whole-cell in vitro recordings from IPN neurons. 500 ms pulses of green light reliably evoke hyperpolarizing currents (500 ms, 100 pA scale). Delivery of green light (15 s) halts action potentials in a tonically firing neuron. C) Depiction of the CPP apparatus. The conditioned side was always paired with light (e.g. nicotine + light or vehicle + light always = conditioned side).

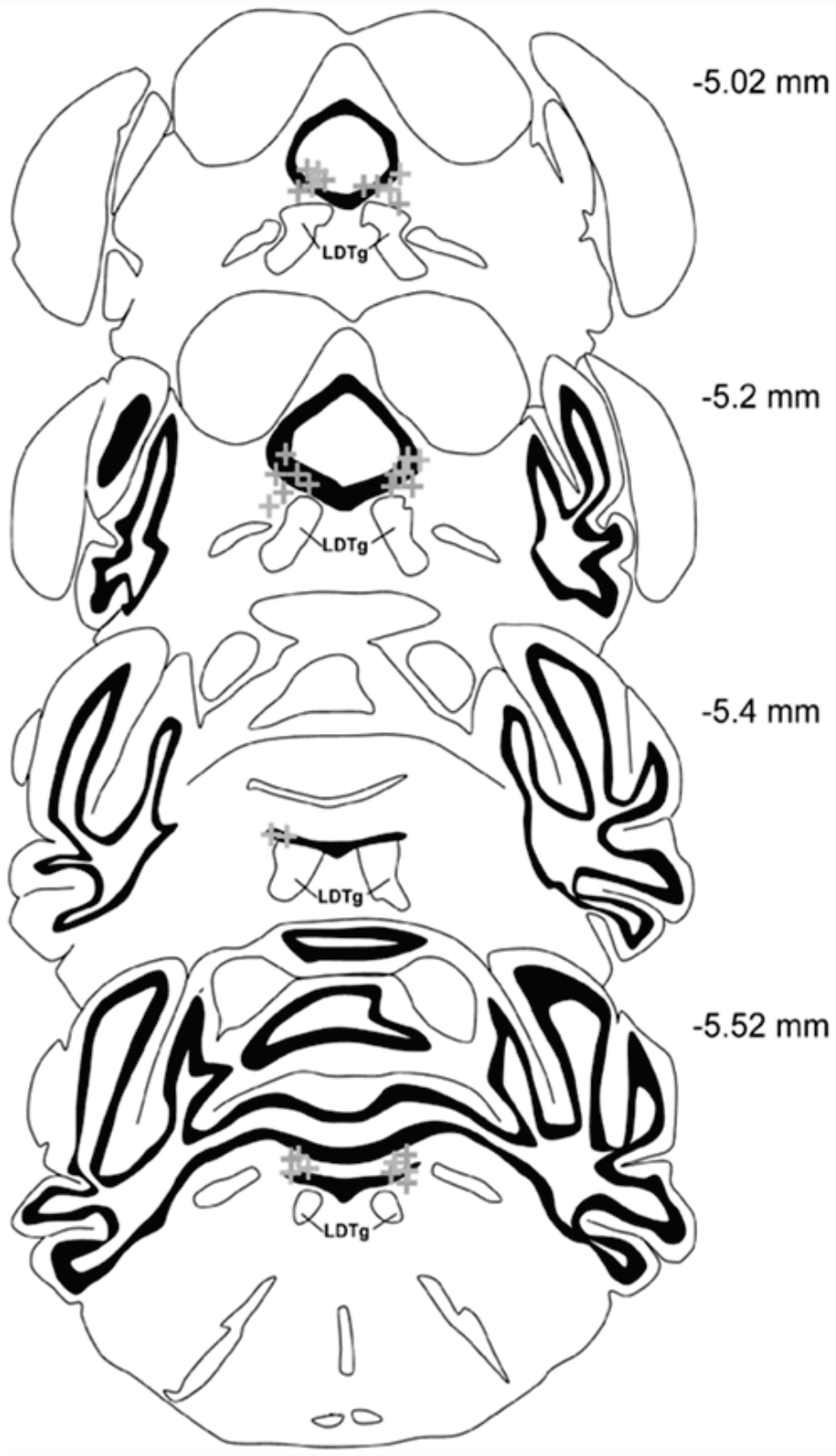

Figure 5.2- Locations of fiber optic placements. Crosses show the termination points of permanently implanted fiber optics. Placements for both ARCH and EYFP groups are included.

compartments of the apparatus. On days 2-4, animals were exposed to two daily conditioning sessions- one in the morning and one in the afternoon. A high dose of nicotine was chosen, generally considered to be aversive (1.5 mg/kg i.p., 10 ml/kg), and nicotine was always administered during the afternoon session, to increase the time between the last nicotine dose and the next conditioning session. Nicotine administration was always paired with green light delivery to IPN terminals in the LDTg. In total, mice had 3 vehicle + no light conditioning sessions and 3 nicotine + light conditioning sessions. On the test day (day 5), animals were allowed free access to all three compartments. Any time the animals were exposed to the conditioning apparatus, the fiber optic cable connected to the laser was attached to their permanent fiber optic implants. Figure 5.1C shows a diagram of the CPP apparatus. Horizontal and vertical striped sides were counterbalanced for morning and afternoon conditioning sessions.

Mice that expressed EYFP only developed a small, non-significant aversion to the side of the apparatus paired with a high dose of nicotine. Mice that expressed ARCH, however, developed a remarkably strong preference for the side of the apparatus paired with the same high dose of nicotine. Preference and aversion were calculated as preference scores, in which time spent on the no light-paired side is subtracted from time spent on the light-paired side. Thus, positive numbers reflect preference, while negative numbers reflect aversion. The change in preference score within groups from the pre-conditioning test to the post-conditioning test was also calculated. Mice in the EYFP + nicotine group shifted their preference scores to more negative numbers (aversion), while the ARCH + nicotine group shifted their preference scores to more positive numbers (preference). Despite the small effect size of the nicotine aversion in the EYFP group, this is likely an aversive dose of nicotine. The modest effect sizes in nicotine conditioning experiments are well-documented (Risinger and Oakes, 1995), and we found that a

similar dose (2 mg/kg) of nicotine was aversive in control animals that had not undergone surgical manipulations. These results are shown in Figure 5.3. The nicotine dose was decreased in these experiments to minimize adverse side effects, such as seizures, that would confound interpretation of the results. Even at this lower dose, mice are almost completely immobilized after nicotine administration. This dose has also been reported to elicit CPA by other groups (Grieder et al., 2012b).

(5e) Optogenetic Inhibition of IPN Terminals in the LDTg Elicits Neither Preference Nor Aversion in the Absence of Nicotine.

Given that inhibition of IPN terminals in the LDTg was able to shift aversion to nicotine to preference, it was important to understand whether optogenetic manipulation resulted in behavioral effects on its own. If this inhibition results in reward-related behaviors independent of nicotine, then perhaps nicotine aversion is not mediated by the IPN-LDTg connection, but rather overshadowed by the rewarding effects of inhibiting this connection. To test this, either archaerhodopsin (ARCH), which is a light-driven proton pump, or EYFP only were again expressed in the IPN of adult mice. Fiber optic implants were again placed unilaterally above the LDTg, as shown in Figure 5.1. These mice underwent a CPP testing as described in section 5d, except that instead of receiving a high dose of nicotine paired with one side of the apparatus, these mice received vehicle injections on both the light-paired and no-light paired sides of the apparatus.

Mice in the ARCH + vehicle group developed neither preference nor aversion to light delivery in the LDTg. Mice in the EYFP + vehicle group performed similarly, and no significant differences in distance traveled were seen in any groups. These data are shown in Figure 5.4.

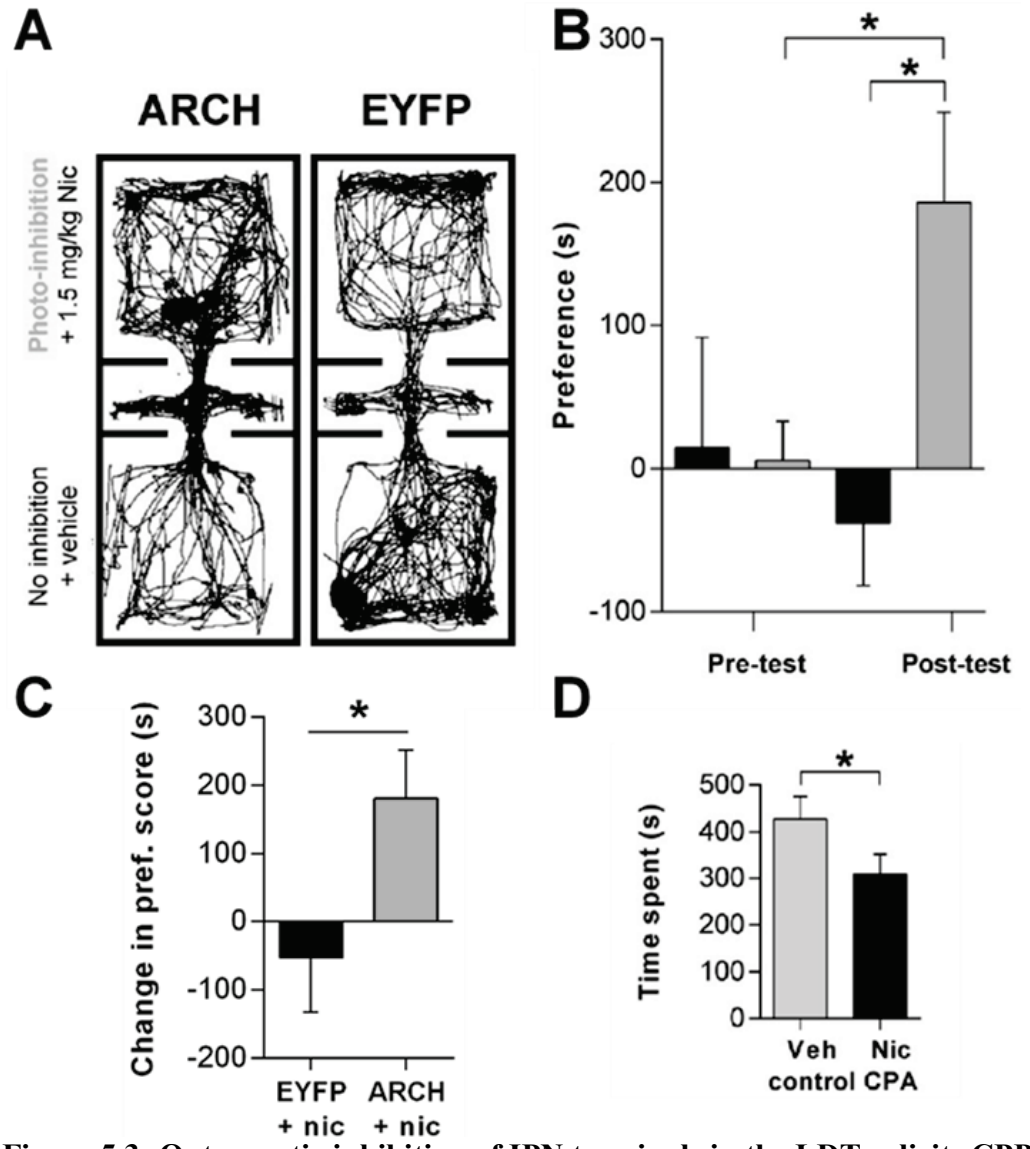

Figure 5.3- Optogenetic inhibition of IPN terminals in the LDTg elicits CPP to a high dose of nicotine. A) Sample traces of activity from ARCH or EYFP control mice during the post-conditioning test. Lines show center point of mouse over the course of the 20 min test. ARCH mice spent more time on the nicotine and light-paired side of the apparatus, while EYFP mice spent less. B) Preference scores pre- and post-conditioning for ARCH (gray) and EYFP (black) groups. Preference score calculated as time spent in unconditioned side subtracted from time spent in the conditioned side. EYFP mice developed a more negative preference score post-conditioning (not significant). ARCH mice developed a significantly more positive preference score post-conditioning compared to their pre-conditioning preference score and compared to the post-conditioning preference scores of EYFP mice (one-way ANOVA, $P<0.05$, Holm-Sidak test for multiple comparisons). C) Change in preference scores from pre- to post-conditioning. Preference scores of EYFP mice became more negative, while those of ARCH mice became more positive (unpaired t-test, $P<0.05$). D) Nicotine CPA in wt mice with no surgical manipulations. A similar dose of nicotine (2.0 mg/kg) to that used in the ARCH experiments was used. Wild-type + nicotine mice (black) spent significantly less time on the conditioned side than did wild-type + vehicle mice (gray) post-conditioning (unpaired t-test, $P<0.05$).

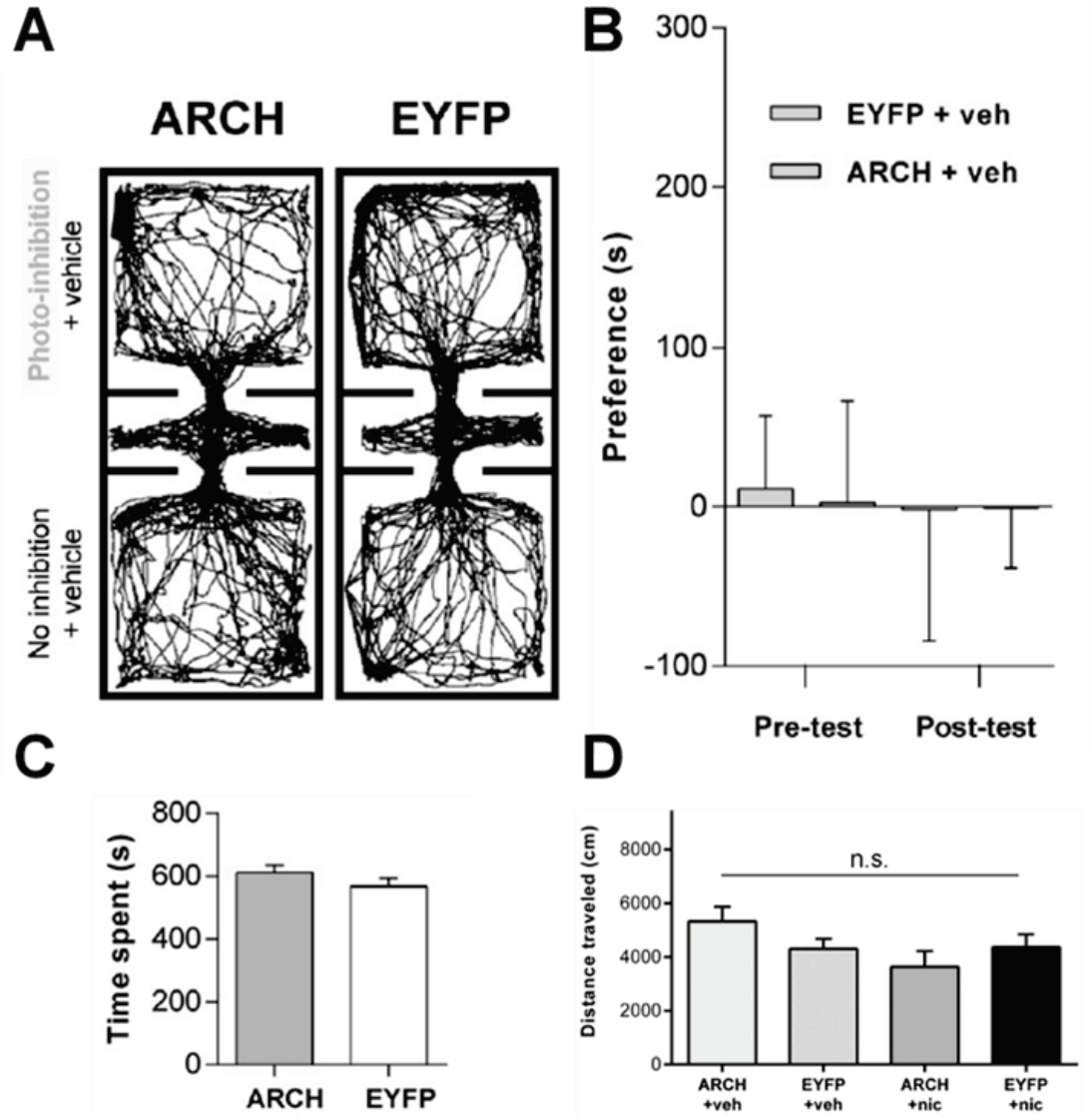

Figure 5.4- Optogenetic Inhibition of IPN Terminals in the LDTg has no Effect on CPP in the Absence of Nicotine. A) Sample traces of activity from ARCH or EYFP control mice during the post-conditioning test. Lines show center point of mouse over the course of the 20 min test. No preference for either side was observed for either group. B) Preference scores in the CPA assay pre- and post-conditioning for ARCH + vehicle and EYFP + vehicle groups. In the absence of nicotine, light conditioning had no impact on the preference scores of either ARCH or EYFP-expressing mice. C) Real-time place preference test of the effects of inhibition of IPN terminals in the LDTg. ARCH- and EYFP-expressing mice both spent equal times on the light-paired side in the absence of nicotine. D) Distance traveled during post-conditioning test for all groups tested. No manipulation affected gross locomotor behavior.

(5f) Discussion.

Though the MHb projection to the IPN has been implicated in the regulation of reward and aversion to nicotine, the downstream targets of this connection and the mechanisms by which this pathway mediates aversion have remained unknown. These experiments demonstrate that although unilateral optogenetic inhibition of IPN terminals in the LDTg results in neither place preference nor aversion on its own, this same inhibition elicits conditioned place preference to a high dose of nicotine which would otherwise produce conditioned place aversion or no change in preference (Risinger & Oakes 1995; Korkosz et al. 2006; Grieder et al. 2012b). These findings suggest that one way in which the MHb-IPN pathway mediates aversion to nicotine is by inhibiting LDTg neurons, and that IPN activity can impede reward-related circuitry and produce aversion.

These data support the idea that reward and aversion are opposite ends of a spectrum of potential affective states and that this state is mediated by the interactions between reward- and aversion-related circuitries rather than by independent, segregated pathways. Excitation of the MHb-IPN pathway can shift the balance to aversion, while inhibition of that excitation can allow the LDTg-VTA circuit to shift the balance to reward.

This particular inhibitory opsin was chosen for a variety of reasons that are described in *Chapter 2*, but the choice of an inhibitory opsin in general was made so as to avoid two potential confounds. One of those is the confound of antidromic action potentials when using ChR2. Exciting terminals may result in action potentials that back-propagate to the somata, and these neurons may collateralize. If these neurons do collateralize, these antidromic action potentials eliminate the spatial specificity desired for terminal stimulation. Use of an inhibitory opsin eliminates this confound.

The other was to avoid activating two distinct aversive pathways that would result in summated aversion. Had the experiment tested whether activation of IPN terminals in the LDTg can enhance the aversive effects of nicotine, one possible interpretation would be that nicotine may have exerted its aversive effects via some unknown pathway, while the aversive experience mediated by IPN inputs to LDTg was also occurring. If two separate but aversive stimuli occur simultaneously, they might be expected to enhance aversion compared to either on its own, and conclusions cannot necessarily be drawn about the underlying circuitry. Because inhibition of these terminals resulted in no behavioral effect on place preference alone, the ability of this manipulation to alter the behavioral effects of nicotine strongly indicates that the IPN-LDTg pathway is important in mediating the aversive effects of nicotine.

As with all experimental tools, there are some limitations to the use of archaerhodopsin that should be considered. One potential confound associated with the use of ARCH is that because it is a proton-pump, activation of these channels likely results in pH changes in the neurons that express them. The magnitude and effects of this possible change in pre-synaptic terminals remains unclear. It is possible that transient changes in the pH of pre-synaptic terminals might only serve to enhance light-induced inhibition, given that pH affects packaging of neurotransmitters into vesicles (Reimer et al., 1998). Electrical gradient across the vesicular membrane can also influence packaging of neurotransmitters, and any inhibition or excitation of these terminals could potentially alter vesicular transport. Still, the possibility that changes in pH result in changes that are problematic to interpreting results should still be considered. However, the finding that ARCH-mediated inhibition of IPN terminals in the LDTg in the absence of nicotine results in no observed behavioral effect suggests that pH changes likely do not drive the behavioral changes observed during nicotine CPA.

Another consideration in these experiments is whether or not ARCH activation of IPN terminals is actually able to inhibit action potential-mediated neurotransmitter release. Although ARCH activation on somatodendritic regions of the neuron can halt tonic action potential firing, the ability of similar ARCH activation in nerve terminals to prevent coordinated neurotransmitter release is challenging to test, and many researchers remain skeptical. However, there are studies that have provided evidence that inhibitory opsins expressed on terminals can inhibit sEPSCs or electrically-evoked IPSCs (Tye et al., 2011; Stefanik et al., 2013). Therefore, while ARCH-mediated inhibition of terminals may not completely eliminate neurotransmitter release, it seems that even coordinated release can be effectively inhibited by inhibitory opsins.

An unfortunate issue with inhibitory opsins is that often prolonged light delivery is required to maintain inhibition over the course of a behavioral assay. Prolonged light delivery could result in heating of the tissue, which might change synaptic activity and/or behavioral outcomes. Although not ideal, this problem can largely be overcome with attention to control groups. In the studies presented in this chapter, EYFP animals were exposed to the same light intensity and duration as the ARCH animals were. Therefore, if prolonged light delivery were responsible for the behavioral effects observed, the EYFP mice would have also displayed these changes.

A limitation to all the *in vivo* optogenetic experiments presented in this thesis is the lack of specificity for the projection targets of LDTg neurons that are impacted by IPN changes in activity. As discussed in *Chapter 3,* it is possible that these behavioral effects are driven by LDTg projections to targets other than the VTA.

It is also challenging to causally link the pre-synaptic nAChRs described in *Chapter 4* to the behavioral outcomes described in this chapter. Nicotine was delivered systemically, and so

the functional relevance of the pre-synaptic nAChRs expressed on IPN terminals cannot be assessed *in vivo*. However, given the dose-dependent effects of nicotine behaviors and nicotinic modulation of the IPN-LDTg synaptic connection, it seems reasonable to conclude that these receptors likely participate in the distinction between aversive and rewarding effects of nicotine.

Finally, as discussed at length in *Chapter 2,* the conditioned place preference test yields results can be challenging to interpret. It has been reported that a biased design (i.e. the animal receives a rewarding dose of nicotine on its initially less-preferred side) is more effective at conditioning place preference and aversion to nicotine in rodents (Tzschentke, 2007; Brielmaier et al., 2008). However, an unbiased design (i.e. the experimenter randomly assigns the drug-paired side regardless of an individual's initial preference) was used in these experiments. This was chosen because although high doses of nicotine were expected to condition aversion in EYFP control mice, the effects of ARCH on nicotine-induced CPA were unclear. It was hypothesized that ARCH inhibition of IPN terminals in the LDTg would reduce CPA, but it was also possible that CPA would be shifted to CPP. Therefore, in an effort to not obscure any possible outcomes, an unbiased procedure was used. The use of an unbiased design likely explains the small effect size seen for CPA for EYFP control mice (Brielmaier et al., 2008).

In summary, these data have demonstrated the behavioral relevance of the IPN-LDTg connection. These experiments have shown how modulating this connection can shift the scales from aversion to reward, and these data support the larger hypothesis that aversion plays a critical role in the regulation of nicotine-related behaviors (Fowler et al. 2011; Frahm et al. 2011; Zhao-Shea et al. 2013).

Chapter 6

Discussion

(6a) Summary.

Though the MHb projection to the IPN has been implicated in the regulation of reward and aversion to nicotine, the downstream targets of this connection and the mechanisms by which this pathway mediates aversion have remained unknown. Our investigations into the IPN projections to the LDTg provide new insights into how aversion-related circuitry interacts with reward-related circuitry. Here we report that direct, optogenetic stimulation of IPN neurons results in behavioral avoidance and that the IPN sends GABAergic projections to LDTg neurons which in turn project to the VTA. We have also shown that photo-stimulating the IPN-LDTg connection results in behavioral avoidance. Our data also demonstrate that the GABAergic drive from the IPN to LDTg-projecting VTA neurons is selectively augmented by a high concentration of nicotine due to the expression of presumably low affinity nAChRs on the presynaptic terminals of these IPN neurons. Perhaps most remarkably, we have demonstrated that although unilateral optogenetic inhibition of IPN terminals in the LDTg results in neither place preference nor aversion on its own, this same inhibition elicits conditioned place preference to a high dose of nicotine which would otherwise produce conditioned place aversion or no change in preference (Risinger and Oakes, 1995; Korkosz et al., 2006; Grieder et al., 2012b). Taken together, our findings suggest that one way in which the MHb-IPN pathway mediates aversion to

nicotine is by inhibiting LDTg neurons, and that IPN activity can impede reward-related circuitry and produce aversion.

In summary, we have clarified the nature of an important IPN projection and demonstrated the behavioral relevance of the IPN-LDTg connection. We have shown how modulating this connection can shift the scales from aversion to reward, and our data support the larger hypothesis that aversion plays a critical role in the regulation of nicotine-related behaviors (Fowler et al., 2011; Frahm et al., 2011; Zhao-Shea et al., 2013). We have also proposed a mechanism by which interactions between the MHb-IPN pathway and the LDTg-VTA pathway serve to differentiate the reward valence of different doses of nicotine. Exploiting these findings may lead to novel therapeutic strategies that can help smokers quit.

(6b) MHb-IPN Pathway Mediates Aversion.

Our finding that optogenetic activation of IPN somata results in behavioral avoidance is in agreement with the existing evidence. Selective activation of GABAergic neurons in the IPN produces a nicotine withdrawal-like phenotype even in nicotine-naïve mice, and nicotine withdrawal corresponded to more activity in the IPN, as shown by c-fos expression (Zhao-Shea et al., 2013). Nicotine withdrawal has been repeatedly shown to be aversive in both humans and animals (Kenny and Markou, 2001). Additionally, enhancing the sensitivity of the MHb to nicotine results in augmented MHb activity in response to low concentrations of nicotine, and this results in aversion to doses of nicotine that were previously neutral or rewarding (Risinger and Oakes, 1995; Frahm et al., 2011). Because the MHb sends the vast majority of its excitatory projections to the IPN, it follows that the enhanced activity in the MHb would also lead to

enhanced activity in the IPN, and through one or many of the IPN's various projection targets (Klemm, 2004), this pathway mediates aversion.

(6c) IPN Inhibition of LDTg Mediates Aversion.

Our results indicating that the inputs to VTA-projecting LDTg neurons from the IPN are GABAergic is also in line with the existing literature. Selective activation of GABAergic neurons in the IPN results in a nicotine withdrawal-like phenotype, and the IPN has an abundance of GABAergic neurons (Kawaja et al., 1989; Zhao-Shea et al., 2013). Additionally, an anatomical study tracing the projections of α5 nAChR-expressing IPN neurons found that these neurons, including those that project to the LDTg, are largely GABAergic (Hsu et al., 2013b). However, the IPN is a very heterogeneous nucleus which remains relatively poorly understood (Klemm, 2004), and the α5 nAChR expression in the MHb, rather than in the IPN, has been the focus of nicotine-related behavioral inquiry (Fowler et al., 2011; Frahm et al., 2011). Our data clarify the nature of the IPN projection to the LDTg, and also specifically identify the nature of this connection in the context of reward-related circuitry.

Our findings that optogenetic activation of the IPN inputs to the LDTg results in behavioral avoidance and that optogenetic inhibition of these inputs results in conditioned place preference to a high dose of nicotine demonstrate the behavioral relevance of this connection. The LDTg is known to control firing patterns of VTA DA neurons (Lodge and Grace, 2006), and enhanced GABAergic drive to the LDTg may be reducing the firing rate or prohibiting phasic firing of these neurons. Pauses in VTA DA neuron firing correspond to aversive events (Cohen et al., 2012), suggesting that IPN-mediated inhibition of LDTg neurons is indeed affecting VTA DA neuron firing rates. However, IPN-mediated inhibition of LDTg neurons may be modulating

behavior in more complex ways. It has been reported that phasic VTA DA neuron firing is required for the expression of nicotine conditioned place aversion (Grieder et al., 2012b), so IPN inhibition of LDTg neurons may serve to alter the timing or pattern of VTA DA neuron firing in more subtle ways. It should also be kept in mind, however, that even VTA DA neurons appear to have a variety of functions, and those that receive input from the LDTg have been shown to participate in reward-related behaviors (Lammel et al., 2012). Therefore the simplest hypothesis would be that by inhibiting the LDTg, the IPN is indirectly inhibiting those reward-facilitating VTA DA neurons. Studies that directly assess these possible effects of the IPN-LDTg connection on VTA DA neuron activity are currently underway.

(6d) IPN-LDTg Connection and Nicotine.

We have also identified a potentially important mechanism by which different concentrations of nicotine can differentially result in reward or aversion. A high concentration of nicotine enhances the amplitude of light-evoked currents from the IPN in VTA-projecting LDTg neurons. A lower concentration of nicotine fails to do so. This is consistent with reports that high doses of nicotine result in aversion while lower doses result in reward (Risinger and Oakes, 1995; Rose and Corrigall, 1997). It has also been reported that higher doses of nicotine activate the IPN, while lower doses fail to do so. Furthermore, higher doses of nicotine fail to activate the IPN in α5 nAChR KO mice which self-administer much higher doses of nicotine than their wild-type counterparts (Fowler et al., 2011). These effects of nicotine on the IPN have been studied in the context of nicotine-sensitivity in the MHb (Fowler and Kenny, 2012b), but taken together, these previous findings and our results point to multiple potential sites of nAChR expression that may be separately and in conjunction mediating the aversive effects of high concentrations of

nicotine. Perhaps enhanced MHb activity in response to a high dose of nicotine leads to enhanced IPN activity, and the inhibitory effects of this IPN excitation are maximized by nAChRs expressed pre-synaptically on IPN terminals in the LDTg.

We propose a simplified model to summarize our findings, shown in Figure 6.1. Tonic activity of the IPN, LDTg, and VTA sustain a neutral state. In the presence of a low, rewarding concentration of nicotine, the high affinity nAChRs found on LDTg and VTA neurons are activated, while the IPN is unaffected. In the presence of a high, aversive concentration of nicotine, the lower affinity nAChRs found pre-synaptically on IPN terminals in the LDTg are activated (as well as those located on MHb neurons), resulting in inhibition of LDTg neurons, and thus potentially VTA DA neurons. In this way, the canonical reward circuit is gated by the MHb-IPN pathway, and the reward valence of nicotine is determined. Our data support the idea that reward and aversion are opposite ends of a spectrum of potential affective states and that this state is mediated by the interactions between reward- and aversion-related circuitries rather than by independent, segregated pathways. Excitation of the MHb-IPN pathway can shift the balance to aversion, while inhibition of that excitation can allow the LDTg-VTA circuit to shift the balance to reward.

(6e) Heterogeneity of the MHb and IPN.

Future studies are required to determine how somatic and pre-synaptic nAChRs in the MHb and IPN participate in the balance between reward and aversion. Both regions remain relatively poorly understood both in terms of normal function and in terms of responses to nicotine-taking, and various sub-regions of the MHb and IPN may contribute in very different ways to the behavioral outcomes associated with nicotine. There are also many neuromodulators

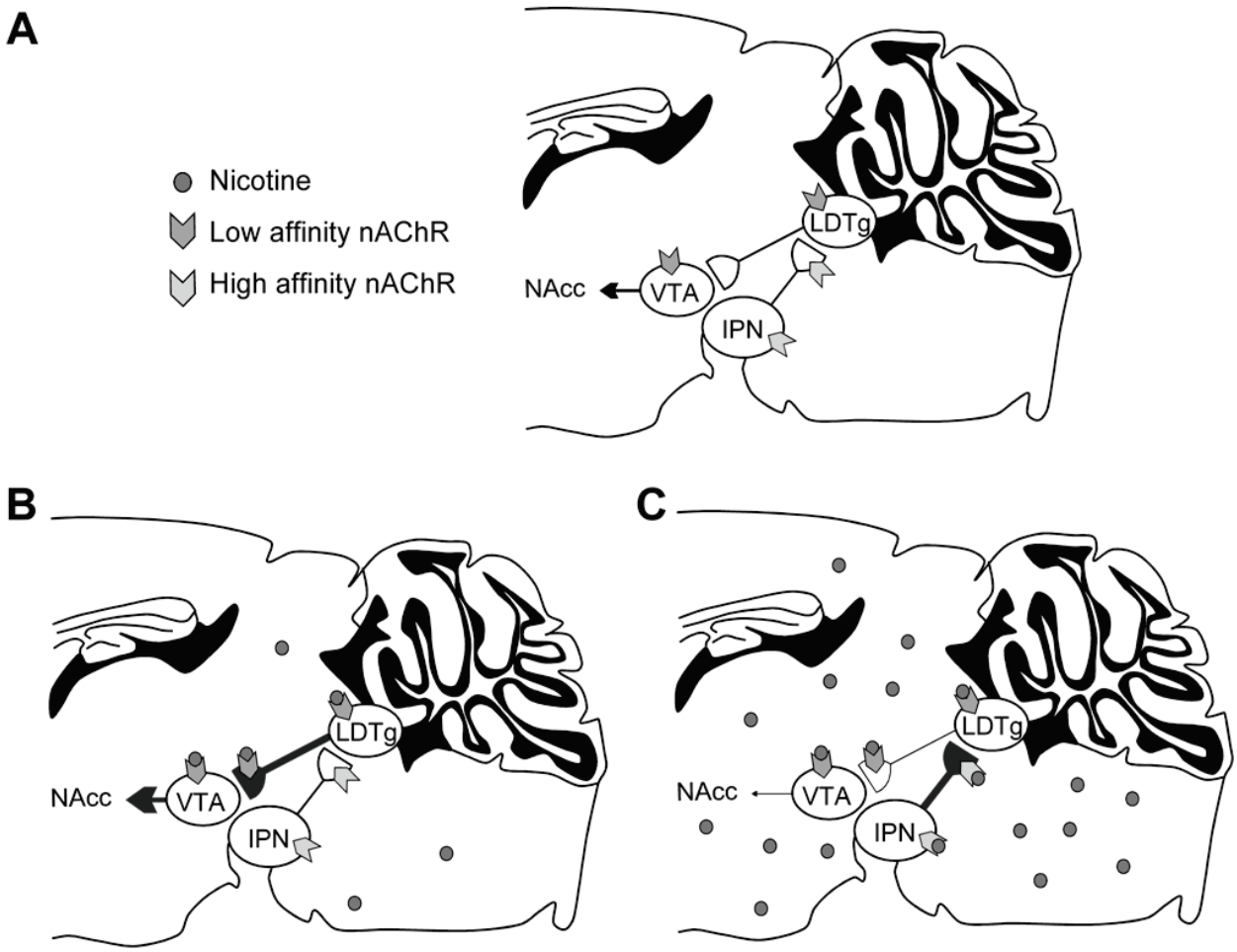

Figure 6.1- Proposed Model. A) In the absence of nicotine or other motivational stimuli, aversion- and reward-related circuits (MHb-IPN and LDTg-VTA-NAcc) are active at basal levels, maintaining a neutral state. B) In the presence of a low, reinforcing dose of nicotine, high affinity nAChRs that are expressed in reward-related areas are activated. This results in enhanced activity of this pathway and enhanced DA release in the NAcc. C) In the presence of a high, aversive dose of nicotine, lower affinity nAChRs that are expressed in aversion-related areas are now also activated. This results in enhanced activation of the aversive pathway, which in turn inhibits the LDTg-VTA-NAcc pathway. Thus, IPN activity gates the rewarding effects of nicotine.

expressed in these various sub-regions, and understanding the contributions of these neuropeptides could prove enlightening. Figure 6.2 shows a simplified diagram of some known MHb and IPN sub-regional connections.

My thesis work focused on IPN neurons regardless of sub-region localization or input specificity, although viral expression was targeted to the ventromedial portion of the IPN, which receives cholinergic and glutamatergic input from the MHb. It has been reported, though, that α5-expressing neurons in this region of the IPN do not project to the LDTg (Hsu et al., 2013a). However, although α5-expressing neurons in both the MHb and IPN are potentially important cell-types for mediating nicotine behaviors (Fowler et al., 2011), other cell-types, particularly in the IPN, may be equally important. IPN neurons from various sub-regions have been reported to project to the LDTg (Groenewegen et al., 1986), and it may be through these neurons that the MHb-IPN pathway modulates LDTg activity. Furthermore, even if our results are largely a result of IPN projections from lateral sub-regions, it is possible that cholinergic drive from the MHb is modulating these neurons, even though they do not directly receive dense innervations, as acetylcholine in the IPN is thought to function through volume transmission (Ren et al., 2011).

The MHb can be broadly broken into two main sub-divisions, the ventral MHb (vMHb) and the dorsal MHb (dMHb). The MHb receives dense innervations from the septum, in particular the posterior septum, which can be broken down into the triangular septum (TS) and the bed nucleus of the anterior commissure (BAC). The TS is thought to send glutamatergic projections predominantly to the vMHb to promote anxiety, while the BAC sends non-glutamatergic projections to the dMHb. Ablation of BAC inputs to the dMHb results in reduced fear conditioning (Yamaguchi et al., 2013). The importance of these inputs in relation to nicotine-related behaviors is unknown and requires further investigation. Additionally, both the

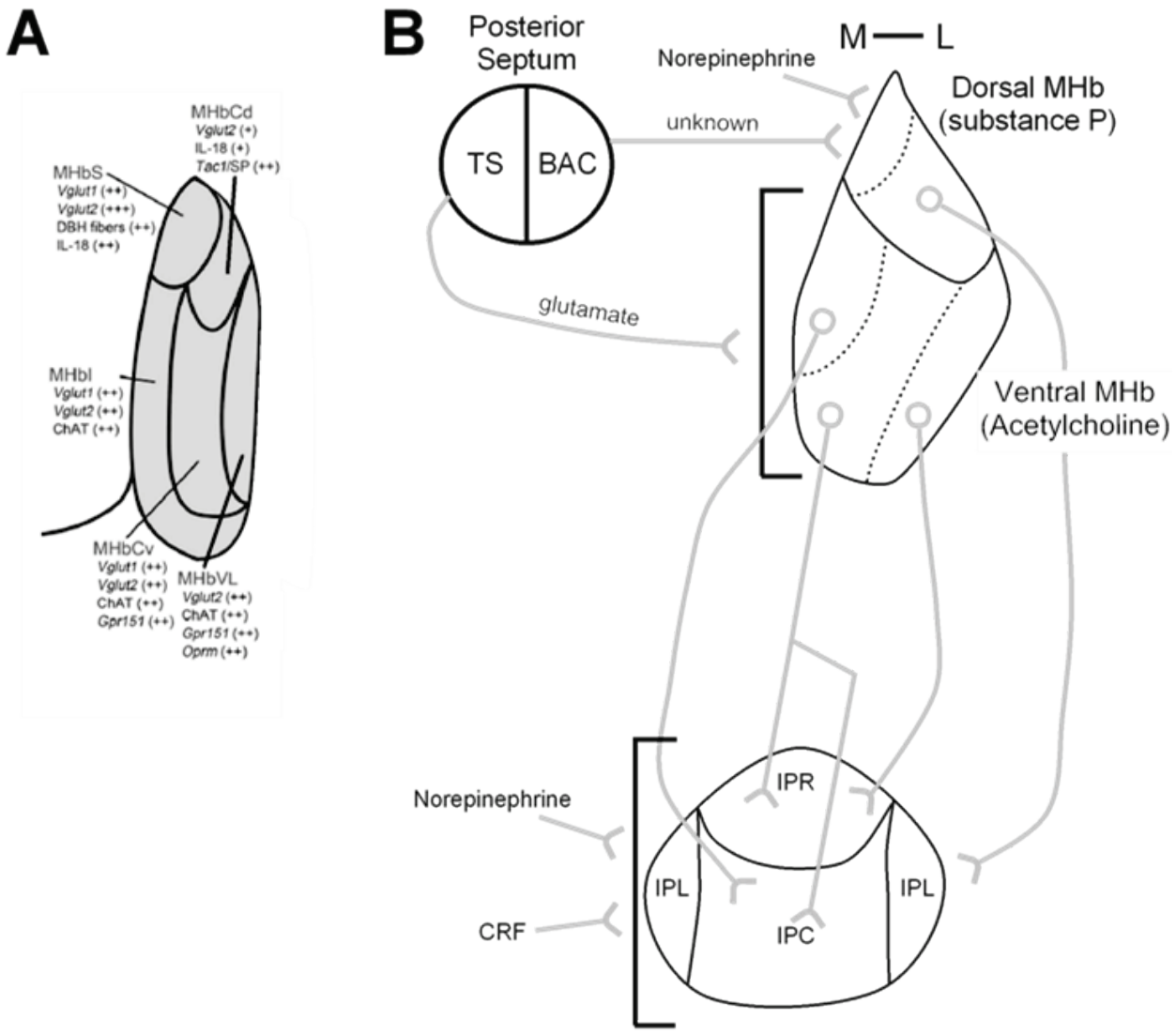

Figure 6.2- Simplified Diagram of Sub-Regional Connectivity in the MHb and IPN. A) MHb can be divided into sub-regions with substantial heterogeneity in mRNA expression. Adapted from (Aizawa et al., 2012). B) Different regions of the posterior septum preferentially target different sub-regions of the MHb. Different sub-regions of the MHb preferentially target different sub-regions of the IPN. All MHb inputs to IPN are thought to release glutamate, but the presence of substance P and ACh varies between regions. Adapted from (Yamaguchi et al., 2013; Shih et al., 2014; Zhao-Shea et al., 2015).

dMHb and vMHb can be further divided into more sub-regions, and the differences in innervations received by those regions remains unknown. These regions also target different sub-regions of the IPN.

The dMHb preferentially projects to the lateral IPN and has been shown to consist of substance P and glutamatergic afferents (Qin and Luo, 2009). Substance P in the MHb has also been implicated in nicotine-induced increases in excitability of MHb neurons and in the aversive effects of nicotine withdrawal, suggesting that cholinergic neurons in the MHb may not be the only critical mediators of nicotine aversion (Dao et al., 2014). However, this region has also been associated with enhanced reward-related behaviors (Hsu et al., 2014). Mice with genetic lesions of dMHb engage in less voluntary wheel-running than controls, and normal mice with ChR2 expressed in the dMHb will self-stimulate these neurons by running on a wheel more than controls. Inhibition of these inputs to the IPN results in real-time place avoidance (Hsu et al., 2014).

These results are challenging to interpret for a number of reasons. The stimulation protocol used in the wheel-running study was also shown to cause pauses in dMHb firing after a light pulse. The inhibition light delivery protocol also used pulses, so light turning off may have caused rebound excitation. It is also challenging to parse the rewarding effects of dMHb stimulation itself and the increased motivation it drives for wheel-running behavior. Additionally, the dMHb can be divided into sub-regions, one of which contains substance P markers, the other of which contains only glutamate markers (Aizawa et al., 2012). These distinct sub-regions of the MHb may differentially contribute to different behavioral outcomes.

Additionally, IPN microcircuitry might respond seemingly counter-intuitively to enhanced or diminished substance P input from the MHb. It has recently been shown that knock-

out of CB1 receptors selectively from MHb neurons reduces conditioned fear responding. Selective antagonism of intra-IPN nAChRs recovered these responses, suggesting that CB1 modulation of MHb cholinergic inputs to the IPN are required for this type of aversive responding (Soria-Gómez et al., 2015). CB1 activation on pre-synaptic terminals is generally thought to inhibit neurotransmitter release, and bath application of a CB1 antagonist facilitates ACh release from the MHb to the IPN (Soria-Gómez et al., 2015). However, CB1 activation has also been shown to facilitate LTP induction, particularly that which is thought to contribute to rapid learning (Cui et al., 2015). Therefore, CB1 signaling can dynamically regulate synapses, and dysregulation of cholinergic drive in the MHb is associated with deficits in aversive learning (Soria-Gómez et al., 2015).

It is also unclear how much overlap exists between fear-related behaviors and more general forms of aversion and their underlying neural circuits. Expression of a conditioned fear response might be rewarding for an animal, and might thus be expected to be mediated by diminished cholinergic drive from the MHb. There is also a variety of receptor classes expressed in the IPN, including muscarinic receptors, which could mediate inhibition of IPN neurons in response to enhanced cholinergic drive.

While much remains to be elucidated in MHb and IPN signaling, the experiments presented in this thesis and the literature presented in *Chapter 1* do support the idea that enhanced activity in the MHb-IPN circuit relates to enhanced aversion. Our data clarify some questions concerning the nature of IPN projections to the LDTg, including that the functional effect of IPN neuron activation on VTA-projecting LDTg neurons is inhibitory and that this connection functions by interacting with reward-related circuitry. Future work may focus on identifying the sub-regions of the IPN that constitute these projections and on identifying any

additional neurotransmitters they might contain. Additionally, although some work has examined changes induced by nicotine in these critical regions, a comprehensive understanding of the effects of nicotine on MHb-IPN circuitry is lacking. The IPN inputs to the LDTg may undergo long term modulations that might impact escalation and continuation of nicotine taking.

(6f) Potential Role of LHb in Nicotine Behaviors.

The LHb and MHb are vicinal but distinct nuclei that receive different afferent projections and send efferents to different targets (Herkenham, 1977; Herkenham & Nauta, 1979). Though both have been implicated in aversion, (Frahm et al., 2011; Lecca et al., 2011; Salas et al., 2010; Stamatakis & Stuber, 2012) much more is known about the network functions of the LHb than is known about its role in nicotine-related behaviors. Given the similarities in function between the two regions and their close proximity, it is possible that they interact directly with each other (Kim and Chang, 2005) to facilitate a common endpoint.

The LHb contains mostly glutamatergic neurons which project via the fasciculous retroflexus (FR) to the rostromedial tegmentum (RMTg) (Balcita-Pedicino et al., 2011; Brinschwitz et al., 2010). The RMTg is a ventral midbrain nucleus containing mostly GABAergic neurons that inhibit ventral tegmental area (VTA) dopamine (DA) neurons (Jhou, Fields et al., 2009; Jhou, Geisler et al., 2009). The LHb is excited by aversive stimuli such as a paw pinch and the absence of a predicted reward (Matsumoto & Hikosaka, 2007; 2009). In fact, non-human primates respond to LHb stimulation by learning to delay responses in order to prevent further stimulation of this area (Matsumoto and Hikosaka, 2011). The RMTg neurons that project to the VTA are also activated by reward omission and aversive stimuli (Jhou et al., 2009a; Lecca et al., 2010), and this activation precedes DA neuron inhibition (Matsumoto and

Hikosaka, 2007). Stimulating the LHb activates the RMTg, and this in turn inhibits VTA DA neurons (Lecca et al., 2010). Optogenetic stimulation of the LHb inputs to the RMTg elicit behavioral avoidance, consistent with the idea that diminished VTA DA neuron activity results in aversion (Stamatakis and Stuber, 2012).

The role of the LHb in aversion is supported by human work as well. In response to positive feedback on a task, activation in the nucleus accumbens (NAcc) is observed, and in response to negative feedback, activation of the habenula is observed (Ullsperger and Cramon, 2003; Hennigan et al., 2015). Additionally, the habenula is activated by the absence of a predicted reward in human subjects (Salas et al., 2010). It should be noted that these studies cannot differentiate between the MHb and LHb because the habenula is such a small area of the brain, but progress in imaging has been made, and studies examining the differential activations of MHb and LHb may be forthcoming (Strotmann et al., 2014).

Investigations into the effects of nicotine on LHb are largely lacking. Two studies have investigated LHb-related circuitry in response to acute nicotine. RMTg neurons are excited by paw pinch and inhibited by systemic administration of many abused drugs, including cocaine and morphine; however, nicotine administration results in excitation of RMTg neurons (Lecca et al., 2010).

The counter-intuitive effects of nicotine on RMTg neuron activity are difficult to explain given the lack of attention LHb circuitry has garnered. One possible explanation is that although the dose used in this study was within the range of doses used to condition place preference in rats, the serum levels of nicotine would likely be above 500 nM (Matta et al., 2007). Slice recordings show similar effects of 1 μM nicotine (Lecca et al., 2010), but again, the relevance of this concentration to nicotine reward is unclear. Another explanation could be that the RMTg

may be heterogenous, or may impact a heterogenous group of neurons in the VTA. Some VTA DA neurons may project to the LHb or the PFC, and these appear to mediate aversion (Lammel et al., 2012; Stamatakis et al., 2013). Alternatively, perhaps nicotine enhances activity of these neurons, but excitatory inputs to VTA DA neurons overcome RMTg-mediated inhibition to result in nicotine reward. For example, if the RMTg and LDTg are both excited by the arrival of nicotine, then it is possible that through somatic and pre-synaptic nAChR activation, excitation of VTA DA neurons prevails. In the event of high doses of nicotine, IPN-mediated inhibition of LDTg activity may shift the balance to reduced DA efflux in the NAcc and increased aversion. More work is clearly required to understand what, if any, role the LHb-RMTg pathway plays in modulating nicotine-related behaviors.

(6g) Effects of Nicotine in the NAcc.

The NAcc, and the striatum as a whole, is populated by three main classes of cells: medium spiny neurons (MSNs), cholinergic interneurons (ChINs), and fast-spiking GABAergic interneurons (FSINs). MSNs generally fall into two classes based on the type of dopamine receptor class they express, either D1 or D2 receptors. Although the ChINs make up only ~1-2% of neurons in the striatum, they are responsible for the prevalence of ACh throughout the region (Butcher and Butcher, 1974; Pisani et al., 2001). These neurons function both via synaptic transmission and volume transmission (Koós and Tepper, 2002). ChINs can express both D1-type and D2-type DA receptors, which increase and inhibit their firing rates, respectively (Alcantara et al., 2003; Berlanga et al., 2005; Bertorelli & Consolo, 1984; Consolo et al., 1999). Additionally, nAChRs localized to DA neuron terminals (Jones et al., 2001) are tonically activated by ChINs, which facilitates DA release (Zhou et al., 2001). Acetylcholinesterase

(AChE) activity prevents endogenous ACh from desensitizing these pre-synaptic receptors (Zhou et al., 2001).

Nicotine results in phasic DA release in the NAcc by exciting VTA DA neurons (Mansvelder and McGehee, 2002). Nicotine also enhances synchrony between the DA neurons in the VTA (Li et al., 2011b), maximizing the levels of NAcc DA. Additionally, nicotine modulates DA release in the NAcc by binding to pre-synaptic nAChRs, inhibiting tonic DA release and facilitating phasic DA release. This enhances the phasic DA signal compared to baseline (Rice and Cragg, 2004). Large, phasic increases in DA in the NAcc are required to activate the low-affinity D1Rs, while tonic firing is sufficient to activate the D2Rs. The D1-containing MSNs are part of the direct pathway that seems to facilitate reward, while the D2-containing MSNs are part of the indirect pathway that facilitates punishment (Volkow and Morales, 2015). Therefore, when large increases in DA occur in the NAcc, D1-MSNs are active and reward is facilitated, while D2-MSNs are inhibited and punishment is avoided. During pauses in tonic firing, the D2-MSNs become disinhibited, and punishment is facilitated (Volkow and Morales, 2015). Despite the over-simplified nature of this discussion (e.g. core vs. shell, overlap between D1- and D2-MSNs in the direct and indirect pathways, and many other topics were not covered), this description is generally not contested.

However, the role of ChINs in the NAcc remains less clear. ChINs express a wide variety of receptor types, including nAChRs, and receive a wide variety of inputs (Lim et al., 2014). One hypothesis concerning ChINs in the NAcc is that the ACh-DA balance in the NAcc determines the rewarding or aversive state, such that higher ACh levels relative to DA levels mediate aversion, while higher DA levels relative to ACh levels mediate reward (Hoebel et al., 2007). Support for this hypothesis comes from a variety of sources. When rats are initially given

saccharin, DA levels are increased in the NAcc. After conditioned taste aversion to saccharin, saccharin delivery resulted in decreased DA levels and an increase in ACh levels (Mark et al., 1991; Mark et al., 1995). Enhanced cholinergic activity in the NAcc is also sufficient to produce a conditioned taste aversion to saccharin (Hoebel et al., 2007). Rats will also work to escape an intra-cranial stimulation that elicits enhanced ACh levels in the NAcc (Rada and Hoebel, 2001), and ChINs in the striata of non-human primates have been shown to pause their firing in response to reward and reward-related cues (Apicella, 2007; Joshua et al., 2008; Morris et al., 2004). In response to reward omission, ChINs maintain their firing rates while DA neurons decrease firing (Morris et al., 2004). In response to an aversive event, ChINs increase their firing (Joshua et al., 2008). These findings suggest that increased ACh levels in the NAcc correspond to an aversive state, while increased DA levels correspond to a rewarding state.

However, an alternative hypothesis suggests that ChINs facilitate associative learning. GABAergic neurons from the VTA project specifically to the ChINs in the NAcc, and activation of these neurons reliably causes the ChINs to pause (Brown et al., 2012). Optogenetic activation of these GABAergic projection neurons during fear conditioning resulted in enhanced ability to discriminate between the predictive cue and a neutral cue (Brown et al., 2012). Additionally, it has been shown that cocaine activates the ChINs, and while direct optogenetic inhibition of the ChINs alone was not aversive, this inhibition prevented the acquisition of cocaine-induced CPP (Witten et al., 2010). These studies contradict the ACh-DA balance hypothesis. If enhanced fear conditioning could be considered to be an aversive state, then this aversive state is enhanced by decreased ACh release in the NAcc. CPP with cocaine is a reward-related learning phenomenon, and diminished ACh reduces this learning. These studies instead suggest that ChINs might dynamically alter firing rate to maximize associative learning, whether to avoid something

dangerous or to pursue something pleasurable. Indeed, recent work has shown that VTA DA neurons project to ChINs and co-release glutamate in the shell of the NAcc. Activation of these inputs consistently results in a short burst of activity followed by a pause in firing (Chuhma et al., 2014). The exact means by which ChIN firing patterns regulate various types of learning remains unclear, but it seems that optogenetic tools and increasing attention to heterogeneity between different sub-regions of nuclei will provide more clear answers to these questions.

The role of DA and ACh interactions in the NAcc in nicotine-related behaviors is also unclear. ChINs express nAChRs, but the types of nAChRs are largely unknown (Lim et al., 2014). It has been reported that nAChR activation can cause ChINs to release ACh, but only when relieved of D2R activation that provides intense inhibition (Sandor et al., 1991). If true, then perhaps direct nAChR activation by nicotine would have little effect on ChIN firing patterns, and the effects of nicotine reward will be similar to those of other rewards. One study reports that ChIN firing enhances DA release, and that low concentrations of nicotine inhibit this facilitation. In doing so, nicotine prevents depletion of DA from VTA terminals, which ensures DA-containing vesicles can be released during phasic bursts (Wang et al., 2014a). The effects of nicotine on ChINs in the NAcc have largely been ignored, so understanding the way that nicotine reward might be facilitated by NAcc signaling will require much more work. Investigations into the differential impacts of high and low doses of nicotine on ChINs or NAcc circuitry in general would also be interesting. It does seem, however, that nicotine inhibits DA release in the NAcc except for during phasic bursts. We hypothesize that IPN inhibition of the LDTg will reduce burst firing of VTA DA neurons, so pre-synaptic nicotinic signaling in the NAcc may serve to further reduce DA release in the NAcc. If this were true, perhaps the function of nicotinic signaling in the NAcc is to amplify the motivational signal that the VTA sends.

(6h) Nicotine Induces Changes in Relevant Brain Regions.

Even brief exposure to nicotine can result in long-term plasticity (LTP) in VTA DA neurons (Saal et al., 2003). Nicotine-induced LTP is mediated by somatic NMDARs, α4β2 receptors, and D5 DA receptors, and by pre-synaptic α7 receptors on glutamatergic inputs to VTA DA neurons (Mao et al., 2011). A6 subunit-expressing VTA DA neurons may also be important for nicotine-induced LTP and associated behaviors (Berry et al., 2015). Nicotine-induced LTP has not been extensively examined in other relevant brain regions of previously nicotine-naïve mice.

Chronic nicotine exposure results in additional changes in relevant brain regions. During chronic exposure to nicotine, nAChRs are up-regulated (Pistillo et al., 2015). This up-regulation is thought to be a homeostatic response to nAChR desensitization that occurs due to the relatively slow degradation of nicotine compared to ACh. Different receptor types are differentially up-regulated depending on the brain region and route of nicotine delivery (Moretti et al., 2010). For example, chronic nicotine results in functional up-regulation of α4-containing nAChRs selectively in the GABAergic neurons of the VTA. This results in increased basal firing rates of GABA neurons in the VTA, correspondingly decreased basal firing rates of VTA DA neurons, and diminished responses of VTA DA neurons to nicotine (Nashmi et al., 2007). It is thought that during smoking, these receptors become desensitized, so functional inhibition of VTA DA neurons is reduced. During abstinence, GABA activity via α4β2 function is restored and enhanced compared to pre-smoking, and this may contribute to negative affect during withdrawal (Pistillo et al., 2015). There are likely more complexities involved in this process; for example, expression of α4 subunits with enhanced sensitivity to nicotine selectively in GABA neurons of the VTA result in enhanced rewarding effects of nicotine, possibly due to GABAergic

projections to aversive-related regions like the LHb (Ngolab et al., 2015). Withdrawal will be discussed further in section 6i.

While more work is required to fully clarify the effects of chronic nicotine on VTA circuitry, the effects of chronic nicotine on other relevant brain regions is largely lacking. Although the LDTg has been implicated in nicotine- and reward-related behaviors (Lança et al., 2000b; Alderson et al., 2005; Lodge and Grace, 2006; Ishibashi et al., 2009; Lammel et al., 2012), there have been no investigations into nicotine-induced plasticity to my knowledge.

A few studies have investigated the effects of chronic nicotine on MHb neurons. One study reports that chronic nicotine results in up-regulation of β4 subunits in the habenula, although the functional relevance of this change is not examined (Meyers et al., 2015). A different study reports that nicotine enhances excitability in MHb neurons by augmenting neurokinin signaling, and that this augmentation requires α5-containing nAChRs. Nicotine effects on neurokinin signaling are attenuated by chronic nicotine treatment, and direct infusion of neurokinin receptor antagonists into the MHb are sufficient to precipitate withdrawal (Dao et al., 2014). It is suggested that chronic nicotine leads to enhanced desensitization of α5-containing nAChRs, and that withdrawal-induced aversion may be related to diminished nicotine-induced excitability of MHb neurons. However, it has also been shown that different sub-regions of the MHb respond to chronic nicotine differently. MHb neurons in the ventrolateral sub-region show no changes in excitability or nAChR function after chronic nicotine, but those in the ventroinferior sub-region exhibit hirer basal firing rates and a blunted response to nicotine after chronic nicotine (Shih et al., 2015). These results may have been due to slices experiencing abrupt removal of nicotine, as nicotine was not included in the bath, but they

nevertheless highlight the importance of understanding the effects of nicotine on the various sub-regions of critical brain areas.

The effects of chronic nicotine on IPN neurons also remain largely unexplored. It has been shown that IPN neurons that receive inputs from the ventrolateral region of the MHb can be grouped into two classes based on electrophysiological responses to glutamatergic and nicotinic activation (Shih et al., 2014), but the ways in which excitability might be changed during chronic nicotine was not explored. Another study reports that CRF release from the VTA is enhanced in the IPN during nicotine withdrawal, and that this causes increased excitation in the IPN neurons, promoting anxiogenic effects of withdrawal (Zhao-Shea et al., 2015). However, plasticity or nicotine-induced adaptations have not been explored, and activity in the IPN has largely been assessed using indirect markers rather than electrophysiological measurements.

As more information about the changes that nicotine causes in these various brain regions emerges, it will be interesting to see how the IPN projection to the LDTg might contribute to the behavioral effects of nicotine at different stages of drug-taking. It is possible that the MHb-IPN pathway is most sensitive to nicotine in the initial stages of drug-taking, and that inhibitory drive from this pathway to the LDTg diminishes with repeated exposures. It has been found that during withdrawal (Ngolab et al., 2015), nicotine can become more rewarding, and perhaps this is due to dampening of IPN drive to the LDTg.

(6i) Nicotine Withdrawal.

The MHb-IPN circuit has also been implicated in many aspects of nicotine withdrawal (De Biasi and Dani, 2011; Zhao-Shea et al., 2013, 2015). The IPN-LDTg connection may also be important in regulating the reward and aversion during chronic nicotine-taking and withdrawal.

Nicotine withdrawal is an aversive state that results at least in part from nicotine-induced homeostatic neuroadaptaions (Edwards, 2010; De Biasi and Dani, 2011). Nicotine withdrawal can result in aversive symptoms such as anxiety, negative affect, sleep disturbance, impaired concentration, and increased appetite (Hughes, 2007; Piper, 2015), and these symptoms are accompanied by craving for nicotine (Sweitzer et al., 2012). Although the role of this negative withdrawal syndrome in addiction remains heavily debated (Wise and Koob, 2014), it does seem that severity of withdrawal symptoms, particularly craving and negative affect, can predict relapse, especially within the first two weeks of quitting (Piper, 2015). Therefore, understanding the neural mechanisms that underlie nicotine withdrawal may lead to treatment strategies that can facilitate cessation.

Rodents also display a measurable withdrawal syndrome during both spontaneous withdrawal and mecamylamine (MEC)-precipitated withdrawal (Malin and Goyarzu, 2009). Withdrawal-induced behaviors include increased chewing, shaking, and anxiety (Salas et al., 2004; Salas et al., 2009). This withdrawal syndrome is aversive to rodents, producing CPA and increasing the reward threshold of ICSS (Watkins et al., 2000).

There is substantial evidence to suggest a role for the MHb-IPN pathway in this aversive withdrawal syndrome (Mclaughlin et al., 2015). Mice null for the β4 nAChR subunit, which is highly expressed in this pathway, show greatly diminished signs of somatic withdrawal from chronic nicotine (Salas et al., 2004), and selective antagonism of α3β4 receptors reduces the physical signs of MEC-precipitated withdrawal. Mice lacking the α2, which is highly and almost exclusively expressed in the IPN, or α5 nAChR subunit experienced no somatic withdrawal symptoms when challenged with MEC after chronic nicotine (Salas et al., 2009). Furthermore, MEC delivery directly to the MHb or IPN was sufficient to precipitate withdrawal from nicotine

(Salas et al., 2009; Zhao-Shea et al., 2015). Direct optogenetic stimulation of GABAergic neurons in the IPN also precipitates nicotine withdrawal, and can even induce withdrawal-like behaviors in nicotine-naïve mice (Zhao-Shea et al., 2013). Inhibition of MHb cholinergic inputs to the IPN during withdrawal reduces withdrawal-induced anxiety (Zhao-Shea et al., 2015). Together, these findings suggest that activity in the MHb-IPN pathway is a critical mediator of the nicotine withdrawal syndrome.

Given the apparent overlapping mechanisms between acute nicotine aversion and nicotine withdrawal, it seems that the MHb-IPN connection may contribute to general aversive effects associated with nicotine. However, many of the findings concerning the MHb-IPN pathway in withdrawal seem contradictory, which is similar to the state of the literature examining the acute effects of nicotine on this pathway. The knock-out and optogenetic studies suggest that enhanced activity in the MHb-IPN pathway results in enhanced withdrawal symptoms; however, MEC infusion into the MHb or the IPN can precipitate withdrawal. MEC would presumably decrease activity in these regions. However, it has been shown that MEC infusion into the IPN to precipitate withdrawal results in increased activation of GABA neurons in the IPN, potentially by inhibiting nicotinic-mediated excitation of GABAergic interneurons (Zhao-Shea et al., 2013). Nicotinic activation of these interneurons may represent one way that continued nicotine-taking prevents the aversive withdrawal state.

Given the overlapping role of the α5 nAChR subunit in the MHb-IPN in mediating both acute aversion and withdrawal from chronic nicotine, it is possible that the human genetic variant of the α5 subunit that confers increased risk for addiction also has implications for withdrawal from nicotine. An enhanced withdrawal syndrome may relate to an enhanced propensity to relapse in a smoker who has quit, especially shortly after quitting (Piper, 2015). In fact,

individuals with the *CHRNA5* risk variant have delayed smoking cessation and more cessation failures compared to those with the normal variant (Belsky, 2013; Chen et al., 2015). However, the causal role of a more severe withdrawal syndrome and trouble quitting has not been conclusively established (Piper, 2015).

The role of the MHb-IPN pathway in both acute and withdrawal-induced aversive effects of nicotine also suggests that the IPN-LDTg connection may be a downstream target of the IPN that is also important in mediating nicotine withdrawal. Indeed, there is evidence to suggest that DA transmission is altered during the withdrawal syndrome (Mclaughlin et al., 2015). Nicotine withdrawal results in basal hypodopaminergic levels in the NAcc, such that in response to nicotine, the ratio of phasic to tonic dopamine levels is higher than for nicotine-naïve animals (Zhang et al., 2012). This diminished background DA signal may facilitate relapse by enhancing the reward value of smoking. In fact, very low doses of nicotine that nicotine-naïve mice will not work for are readily self-administered during withdrawal (Tammimäki et al., 2008). Because the LDTg gates the activity of VTA DA neurons (Lodge and Grace, 2006; Paladini and Roeper, 2014), the IPN projection to the LDTg might contribute to the effects of withdrawal on NAcc DA levels. Therefore, the connection between the IPN and LDTg described in this thesis may have relevance not only for development, but also for maintenance of nicotine dependence through mediation of aversive withdrawal effects.

(6j) Treatments for Nicotine Dependence.

Despite decades of investigation into the rewarding effects of nicotine, treatment strategies for smokers who want to quit remain marginally effective at best (Harmey et al., 2012; Prochaska and Benowitz, 2016). The main lines of treatment for smoking cessation are nicotine

replacement therapy (NRT), buproprion, and varenicline (McDonough, 2015; Prochaska and Benowitz, 2016). Buproprion inhibits re-uptake of dopamine and norepinephrine and can act as a nAChR antagonist (Harmey et al., 2012; Prochaska and Benowitz, 2016).

Burproprion is thought to facilitate smoking cessation by mimicking the effects of nicotine on the dopamine system, thus limiting cravings and withdrawal; however, the exact mechanisms by which buproprion reduces smoking remain unclear. Varenicline is a partial agonist at α4β2 receptors and a full agonist at α7 receptors. Varenicline is thought to facilitate smoking cessation by reducing the ability of nicotine to cause dopamine efflux in the NAcc. This is achieved by varenicline occupying binding sites on nAChRs so that nicotine binding is limited. α4β2 receptors are critically important in mediating the rewarding effects of nicotine, and reducing activation of these receptors is thought to decrease the reward associated with continued smoking. Because varenicline is a partial agonist at these receptors, it is thought to prevent the depletion of dopamine levels in the NAcc, and thus reduce some of the negative effects associated with withdrawal (Harmey et al., 2012; Prochaska and Benowitz, 2016).

These treatments are useful in helping smokers quit compared to placebo treatments, but long-term quit rates remain low (~30%) (Prochaska and Benowitz, 2016). There have been a couple of studies to investigate the effects of buproprion and varenicline treatment on the subjective effects of nicotine in smokers. Buproprion increases ratings of post-smoking satiety, and varenicline reduces the pleasant feelings associated with smoking (Sofuoglu et al., 2009; Hussain et al., 2010). If reward and aversion can be considered on a spectrum, then an alternative approach to diminishing the rewarding effects of nicotine would be to enhance the aversive effects of nicotine. The strong genetic and pre-clinical studies implicating nAChRs that are densely expressed in the MHb and IPN in aversion to nicotine and vulnerability to nicotine

dependence suggest that treatments along these lines may be efficacious. For example, it has been proposed that a positive allosteric modulator for α5-containing nAChRs could be a promising therapeutic (Harmey et al., 2012).

Pharmacotherapies that result in aversion generally have poor compliance, limiting their usefulness in treating patients (Franck and Jayaram-Lindström, 2013). However, aversive circuits could in theory be activated in such a way that the rewarding effects of nicotine were reduced without shifting the balance completely to aversion. This type of treatment might work more similarly to a satiety signal rather than a fully aversive signal and may therefore hold promise (Fowler and Kenny, 2014). Understanding the pathways by which aversive circuitry influences reward circuitry can help direct future approaches.

(6k) Conclusions.

The habenulointerpeduncular pathway has been implicated in a variety of behaviors and affective states, including pain, aversion, anxiety, fear, depression, addiction, and other psychiatric disorders (Hikosaka, 2010; Fowler and Kenny, 2012b; Nakajima et al., 2013; Yamaguchi et al., 2013; Mirrione et al., 2014; Zhao-Shea et al., 2015). We have identified a point of convergence between this epithalamic region and the mesolimbic dopamine system that contributes to the computation of gestalt motivational valence, and this connection between the IPN and LDTg may play an important role in related behavioral states. Understanding the impact of IPN inhibition of LDTg neurons in many behavioral and disordered contexts could lead to new insights into pathophysiology and novel treatment targets.

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