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The Role of VTA Gabaergic Nicotinic Acetylcholine Receptors Containing the α4 Subunit in Nicotine Dependence: A Dissertation

Jennifer Ngolab

University of Massachusetts Medical School

THE ROLE OF VTA GABAERGIC NICOTINIC ACETYLCHOLINE RECEPTORS CONTAINING THE α4 SUBUNIT IN NICOTINE DEPENDENCE

A Dissertation Presented

By

Jennifer Ngolab

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The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

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Andrew R. Tapper/ Paul D. Gardner, Thesis Advisors

Michael Francis, Member of Committee

Fen-Biao Gao, Member of Committee

Jean King, Member of Committee

Ralph Loring, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

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David Weaver, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Neuroscience

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ABSTRACT

Nicotine dependence is hypothesized to be due to neuroadaptations that ultimately drive compulsive nicotine use. The studies in this thesis aim to understand how the "upregulation" of nicotinic acetylcholine receptors (nAChRs) caused by chronic exposure to nicotine contributes to nicotine reward and nicotine withdrawal. Previous studies have shown that chronic nicotine induces upregulation of nAChRs containing the α4 subunit (α4* nAChR) within the Ventral Tegmental Area (VTA), a brain region critical for the rewarding properties of all illicit drugs. Curiously, α4* nAChR upregulation occurs specifically in the inhibitory GABAergic neuronal subpopulation of the VTA. To determine if increased expression and activation of α4* nAChRs in VTA GABAergic neurons contributes to nicotine dependence behaviors, I devised a viral-mediated, Cre-regulated gene expression system that selectively expressed α4 nAChR subunits containing a "gain-of-function" point mutation (a leucine mutated to a serine residue at the TM2 9′ position: Leu9′Ser) in VTA GABAergic neurons of adult mice. Sub-reward doses of nicotine were sufficient to activate VTA GABAergic neurons in mice expressing Leu9′Ser α4 nAChR subunits in VTA GABAergic neurons (Gad2VTA: Leu9′Ser mice) and exhibited acute hypolocomotion upon initial injection of low doses of nicotine that developed tolerance with subsequent nicotine exposures compared to control animals. In the conditioned place preference procedure, nicotine was sufficient to condition a significant place preference in $Gad2^{VTA}$: Leu9′Ser mice at low nicotine doses that failed to condition control animals. I conclude from these data that upregulating α4* nAChRs on VTA GABAergic neurons increases sensitivity to nicotine reward. In a separate study testing the hypothesis that overexpression of Leu9′Ser α4* nAChRs in VTA GABAergic neurons disrupts baseline behavior and promotes anxiety-like behaviors, I found that overexpressing Leu9′Ser α4* nAChRs in VTA GABAergic neurons had a minimal effect on unconditioned anxiety-like behaviors. Drug naïve Gad2VTA: Leu9′Ser and control mice failed to exhibit any behavioral differences in the open-field, marble burying test and elevated plus maze compared to control.

Together, these data indicate that overexpression of the "gain-of-function" α4* nAChRs in VTA GABAergic neurons contributes to reward sensitivity without increasing susceptibility to nicotine withdrawal symptoms. My data indicates that nAChRs expressed in VTA GABAergic neurons may be a suitable target for the development of better smoking cessation aids.

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LIST OF ABBREVIATIONS

5-HT – 5-hydroxytryptamine, Serotonin
aca – Anterior Commissure
ACh – Acetylcholine
AAV – Adeno Associated Virus
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ca^{++} – Calcium
CPP – Conditioned Place Preference
Cre – Cre Recombinase/ Causes Recombination
CRF – Corticotropin Releasing Factor
Cys - Cysteine
DA – Dopamine
DAergic – Dopaminergic
DHβE – Di-Hydro-Beta-Erythodine
Ef1a – Elongation Factor 1 Alpha
EPM – Elevated Plus Maze
FDA – Food and Drug Administration
GABA – γ-Aminobutyric Acid
Gad1 – Glutamic Acid Decarboxylase 1
Gad2 – Glutamic Acid Decarboxylase 2
$Gad2^{VTA}$: Leu9´Ser – Gad2-Cre mice expressing Leu9´Ser α4* nAChRs in the VTA
GFP – Green Fluorescent Protein
ICSA – Intracranial Self-Administration
ICSS – Intracranial Self-Stimulation
IPN – Interpeduncular Nucleus
IVSA – Intravenous Self-Stimulation
KO – Knock Out
LDB – Light/Dark Box
LDTg – Laterodorsal Tegmentum
Leu9´Ser – point mutation of the TM2 9′ Leucine to Serine
LHb – Lateral Habenula
Mec – Mecamylamine
mg/kg – Milligrams per Kilogram
MHb – Medial Habenula
ml – Mammillary Nucleus
MLA= Methyllcaconitine
µm - Micrometer
Na^{+} – Sodium
NAc – Nucleus Accumbens
nAChR – Nicotinic Acetylcholine Receptor
Nic – Nicotine
NMDA - N-methyl-D-aspartate
NRT – Nicotine Replacement Therapy
n.s. – Non-Significant

OFT – Open Field Test
PAS – Photobeam Activity System
PFC – Prefrontal Cortex
PPI – Prepulsed Inhibition
pVTA – posterior Ventral Tegmental Area
RMTg – Rostromedial Tegmentum
s.c. – Subcutaneous
SEM – Standard Error of the Mean
TH – Tyrosine Hydroxylase
TM1 – First Transmembrane Domain
TM2 – Second Transmembrane Domain
VGluT – Vescicular Glutamatergic Transporter
VTA – Ventral Tegmental Area
YFP – Yellow Fluorescent Protein

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CHAPTER I.

INTRODUCTION

I.A. – Nicotine Dependence

Long-term cigarette smoking reduces the quality of life for the smoker and those around them (Jha et al., 2013; USDHHS, 2014). Multiple studies indicate that smoking increases the risk for diabetes, lung cancer and coronary maladies. In fact, it is estimated that every year more than 5 million deaths are attributed alone to smoking related complications (USDHHS, 2014). It is estimated that smoking subtracts a decade from total life expectancy while quitting can restore 5 years (Jha et al., 2013). Second hand smoke can cause sudden infant death syndrome in children and contribute to the risk of developing coronary heart disease and lung cancer in adults (USDHHS, 2014). Despite being the leading preventable cause of death and disability and 50 years of advocating against the practice, around 42.1 million people in the United States still smoke (USDHHS, 2014).

Nicotine is the addictive substance in tobacco (Stolerman and Jarvis, 1995). It is as addictive, if not more, as heroin and alcohol and takes as few as 100 cigarettes to develop dependence (USDHHS, 2014). Humans experience a mild positive reinforcing response to nicotine, described to be slightly euphoric and relaxing (Henningfield et al., 1985; Pomerleau, 1992; Stolerman and Jarvis, 1995; Benowitz, 1996). Nicotine readily enters the bloodstream through the lungs and crosses the blood brain barrier within 8 to 10 seconds after inhalation (Benowitz and Jacob, 1990; Matta et al., 2007). One inhalation of a cigarette induces a rapid influx of nicotine into the blood (Henningfield and Benowitz, 1995; Nides, 2008). While nicotine routinely remains in the plasma for 3 hours,

smokers can retain nicotine in the plasma for up to 20 hours (Matta et al., 2007). Smokers are able control the amount of nicotine through the amount of puffs they inhale from a cigarette, giving them the ability to control the buzz they experience.

Nicotine dependence is a complicated behavior composed of three-phases: 1) acquiring and maintaining constant nicotine-taking behavior, 2) exhibiting negative withdrawal symptoms while not using and 3) increasing vulnerability to relapse (D'Souza and Markou, 2011). These stages presumably reflect neurobiological adaptations in response to constant nicotine consumption. While nicotine has positive properties, it is hypothesized that the adverse symptoms experienced during acute nicotine withdrawal primarily motivate relapse. The onset of symptoms ranges from 4 to 24 hours after the last cigarette consumed (McLaughlin et al., 2015). Somatic or physical symptoms include dry mouth and body tremors while affective or psychological symptoms include anxiety, irritability and inability to focus (Hughes, 1999). Nicotine withdrawal symptoms peak within the first week and can last up to four weeks (Hughes, 2007). The intensity of negative affective symptoms experienced during withdrawal can predict the likelihood to relapse (Piasecki et al., 2000). While long-term nicotine use causes changes in the brain such as changes in neurotransmission and receptor expression, it is unknown how these neuroadaptations contribute to negative withdrawal symptoms. Another line of evidence for long-term neuroadaptations following chronic nicotine exposure is the susceptibility to relapse even after years of abstinance.

Negative stressors, or stimuli that are perceived to be harmful or challenging, are a major contributor to drug relapse (Sinha, 2008, 2009; Bruijnzeel, 2012). Studies in humans and animals have determined that chronic stress coincided with heightened vulnerability to drug dependence, especially in adolescence (Sinha et al., 2001, 2008; Burke and Miczek, 2014). The emotional value of a particular stimulus can trigger relapse. Stressful cues induced drug craving, both in cocaine (Sinha et al., 1999) and nicotine dependent individuals (Michalowski and Erblich, 2014). Furthermore, laboratory experiments that aggravated stress in withdrawn smokers observed a decreased resistance to smoke and longer smoking consumption (Mckee et al., 2011). Chronic nicotine can also heighten physiological response to stress during withdrawal (Tsuda et al., 1996). Due to the contributions of stress to relapse, it is important to also understand how nicotine factors into the mechanisms behind stress-induced relapse.

Maintaining abstinent from cigarettes is often difficult for smokers, despite their well-meaning intents. In a 2010 survey, 68.8% of smokers expressed the intention to quit; yet only 6.2% of those surveyed reported to have quit within the year (CDC, 2011). 51.2% of smokers that attended a smoking cessation clinic relapsed within a year, further emphasizing the difficulty of remaining abstinent even after seeking help (Kocak et al., 2015).

Current first-line therapeutics available to help aide smokers to quit includes nicotine replacement therapies (NRT), buproprion (Zyban®) and varenicline (Chantix®). Nicotine replacement therapies administer nicotine at concentrations lower than what is obtained through cigarettes to wean a smoker from the

reinforcing effects of nicotine. NRTs come in different forms, from lozenges to skin patches, with similar efficacy (Nides, 2008). However, NRTs do not deliver nicotine at the same rate as smoking a cigarette, which may account for the dissatisfaction that smokers express when on NRTs and could discourage continued use (Henningfield, 1995). With non-nicotine therapies, as many as 44% of smokers remained abstinent after 12 weeks on varenicline compared to 28.9% on buprorion and 17.7% on placebo (Gonzales et al., 2006; Jorenby et al., 2006). However, the percentage of smokers that remained abstinent while on varenicline decreased to 20-25% within one year of treatment (Gonzales et al., 2006; Jorenby et al., 2006). Furthermore, current smoking cessation therapeutics are minimally successful in mitigating nicotine withdrawal symptoms (Cinciripini et al., 2013; Foulds et al., 2013; Turner et al., 2013). Even with the Food and Drug Administration approval of varenicline in 2006 as a smoking cessation agent, annual smoking cessation rates changed from 4.5% in 2003 to 4.7% in 2010 (Zhu et al., 2015). Overall, the available therapies provide temporary relief, but are not sufficient to maintain long-term abstinence. Better understanding of the biological mechanisms behind nicotine dependence, such as the substrates nicotine binds onto, would help with the development of better aids for smoking cessation.

I.B. - Nicotinic acetylcholine receptors

The nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that are endogenously bound by the neurotransmitter acetylcholine as well as nicotine. They are a part of the Cys-loop receptor superfamily that also contains

the $GABA_A$, glycine and the $5\text{-}HT_3$ receptor. nAChRs are composed of five subunits arranged to form a pore that permits ions to flow down their electrochemical gradient. Twelve unique nAChR subunits have been identified, including nine α subunits ($\alpha2$-$\alpha10$) and three β subunits ($\beta2$-$\beta4$) (Luetje and Patrick, 1991; Covernton et al., 1994; Chavez-Noriega et al., 1997; Kuryatov and Lindstrom, 2011). Each subunit contains an amino extracellular terminus that contributes to the ligand binding pocket, four membrane spanning domains, of which the second transmembrane domain (TM2) lines the pore and forms the channel gate, and a large variable intracellular loop (Figure I-1 A) (Albuquerque et al., 2009). There are specific cysteine residues along the extracellular amino terminus that play an important role in the identification and the function of the subunit. The ligand-binding site is composed of the hydrophobic pocket formed by a cysteine disulfide bond on the alpha subunit and the backside of the adjacent subunit (Albuquerque et al., 2009) (Fig I-1B, red triangles). A pair of cysteine residues in the extracellular amino terminus close to the first transmembrane domain (TM1) distinguishes an alpha subunit from a beta subunit (Lukas et al., 1999) (Fig I-1A, red dots). The nAChR subunits can coalesce with either five identical subunits to form homomeric receptors, or combine with both alpha and beta subunits to create heteromeric receptors (Figure I-1 B) (Cooper et al., 1991). The receptor subunit combination determines the biophysiological and pharmacological properties. For example, $\alpha4\beta2$* nAChRs can exist either as $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ stoichiometry (the asterisk denote that other subunits may be present in the receptor) (Nelson et al.,

2003; Kuryatov et al., 2005). Studies done in *Xenopus* oocytes determined the former stoichiometry forms low sensitivity receptors and the latter confers high

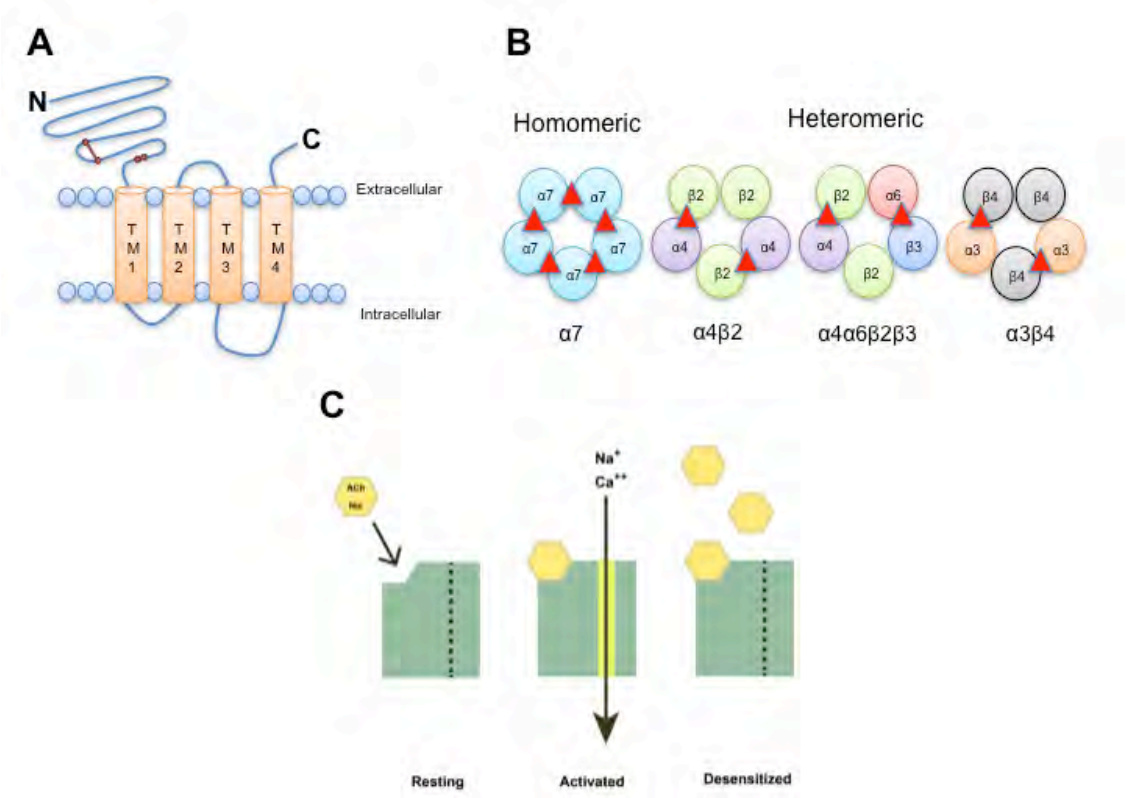

Figure I-1. Neuronal nAChRs structure and function A) Structure of the nAChR subunit. Each subunit is composed of four transmembrane domains (TM1-TM4), a large extracellular amino tail that is responsible for ligand binding, and an intracellular variable loop connecting TM3 to TM4. Highlighted in red are cysteine residues on the extracellular amino tail that form a cys-loop found in all subunits and the cysteine doublet that distinguishes α from β nAChR subunits. B) Representative nAChR subtypes, including receptors composed of identical nAChR subunits (homomeric) and nAChRs composed of a variety of nAChR subunit (heteromeric). The red triangles represent the binding site formed by the α nAChR cys-loop and the adjacent subunit. C) Possible conformations of nAChR. Receptors that are not bound by agonist are considered to be in the 'resting state', where the pore of the channel is closed. When agonist such as acetylcholine or nicotine (represented by the yellow hexagon) binds onto a nAChR, the receptor is 'activated' the pore is open. The 'desensitized' state is when the pore remains closed despite being bound to agonist (Figures 1A and B were adapted from Hendrickson et al., 2013, 1C is adapted from Changeux, 1984).

sensitivity receptors (Nelson et al., 2003; Tapia et al., 2007). Furthermore, different nAChR combinations, or subtypes, are expressed in selected subpopulations in the brain. While $\alpha4\beta2$* and $\alpha7$ nAChRs are ubiquitously expressed throughout the brain, nAChR subunits such as the $\alpha6$, $\alpha5$ and $\beta4$ are found in distinct brain regions such as the interpeduncular nucleus (IPN) and medial habenula (MHb) (Klink et al., 2001). These properties of nAChR expression and function provide a challenge to identifying an effective target for nicotine dependence.

Chronic nicotine treatment can alter the expression and function of nAChRs. Prolonged exposure to agonist causes nAChRs to desensitize, or no longer respond to agonist (Quick and Lester, 2002) (Figure I-1 C). Desensitization occurs immediately after channel opening, although activation of the receptor is not always required (Changeux et al., 1985; Giniatullin et al., 2005). The desensitization properties of a nAChR are highly dependent on the nAChR subtype, as $\alpha7$ nAChRs quickly desensitize compared to other subtypes while $\alpha4\beta2$* nAChRs desensitize at low concentrations of agonist (Couturier et al., 1990; Pidoplichko et al., 1997). Ligand binding affinity is higher in desensitized nAChRs compared to their active counterparts (Quick and Lester, 2002). This fundamental characteristic of nAChRs could factor into certain aspects of nicotine dependence. For example, it is hypothesized that smokers continue to smoke in order to maintain nAChR desensitization (Brody et al., 2006).

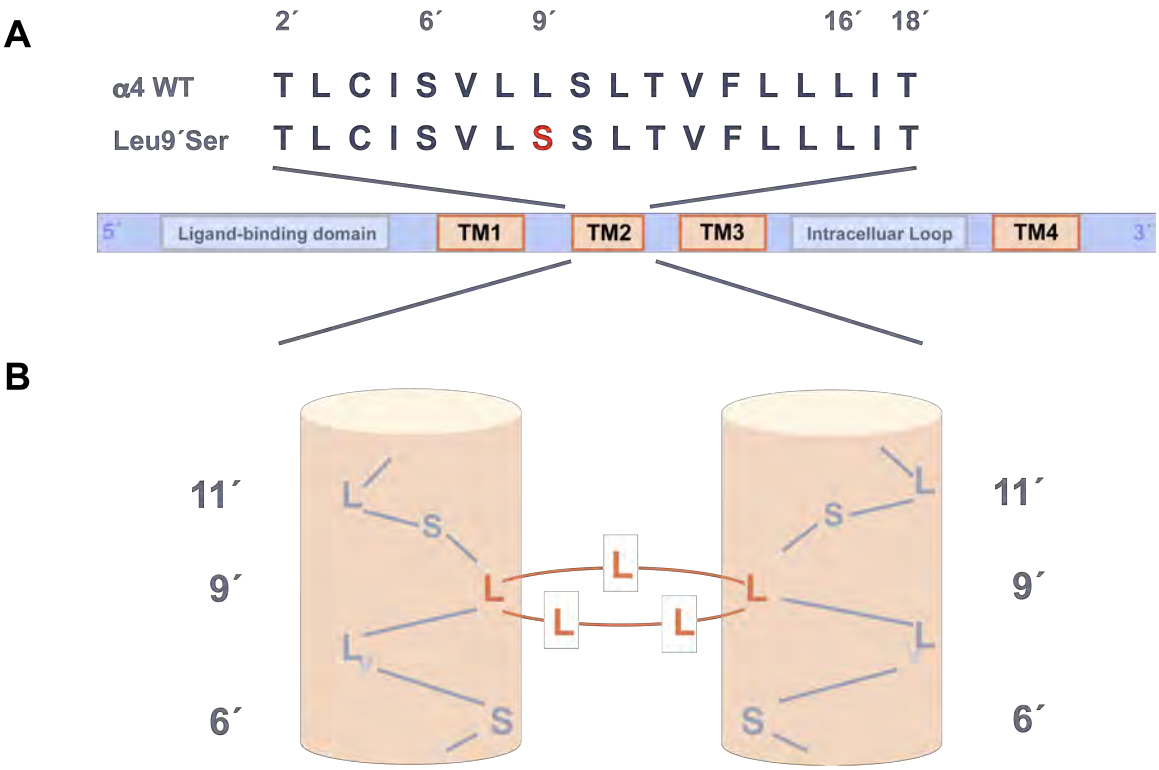

Figure I-2. Sequence and structure of the α4 nAChR subunit TM2 domain. A) Amino acid sequence of the endogenous (α4 WT, top) and Leu9'Ser nAChR (middle) TM2 region of the α4 nAChR subunit. (Bottom) Schematic depicting each nAChR subunit domain along the sequence of the CHRNA4 sequence. B) Proposed alignment of TM2 residues within a nAChR pore. Two α4 nAChR TM2 domains subunits are in view. It is proposed that the 9' leucines from the five nAChR subunits form a gate within the ion pore (Adapted from Revah et al., 1991).

A neuronal compensatory mechanism that occurs with chronic nicotine use is the upregulation of nAChRs. "Upregulation" encompasses all changes in nAChR expression that result in the increased expression and function of nAChRs, including increasing receptor number, decreasing the rate of degradation of particular receptors and changing subunit stoichiometry within a receptor (Nelson et al., 2003; Kuryatov et al., 2005; Srinivasan et al., 2011; Govind et al., 2012). Multiple studies from rodent to human have observed that the subtype commonly upregulated is $\alpha4\beta2^*$ nAChR subtype in the $(\alpha4)_2(\beta2)_3$ conformation (Flores et

al., 1992; Nashmi et al., 2007; Staley et al., 2006; Muhkin et al., 2008). Increased nAChR expression due to chronic nicotine can last up to 7-21 days after the last nicotine exposure (Marks et al., 1983; Schwartz and Kellar, 1985; Staley et al., 2006; Hilario et al., 2012). Much is known about the molecular and cellular underpinnings of nAChR upregulation (Henderson and Lester, 2015), yet how upregulation of nAChRs contributes to nicotine dependence behaviors is not well understood. When factoring in the contributions of desensitization of nAChRs and nAChR upregulation, one can appreciate the level of complexity of nicotine dependence at the receptor level.

To determine where the functional domains on the nicotinic acetylcholine receptor subunit reside, individual residues along the amino acid sequence were mutated via site directed mutagenesis. Viable mutant receptors were then assessed through biophysical techniques *in vitro* to determine how the mutated residue affected receptor dynamics. Point mutations to the TM2 of the α1 nAChR decreased binding of QX-122, an open-pore blocker, highlighting the role of these particular residues in forming the nAChR pore (Leonard et al., 1988). Another study mutated the 9′ leucine in TM2 of the α7 nAChR subunit to a serine and observed decreased receptor desensitization rates, increased sensitivity to agonist and the lack of a rectifying current (Revah et al., 1991). Subsequent 9′ leucine to serine mutation (Leu9′Ser) in muscle and α4* nAChRs conferred nAChRs with similar characteristics (Labarca et al., 1995, 2001) (Figure I-2A). Together, these studies helped identify key regions of the nAChR important for receptor function. The 9′ leucine is highly conserved amongst nAChR subunits

(Lester 1992; Betz 1990) and is thought to form the gate of the ion pore (Figure I-2 B). Mutating the 9´ leucine increases the permeability of the channel, therefore allowing ions to move along their electrochemical gradient while in the high affinity desensitized state (Revah et al., 1991). This confers nAChRs with the Leu9´Ser mutation hypersensitive to agonist. These data paved the way for the development of a novel method to study specific nAChR receptor function *in vivo* through genetic mouse models (Labarca et al., 2001; Tapper et al., 2004; Fonck et al., 2005).

I.C. Mesocorticolimbic Dopaminergic Pathway

It has been well established that the mesocorticolimbic DAergic pathway is a major component in goal-oriented behavior (Wise, 2004). Composed of the ventral tegmental area, the nucleus accumbens (NAc) and the prefrontal cortex (PFC), this pathway critical in the development of nicotine dependence (D'Souza and Markou, 2011) (Figure I-3). Multiple drugs of abuse, including nicotine, manipulate the activity of the mesocorticolimbic DAergic pathway through the ventral tegmental area to increase DA concentration in the NAc, which is considered a hallmark of brain reward (Di Chiara and Imperato, 1988).

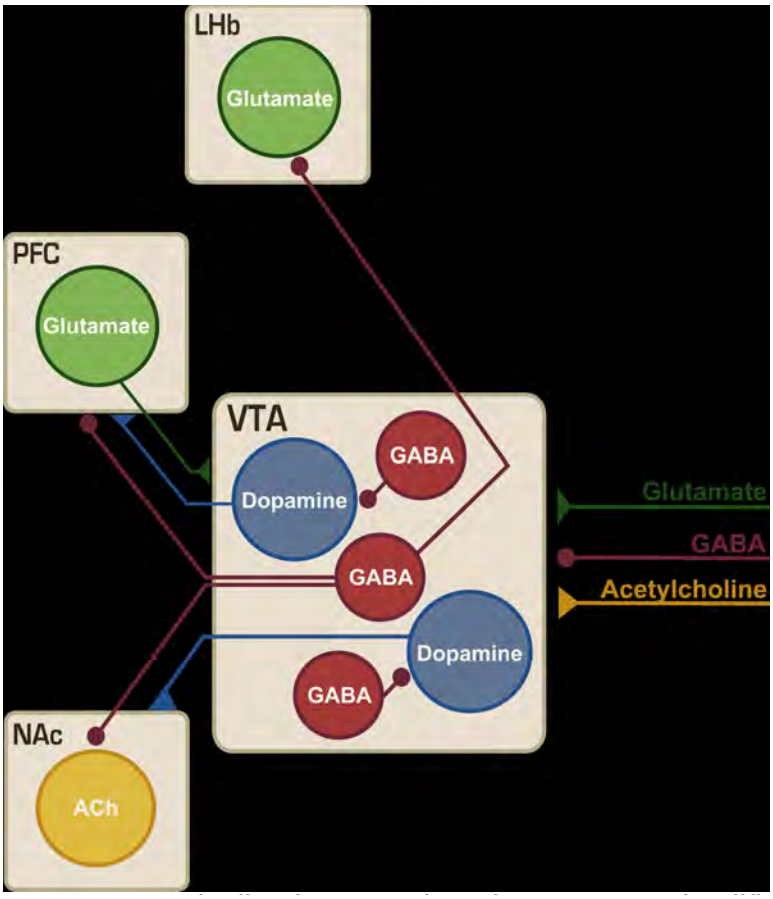

Figure I-3. The mesocorticolimbic dopaminergic pathway. Simplified schematic of the Ventral Tegmental Area (VTA) microcircuitry, with emphasis on the neuronal subpopulations within the area. The VTA receives glutamatergic, GABAergic and cholinergic (ACh) inputs from multiple brain regions. These inputs can innervate the dopaminergic (DA) and GABAergic subpopulations of the VTA. The DAergic neurons project to the nucleus accumbens (NAc), forming the mesolimbic DAergic pathway, or the prefrontal cortex (PFC), forming the mesocortical DAergic pathway. Glutamatergic projections from the PFC have been found to influence VTA activity. The GABAergic portion of the VTA is comprised of interneurons that influence DAergic activity or projection neurons that act upon the NAc, PFC and lateral habenula (LHb).

The Ventral Tegmental Area

The Ventral Tegmental Area (VTA) is a heterogeneous population of neurons located in the ventral midbrain (Roeper, 2013; Pistillo et al., 2015). This dense

group of cells is composed of dopamine (DA) neurons as well as neurons that release Y-aminobutyric acid (GABA) (Dahlstroem and Fuxe, 1964; Olson and Nestler, 2007; Nair-Roberts et al., 2008; Yamaguchi et al., 2011) (Figure I-3 A). It is becoming increasingly apparent that different groups of DAergic neurons are segregated according to location (Lammel et al., 2012, 2015) while there is no consistent subpopulation organization in GABAergic neurons (Chieng et al., 2011). Recent studies identified a subset of VTA neurons, including DAergic neurons, express vesicular glutamatergic transmitter 2 (VGLUT2) and therefore can release the major excitatory neurotransmitter glutamate (Yamaguchi et al., 2007, 2011, 2015; Hnasko et al., 2010, 2012). Studies are ongoing to discern how VTA glutamatergic neurons modulate motivated behavior (Yoo et al., 2015). Distinct nuclei reside within the VTA, including the paranigral nucleus, the parabrachial pigmented nucleus and the interfasicular nucleus (Ikemoto, 2007). Furthermore, the VTA can be roughly sectioned into three subregions along the coronal plane- the anterior, posterior and tail (or rostromedial tegmental nucleus-RMTg) (Perrotti et al., 2005; Ikemoto, 2007; Jhou et al., 2009, Sanchez-Catalan, 2014), with distinct nuclei as well as the set of afferents and efferents distinguishing each VTA subregion (Pistillo et al., 2015).

VTA Dopaminergic Neurons

Dopaminergic neurons comprise about 60-65% of the VTA neuronal population (Yetnikoff et al., 2014; Pistillo et al., 2015). Neurons in this subpopulation are identified through a distinct set of electrophysiological characteristics, such as

currents that contain a voltage sag, or an I_h current and spontaneous tonic pace-maker firing activity as well as tyrosine hydroxylase (TH) immunoreactivity (Grace and Onn, 1989; Lacey et al., 1989). Yet recent studies indicate that DAergic neurons in the VTA are not homogeneous (Margolis et al., 2008; Ford et al., 2006; Lammel et al., 2012, 2014). The diversity of DAergic neurons provides a better framework for understanding how the VTA can be activated by rewarding, aversive and neutral stimuli (Schultz, 2010).

Classic electrostimulation studies in rats have indicated the midbrain, particularly the DAergic neuron rich area, as an important region in reward and reinforcement behavior (Olds and Milner, 1955; Crow, 1971). Complimenting these findings, studies in monkeys established that stimuli preceding reward activate midbrain DAergic neurons, specifically the A10 DAergic neurons (Schultz, 1986; Schultz et al., 1993). Furthermore, self-administration studies have pinpointed the posterior VTA (pVTA), but not the aVTA, is important for reward signaling, for infusing drug into the pVTA increased reward behavior (Zangen et al., 2000; Rodd et al., 2005; Ikemoto et al., 2006). Presentation of a reward stimulus caused DAergic neurons to exhibit bursts of activity, a classic pattern of activity often accompanied by dopamine release (Schultz, 1986; Schultz et al., 1993; Wightman and Robinson, 2002; Tsai et al., 2009). DAergic neurons in the VTA project to other brain regions such as the NAc and PFC (Laviolette and van der Kooy et al., 2004; Ikemoto et al., 2007) (Figure I-2 B). Increased dopamine concentration in the NAc occurs in response to naturally occurring reward, VTA self-stimulation and drugs of abuse (Di Chiara et al.,

1988; Hernandez et al., 1988; Damsma et al., 1992; Pfaus et al., 1993; Fiorino et al., 1993). Stimulating of VTA DAergic neurons at a frequency that mimics burst firing leads to DA release and conditioned place preference, further bolstering the argument that VTA DAergic neurons transduce reward (Tsai et al., 2009). These data support the hypothesis that inducing burst-firing activity in the DAergic neurons of the VTA results in an increase of dopamine and reward signaling.

VTA GABAergic neurons

Neurons that release the neurotransmitter GABA, the major inhibitory neurotransmitter in the central nervous system make up about 20-35% of VTA (Chieng et al., 2011; Nair-Roberts et al., 2008; Yamaguchi et al., 2007). GABAergic neurons in the VTA have distinct biophysical characteristics that set them apart from DAergic neurons, such as fast action potential duration compared to DAergic neurons and do not have a slow depolarizing current seen in VTA DAergic neurons (Lacey et al., 1989, Johnson and North). Recent studies assessing the electrophysiological characteristics of VTA GABAergic neurons confirmed these criteria held true for the majority of GABAergic neurons with a minority of GABAergic neurons exhibiting an I_h current (Chieng et al., 2011). The authors rationalize the electrophysiological differences may be due to location within the VTA, similar to what has been observed in VTA DAergic neurons (Lammel et al., 2014). The VTA receives cholinergic innervation primarily from the LDTg, with 65% of the cholinergic terminals forming synapses onto non-DAergic neurons (Oakman et al., 1995; Garzon et al., 1999). Therefore,

cholinergic innervation of GABAergic neurons plays a role in behaviors mediated by the LDTg. About 25% VTA GABAergic neurons are projection neurons targetting other brain regions, such as the NAc, the PFC and the lateral habenula (LHb) (Carr and Sesack, 2000; Taylor et al., 2014) (Figure I-3 C).

Much of what is known about VTA GABAergic neurons in the mesocorticolimbic DAergic pathway comes from experiments using either various GABAergic signaling blockers or opioid receptor antagonists. Opioids specifically reduce activation of non-DAergic neurons *in vivo* (Johnson and North, 1992). These and other studies lead to the 'disinhibition' hypothesis: that is, when GABAergic neurons are inactivated to disinhibit DAergic neurons and promote reward signaling (Johnson and North, 1992; Mansvelder et al., 2002; Creed et al., 2014). Recent technological advances have allowed for the specific activation of GABAergic neurons located within the VTA to assess their role in behavior. Optogenetic activation of VTA GABAergic neurons disrupts reward behavior (van Zessen et al., 2012) elicits aversion (Tan et al., 2012) and modulates acquisition of reward (Brown et al., 2014). Inhibiting VTA GABAergic neurons through halorhodopsin resulted in the disinhibition of DA neurons (Bocklisch et al., 2013). Furthermore, GABAergic neurons exhibit an intense spike in activity immediately after punishment, which coincided with an immediate decrease in VTA DAergic firing frequency (Cohen et al., 2012). Overall, these data highlight a regulatory role for GABAergic neurons in the VTA in modulating DAergic transmission.

Afferents to the VTA

The VTA receives glutamatergic, cholinergic and GABAergic input that regulates DAergic neurons and ultimately modulate motivated behaviors (Fig I-2) (Paolini et al. 2011; Li et al., 2014; Pistillio et al., 2015). Glutamate into the VTA can increase DAergic neuronal activity; initiate DAergic burst firing *in vivo* and increase DA concentrations in the NAc (Geisler et al., 2007; van Huijstee et al., 2015). Innervation by glutamatergic projections from the PFC, the Laterodorsal Tegmentum (LDTg) and LHb activates VTA DA neurons (Murase et al., 1993; Lodge and Grace 2006; Lammel et al., 2012). Additionally, the infusion of glutamate agonists into the VTA elevated DAergic firing frequency and incites DA burst activity (Johnson et al., 1992b; Chergui et al., 1993). Drugs of abuse including nicotine increase glutamatergic receptors, particularly the ionotropic AMPA receptors. As a result, the AMPA/NMDA ratio, a measure of synaptic strength, was increased in DAergic neurons 24 hours after drug administration (Saal et al., 2003). However, inactivation of the LDTg reduced DA burst firing even with the infusion of additional glutamate, suggesting other neurotransmitters from the LDTg such as acetylcholine contribute to DAergic burst activity (Lodge and Grace, 2006). The majority of VTA cholinergic projections originate in the LDTg (Cornwall et al., 1990; Oakman et al., 1995; Maskos, 2008). Infusions of acetylcholine receptor antagonists into the VTA lessened NAc DA concentrations during LDTg electrostimulation, implying cholinergic transmission from the LDTg shapes VTA DAergic neurons (Forster and Blaha, 2000). Delivering cholinergic antagonists into the VTA also decreases reward signaling (Corrigal et al., 1998;

Shinohara et al., 2015) and precipitated withdrawal, heavily implicating the importance of cholinergic transmission in affective behaviors. The VTA also receives GABAergic innervation from the RMTg and the LDT (Perotti et al., 2005, Jhou et al., 2005). RMTg neurons are activated by aversive stimuli, such as foot shock and food deprivation (Jhou et al., 2009). Furthermore, RMTg GABAergic neurons synapse onto DAergic neurons, suggesting that RMTg neuronal activation decreases DAergic activation (Jhou et al., 2009). It is interesting to note that psychostimulants activate the RMTg as determined by Fos immunoreactivity (Perrotti et al., 2005), for it suggests inhibitory input into the VTA contributes to the rewarding effects of these drugs. Overall, afferents to the VTA can influence the VTA microcircuitry and drive DAergic activity to induce reward, while various drugs of abuse can alter neurotransmission to promote reward.

Neuroadaptations within the VTA caused by chronic nicotine

Chronic nicotine use instigates brain-wide neuroadaptations thought to drive the persistent consumption of nicotine (Koob and Volkow et al., 2008; D'Souza et al., 2011; Jackson et al., 2012). Changes in receptor expression and neurotransmitter release within the VTA instigated by chronic nicotine use result in modified VTA DA transmission. NAc DA concentrations were significantly lower in nicotine-withdrawn rat brain slices compared to control (Zhang et al., 2012). Decreased NAc DA concentration due to hypofunctional DAergic neurons resulted in an anhedonic state that could factor into increased drug seeking

(Epping-Jordan et al., 1998; Tye et al., 2012). Furthermore, an acute nicotine exposure induced a significant increase of DA released into the NAc of withdrawn rats compared to control (Zhang et al., 2012). Chronic nicotine leads to increased expression of specific nAChR subunits in VTA DAergic neurons as well as glutamatergic receptors in the VTA and the NAc (Nashmi et al., 2007; Kenny et al., 2009; Baker et al., 2012; Liechti et al., 2007a,b). The overall result of the molecular and cellular changes brought forth by chronic nicotine exposure could induce a rapid influx of DA in a DA-deprived NAc that results in an intense rewarding signal that strongly drives relapse. Precipitating nicotine withdrawal increases expression of Corticotrophin Release Factor (CRF), a neurotransmitter involved in stress signaling, within the central amygdala and the VTA (George et al., 2007; Grieder et al., 2014). It is hypothesized that CRF acts upon the IPN to elicit anxiety (Zhao-Shea et al., 2014), which would explain the increased negative affective symptoms experienced during nicotine withdrawal.

nAChRs and the VTA

Various drugs of abuse act upon presynaptic and post-synaptic components of the mesocorticolimbic pathway to increase dopamine concentrations (Luscher and Malenka 2011). Nicotine acts upon the VTA to ultimately induce DAergic release into the NAc (Di Chiara and Imperato, 1988). Expression of $\beta 2$ and $\alpha 4$ expression is necessary for nicotine-mediated DAergic activation (Picciotto et al., 1998; Exley et al., 2011) and nicotine reward (Maskos et al., 2005; Tapper et al., 2007; Pons et al., 2008; McGranahan et al., 2011). However, various nAChR

subtypes are expressed on both the DAergic neurons and inhibitory GABAergic neurons within the VTA (Klink et al., 2001) (Figure I-4). Therefore, the mechanism by which nicotine activates the VTA to ultimately lead to increased DA in the NAc and promote reward is not clear.

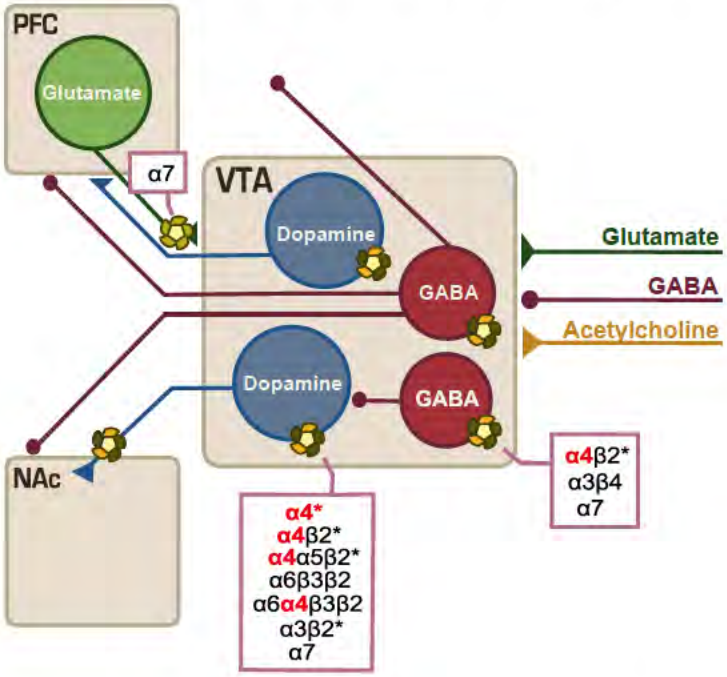

Figure I-4. Expression pattern of nAChR subunits within the VTA. Different subunits are expressed within the VTA DAergic and GABAergic subpopulations. nAChRs can be found on the soma and the terminals of a neuron. Furthermore, projections from other regions, such as the PFC, express nAChRs. (Adapted from Hendrickson et al., 2013; Yang et al., 2011).

While the effect of nicotine on specific neuronal subpopulations in the VTA is not clear, how nicotine modulates VTA DAergic activity through nAChRs is well characterized. Directly infusing nicotine and nicotinic receptor antagonists into the VTA demonstrates the necessity of the VTA in the rewarding properties of nicotine (Imperato and Di Chiara, 1988; Corrigall and Coen, 1994). Administering dopaminergic antagonists prior to nicotine exposure abolishes both nicotine-induced dopamine release and nicotine self-administration, indicating

nicotine reward is the result of manipulation of dopaminergic neuronal activity (Laviolette and van der Kooy, 2004). Furthermore, studies in mice that lack the β2 nAChR subunit indicated nicotine acts upon β2* nAChRs on DAergic neurons to initiate burst firing activity and induce dopamine release (Picciotto et al., 1998a; Mameli-Engvall et al., 2006). Mice lacking the α4 nAChR exclusively in dopaminergic neurons failed to exhibit nicotine place preference but retained preference conditioned by cocaine (McGranahan et al., 2011). Furthermore, re-expression studies in mice lacking α4 nAChR demonstrated the α4 nAChR subunit is also necessary for burst firing activity (Exley et al., 2011). Nicotine activates DAergic neurons in the posterior VTA, specifically through α4* nAChRs, to induce reward (Ikemoto et al., 2006; Zhao-Shea et al., 2011). Ethanol also leads to the activation of DAergic neurons through α4* nAChRs on pVTA DAergic neurons (Hendrickson et al., 2010). Nicotinic receptors containing the α6 subunit on DAergic neurons remain activated by nicotine (Liu et al., 2012), while chronic nicotine leads to the upregulation of α6* nAChR in VTA DAergic neurons (Baker et al., 2013). However, studies in mice that express hypersensitive α6 nAChR subunits but lack α4 nAChR subunits indicate that α4 nAChRs are required for nicotine-mediated behaviors (Drenan et al., 2010). In summary, it is hypothesized that nicotine acts upon VTA DAergic neurons through the α4α6β2* nAChR subtype to initiate its psychotropic effects (Figure I-4).

Few studies have been done to examine how nAChRs affect VTA GABAergic activity. In contrast with the VTA DAergic neurons, VTA GABAergic neurons express a discrete number of nAChRs subtypes: α3, α4, β2, α7 and possibly α6,

though this may be restricted to interneurons (Charpantier et al., 1998; Klink et al., 2001; Yang et al., 2011) (Figure I-4). Electrophysiological studies have indicated that $\alpha4\beta2$* nAChRs on GABAergic neurons of the VTA are desensitized with chronic nicotine exposure (Mansvelder and McGehee, 2002), which falls in line with the accepted 'disinhibition' hypothesis. A recent study that rescued $\beta2$ nAChR subunit expression in either DAergic or GABAergic VTA neurons suggests $\beta2$* nAChRs expression in VTA DAergic and GABAergic neurons shapes the burst-firing activity often observed in reward signaling (Tolu et al., 2013), indicating that VTA GABAergic neurons may modulate nicotine reward signaling rather than solely restricting DAergic activity.

I.D. Rodent models used to study nAChR subunits in the VTA

Our understanding of the neurobiological contributions of nAChR subunits greatly benefited from rodents experiments implementing pharmacological reagents, genetic-engineered mice and viral-delivered strategies (Table I-1). Studies discussed in this section will focus on nAChRs found within the VTA (Fig I-4), with particular emphasis on the $\alpha4$ and $\beta2$ subunits, due to multiple studies indicating the importance of these subunits to the reinforcing and rewarding properties of nicotine.

Pharmacology

Pharmacological reagents have been used to assess nAChRs function on behavior. Mecamylamine (Inversine), a non-selective and non-competitive

nAChR antagonist (Takayama et al., 1989), has been used for more than twenty years to alter nAChR activity. First prescribed as an antihypertensive, mecamylamine readily crosses the blood brain barrier to allosterically inhibit nAChR activation. Administration of mecamylamine either through subcutaneous injection or infusion into the VTA reduced nicotine self-administration (Nisell et al., 1994; Watkins et al., 1999) and diminished cue instated nicotine reward (Liu et al., 2007, 2010), confirming that nAChR activation is necessary for the rewarding and reinforcing properties of nicotine. Mecamylamine precipitated withdrawal symptoms in chronically nicotine treated animals (Hildebrand et al., 1999; Watkins et al., 2000), further implicating deactivation of nAChRs contributes to nicotine withdrawal syndrome. Because different nAChR subtypes can have varying reactions to nicotine, it is important to take into account nAChR composition. Dihydro-beta-erythrodine (DHβE) is a nAChR direct antagonist that is selective to α4* and α3β4* nAChRs (Damaj et al., 1995; Harvey et al., 1996). Administering DHβE either systemically or intracranially prior to nicotine self-administration diminishes nicotine's reinforcing effects (Corrigall et al., 1994; Watkins et al., 1999), further narrowing down the potential nAChR subtypes important for nicotine reward and reinforcement. Receptor function can be inferred indirectly using drugs that inactivate certain nAChR subtypes. Varenicline (Chantix), a partial agonist for α4β2* nAChRs as well as a full agonist for α7 nAChRs (Coe et al., 2005; Mihalak et al., 2006) reduced nicotine self-administration in rats (Rollema et al., 2007) and alcohol reward in mice (Hendrickson et al., 2010).

Knock out	Behavioral Test(s)	Phenotype	References
α2	Mecamylamine-preciptated nicotine somatic withdrawal	Decreased somatic withdrawal symptoms.	Salas et al. 2009
α3	N/A – Lethal at 6-8 weeks postnatal	Autonomic nervous system defects.	Xu et al., 1997
α4	Rotorod, EPM, acute nicotine exposure, Yoked IVSA	Increased rotorod performance. Increased anxiety. Decreased nicotine-induced nociception. Decreased nicotine IVSA.	Marubio et al., 1999; Ross et al., 2000; Pons et al. 2008
α5	Locomotor activity, Nicotine-induced Seizures Mecamylamine-preciptated nicotine somatic withdrawal	Decreased somatic withdrawal symptoms. Protection against nicotine-induced hypolocomotion and seizures.	Salas et al., 2003, 2009
α6	Yoked IVSA	Decreased nicotine IVSA.	Champtiaux et al., 2002; Pons et al., 2008
α7	Yoked IVSA, MLA precipitated nicotine withdrawal	No change in Nicotine IVSA. MLA-precipitated withdrawal.	Orr-Urtreger et al., 1997; Pons et al., 2008; Salas et al. 2007
α9	Cochlear cell innervation	Deceased suppression of cochlear activation.	Vetter et al., 1999
α10	Cochlear cell innervation	Decreased suppression of cochlear activation.	Vetter et al., 2007
β2	Yoked IVSA, Open Field, Passive Avoidance, Mecamylamine-precipitated withdrawal	Decreased nicotine IVSA. Decreased locomotor activity in familiar environments. Increased Passive Avoidance performance. No effect on mecamylamine-precipitated withdrawal.	Picciotto et al., 1995 1998 Xu et al., 1999; Salas et al., 2004; Pons et al., 2008
β3	Open Field PPI EPM Light/Dark Box, Mirror chamber	Increased locomotor activity. Increased startle response. Decreased anxiety.	Cui et al. 2003; Booker et al., 2007
β4	Mecamylamine-precipitated withdrawal	Decreased nicotine-induced hyperplalgesia. Decreased somatic withdrawal symptoms	Xu et al., 1999; Salas et al., 2004
β2β4	N/A – Lethal at 1-3 weeks postnatal	Autonomic nervous system defects: Enlarged bladder.	Xu et al., 1999

α5β4	Nicotine-induced seizures	Decreased latency to seizure.	Kedmi et al., 2004

Hypersensitive nAChR	Mutation	Location of Mutation	Method	Behavioral Test(s)	Effect of Hypersensitive nAChR	References
α4	L9 A* (L250A)^	TM2 9	Homologous recombination	Locomotor activity, CPP	Increased nicotine sensitivity. Increased Place Preference to nicotine and alcohol.	Tapper et al 2004, 2007; Hendrickson 2009;
α4	L9 S (L250S)	TM2 9	Homologous recombination	Locomotor Activity, Elevated Plus Maze, Mirror Chamber, Nicotine-induced Seizures, Pain	Lethality in homozygous mice. Impaired motor learning. Increased anxiety straub tail and seizures.	Labarca et al., 1995, 2001; Orb et al., 2004; Fonck et al., 2005
α4	S6 F (S247F)	TM2 6	Homologus recombination	Self-administration, CPP	Distonic arousal complex. Resistant to mecamylamine.	Teper et al., 2007; Cahir et al., 2011; Madsen et al., 2014
α4	T529A	Cytoplasmic loop	Homologous recombination	Body Temperature, Locomotor Activity, CPP	Greater change in body temp. CPP with low dose of nicotine but not ethanol.	Wilking et al., 2010; Butt et al., 2005
α6	L9 S (L250S)	TM2 9	BAC	Locomotor activity	Increased basal locomotor activity compared to wildtype littermates.	Drenan et al., 2008;
α7	L9 T (L250T)	TM2 9	Homologous recombination	Open Field, Light/Dark Box Rotarod Test, Prepulse inhibition, Acoustic Startle Response, Conditioned Fear Response, Morris Water Maze	Lethality in lomozygous mice. "Head bobbing". Increased sensitivity to nicotine-induced seizures.	Orr-Urtreger et al., 2000; Broide et al., 2001
α9	L9 T (L250T)	TM2 9	Homologous recombination	Acoustic overexposure	Protection from hearing loss due to intense sounds.	Taranda et al., 2009

Re-Expression

Mouse Line	Brain region	nAChR Re-Expressed	Viral Vector/ Expression Construct	Behavioral Test(s)	Effect of Re-Expression	References
$\beta2^{-/-}$	VTA	β2	Lentivirus/ PGK-Beta2-IRES-eGFP	Open Field, ICSA	Restored VTA DA firing activity and DA release.	Maskos et al., 2008

					Rescued rate of ICSA and exploratory behavior	
$\alpha4^{-/-}$, $\alpha6^{-/-}$, $\alpha7^{-/-}$, $\beta2^{-/-}$	VTA, SN	α4, α6, α7, β2	Lentivirus/ PGK-nAChR-IRES-eGFP	Yoked IVSA	Restored IVSA.	Pons et al., 2008
$\alpha4^{-/-}$,$\alpha6^{-/-}$	VTA	α4	Lentivirus/ PGK-alpha4- RES-eGFP	ICSA	Restored Nicotine-activated DAergic spike bursting. Rescued short term ICSA.	Exley et al., 2011
$\alpha5^{-/-}$	MHb	α5	Lentivirus/ CMV-CHRNA5-EF1a-GFP	IVSA	Reduced nicotine consumption at high doses.	Fowler et al., 2011
$\beta2^{-/-}$ x DAT-Cre/ GAD-Cre	VTA	β2	Lentivirus/ PDGFβ-floxed mCherry-β2/GFP	ICSA, Y-maze	Re-expression in DA neurons increased ICSA. Re-expression in GABA neurons decreased ICSA. Nicotine-induced DAergic spike bursting rescued when β2 was rescued in both VTA subpopulations	Tolu et al., 2012
$\alpha5^{-/-}$ and $\alpha5^{-/-}$ x DAT-Cre	VTA	α5, α5 D398N	Lentivirus/ PGK-alpha5/alpha5397N-IRES-eGFP	Yoked IVSA	α5 D398N re-expression increased nicotine consumption at higher doses	Morel et al., 2013

Table I-1. Genetic manipulations in mice to study mammalian nAChR subunits
* Pertaining to residues of TM2 (As defined by Charnet et al., 1990)
^ Relative to the cysteine doublet location on Torpedo AChR (As defined by Kao et al., 1984)
BAC= Bacterial Artificial Chromosome; CPP= Conditioned Place Preference; EPM = Elevated Plus Maze, ICSA = Intracranial Self-Administration; IVSA = Intravenous Self-Administration; MLA= Methyllcaconitine; PPI = Prepulsed Inhibition; TM2 = Transmembrane domain 2
[Adapted from Cordero-Erasquin et al., 2000; Champtiaux and Changeux, 2004; Zhang, 2006; Drenan and Lester, 2012]

Out of the three drugs mentioned, varenicline is the only FDA approved nAChR modulator approved for smoking cessation, having shown modest effects in early smoking cessation (Gonzalez et al., 2006; Jorenby et al., 2006). Together, these studies point to the possibility that nAChRs containing the α4 subunit are

important for nicotine dependence behaviors. However, because these drugs target other nAChR subunits that are expressed within the VTA, these results leave open the possibility that other receptor subtypes can also contribute.

Genetically Engineered Mouse Strains

The advent of rodent genetic engineering brought forth a whole new avenue for studying nAChR subtypes through genome modification. Because the diversity of nAChR subtypes expressed throughout the brain and the lack of reagents that target a discrete nAChR subtype, the generation of animals lacking a particular nAChR subunit, or nAChR knock-out (KO) mice, provided a novel way to determine the physiological function of each nAChR subunit (Cordero-Erausquin et al., 2000; Zhang et al., 2006). The nAChR KO mouse lines provide a way to access the physiological necessity of a nAChR subunit both in development and in behavior. A nAChR mouse KO line exists for each mammalian nAChR subunit, with the addition of several double KO mouse lines (Table I-1). Deletion of a subunit yielded obvious developmental phenotypes, as α3 nAChR KO mice failed to live pass 7 days and exhibited gross autonomic nervous system abnormalities, such as dialated pupils, extended bladders and poor bladder control (Xu et al., 1999a). Interestingly, mice lacking both the β2 and β4 subunit were non-viable and exhibited a similar fate to the α3 KO mouse line (Xu et al., 1999b), hinting at compensatory mechanism between beta nAChR subunits, as the single nAChR KO mice are viable (Picciotto et al., 1998a; Xu et al., 1999b). Development of KO mice also identified the function of the subunit within a

system, such as the α9 and α10 nAChR subunits (Vetter et al., 1999; 2007)(Table I-1). Other nAChR KO mice were viable, providing a stable system to further determine the role of the subunit in basic behavior and with nicotine exposure (Cordero-Erausquin et al., 2000). Mice from the α7 KO line still consumed nicotine, which suggests that α7 nAChRs are not necessary for nicotine reward (Pons et al., 2008). Mice lacking the β2 nAChR self-administered nicotine significantly less than control mice and midbrain dopaminergic neurons in these mice failed to respond to nicotine (Picciotto et al., 1998). This study confirmed the importance of the β2 nAChR subunit in the rewarding and reinforcing effects of nicotine. The β2 nAChR subunit is expressed in 90% of the brain and is commonly co-expressed with the α4 nAChR subunit (Perry et al., 2002; Gotti et al., 2009). Other studies into nAChR KO mice have implicated the necessity of the α4 and the α6 nAChR subunits in nicotine-mediated dopamine transmission (Marubio et al., 2003; Liu et al., 2012), while α7 nAChR KO did not exhibit rapidly desensitizing nicotine currents (Orr-Urterger et al., 1997). Mouse strains lacking the β2, α4 and α6 nAChR subunit fail to self-administer nicotine, further indicating the role of these receptors in mediating nicotine reward (Maskos et al., 2005; Pons et al., 2008; Exley et al., 2011). Together, nAChR KO mice provided much insight into the necessity of each nAChR subunit in regards to overall development and physiological function. However, developmental issues due to the lack of a particular nAChR subunit may result in differences in adult behavior that do not reflect physiological conditions (Picciotto, 1998b; Muller, 1999; Changeux and Champtiaux 2004).

Furthermore, KO models do not assess if a nAChR is sufficient to drive a particular behavior.

To address the question of sufficiency, genetically engineered mice expressing nAChR subunits harboring a point mutation at the 9´ leucine of the TM2 domain were generated. As previously described, this mutation conferred “gain-of-function” nAChRs *in vitro* (Revah et al., 1991; Labarca et al., 1995). Hypersensitive nAChR mice provided a novel method to preferrentially activate nAChRs containing the mutated nAChR subunit and observe the effects on firing frequency, neurotransmitter release and ultimately behavior. Currently, mice expressing “gain-of-function” α4, α6, α7 or α9 nAChR subunits are available (Drenan and Lester, 2012) (Table I-1). The majority of these mice were generated through homologous recombination (Orr-Urtreger et al., 2000; Labarca et al., 2001; Tapper et al., 2004; Taranda et al., 2009), with the exception of the α6 Leu9´Ser mouse line that utilized a bacterial artificial chromosome to express α6 Leu9´Ser nAChR subunits (Drenan et al., 2008). Selectively activating α4* nAChRs using mice expressing α4* nAChRs containing a leucine to alanine point mutation at the 9´ residue at TM2 (Leu9´Ala) conferred Conditioned Place Preference with a sub-threshold dose of nicotine (Tapper et al., 2004), confirming that α4* nAChRs are sufficient to elicit nicotine reward. However, one study utilizing a mouse line harboring an α4 nAChR mutation found in patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) suggested the α4 nAChR is critical in the hypolocomotor properties of nicotine, but only plays a modulatory role in nicotine reinforcement (Cahir et al., 2011). Other

knock-in mouse line harboring a leucine to serine 9´ mutation on the α4 nAChR TM2 (Leu9´Ser) had elevated anxiety-like behavior and altered hippocampal theta rhythms (Fonck et al., 2003). This suggests that α4* nAChR may also be involved in nicotine withdrawal syndrome. Mice expressing α6 Leu9´Ser nAChR mice have also been bred to α4 KO mice to determine the sufficiency of α6* nAChR receptors in the absence of the α4 nAChR subunit. This study showed that α4 nAChR expression is necessary for the behavioral effects observed in α6 Leu9´Ser nAChR mice (Drennan et al., 2010). Despite the power of the technique, expression of hypersensitive mutant nAChR subunits also resulted in heightened mortality in homozygous mice expressing either mutant α4 or α7 nAChR subunits (Orr-Urtreger et al., 2000; Labarca et al., 2001). *In vitro* studies have observed differences in receptor dynamics in the mutant subunits, such as the slowing of desensitization rates (Revah et al., 1995) and increasing of receptor opening time (Labarca et al., 1999). How this is translated to neuronal activity in *in vivo* models has not been thoroughly investigated. Overall, mice expressing hypersensitive nAChRs provide a “gain-of-function” assessment of nAChR subunits that compliments the “loss-of-function” studies, by providing context into the sufficiency of the nAChR subunit in nicotine-mediated behaviors.

Although KO mice address the issue of necessity and the hypersensitive mice addresses the issues of sufficiency, nAChRs are expressed in various brain regions (Gotti and Clementi, 2004; Changeux 2010; Pistillo et al., 2014). Neither KO mice nor hypersensitive nAChR mice provide a way to assess how a particular nAChR receptor affects a specific brain region. To address this

question, lentiviral-mediated nAChR expression systems were developed to rescue nAChR subunit expression in specific brain areas of KO mice. Results of these studies further highlighted the importance of nAChR expression within the region and the sufficiency of the region to mediate behavior. The first of these experiments rescued the expression of β2 nAChR subunits in the VTA of β2 KO mice, which resulted in the reinstatement of nicotine self-administration (Maskos et al., 2005). Re-expressing α4 and α6 nAChRs specifically within the VTA and β2 nAChRs within the VTA and substantia nigra led to similar results (Pons et al., 2008). Further studies of selective re-expression of α4* nAChRs in the VTA rescued intracranial self-administration and restored nicotine-induced burst firing activity in VTA DAergic neurons (Exley et al., 2011). Together, these data further implicate the necessity of high-affinity nAChRs, particularly α4* nAChRs within the VTA as important for nicotine consumption. However, viral particles can infect multiple cellular populations within the brain, including nearby astrocytes. Therefore, it is difficult to conclude the re-expression of the nAChR subunit specifically addresses DAergic neuronal activity when astrocytes, DAergic and GABAergic neurons are all present. Furthermore, the lack of restricting expression to a specific cell population can lead to the formation of non-physiological nAChRs. To understand the contributions of high-affinity nicotinic receptors within the subpopulations of the VTA, Tolu and colleagues (2102) utilized a Cre-restricted lentiviral expression system to selectively re-express β2 nAChRs in DAT-expressing or GAD-expressing neurons. These studies found that expression of high-affinity nicotinic receptor are necessary on both DAergic

and GABAergic neurons to shape the burst firing of DAergic neurons (Tolu et al., 2012), providing insight into how high-affinity nAChRs on GABAergic neurons affect DAergic activity. Furthermore, re-expressing α5 nAChR subunits containing a mutation commonly associated with smokers in the VTA resulted in increased consumption and decreased activation of VTA DAergic neurons by nicotine (Morel et al., 2013). Decreased DAergic neuron activation was observed to promote drug seeking, which can explain sustained nicotine use (Epping-Jordan et al., 1999). Lentiviral-mediated gene expression provides precision in determining the necessity of nAChR subunit expression within a particular brain region, but is dependent on KO mice. Therefore, developmental issues from compensatory mechanisms potentially confound the results. The lentiviral vector integrates the gene of interest randomly into the genome, which increases the susceptibility of the target gene to various genome silencing mechanisms. Furthermore, high protein expression is achieved by a high concentration of viral particles; therefore, high viral titers are necessary for sufficient expression. An alternative viral vector recently gaining favor is the adeno associate virus (AAV). Unlike lentiviruses, AAV genomes form an episome upon entry into the cell instead of integrating into the host genome, therefore bypassing gene silencing through methylation and unwanted complications due to insertional mutagenesis (Davidson et al., 2011). Furthermore, AAV is considered Risk Group 1 agent and can be use without additional accomodations or special facilities. High AAV viral titers are also easier to achieve compared to lentivirus, further increasing the chances for robust gene expression. AAV has been used to reconstitute gene

expression in rodent models of neurological disorders, such as Canavan's, Parkinson's and Alzheimer'sDisease (McPhee et al., 2004; Cederfjäll et al., 2012; Fol et al., 2015). In addition, the viral vector has been used to deliver genes in a Cre-dependent manner without any complications (Atasoy et al., 2008). Regardless, these previous studies further reinforce the idea that α4* nAChRs are crucial for nicotine reward.

It is unclear how nAChR upregulation contributes to the development of nicotine dependence. Several studies assessed how withdrawing from nicotine affected the reinforcing responses of the drug. Mice chronically treated with nicotine exhibited a stronger place preference when repeatedly withdrawn from nicotine, and repeated exposures of nicotine led to the upregulation of high-affinity nAChRs (Hilario et al., 2012). Whether these upregulated nAChRs play an active role in initiating these behaviors is unknown. Upregulation of nicotinic receptors, in particular high affinity nAChRs, occurs in distinct parts of the brain (Marks et al., 1983; Nashmi et al., 2007; Muhkin et al., 2008; Hilario et al., 2012). Chronic nicotine exposure results in the upregulation of functional α4* nAChRs in VTA GABAergic neurons (Nashmi et al., 2007, Renda and Nashmi, 2014). A recent study observed that α4* nAChRs were upregulated with chronic nicotine treatment, which was correlated with increased consumption of nicotine-laced water (Renda and Nashmi, 2014). This is in contrast with optogenetic studies indicating VTA GABAergic activation elicits aversion (Tan et al., 2012; Van Zessen et al., 2012; Cohen et al., 2012). However, unlike optogenetic stimulation, nAChR activation is thought to be more modulatory than stimulatory

(Picciotto, 2003). Furthermore, high-affinity nicotinic receptors on both VTA dopaminergic and GABAergic neurons are necessary to shape the burst firing activity of DAergic neurons associated with reward signaling (Tolu et al., 2013), indicating that nAChR activation on VTA GABAergic neurons does not necessarily elicit aversion. Interestingly, ex-smokers exhibit an increase in $GABA_A$ receptors in areas involved in nicotine reward (Stokes et al., 2013), which is hypothesized to be due to a compensatory mechanism for the lack of GABA released during smoking. Many of these regions also receive GABAergic projections from the VTA, such as the NAc (Taylor et al., 2014). It is quite possible that directly activating VTA GABAergic neurons through nicotine can affect these regions to promote nicotine dependence, seeing that these regions are heavily implicated with reward and associative learning (Brown et al., 2012).

I.E. The Habenula, Interpeduncular Nucleus and Nicotine Withdrawal

The role of stress in drug abuse is well established and heavily implicated in increasing craving and promoting relapse (Sinha, 2008; 2009). Stressful stimuli can increase negative affective symptoms, as stressed smokers withdrawn from nicotine exhibited heightened negative affect (McKee et al., 2011). The release of CRF increased anxiety-like behavior through the VTA to instigate drug relapse (Wise, 2010). Footshock induced CRF transmission within the VTA in both cocaine naïve and cocaine exposed rodents, indicating that CRF release was dependent only on stressful stimuli (Wang et al., 2005). CRF release into the VTA can originate from various regions as well as occur locally. Chronic nicotine

exposure led to increased CRF transcript expression in VTA DAergic neurons, while infusing CRF1 receptor antagonists in the pVTA of nicotine withdrawn mice abolished aversion (Grieder et al., 2014), implicating a potential role of the pVTA in nicotine withdrawal symptoms. Another study silenced CRF production in the VTA and observed decreased activation of the IPN and decreased anxiety, highlighting a possible VTA to IPN pathway that nicotine induces withdrawal symptoms and potentially be involved in signaling stress (Zhao-Shea et al., 2014).

There has been a recent surge of interest in the habenula and the IPN in regards to nicotine withdrawal. The habenula is a small symmetrical brain region surrounded by the third ventricle and located dorsal to the posterior thalamus. It is further portioned into medial (MHb) and lateral (LHb) subnuclei due to differences in cytostructure and genetic profile. The LHb is involved in negative reward signaling and indirectly inhibits the VTA via the RMTg while the MHb is implicated in anxiety and nicotine withdrawal. Furthermore, the MHb is further organized into subsections based on neuronal subpopulation and projection targets. The MHb contains a high concentration as well as a wide range of nAChR subunits, many associated with aversive nicotine withdrawal symptoms (Connolly et al., 1995; Quick et al., 1999; Salas et al., 2004; 2009; Viswanath et al., 2014).

The IPN is a midbrain region situated along the midline and ventral to the VTA. It is comprised of 4 paired and 3 unpaired subnuclei. Neurons within the IPN express glutamate decarboxylase, the enzyme that catalyzes GABA; therefore,

the neurons within the IPN are thought to be mostly GABAergic (Kawaja et al., 1989; Zhao-Shea et al., 2013). Like the MHb, the IPN also expresses nAChR subunits associated with nicotine withdrawal (Salas et al., 2004; 2009; Velasquez et al., 2014). Optical activation of GABAergic neurons within the IPN induces somatic stereotypical withdrawal behaviors in chronic nicotine treated mice, suggesting the IPN is involved in the expression of withdrawal symptoms. Additionally, blockage of β4 nAChRs within the IPN leads to increased instances of somatic symptoms (Zhao-Shea et al., 2015). The IPN also contains CRF1 receptors possibly innervated by CRF produced in the VTA (Greider et al., 2014; Zhao-Shea et al., 2015).

The majority of the projections from the MHb innervate the IPN via the fasiculus retroflexus. The IPN receives projections from the MHb in a topographical manner; that is, nuclei situated in the midline receive innervation from the ventral MHb, a region consisting of ChAT-expressing neurons. Optogenetic studies demonstrated that the neurons in the MHb release both acetylcholine and glutamate, depending on the frequency of stimulation (Ren et al., 2011). These data suggest the MHb-IPN circuit could possibly works in opposition to the mesocorticolimbic pathway to reduce reward and promote withdrawal symptoms in order to maintain nicotine dependence (Koob and Volkow, 2008).

Nicotine withdrawal can be instigated in the laboratory setting by either withdrawing a mouse from the source of nicotine for 24 hours or administering a nicotinic receptor antagonist (Damaj et al., 2003; Salas et al., 2004., Salas et al., 2009). An injection of either DHβE, mecamylamine or MLA to nicotine-adapted

rodents induced somatic withdrawal symptoms (Hildebrand et al.,1997, Epping-Jordan et a., 1998, Damaj et al., 2003). The specificity of these nicotinic receptor antagonists suggests nAChRs containing the α3, α4, β2 and α7 nAChR subunits are involved in nicotine withdrawal. Infusion of mecamylamine into the VTA was sufficient to precipitate somatic withdrawal symptoms (Hildebrand et al., 1997), suggesting that the VTA as a candidate region involved in nicotine withdrawal. However, it is possible that mecamylamine also affected nAChRs within the IPN, due to the proximity of the nucleus to the VTA.

Although not as extensively studied as nicotine reward, nAChRs located within the MHb and the IPN have been linked to behaviors involved in nicotine dependence. A series of studies have screened each nAChR KO line and concluded that the subunits implicated in somatic symptoms of nicotine withdrawal were α2, α5, β4 but not α7 or β2 (Salas et al., 2004, 2009; Jackson et al., 2012). The α2, α5, β4 nAChR subunits are extensively expressed in the MHb and the IPN. Mice lacking the α2, α5 or β4 nAChRs failed to exhibit nicotine withdrawal symptoms while α7 and β2 KO mice exhibited withdrawal symptoms when given MLA or mecamylamine, respectively (Salas et al., 2004, 2009). Viral-mediated re-expression studies further implicated the brain regions involved in nicotine consumption. Intravenous self-administration studies found that α5 KO mice administered nicotine readily at high doses, and reinstating α5 nAChR subunits in the MHb lowered the preferred dose (Fowler et al., 2011). The authors proposed α5 nAChRs within the MHb are involved in an inhibitory

circuit that restricts continued nicotine consumption. Dysregulation of this circuit could instigate behaviors involved in nicotine withdrawal.

I.F. Thesis Overview

The overall goal of the work described within this thesis is to understand the impact of nicotinic acetylcholine receptor “upregulation”- a well-characterized neuroadaptation that occurs with chronic nicotine consumption- on a classical pathway involved in nicotine-mediated behaviors. I developed a gene expression system in order to mimic the upregulation of α4* nAChRs. This gene expression system conferred α4 nAChR subunits with a 9´ leucine to serine point mutation (Leu9´Ser) in a Cre-restricted manner to generate “gain-of-function” α4* nAChRs in discrete neuronal subpopulations. This experimental design allows to distinguish the contribution of “upregulated” α4* nAChR from baseline α4* nAChR, as well as discern the impact of activation of VTA GABAergic α4* nAChRs on nicotine dependence behaviors. Expression and function of viable Leu9´Ser α4* nAChRs was verified through immunohistochemical and electrophysiological techniques. Furthermore, expressing these Leu9´Ser α4* nAChRs did not change locomotor activity or induce anxiety. Finally, expression of Leu9´Ser α4* nAChRs in VTA GABAergic neurons increased sensitivity to nicotine reward.

CHAPTER II.

FUNCTIONAL UPREGULATION OF α4* NICOTINIC ACETYLCHOLINE RECEPTORS IN VTA GABAERGIC NEURONS INCREASES SENSITIVITY TO NICOTINE REWARD

CONTRIBUTIONS TO CHAPTER II.

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Author Contributions

Ngolab J – Designed and performed experiments, analyzed data, prepared figures, wrote manuscript

Liu L – Designed and performed electrophysiological recording, prepared figures, wrote manuscript

Zhao-Shea R– Designed and performed single-cell RTPCR experiments, prepared figures

Gao G – Provided AAV2 viral particles packaged with pAAV-EF1a-Leu9Ser-a4-YFP plasmid

Gardner P.D. – Provided feedback on experimental designs and edits to manuscript

Tapper A.R. – Designed experiments, analyzed data, prepared figures, co-wrote manuscript

Other contributions

Deisseroth K – provided the AAV-Ef1a-DIO-ChR2-eYFP plasmid.

ABSTRACT

Chronic nicotine exposure increases sensitivity to nicotine reward during a withdrawal period which may facilitate relapse in abstinent smokers, yet the molecular neuroadaptation(s) that contribute to this phenomenon are unknown. Interestingly, chronic nicotine use induces functional upregulation of nicotinic acetylcholine receptors (nAChRs) in the mesocorticolimbic reward pathway potentially linking upregulation to increased drug sensitivity. In the ventral tegmental area (VTA) functional upregulation of nAChRs containing the a4 subunit (α4* nAChRs) is restricted to GABAergic neurons. To test the hypothesis that increased functional expression of α4* nAChRs in these neurons modulates nicotine reward behaviors, we engineered a Cre recombinase-dependent gene expression system to selectively express α4 nAChR subunits harboring a "gain-of-function" mutation (a leucine mutated to a serine residue at the 9´ position: Leu9´Ser) in VTA GABAergic neurons of adult mice. In mice expressing Leu9´Ser α4 nAChR subunits in VTA GABAergic neurons (Gad2VTA: Leu9´Ser mice), sub-reward threshold doses of nicotine were sufficient to selectively activate VTA GABAergic neurons and elicit acute hypolocomotion which developed tolerance with subsequent nicotine exposures compared to control animals. In the conditioned place preference procedure, nicotine was sufficient to condition a significant place preference in Gad2VTA: Leu9´Ser mice at low nicotine doses that failed to condition control animals. Together, these data indicate that functional upregulation of α4* nAChRs in VTA GABAergic neurons confers increased sensitivity to nicotine reward and points to nAChR subtypes

specifically expressed in GABAergic VTA neurons as molecular targets for smoking cessation therapeutics.

INTRODUCTION

Chronic exposure to tobacco smoke accounts for ~5 million deaths per year making health complications from smoking the primary cause of preventable mortality in the world (Harris and Anthenelli, 2005). Nicotine, the addictive component of tobacco, binds to and activates neuronal nicotinic acetylcholine receptors (nAChRs), ligand-gated cation channels that are normally activated by the endogenous neurotransmitter, acetylcholine (ACh). Nicotine initiates dependence by activating neurons within the ventral tegmental area (VTA) of the mesocorticolimbic reward circuitry, ultimately driving the release of dopamine (DA) within the nucleus accumbens (NAc), a phenomenon widely associated with the rewarding or reinforcing value of nicotine (De Biasi and Dani, 2011). A large variety of nAChR subunit genes are expressed in both VTA DAergic projection neurons and GABAergic neurons (Klink et al., 2001, Wooltorton et al., 2003).

Neuronal nAChRs are pentameric receptors consisting of homologous or heterologous combinations of subunits. Twelve genes encoding twelve individual nAChR subunits have been identified, accounting for a vast array of nAChR subtypes each with distinct pharmacological and biophysical properties. A great deal of effort has focused on identifying nAChR subtype expression within the VTA to determine which subtypes, when activated, are necessary and sufficient for nicotine reinforcement and/or reward (Picciotto et al., 1998, Tapper et al., 2004, Maskos et al., 2005, Pons et al., 2008). From these studies, a general consensus is that expression of nAChRs containing α4 and β2 subunits in the VTA are both necessary and sufficient for nicotine reinforcement with at least

some contribution of the α6 subunit (Pons et al., 2008, Brunzell et al., 2010, Gotti et al., 2010). However, α4 and β2 subunits are expressed in both VTA DAergic neurons and GABAergic neurons (Klink et al., 2001, Wooltorton et al., 2003). While nicotine can directly activate VTA DAergic neurons, recent studies suggest that activation of GABAergic neurons may also modulate DAergic neuron activity and is required for nicotine reinforcement (Tolu et al., 2012).

Unlike other drugs of abuse, chronic use of nicotine leads to increased expression or "upregulation" of α4β2* nAChRs ("*" denotes that nAChR subunits in addition to α4 and β2 may be assembled in the nAChR complex) in the mesocorticolimbic pathways in addition to other brain regions. While upregulation of nAChRs is a hallmark of chronic nicotine exposure, the behavioral consequence of this phenomenon and how it relates to nicotine dependence is unknown (Wonnacott, 1990). Interestingly, functional upregulation of α4* nAChRs in the mesolimbic pathway appears to be restricted to midbrain GABAergic neurons including those of the VTA (Nashmi et al., 2007, Xiao et al., 2009). In addition, the rewarding properties of nicotine have been shown to increase in chronic nicotine-exposed mice, perhaps linking upregulation and increased functional α4* nAChR expression in VTA GABAergic neurons with reward behavior (Hilario et al., 2012). We sought to test the hypothesis that increased functional expression of α4* nAChRs selectively in VTA GABAergic neurons will increase sensitivity to nicotine reward.

MATERIALS AND METHODS

Mice. C57BL/6J and Gad2-Cre (strain B6N.Cg-Gad2$^{tmz(cre)Zhj}$/J) male mice on a C57BL/6J background were used in this study (Jackson Lab, Bar Harbor, ME, USA)(Taniguchi et al., 2011). Adult (8 to 10 weeks old) Gad2-Cre mice were injected with viral particles and used for behavioral experiments 4-6 weeks post-infection. All mice were kept on a 12-hour light-dark cycle with lights on at 0700 hours and off at 1900 hours. All mice were given food and water *ad libitum*. All procedures were performed in compliance with the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs. For acute treatments, nicotine hydrogen tartrate, mecamylamine hydrocholoride (Sigma-Aldrich, St. Louis, MO, USA) and dihydro-β-erythroidine hydrobromide (DHβE, Tocris Bioscience, Bristol, UK) were dissolved in sterile 0.9% phosphate buffered saline (PBS) (Hospira, Lake Forest, IL, USA). Nicotine was titrated to physiological pH (7.4) before being administered to mice. Vials containing nicotine solutions were wrapped in aluminum foil to prevent degradation by light exposure. For chronic exposure studies, nicotine dihydrate ditartrate (200 µg/ml, Acros Organics, Geel, Belgium) and L-tartaric acid (300 µg/ml, Sigma-Aldrich) were dissolved in water. Saccharin sodium (3 mg/ml, Fisher Scientific) was added to both solutions to increase palatability. Doses for nicotine, mecamylamine, and DHβE were calculated as free base. All injections were administered subcutaneously (s.c.).

Viral Plasmid Engineering. The mouse α4-YFP nAChR subunit cDNA was obtained from Addgene (plasmid 15245) and has been described previously (Nashmi 2003). Using the Quickchange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA), PCR mutagenesis was done to convert the 9′ leucine of the M2 pore domain to a serine (changing the codon from CTT to TCT). The resulting construct contained an α4-YFP cDNA with the leucine to serine mutation (Leu9′Ser α4-YFP). The Leu9′Ser α4-YFP cDNA was sub-cloned into the AAV expression vector pAAV-EF1a-DIO-WPRE-pA (Tsai et al., 2009).

Chronic Nicotine Exposure. Animals were restricted to drinking either control (tartaric acid, 300 µg/ml and 3 mg/ml saccharin) or nicotine- (200 µg/ml and 3 mg/ml saccharin) treated water through a 250-ml opaque water bottle placed in the home cage for 6-8 weeks. To induce withdrawal, the nicotine bottle was replaced with a water bottle (Zhao-Shea et al., 2013).

Viral-Mediated Gene Delivery. Both the pAAV-EF1a-Leu9Ser-a4-YFP (Leu9′Ser α4-YFP) and pAAV-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA (control) constructs were packaged into AAV2 viral particles by the University of Massachusetts Medical School Viral Vector Core. Viral titers were 10^{12} viral particles/µl with 1 µl of viral supernatant bilaterally injected into the VTA. Gad2-Cre animals were anesthetized with ketamine/xylazine (0.1 ml/10g body weight, 10 mg/ml ketamine, 1 mg/ml xylazine). To prep the surgical area, fur was

shaved and skin was disinfected with 10% povidone-iodine. The VTA was located with a stereotaxic frame (Stoelting, Wood Dale, IL, USA) using the following coordinates from bregma: -3.3(AP), +/- 0.3 (ML), -4.0 (DV). Viral particles were infused into the brain using an injection syringe (Hamilton, Reno, NV, USA) attached to a syringe pump (Stoelting Quintessential Stereotaxic Injector, Stoelting) at a rate of 0.25 ml/min. The injection needle was left in place for 10 min after each injection and then slowly retracted. Mice were given 5% glucose and 15 mg/kg ketaprofen after fully regaining consciousness. Mice were given four weeks for recovery and to allow for expression of the viral particles prior to each experiment.

Immunofluorescence. For c-Fos immunolabeling, animals received s.c. saline injections three days prior to each experiment to reduce possible effects of stress and/or handling on neuronal activity. To assess c-Fos expression, Gad2-Cre male mice infected with either control or Leu9´Ser α4-YFP were injected either with saline or 0.09 mg/kg nicotine. Ninety minutes after drug administration, brains were harvested for slice preparation. Animals were anesthetized with 200 mg/kg sodium pentobarbital (interperitoneal injection) and transcardiacally infused with 10 ml of chilled 0.1 M PBS following with chilled 4% (w/v) paraformaldehyde dissolved in 0.1 M PBS. The brains were harvested and placed in cold 4% paraformaldehyde for 2 h before submerging in 30% sucrose. Brains were then sectioned into 30-mm slices using a microtome (Leica, Buffalo Grove, IL, USA) and immunolabeled via free-floating sections. Slices were

washed in PBS for 5 min, permeabilized in 0.2% (v/v) Triton-X (Sigma-Aldrich) for 5 min, and blocked with 2% Bovine Serum Albumin (BSA) (Fisher Scientific, Waltham, MA, USA) for 30 min before incubation with primary antibody in 2% BSA overnight at 4 C°. Primary antibodies used were: mouse anti-TH MAB318 (1:1000, lot number: 2499557, Millipore, Billerica, MA, USA), rabbit anti-Gad1/2 (1:2000, lot number: 122M4761, Sigma-Aldrich), rabbit anti-c-Fos (1:1000, lot number: F2510, Santa Cruz, Santa Cruz, CA, USA), rabbit anti-GFP (1:4000, lot number: GR158277-1, Abcam, Cambridge, MA, USA), mouse anti-calbindin (1:3000, lot number: 052M4833, Sigma-Aldrich), rabbit anti-calretinin (1:1000, lot number: AB5054, Millipore), rabbit anti-parvalbumin (1:1000, lot number: ab11427, Abcam) and goat anti-somatostatin (1:100, lot number: sc-789, Santa Cruz). Secondary antibodies were Alexa Fluor 405 (lot number: 1126599), 488 (lot number: 1608521) and 594 (lot numbers: 1431805, 1003216 and 1602780) (1:1000, Life Technologies, Grand Island, NY, USA). An AxioCam MRm camera (Carl Zeiss Microscopy GmbH, Jena, Germany) attached to a Zeiss Axiovert inverted fluorescent microscope equipped with Zeiss filter sets 38HE, 49, and 20 was used to acquire fluorescent images. Zeiss objectives A-plan 10x, EC-Plan-NEOFLUAR 20x and Plan-APOCHROMAT-63x were used to view and capture images. Images were processed using Axiovision v.4.8.2. For quantification and co-localization analysis, images were deconvoluted and segmented using the segmentation and quantification of subcellular shapes (Squassh) software plugin through ImageJ. At least 10 slices/mouse brain that spanned the entire VTA were analyzed. The VTA was located using TH staining and morphology of

nearby brain regions as previously described (Zhao-Shea et al., 2011). Areas of interest for each slice were identified through TH fluorescence and measured in ImageJ using the “Analyze Particles” option to account for differences between slices.

Electrophysiological Recordings. Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and then decapitated. Slices were prepared as previously described (Zhao-Shea et al., 2011). Briefly, brains were quickly removed and placed in an oxygenated ice-cold high sucrose artificial cerebrospinal fluid (SACSF) containing kynurenic acid (1 mM, Sigma-Aldrich). Brain slices (180 - 200 µm) were cut using a Leica VT1200 vibratome (Leica Microsystem Inc.). The brain slices were incubated in oxygenated Earl’s balanced salt solution (EBSS) supplemented with glutathione (1.5 mg/ml, Sigma), N-ω-nitro-L-arginine methyl ester hydrochloride (2.2 mg/ml, Sigma), pyruvic acid (11 mg/ml, Sigma) and kynurenic acid (1 mM) for 45 min at 34°C. Slices were transferred into oxygenated ACSF at room temperature for recording. SACSF solution contained (in mM): 250 sucrose, 2.5 KCl, 1.2 $NaH_2PO_4•H_2O$, 1.2 $MgCl_2•6H_2O$, 2.4 $CaCl_2•2H_2O$, 26 $NaHCO_3$, 11 D-Glucose. Single slices were transferred into a recording chamber continually superfused with oxygenated ACSF. The junction potential between the patch pipette and bath ACSF was nullified just prior to obtaining a seal on the neuronal membrane. Action potentials and currents were recorded at 32°C using the whole-cell configuration of a Multiclamp 700B patch-clamp amplifier *(*Molecular Devices).

Action potentials measured in cell-attached mode were recorded at 31-32 ºC. Action potentials were obtained using a gap-free acquisition mode and Clampex software (Molecular Devices). I_h currents were elicited every 5 sec by stepping from -60 mV to a test potential of -120 mV for 1 sec using Clampex. Input resistances were calculated using steady state currents elicited by 5 mV hyperpolarizing pulses. Signals were filtered at 1 kHz using the amplifier's four-pole, low-pass Bessel filter, digitized at 10 kHz with an Axon Digidata 1440A interface and stored on a personal computer. Potential VTA GABAergic neurons were selected for recording based on fluorescent signal and further verified by action potential frequency (~10-20 Hz) and lack of I_h expression. At the end of recording, the neuronal cytoplasm was aspirated into the recording pipette and the contents were expelled into a microcentrifuge tube containing 75% ice-cold ethanol and stored at -20°C for at least 2 h before single-cell RT-PCR experiments to verify expression of GAD 1 and 2. ACh chloride (Sigma) was dissolved in ACSF. Whole cell ACh responses were recorded in the presence of TTX (0.5 mM), atropine (1 mM), bicuculline (20 mM), and CNQX (10 mM). Drugs were applied to slices by gravity superfusion.

Nicotine Tolerance. Mice received s.c. saline injections three days before testing to habituate to handling. Locomotor activity was recorded as beam breaks using the Photobeam Activity System (PAS) (San Diego Instruments, San Diego, CA, USA). To determine baseline activity, mice were injected with saline and immediately placed into a novel cage inside the PAS locomotor chamber.

Activity was quantified for 15 min before mice were returned to their home cage. On the second day, mice received an s.c. injection of nicotine and immediately placed into a novel cage within the locomotor activity chamber. Activity was measured for 15 min. Mice were subsequently injected with nicotine each day for six more days, with locomotor activity recorded on the fourth and seventh day of nicotine challenge.

Conditioned Place Preference. A three-chamber conditioned place preference (CPP) apparatus (Med Associates, St. Albans, VT) was used to measure nicotine reward. Briefly, at least three days prior to testing, mice were habituated to the test room and handling by picking them up once a day by the scruff of their neck. The testing protocol was comprised of three phases. In the pre-training phase, mice were placed into the CPP apparatus and allowed to freely explore all three chambers for 15 min. Time spent in each chamber was quantified using MEDPC IV software (Med Associates). The training phase lasted four days, where each day the mice was twice confined to a chamber for 30 min. In the morning session, mice were given an s.c. injection of sterile saline and placed in the chamber assigned as the saline-paired chamber. Five hours later, mice were given an s.c. injection of drug (i.e. nicotine) and placed in the opposite, drug-paired chamber. The training phase was counterbalanced for each group in that approximately half the number of animals received nicotine paired with the white chamber while the other half received nicotine paired with the black chamber. Drug was paired with the least preferred chamber. Mice that spent greater than

70 % of pre-training in any one chamber were not included in the analysis. The post-training phase was identical to the pre-training phase. Difference scores were measured by calculating the difference between the time spent in a chamber during the post-training phase and during the pre-training phase. For CPP experiments testing nicotine reward in control and Leu9´Ser α4-YFP-infected mice (as described below in Fig. 3 and Table 1), the CPP procedure was initiated 4-6 weeks post-infection. Mice received saline in both chambers, 0.09 mg/kg nicotine, or 0.5 mg/kg nicotine (n = 8-10 mice/group) during training. Additional groups of control and Leu9´Ser α4-YFP-infected mice were tested for CPP to 1 mg/kg mecamylamine (n = 8-10 mice/group), or 0.5/2.0 mg/kg DHβE (n = 6-8 mice/group) as described below in Fig. 4 A-B and Table 1. For this set of experiments, CPP was performed exactly as described above except instead of nicotine during training, mice received mecamylamine or DHβE at the indicated dose. For CPP in nicotine-dependent mice (Fig. 3C, inset and Table 1), mice were exposed to nicotine in their drinking water for 8 weeks. Water bottles were swapped for untreated drinking water after the completion of the CPP pre-training phase and CPP was performed as above using 0.5 mg/kg nicotine during training (n = 11). An additional group of control nicotine-naïve animals were also tested in the CPP procedure using 0.5 mg/kg nicotine (n = 11).

Statistics. Normality of data were tested prior to analysis. Locomotor activity data were analyzed using repeated measure one-way analysis of variance (ANOVA). CPP data were analyzed using two-way ANOVA with drug dose and

paired-chamber as main factors followed by Bonferroni post-hoc analysis as indicated. c-Fos data were analyzed using two-way ANOVA with virus and drug treatment or neuron sub-type as main factors as indicated. Fold-change differences in inward current were analyzed used a two-tailed t-test. Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

Expression of "gain-of-function" α4 nicotinic receptor subunits in VTA GABAergic neurons. To understand how increased functional expression of α4* nAChRs in VTA GABAergic neurons affect behavior, we developed a viral-mediated gene delivery system to express α4* nicotinic receptor subunits with a "gain-of-function" mutation in select neuronal populations allowing for selective activation of neurons expressing this subunit with low doses of nicotine that minimally activate non-α4* nAChRs. We engineered an AAV plasmid construct containing cDNA encoding the α4 nAChR subunit with a single point mutation, a leucine mutated to serine, at the 9´ residue of the pore-forming, M2 domain (Leu9´Ser, Figure II-1 A), which renders nAChRs that incorporate this subunit significantly more sensitive to nicotine (Labarca et al., 2001). To visualize subunit expression, a YFP tag was included in the M3-M4 intracellular loop where it does not interfere with receptor assembly or function (Leu9´Ser α4-YFP) (Nashmi et al., 2003, Nashmi et al., 2007). The cDNA encoding Leu9´Ser α4-YFP was positioned within the AAV expression vector in the antisense orientation and flanked by two pairs of distinct Lox sites (Figure II-1 A). These Lox sites

regulate Leu9´Ser α4-YFP expression by directing recombination of the cDNA cassette to the sense orientation in the presence of Cre recombinase (Figure II-1 A) (Tsai et al., 2009).

The expression vector was packaged into AAV2, a serotype that will infect a brain region locally, and viral particles were injected into the VTA of glutamate decarboxylase 2 (Gad2)-Cre mice (Gad2VTA: Leu9´Ser) for expression of Leu9´Ser α4-YFP subunits selectively in GABAergic neurons. To verify subunit expression in GABAegic neurons, Gad2VTA: Leu9´Ser midbrain slices were immunolabeled with either an anti-TH or Gad1/2 antibody. VTA of infected mice exhibited robust expression of Leu9´Ser α4-YFP subunits as indicated by strong YFP fluorescent signal selectively in non-DAergic neurons (Figure II-1 B). To determine functional expression of Leu9´Ser α4-YFP subunits in GABAergic VTA neurons, patch-clamp recordings were made in Gad2VTA: Leu9´Ser and control midbrain slices. Control Gad2-Cre animals were infected with AAV2 particles containing channelrhodopsin within the same vector so that GABAergic neurons from control mice will express a non-nAChR membrane protein insensitive to nicotinic agonists. The electrophysiological characteristics of infected neurons, as identified by YFP fluorescent signal, were analyzed to confirm incorporation of the Leu9´Ser α4-YFP subunit into a nAChR. Leu9´Ser α4-YFP-infected neurons exhibited fast-spiking spontaneous action potentials and lacked an I_h current, both characteristics of VTA GABAergic neurons (Figure II-1 C) (Johnson and North, 1992). Single-cell RT-PCR from the cytoplasm of recorded neurons

confirmed Gad1 and Gad2 expression in YFP-positive neurons (Figure II-1 D). To test for functional expression of the Leu9´Ser α4-YFP subunit, whole cell current responses to bath application of 1 mM ACh were recorded in infected Gad2-Cre midbrain slices. ACh elicited robust inward currents in Leu9´Ser α4-YFP subunit-expressing VTA GABAergic neurons that were significantly larger compared to responses from neurons recorded from control slices (Figure II-1 E). Together, these data suggest that Gad2VTA: Leu9´Ser mice express the Leu9´Ser α4-YFP subunit in GABAergic neurons and the subunit co-assembles with endogenous subunits to form “gain-of-function” nAChRs.

Selective activation of VTA GABAergic neurons in Gad2VTA: Leu9´Ser mice. To test the hypothesis that a low dose of nicotine selectively increased activation of VTA neurons in nicotine-naïve Gad2VTA: Leu9´Ser mice compared to control animals, we challenged each group with saline or an acute dose of 0.09 mg/kg nicotine and immunolabeled VTA slices for c-Fos expression, an immediate early gene that is a marker for neuron activation (Cole et al., 1989). Two-way ANOVA indicated a significant main effect of virus expression ($F_{(1,22)}$ = 29.96, $p < 0.001$) and nicotine treatment ($F_{(1,22)}$ =31.62, $p < 0.001$), and a significant virus expression x nicotine treatment interaction ($F_{(1,22)}$ =22.23, $p < 0.001$) (Figure II-2 A). Post-hoc analysis indicated that there was a statistically significant increase of c-Fos immunopositive VTA neurons from Gad2VTA: Leu9´Ser mice receiving 0.09 mg/kg nicotine compared to Gad2VTA: Leu9´Ser mice receiving saline injection ($p < 0.0001$). In addition, the number of c-Fos

immunopositive VTA neurons in Gad2VTA: Leu9´Ser mice was also significantly greater than the number of c-Fos immunopositive VTA neurons in nicotine-treated control animals. In control mice, 0.09 mg/kg nicotine did not significantly increase the number of c-Fos immunopositive VTA neurons compared to saline. To determine if nicotine-activated neurons were predominantly non-DAergic, we challenged control and Gad2VTA: Leu9´Ser mice with 0.09 mg/kg nicotine and double immunolabeled for c-Fos and TH (Figure II-2 B, C). Two-way ANOVA revealed a significant main effect of virus expression ($F_{(1,22)}$ =5.3, $p < 0.05$) and neuron sub-population ($F_{(1,22)}$ =10.0, $p < 0.01$), and a virus expression x nicotine treatment interaction ($F_{(1,22)}$ =4.73, $p < 0.05$). Post-hoc analysis indicated that the number of TH immunonegative, c-Fos immunopositive neurons in Gad2VTA: Leu9´Ser was significantly larger than TH immunopositive, c-Fos immunopositive neurons after nicotine challenge ($p < 0.01$). The number of TH immunonegative, c-Fos immunopositive neurons in Gad2VTA: Leu9´Ser was also larger than the number of TH immunonegative, c-Fos immunopositive neurons in control mice after nicotine challenge ($p < 0.05$). The number of TH immunopositive, c-Fos immunopositive neurons in Gad2VTA: Leu9´Ser mice after nicotine challenge was small and not significantly different from control mice. In addition, YFP signal could be detected in c-Fos immunopositive neurons in Gad2VTA: Leu9´Ser mice but not in control mice (Figure II-2 B). Together, these data indicate that 0.09 mg/kg nicotine selectively activates non-DAergic (i.e., GABAergic) neurons in Gad2VTA: Leu9´Ser mice.

Nicotine activation of α4* nicotinic receptors in VTA GABAergic neurons: Locomotor effects. To test the hypothesis that functional upregulation of nAChRs in GABAergic neurons may be involved in nicotine tolerance (Nashmi et al., 2007), we measured nicotine-induced locomotor activity in response to single daily injections of the drug for seven days in Gad2VTA: Leu9´Ser and control animals. Mice were challenged with 0.09 mg/kg nicotine delivered s.c., a dose that activated GABAergic neurons in Gad2VTA: Leu9´Ser but had little effect on neuronal activation in control mice. In control mice, 0.09 mg/kg nicotine did not significantly modulate locomotor activity compared to saline injection (Figure II-3 A). In Gad2VTA: Leu9´Ser mice, one-way ANOVA revealed a significant main effect of nicotine injections on locomotor activity ($F_{(3,18)} = 7.86$, $p < 0.01$). Post-hoc analysis revealed that nicotine significantly depressed locomotor activity upon first injection compared to saline ($p < 0.01$) and tolerance to this hypolocomotor response occurred with subsequent injections. Thus, acute activation of nAChRs in VTA GABAergic neurons induces hypolocomotor activity which triggers tolerance after subsequent nicotine exposures.

Sensitivity to nicotine reward is modulated by activation of VTA GABAergic α4* nicotinic receptors. To test the hypothesis that functional upregulation of VTA GABAergic neurons modulates nicotine reward, the ability of nicotine to condition a place preference in Gad2VTA: Leu9´Ser and control mice was measured using the CPP procedure. During training, mice were challenged with saline, 0.09 mg/kg or 0.5 mg/kg nicotine (Figure II-3 C, D, Table II-1) delivered

s.c. In control animals, nicotine did not condition a significant place preference in response to either dose (Figure II-3 C) similar to previous reports delivering s.c. nicotine injections with the CPP procedure in C57BL/6J mice (Hilario et al., 2012), the background strain of the Gad2-Cre mice. However, a significant CPP in response to 0.5 mg/kg was observed in this strain during withdrawal from 6 weeks chronic nicotine treatment (Figure II-3 C, inset, significant main effect of drug-paired chamber, $F_{(1,20)} = 5.97$, $p < 0.05$ and a significant drug-paired chamber × chronic treatment interaction, $F_{(1,20)} = 10.10$, $p < 0.01$, two-way ANOVA on difference scores: Significant increase in difference score in the nicotine-paired chamber between nicotine-dependent and nicotine-naïve mice, $p < 0.01$. Also see Table 1). In Gad2VTA: Leu9´Ser mice, two-way ANOVA of difference scores (Figure II-3 D) indicated a significant main effect of drug ($F_{(1,26)} = 18.64$, $p < 0.001$) but not training chamber ($F_{(2,46)} = 0.6807$, $p > 0.05$), and a significant drug x training chamber interaction ($F_{(2,46)}=7.086$, $p < 0.01$). Post-hoc analysis revealed a significant difference between difference scores in the nicotine-paired chamber at the dose of 0.09 mg/kg, but not 0.5 mg/kg, nicotine in Gad2VTA: Leu9´Ser mice compared to saline (Figure II-3 D). Repeated measures two-way ANOVA of time spent in the drug and saline-paired chamber pre- and post-training with 0.09 mg/kg nicotine (Table 1) did not indicate significant main effects of training or time spent in either chamber, but did reveal a significant training x chamber interaction ($F_{(1,8)} = 25.48$, $p < 0.001$). Post-hoc test indicated a significant increase in time spent in the nicotine-paired chamber post-training compared to pre-training. Finally, two-way ANOVA of time spent in

the drug-paired chamber pre- and post-training in Gad2VTA: Leu9´Ser mice that received 0.09 mg/kg nicotine during training compared to mice that received saline in the drug-paired chamber (Table II-1) indicated a significant main effect of drug ($F_{(1,15)} = 10.32$, $p < 0.01$) and time spent in the drug-paired chamber ($F_{(1,15)} = 7.97$, $p < 0.05$), and a significant interaction ($F_{(1,15)} = 13.19$, $p < 0.01$). Post-hoc analysis indicated a significant increase in time-spent in the drug-paired chamber post-training with 0.09 mg/kg nicotine compared to saline. Interestingly, after chronic nicotine exposure, Gad2VTA: Leu9´Ser mice did develop a modest CPP to 0.5 mg/kg nicotine (repeated measures Two-way ANOVA: Significant training x chamber interaction, $F_{(1,9)} = 27.61$, $p < 0.001$. Significant increase in time spent in the nicotine-paired chamber after training, $p < 0.05$, Table 1).

While a low dose of nicotine may elicit a CPP in Gad2VTA: Leu9´Ser mice by activating GABAergic neurons, it is also possible that nicotine may be desensitizing Leu9´Ser α4-YFP nAChRs in VTA GABAergic interneurons, reducing endogenous ACh activation of the mutant nAChRs (i.e., blocking GABAergic interneuron activity), and thereby disinhibiting DAergic neurons to promote reward (Mansvelder et al., 2002). We hypothesized that if this were occurring, then a nAChR antagonist would elicit a similar effect by blocking endogenous activity through the Leu9´Ser α4-YFP nAChRs. Thus, we measured the ability of the noncompetitive nAChR antagonist, mecamylamine, to condition a place preference in Gad2VTA: Leu9´Ser and control, nicotine-naïve animals. In both groups, 1.0 mg/kg mecamylamine failed to significantly condition a place preference (Figure II-4 A). In an additional group of Gad2VTA: Leu9´Ser and

control nicotine-naïve animals, we tested whether DHβE, a selective α4β2 competitive antagonist would be sufficient to disinhibit DAergic neurons and condition a place preference. Training with 0.5 or 2.0 mg/kg DHβE, s.c. in control and Gad2VTA: Leu9´Ser mice failed to condition a place preference (Figure II-4 B). These data indicate that selective activation of α4* nAChRs in GABAergic neurons is sufficient for nicotine reward.

Gad2^{VTA}: Leu9´Ser mice α4* nAChRs are expressed in VTA GABAergic neurons that project to the Lateral Habenula (LHb). To gain insight into the neuronal sub-population(s) of VTA GABAergic neurons that may express the Leu9´Ser α4-YFP subunit and elicit nicotine-induced reward in Gad2^{VTA}: Leu9´Ser animals, we immunolabeled C57BL/6J midbrain slices with potential GABAergic neuronal markers calretinin, calbindin, somatostatin and parvalbumin (Figure II-5 A). Interestingly, we did not detect somatostatin nor parvalbumin expression in the area of the VTA that was targeted with our virus expression system (that is, the posterior VTA), although somatostatin expression was observed in the rostral interpeduncular nucleus as previously described (Zhao-Shea et al., 2013). Both calretinin and calbindin immunopositive cells were detected (Figure II-5 A). However, analysis of YFP signal in Gad2^{VTA} : Leu9´Ser mice revealed that calretinin and calbindin immunopositive neurons did not express YFP, consistent with previous reports that the majority of calretinin- and calbindin-expressing VTA neurons are actually DAergic (Olson and Nestler, 2007). We next analyzed YFP signal in known VTA efferent brain regions of Gad2VTA: Leu9´Ser mice including the NAc, PFC, and LHb, for YFP

fluorescence (Figure II-5 B, C). Interestingly, YFP signal was not detected in the PFC or NAc. However, fluorescence was observed in the LHb.

DISCUSSION

We expressed Leu9´Ser α4-YFP nAChR subunits in VTA GABAergic neurons in an effort to understand how functional upregulation of α4* nAChRs in this neuronal subpopulation may contribute to behaviors associated with nicotine dependence. Chronic nicotine upregulates α4* nAChRs selectively in GABAergic neurons of the VTA and this is accompanied by an increase in functional expression as measured by an increase in nicotine activation of these neurons (Nashmi et al., 2007). It is important to note that if chronic nicotine merely upregulated the α4 nAChR subunit and not the β2 subunit then this would result in a change in α4β2 nAChR stoichiometry to the low sensitivity $(\alpha4)_3(\beta2)_2$ subtype (Eaton et al., 2014). However, a functional increase in activation is observed in chronic nicotine-treated animals, suggesting that upregulation of both α4 and β2 subunits occurs (Srinivasan et al., 2011), leading to the observed increase in nAChR function in GABAergic VTA neurons. To mimic this phenomenon, we chose to express “gain-of-function” α4 nAChR subunits in GABAergic VTA neurons instead of wild-type (WT) subunits which would have likely changed the α4β2 nAChR stoichiometry to the low sensitivity $(\alpha4)_3(\beta2)_2$ subtype resulting in a loss of function phenotype.

In $Gad2^{VTA}$: Leu9´Ser mice, a low dose of 0.09 mg/kg nicotine was sufficient to activate GABAergic neurons. This same dose failed to significantly

activate neurons in control animals. Interestingly, there were few DAergic neurons activated in both control and Gad2VTA: Leu9´Ser mice after low-dose nicotine challenge. Importantly, nicotine was delivered s.c. in these experiments; whereas this same dose has been shown to elicit reward in mice when delivered i.p, highlighting that routes of nicotine administration yield differences in bioavailablility of the drug (Brunzell et al., 2009; Alcantara et al., 2014).

Acute nicotine activation of VTA GABAergic neurons induces hypolocomotion. We assessed how functional upregulation of VTA GABAergic neurons may contribute to nicotine tolerance and reward. Acute nicotine induces hypolocomotion in rodents, which is alleviated with multiple nicotine exposures, providing a behavioral measure of tolerance (Tapper et al., 2007). Typically, locomotor suppression has been observed in C57BL/6J mice given a dose of ~0.5 mg/kg nicotine in a novel environment or open field (Salas et al., 2004). A single injection of 0.09 mg/kg nicotine in a novel cage was sufficient to decrease locomotor activity in Gad2 ^{VTA}: Leu9´Ser but had little effect on locomotor activity in control mice. Interestingly, Gad2 ^{VTA}: Leu9´Ser mice developed tolerance to this effect with daily low dose nicotine injections, indicating that acute activation of VTA GABAergic neurons induces hypolocomotion with subsequent exposures eliciting tolerance to this effect. The mechanism underlying nicotine-induced hypolocomotor activity is unknown. Our data indicate that activation of VTA GABAergic neurons may cause the initial nicotine-induced decrease in locomotor activity perhaps by inhibiting DA release into the striatum. However, additional

experiments will be needed to determine if the tolerance to this hypolocomotion involves α4* nAChRs in GABAergic neurons or, alternatively, triggers a non-nAChR mechanism that opposes hypolocomotion.

Selective activation of VTA GABAergic neurons by nicotine is sufficient for reward. Previous studies using optogenetic stimulation have shown that activation of VTA GABAergic neurons can lead to disruption of reward and induce aversion (Tan et al., 2012; van Zessen et al., 2012). Surprisingly, selective activation of VTA GABAergic neurons in $Gad2^{VTA}$: Leu9´Ser mice using a low dose of nicotine conditioned a robust place preference in these animals, suggesting that nicotine activation of these neurons may be sufficient for reward. Conversely, a more typical “rewarding” dose of 0.5 mg/kg s.c. nicotine (Hilario et al., 2012; Smith et al., 2012), failed to elicit a place preference in $Gad2^{VTA}$: Leu9´Ser mice, consistent with a shift in the inverted ‘U’ dose response curve often seen with nicotine reward and reinforcement (Picciotto, 2003). In control mice, nicotine failed to condition a place preference at any of the doses tested. While stress could be a contributing factor to lack of CPP in control animals, this is rendered unlikely as mice were habituated to handling prior to the beginning of the CPP assay. Our results are similar to those of Hilario et al. who demonstrated that withdrawal from chronic nicotine exposure was necessary for the expression of nicotine reward and that this is correlated with nAChR upregulation (Hilario et al., 2012). Verifying these data, we confirmed that control mice withdrawn from chronic nicotine also exhibit a place preference with 0.5

mg/kg nicotine compared to nicotine-naïve mice. Based on data indicating that 1) increased sensitivity to nicotine reward occurs after chronic nicotine exposure and withdrawal, 2) sensitivity to nicotine reward correlates with nAChR upregulation (Hilario et al., 2012), 3) upregulation of α4* nAChRs occurs selectively in VTA GABAergic neurons (Nashmi et al., 2007) and 4) selective activation of functionally upregulated α4* nAChRs in VTA GABAergic neurons elicits reward, we suggest that upregulation of α4* nAChRs specifically in VTA GABAergic neurons increases sensitivity to nicotine reward.

How might activation of GABAergic neurons by nicotine elicit reward? One possibility is that nicotine desensitizes GABAergic nAChRs reducing GABAergic neuron activity and disinhibiting DAergic neurons (Mansvelder et al., 2002). Our data indicate that, at least using our expression system, this possibility is unlikely because 1) we did not observe increased activation of DAergic neurons (as measured by c-Fos induction) after low dose nicotine challenge in Gad2^{VTA}: Leu9´Ser neurons and 2) mecamylamine and DHβE failed to elicit reward in Gad2 ^{VTA}: Leu9´Ser mice. One caveat to these results is that disinhibition of DAergic neurons by nAChR desensitization in GABAergic neurons would require that Leu9´Ser α4-YFP nAChRs are predominantly expressed in GABAergic interneurons. In analyzing VTA neuron sub-populations in the injection area of Gad2^{VTA}: Leu9'Ser mice, which was focused on the posterior VTA, we failed to detect parvalbumin or somatostatin immunopositive neurons; whereas calbindin and calretinin neurons were detected, but did not co-localize with Leu9´Ser α4-YFP expression, consistent with previous studies indicating

that these two populations of neurons are largely DAergic in the VTA (Gerfen et al., 1987, Olson and Nestler, 2007). A more recent study indicates that activation of GABAergic neurons via β2* nAChRs is required for DAergic neuron burst activity and nicotine self-administration (Tolu et al., 2012). Thus, nicotine activation of GABAergic neurons in Gad2^{VTA}: Leu9´Ser mice could lead to increased DAergic neuron bursting and reward. However, as indicated above, increased activation of DAergic neurons, at least with acute nicotine injections, was not observed in these animals. A third and more likely possibility is that a portion of VTA GABAergic neurons expressing Leu9´Ser α4-YFP nAChRs project to brain regions that, when inhibited, promote reward behavior. Indeed, upon analysis of known VTA projection regions, we found Leu9´Ser α4-YFP expression in the LHb. While GABAergic neurons make up ~35% of VTA neurons and little is known about their function within the VTA (Nair-Roberts et al., 2008), recent studies indicate that a portion of VTA GABAergic neurons innervate the LHb and, when activated, elicit reward (Stamatakis et al., 2013; Lammel et al., 2015). This is accomplished by inhibiting LHb glutamatergic inputs to the rostromedial tegmental nucleus (RMTg), which, in turn, disinhibits VTA DAergic neurons to promote reward (Hong et al., 2011; Lecca et al., 2011). Thus, one mechanism by which nicotine activation of VTA GABAergic neurons could elicit nicotine reward is through inhibiting these LHb inputs to the RMTg. Future studies should focus on how VTA GABAergic neuron activation alters the excitability of these downstream brain regions in the context of nicotine-induced reward. Our data suggest that activation of functionally upregulated α4* nAChRs

in VTA GABAergic neurons confers increased sensitivity to nicotine reward. These data indicate that nAChR subtypes specifically expressed in VTA GABAergic neurons may be good molecular targets for therapeutics to aid in smoking cessation.

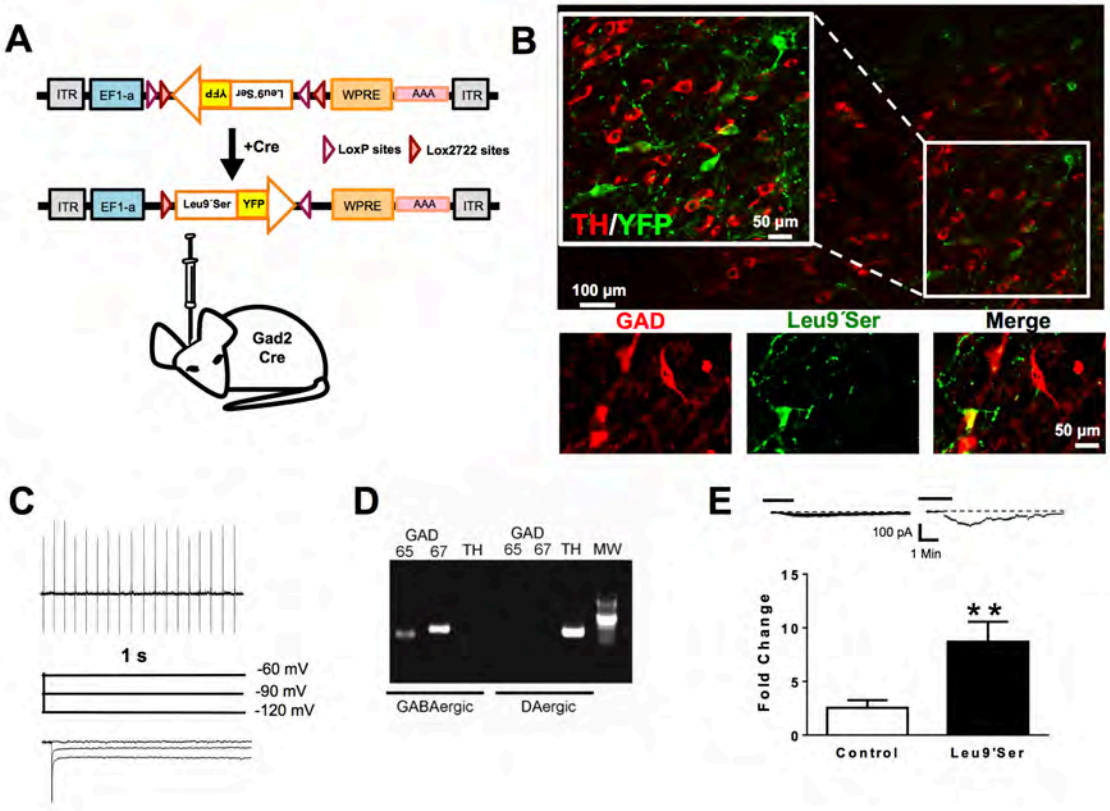

Figure II-1. Viral-mediated gene delivery of "gain-of-function" α4 nAChR subunits in VTA GABAergic neurons. A) Depiction of the Leu9´Ser α4-YFP subunit cDNA viral plasmid. Cre-recombinase flips the Leu9´Ser α4-YFP subunit cDNA in the sense orientation. The viral particles containing this plasmid were intracranially injected into the VTA of Gad2-Cre mice for selective expression in VTA GABAergic neurons. B) Top, expression of Leu9´Ser α4-YFP subunits in non-TH neurons of Gad2-Cre mice. Brain slices from infected mice were immunolabeled for TH to identify DAergic neurons (red, top, left). YFP fluorescence was detected indicating Leu9´Ser α4-YFP subunit expression (green, top, middle). Merged signals revealed little co-localization of Leu9´Ser α4-YFP subunit expression with DAergic neurons (top, right). Photomicrographs (bottom panels) of a representative VTA midbrain section from Gad2-Cre mice expressing Leu9´Ser α4-YFP subunits showing neurons immunolabeled for Gad (left) that also express Leu9´Ser α4-YFP subunits (middle). The merged photomicrograph (right) shows co-localization of GAD and YFP signals. C) Cell-attached (top) and whole-cell voltage-clamp (bottom) recordings from a putative VTA GABAergic neuron from a Gad2-Cre mouse midbrain slice. GABAergic neurons typically have a high frequency of firing (8-20 hz, top) and lack of a hyperpolarizing activated current, I_h (bottom). D) At the end of each recording, single neuron RT-PCR was performed to verify GAD expression in a GABAergic neuron (left lanes) or TH expression in a DAergic neuron (right lanes). E) Whole-cell voltage-clamp recordings from a control VTA GABAergic neuron in Gad2-Cre midbrain slice (top, left) and an Leu9´Ser α4-YFP-expressing GABAergic neuron

in a Gad2-Cre midbrain slice (top, right). ACh was bath applied for 3 min as indicated by the bar above each trace. Average inward current from control infected (n = 5) and Leu9´Ser α4-YFP (n = 8) infected VTA GABAergic neurons. **p < 0.01, two-tailed t-test.

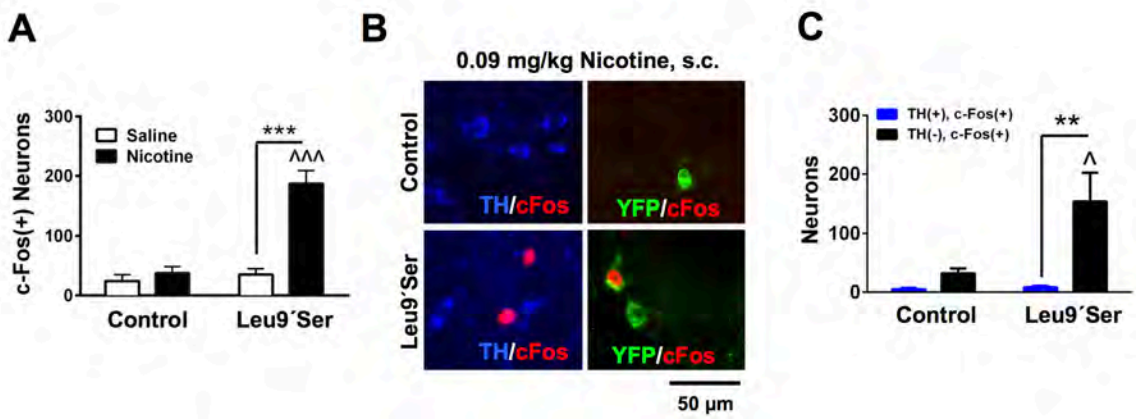

Figure II-2. Selective activation of VTA GABAergic neurons by nicotine in Gad2^{VTA}: Leu9´Ser mice. A) Summed average number of c-Fos immunopositive (c-Fos(+)) neurons in VTA of Control (n = 6/group) and Gad2VTA: Leu9´Ser mice (n = 7/group) after challenge with saline or 0.09 mg/kg nicotine. ***p < 0.001 number of c-Fos(+) neurons in Gad2^{VTA}: Leu9´Ser mice after nicotine challenge compared to saline challenge. ^^^ p < 0.001 number of c-Fos(+) neurons in Gad2^{VTA}: Leu9´Ser mice after nicotine challenge compared to the number of c-Fos(+) neurons in Control mice after nicotine challenge. B) Representative photomicrographs illustrating c-Fos expression (red) in VTA sections taken from Control (top panels) or Gad2^{VTA}: Leu9´Ser mice (bottom panels) mice after 0.09 mg/kg nicotine challenge. Neurons exhibiting TH immunoreactivity are labeled in blue (left panels); whereas YFP expression is labeled in yellow (right panels). C) Summed average number of TH(+), c-Fos(+) and TH(-), c-Fos(+) neurons in the VTA of Control (n = 6) and Gad2^{VTA}: Leu9´Ser mice (n = 7) after challenge with 0.09 mg/kg nicotine. ** p < 0.01 number of TH(+), c-Fos(+) neurons compared to the number of TH(-), c-Fos(+) neurons in Gad2^{VTA}: Leu9´Ser mice after nicotine challenge. ^ number of neurons that are TH(-), c-Fos(+) in Gad2^{VTA}: Leu9´Ser mice after nicotine challenge compared to the number of neurons that are TH(-), c-Fos(+) in Control mice after nicotine challenge.

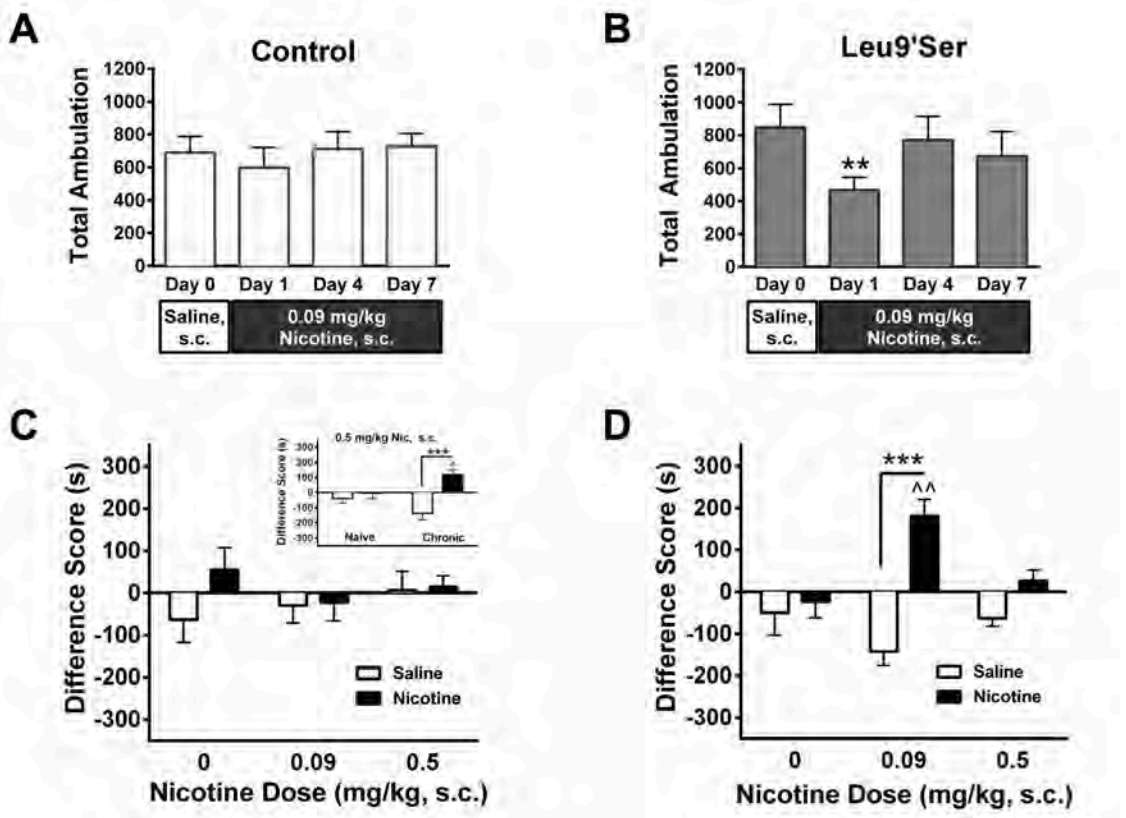

Figure II-3. Selective activation of VTA GABAergic neurons by nicotine is sufficient for nicotine-induced hypolocomotion and reward. A) Summed 15 min total locomotor activity after saline injection, or daily injection of 0.09 mg/kg nicotine in Control mice (n = 7). B) Summed 15 min total locomotor activity after saline injection, or daily injection of 0.09 mg/kg nicotine in $Gad2^{VTA}$: Leu9´Ser mice (n = 7). ** $p < 0.01$ compared to saline challenge. Difference scores indicate the time spent in the nicotine- or saline-paired chamber after training minus the time spent in the nicotine- or saline-paired chamber prior to training in C) Control or D) $Gad2^{VTA}$: Leu9´Ser mice in response to 0 (saline) (n = 10/group), 0.09 (n = 9/group), and 0.5 mg/kg nicotine (n = 8/group). C) Inset: Difference scores in the CPP assay in response to 0.5 mg/kg nicotine from WT mice previously exposed to six weeks of nicotine (n = 8) or vehicle (n = 8). *** $p < 0.001$ nicotine-paired chamber compared to saline-paired chamber, ^ $p < 0.05$ nicotine-paired chamber in dependent mice compared to nicotine-paired chamber in nicotine-naïve mice. ^^ $p < 0.01$ nicotine-paired chamber compared to saline in the drug-paired chamber. Two-way ANOVA, Bonferroni Post-hoc test.

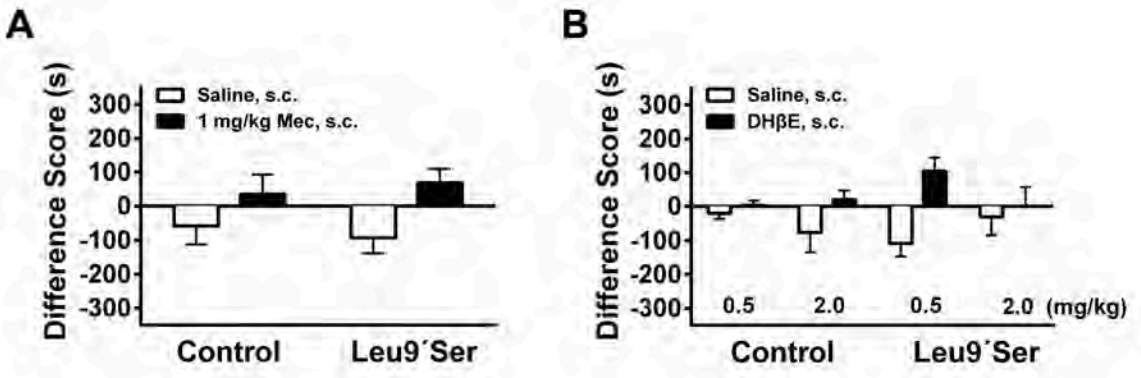

Figure II-4. nAChR antagonists do not condition a place preference in Gad2^{VTA}: Leu9´Ser mice. A) Difference scores in the CPP assay in response to 1.0 mg/kg mecamylamine in Control and Gad2^{VTA}: Leu9´Ser mice (n = 10/group). B) Difference scores in the CPP assay in response to 0.5 or 2.0 mg/kg DHβE in Control mice (n = 7) and Gad2^{VTA}: Leu9´Ser mice (n = 8 and 6, respectively).

Virus (Nic, mg/kg)	Sal (Pre, s)	Sal (Post, s)	Nic (Pre, s)	Nic (Post, s)
Control (Sal)	379.7±20.86	315.7±48.44	280.2±18.88	335.5±45.96
Control (0.09)	339±9.444	309.3±47.33	271.1±14.35	249.5±45.42
Control (0.5)	334.9±32.69	341.1±21.41	284.9±17.82	299.3±24.08
Leu9´Ser (Sal)	365.6±25.91	315.6±53.89	261.7±21.01	239.0-±34.97
Leu9´Ser (0.09)	347.6±21.93	205.9±27.43	257.1±17.7	437.9±37.28**,^^^
Leu9´Ser (0.5)	303.3±24.92	240.4±18.14	293±13.92	319.2±37.3
Leu9´Ser (0.5, Chronic Nicotine)	382±10.11	294.1±19.78	306.8±16.57	376.1±19.33*,^^^
Virus (Mec, mg/kg)	**Sal (Pre, s)**	**Sal (Post, s)**	**Mec (Pre, s)**	**Mec (Post, s)**
Control (1.0)	409.2±28.42	352.5±40.04	282.3±29.93	317.6±43.69
Leu9´Ser (1.0)	386.1±25.30	300.1±36.56	288.2±23.48	354.0±35.28
Virus (DHβE, mg/kg)	**Sal (Pre, s)**	**Sal (Post, s)**	**DHβE (Pre, s)**	**DHβE (Post, s)**
Control (0.5)	363.8 ± 31.05	342.9 ± 24.21	311.7 ± 30.39	315.1 ± 22.15
Control (2.0)	447.6±31.12	332.2±38.96	226.3±32.76	285.9±44.42
Leu9´Ser (0.5)	396.1±23.09	287.7±41.97	244±22.59	348.2±41.85
Leu9´Ser (2.0)	383.1±16.36	352±55.83	309.4±12.95	309.6±55.02
Treatment/Drug	**Sal (Pre, s)**	**Sal (Post, s)**	**Nic (Pre, s)**	**Nic (Post, s)**
Vehicle/Nicotine	367±20.3	328.5±23.48	268.8±18.67	263.7±26.09
Chronic Nicotine /Nicotine	400.2±29.79	263.2±23.39	261.1±15.01	379.6±26.02*,##

Table II-1. Total time spent (in seconds) in nicotine (Nic)- and saline (Sal)-paired chambers of the CPP assay pre-training (Pre) and post-training (Post).

* $p < 0.05$, **$p < 0.01$. Post-training compared to Pre-training, Two-way ANOVA with repeated measures (training and nicotine/saline treatment), Bonferroni post-hoc test. ^^^ $p < 0.001$ time spent in the nicotine-paired chamber post-training compared to time spent in the control nicotine-paired chamber (i.e., group that received saline in the "drug-paired" chamber). Two-way ANOVA with repeated measures (Pre/Post time spent in the Nic-paired chamber). Bonferroni post-hoc. ## $p < 0.01$, time spent in the nicotine-paired chamber post-training in nicotine-dependent animals compared to time spent in the nicotine-paired chamber in nicotine-naïve animals. Two-way ANOVA with repeated measures (Pre/Post time spent in the Nic-paired chamber). Bonferroni post-hoc.

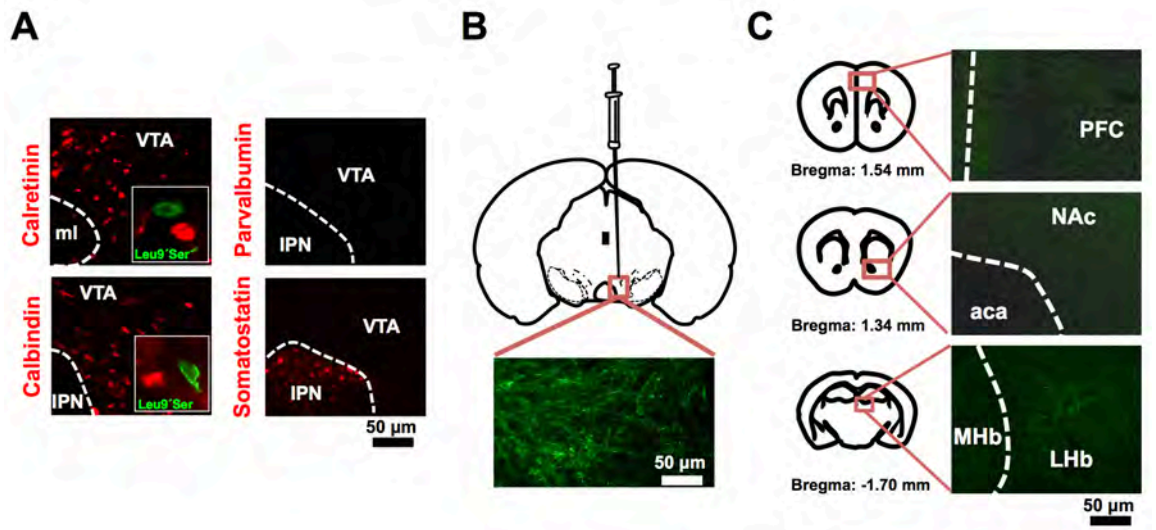

Figure II-5. GABAergic neurons mediating reward in Gad2^{VTA}: Leu9´Ser mice include projection neurons to the lateral habenula. A) Representative photomicrographs illustrating calretinin (top, left panel), calbindin (bottom, left panel), parvalbumin (top, right panel), and somatostatin (bottom, right panel) immunolabeling (red) in C57BL/6J midbrain sections. Insets depict distinct localization of calretinin or calbindin (red) compared to YFP expression (yellow) in Gad2^{VTA}: Leu9´Ser mice. B) Depiction of a coronal section from a Gad2^{VTA}: Leu9´Ser mouse used for analysis of GABAergic projections. The photomicrograph illustrates YFP signal in the VTA. C) Representative photomicrographs from cortical (top), striatal (middle) and epithalamic (bottom) sections from the Gad2^{VTA}: Leu9´Ser mouse in panel B. ventral tegmental area: VTA; interpeduncular nucleus: IPN; mammillary nucleus: ml; prefrontal cortex: PFC; nucleus accumbens: NAc; anterior commissure: aca; lateral habenula: LHb; medial habenula: MHb.

CHAPTER III.

EXPRESSION OF "GAIN-OF-FUNCTION" α4* NICOTINIC ACETYLCHOLINE RECEPTORS IN VTA GABAERGIC NEURONS DOES NOT INDUCE ANXIETY

CONTRIBUTIONS TO CHAPTER III.

This chapter is not published.

Author contributions

Ngolab J – Performed and designed experiments, analyzed data, prepared figures and wrote chapter.

Tapper AR and Gardner PD – helped with designing experiments and data analysis.

Molas SC – Edited chapter and provided insightful input

ABSTRACT

Nicotine withdrawal syndrome is a major contributing factor to smoking relapse. How chronic nicotine consumption changes the neural pathways involved in nicotine withdrawal symptoms such as anxiety remains unknown. Furthermore, chronic nicotine exposure results in the upregulation of high-affinity nicotinic acetylcholine receptors (nAChRs) in the GABAergic neurons of the VTA (Nashmi et al., 2007; Xiao et al., 2009; Renda et al., 2014) neurons implicated in eliciting negative affective behavior (Tan et al., 2012; van Zessen et al., 2012). Yet it is unknown whether nAChR upregulation promotes anxiety in the absence of nicotine. To test the hypothesis that overexpression of high-affinity, α4* nAChRs in VTA GABAergic neurons induces anxiety-like behavior, “gain-of-function” α4* nAChRs (Leu9´Ser α4-YFP) were expressed in VTA GABAergic neurons of nicotine naïve, adult Gad2-Cre male mice (Gad2^{VTA}: Leu9´Ser). A battery of anxiety-related behavioral tests was performed on Gad2^{VTA}: Leu9´Ser and control mice to assess for anxiety-related behavioral differences. The Gad2^{VTA}: Leu9´Ser mice demonstrated no significant changes in behavior from control in the Open Field, Elevated Plus Maze or Marble Burying Test. Together, these results suggest that upregulation of α4* nAChRs in VTA GABAergic neurons does not contribute to anxiogenic behaviors that accompany nicotine withdrawal.

INTRODUCTION

Despite reports that tobacco smoking reducing life expectancy by 10 years, about one in five Americans are active smokers (Jha et al., 2013). One of the barriers to successful smoking cessation is the emergence of negative affective withdrawal symptoms such as increased anxiety. The intensity of an affective withdrawal symptom experienced during abstinence predicts the likelihood to relapse (Piasecki et al., 2000). Furthermore, stressful imagery increased negative affect and nicotine craving in withdrawn smokers (McKee et al., 2011), suggesting that chronic nicotine exposure alters an individual's reaction to stress. How sustained nicotine consumption alters the brain to exaggerate anxiety symptoms during nicotine withdrawal is not well understood.

Nicotine binds onto the nicotinic acetylcholine receptors to modulate neuronal firing activity and ultimately alter behavior (Picciotto et al., 2008). These pentameric ligand-gated cation channels are expressed throughout the brain and modulate a multitude of processes, ranging from attention to learning (Cordero-Erausquin et al., 2000; D'Souza and Markou, 2011). Nicotine exhibits an inverted U-shaped response that is attributed to various factors such as dose administered, specific nAChR subtypes and receptor activation state (Picciotto, 2008; Di Biasi and Dani, 2011). Nicotine induced anxiety-like behaviors in rodents at sub-rewarding doses (Irvine et al., 2001a; Biala and Budzynska, 2006), while rodents withdrawn from chronic nicotine exhibited anxiogenic behavior (Damaj et al., 2003; Salas et al., 2004, 2009; Zhao-Shea et al., 2014).

However, it is not know if changes in nAChRs that occurs with chronic nicotine use are involved in nicotine withdrawal syndrome.

Chronic nicotine use induces upregulation of nAChRs in select areas of the brain including the Ventral Tegmental Area, although the contribution of these upregulated nicotinic receptors in nicotine withdrawal is unknown. Within the VTA, nicotinic receptors are upregulated in both DAergic and GABAergic neurons (Nashmi et al., 2007; Baker et al., 2012; Renda et al., 2014; Henderson et al., 2015). One of the predominant upregulated nAChR subunit combination, or subtype, is the $\alpha4\beta2$* subtype, which has been shown to play a substantial role in mediating reward in the mesocorticolimbic DAergic pathway (Tapper et al., 2004; Maskos et al., 2005; Pons et al., 2008; McGranahan et al., 2011). Previous research suggests that $\alpha4\beta2$* nAChRs are involved in anxiety-like behaviors (Ross et al., 2000; Yohn et al., 2014), but what brain regions are involved are unclear.

The ventral tegmental area, a region often associated with reward signaling and substance abuse, has also been implicated in aversion and anxiety behavior. Although DAergic neurons exhibit increased activity in response to rewarding stimuli, DAergic neurons are also activated with aversive stimuli (Joshua et al., 2008) and project to regions involved in aversion, such as the LHb (Lammel et al., 2014). Optogenetic stimulation of VTA DAergic projections in the PFC identified a subset of DAergic neurons induced anxiety-like behavior (Gunaydin et al., 2014) Furthermore, expression of corticotropin releasing factor (CRF), a neuropeptide involved in modulating stress, is increased in VTA DAergic neurons

during chronic nicotine exposure (Grieder et al., 2014). CRF expression in the VTA is necessary to incite anxiety-like behaviors during nicotine withdrawal through CRF type 1 receptors in the ventral IPN (Zhao-Shea et al., 2015). These data suggest that the VTA can contribute to the emergence of nicotine withdrawal symptoms.

The GABAergic subpopulation of the VTA is also implicated in negative affective behavior. Optogenetic activation of VTA GABAergic neurons disrupts sucrose consumption and conditions place aversion (Tan et al., 2012; van Zessen et al., 2012). Furthermore, inhibition of VTA GABAergic neurons disinhibits DAergic neurons (Bocklisch et al., 2013). VTA GABAergic neurons could contribute to withdrawal symptoms by inhibiting DAergic firing and resulting in an anhedonic state (Tye et al., 2012). While anhedonia does not directly cause anxiety it could coincide with anxiety-inducing behaviors and exacerbate the anxious state (Gold, 2015).

In the previous chapter, mice overexpressing “gain-of-function” Leu9´Ser α4* nAChRs in GABAergic neurons of the VTA (Gad2VTA: Leu9´Ser) exhibited increased conditioned place preference when given nicotine. It is possible the expression of Leu9´Ser α4* nAChRs on VTA GABAergic neurons induced mice into a hyperanxious state. Increased anxiety-like behavior was observed in knock-in mice expressing the Leu9´Ser α4 nAChR subunit (Labarca et al., 2001). The experiments in Chapter II may have inadvertently identified a VTA subpopulation contributing to anxiogenic behaviors. Administering a low dose of nicotine could possibly ameliorate the anxious state.

To assess if Leu9´Ser α4* nAChR expression in the VTA GABAergic neurons contributes to anxiety-like behaviors, Leu9´Ser α4* nAChRs were selectively overexpressed in GABAergic neurons of the VTA of Gad2-Cre male mice. These "gain-of-function" α4* nAChRs are meant to mimic upregulation α4* nAChRs in VTA GABAergic neurons that occurs with chronic nicotine use. Mice were assessed for anxiety-like behavior in the open-field test (OFT), the elevated plus maze (EPM) and the marble burying test (MBT). These tests were chosen because they measure spontaneous responses to a slightly aversive environment. Mice expressing Leu9´Ser α4-YFP* nAChRs in GABAergic neurons of the VTA exhibited a slight trend to be anxiolytic in all three tasks compared to control, without impacting locomotor activity. This suggests that α4 nAChR upregulation in VTA GABAergic neurons has no anxiogenic effects.

MATERIALS AND METHODS

Mice. Genetically engineered male mice containing Cre recombinase coding sequence inserted in the 3'-UTR of the Gad2 gene (B6N.Cg-*Gad2*$^{tm2(cre)/Zjh}$/J) from Jackson Labs (≥ 8 weeks) were used in this study. All mice were group housed, with up to 5 mice per cage, to mitigate anxiety caused by single housing. All animal handling and care was done in accordance to the University of Massachusetts Medical School IACUC.

Stereotaxic Injections Adeno Associated Virus serotype 2 viral particles packaged either with pAAV-zsGreen (control) or pAAV-Ef1a-DIO-L9´S-α4-EYFP

(Leu9´Ser) were obtained through the UMass Medical School Viral Vector Core. Construct engineering has been previously described (Chapter 2). Concentration of virus ranged from 1.0 to 2.5 X 10^12 vp/ml. Viral particles were stereotaxically injected into the VTA of Gad2-Cre mice according to coordinates provided by Franklin and Paxinos (-3.3 (AV); +/-0.3 (ML), -4.0 (DV)). After recovering from anesthesia, mice received an injection of 200 µl 5% glucose and 10 mg/kg ketoprofen to aid in recovery and reduce pain, respectively. Mice were kept under observation for 4 weeks after infection before used in any behavioral assay to allow for optimal gene expression.

Immunofluorescence. After the completion of an anxiety test, animals were anesthetized with 200 mg/kg sodium pentobarbital (intraperitoneal injection) and transcardiacally infused with 10 ml of chilled 0.1 M PBS following with chilled 4% (w/v) paraformaldehyde dissolved in 0.1 M PBS. The brains were harvested and placed in cold 4% paraformaldehyde for 2 h before submerging in 30% sucrose. Brains were then sectioned into 30-micron slices using a microtome (Leica, Buffalo Grove, IL, USA). An AxioCam MRm camera (Carl Zeiss Microscopy GmbH, Jena, Germany) attached to a Zeiss Axiovert inverted fluorescent microscope equipped with Zeiss filter sets 38HE, 49, and 20 was used to acquire fluorescent images. Zeiss objectives EC-Plan-NEOFLUAR 20x were used to view and capture images. Images were processed using Axiovision v.4.8.2. The VTA was located using morphology of nearby brain regions. Mice that did not express YFP fluorescence were excluded from analysis.

Open Field. Experiments were performed in the morning, around 10:00-11:00 am. One hour prior to testing, mice were placed into the experimental room to habituate to their surroundings. Mice were placed in the center of a 40 cm by 36 cm opaque plexiglass arena to assess overall locomotor activity and behavioral responses to open spaces. Before the test, light intensity was measured in the center and the periphery to assure that light intensity was the brightest in the center of the arena. The mouse was placed in the center of the field and allowed to freely explore for 15 minutes. The center of the open field was defined as a 20 cm by 18 cm field in the center of the arena through the Ethovision tracking software (Noldus, Wageningen, Netherlands), while the perimeter was defined as the remaining area around that field. Total distance moved, velocity and time spent in either the center or the perimeter was recorded through the Ethovision software. After each test, the arena was wiped down with 70% ethanol.

Elevated Plus Maze. To reduce anxiety from being placed in a novel environment, mice were placed in the experimental room one hour prior to experiment. A plus-shaped plexiglass platform, elevated 45 cm from the ground (Med Associates, St. Albans, VT) was used for this experiment. Two arms were enclosed with opaque plexiglass walls (30 x 5 x 15 cm), while the other two arms were left exposed (30 x 5 x 0.25 cm). Infrared photobeams were installed at each arm entrance to track entries and amount of time spend in each arm. A 60W fluorescent lamp was placed 100 cm directly over the center of the maze to

discourage time spent in the center. Mice were placed in the center of the maze and allowed to explore the apparatus for five minutes. Number of entries and total time spent in each arm was recorded using the MED-PC IV software (MED Associates, Inc.). The arms of the EPM were wiped clean in between trials.

Marble Burying Test. Two days prior to testing, mice were habituated each day for 45 min to a testing cage containing a 5-6 cm layer of bedding. Fifteen clean 1.5 cm glass marbles were placed on the testing cage floor in a 5 x 3 matrix, 4 cm apart from each other the day of testing. A mouse was placed into a cage and left to explore for 30 minutes. Mice were carefully removed from the test cage to not disturb test cage bedding after 30 minutes and returned to their home cage. Number of marbles buried was assessed, with a marble that was covered at least 2/3 of its depth by bedding being considered buried.

Statistical Analysis. All data sets were tested for normality before statistical analysis. All behavioral experiments were analyzed using two-tailed t-tests. Data was analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

To mimic upregulation triggered by chronic nicotine specifically in VTA GABAergic neurons, male Gad2-Cre mice were stereotaxically injected with viruses packaged with either control or Leu9´Ser constructs. After 4 weeks, both groups of mice were tested in the OFT, the EPM and the MBT. The OFT is a

locomotor activity-based assay that assesses anxiety-like behavior based on the amount of time a mouse spends in the open center versus the perimeter. There was no significant difference in time spent in the center ($p = 0.7121$, non-significant, n.s.) (Figure III-1 A) or time spent in the perimeter ($p = 0.6819$, n.s.) (Figure III-1 B) between $Gad2^{VTA}$: Leu9´Ser and control mice. There were no significant differences in locomotor activity between $Gad2^{VTA}$: Leu9´Ser and control as determined by total distance traveled ($p = 0.8588$, n.s.) (Figure III-1 C) and mean velocity ($p = 0.0651$, n.s.) (Figure III-1 D). These data indicate that increasing $\alpha 4$ nAChR expression does not alter novelty based locomotor activity.

The EPM is considered the standard test in measuring anxiety-like behaviors and the properties of potential anxiety therapeutics. It involves placing a mouse on a raised platform with two extending arms of the platform enclosed and two arms left exposed. A separate cohort of control and $Gad2^{VTA}$: Leu9´Ser was tested on the EPM for height-induced anxiety. Although there was a trend towards $Gad2^{VTA}$: Leu9´Ser mice spending more time in the open arms, there is no significant difference in time spent in the open arms ($p = 0.2168$, n.s.) (Figure III-2 A). There is no significant difference in time spent in the closed arms between $Gad2^{VTA}$: Leu9´Ser mice and control mice ($p = 0.1014$, n.s.) (Figure III-2 B). There is no significant difference in time spent in the junction between $Gad2^{VTA}$: Leu9´Ser mice and control mice ($p = 0.3147$, n.s.) (Figure III-2 C). To account for locomotor activity, total number of entries into each arm was tabulated. There were no significant differences between $Gad2^{VTA}$: Leu9´Ser and control mice in number of arm entries ($p = 0.0629$, n.s.) (Figure III-2 D).

To further compliment the previous anxiety tests, the Marble Burying Test was performed with a separate group of mice. This test is unique from the previous two tests it utilizes the natural tendency for mice to bury objects when stressed. Anxiety levels are assessed by the number of marbles submerged in bedding. There was a non-significant reduction of marbles buried in the Gad2VTA: Leu9´Ser mice compared to control mice ($p = 0.1108$, n.s.) (Figure III-2 E).

After the completion of an experiment, mice brains were assessed for Leu9´Ser α4-YFP or control expression in the VTA through fluorescent microscopy (Figure III-3). Mice that did not exhibit fluorescence in the VTA were excluded from the study.

DISCUSSION

The goal of this study was to investigate how overexpression of “gain-of-function” α4* nAChRs in VTA GABAergic neurons contributes to anxiety during nicotine withdrawal. Increased anxiety is a common affective symptom experienced by smokers during a period of nicotine withdrawal (Hughes, 2007). Upregulation of nAChRs occurs with chronic nicotine use (Staley et al., 2006; Nashmi et al., 2007; Muhkin et al., 2008; Henderson et al., 2015), yet it is unknown if upregulation contributes to nicotine withdrawal symptoms. Activation of VTA GABAergic neurons via optogenetic techniques induces aversion and disrupts appetitive behaviors (Tan et al., 2012, van Zessen et al., 2012), while selectively activating VTA GABAergic neurons with sub-rewarding doses of nicotine

increases reward sensitivity (Ngolab et al., 2015). Furthermore, mice expressing Leu9´Ser α4 nAChRs exhibited anxiety-like behaviors (Labarca et al., 2001).

To test whether overexpresion of α4 nAChRs specifically in VTA GABAergic neurons increases anxiety-like behavior, adult Gad2-Cre male mice transduced with a "gain-of-function" α4 nAChR subunit ($Gad2^{VTA}$: Leu9´Ser) or control virus in VTA GABAergic neurons were subjected to a series of anxiety-like behavioral tests. These tests were chosen because they measure non-conditioned, natural anxiety-like responses (Belzung and Griebel, 2001). Smokers can experience heightened anxiety from nicotine withdrawal without a conditioned response. The anxiety-like tests used in these experiments set two natural tendencies of mice in conflict with one another to measure anxiety-like responses. Mice naturally avoid bright open spaces but also explore novel places. The general consensus is that a mouse spending relatively more time in open, bright spaces is considered to be less anxious. There were no significant differences in anxiety-like behaviors in any of the three tests. Together, these data suggest that Leu9´Ser α4* nAChRs in VTA GABAergic neurons have no significant effect on inducing anxiety. These observations are in agreement with current literature, because α4KO mice exhibit heightened anxiety in the EPM (Ross et al., 2000), suggesting an anxiolytic role for α4* nAChRs. Indeed, anxiety responses were decreased in chronically nicotine treated mice when given an α4* nAChR partial agonist (Yohn et al., 2014). Although not significantly different, $Gad2^{VTA}$: Leu9´Ser mice exhibit anxiolytic tendencies during testing (Fig III-2A, 2E).

All three anxiety-like tests use the motility of a mouse, either to move to a certain area or displace bedding, as a proxy for anxiety. A major variable that can confound results is the overall locomotor activity of a mouse. In the OFT and EPM, no difference in locomotor activity was detected, therefore indicating neither the injection nor overexpression of Leu9´Ser α4* nAChRs damaged the locomotor circuitry. Acute administration of nicotine has been shown to elicit hypolocomotor effects, specifically through activation of α4* nAChRs on VTA GABergic neurons (Tapper et al., 2007; Ngolab et al., 2015). The major contributors of ACh to the VTA are from the laterodorsal tegmentum (LDTg) with 65% of these projections synapsing on to non-DAergic VTA neurons (Garzón et al., 1999). Stimulating LDTg projections to the VTA resulted in increased conditioned place preference (Lammel et al., 2012). Although "novel" environments may cause activation of VTA DAergic neurons (Schultz, 2010), perhaps the exposure to open fields does not provide a strong stimulus to induce acetylcholine release from LDTg neurons and induce hypolocomotion.

One major caveat of these studies is the low number of mice in several of the experiments. Low sample size reduces the statistical power and therefore decreases the possibility of detecting a true effect (Button et al., 2013). Although none of the results were statistically significant in this chapter, the low power due to sample size increases the probability of false negatives confounding the interpretation of these results. Increasing the sample size to a set number determined by power analysis would allow for a statistically robust assessment of the data.

It is possible that Leu9´Ser α4* nAChRs expressed in Gad2^{VTA}: Leu9´Ser mice are not functional. Leu9´Ser α4* nAChRs delivered through this system have been previously characterized to be functional with low doses of nicotine (Chapter II). All mice that were used in this study were verified for Leu9´Ser α4 expression via YFP immunofluorescence. To further improve on this assessment, quantifying the number of YFP(+) GABAergic neurons infected within the VTA would further determine the contribution of the Leu9´Ser nAChR subunit to the behavioral changes.

Recent studies have shown that other pathways are active in the expression of nicotine withdrawal symptoms. The habenulo-peduncular pathway has been highly implicated in the somatic and affective withdrawal symptoms (Salas et al., 2004, 2009; Zhao-Shea et al., 2013, 2015). Furthermore, a recent study indicates that CRF from the VTA can influence the activity of the IPN, a critical nucleus for the expression of nicotine withdrawal symptoms (Zhao-Shea et al., 2015). It has been hypothesized that nicotine dependence is the result of neurological adaptations that occur to facilitate drug consumption (Koob and Moal, 2005; D'Souza and Markou, 2011). There are other neuroadaptations that occur with chronic nicotine, such as increased CRF production in DAergic neurons (Grieder et al., 2014). It is possible that increasing activation of α4* nAChRs on VTA GABAergic neurons through upregulation could increase inhibition of DAergic neurons. Inhibition of DAergic neurons by VTA GABAergic neurons could possibly trigger CRF production and release. Additionally, these data suggest possible segregation of nAChR subunits involved in nicotine reward

and withdrawal, or at least neuronal subpopulations within specific regions that could be involved in withdrawal. VTA GABAergic neurons may not specifically contribute to withdrawal through α4* nAChRs, and it could be that upregulation of these α4* nAChRs could be a compensatory mechanism in response to the overactivation of pathways involved with withdrawal, such as the habenula-peduncular pathway. Understanding the dynamics behind nicotinic receptor upregulation may help with developing efficacious therapies.

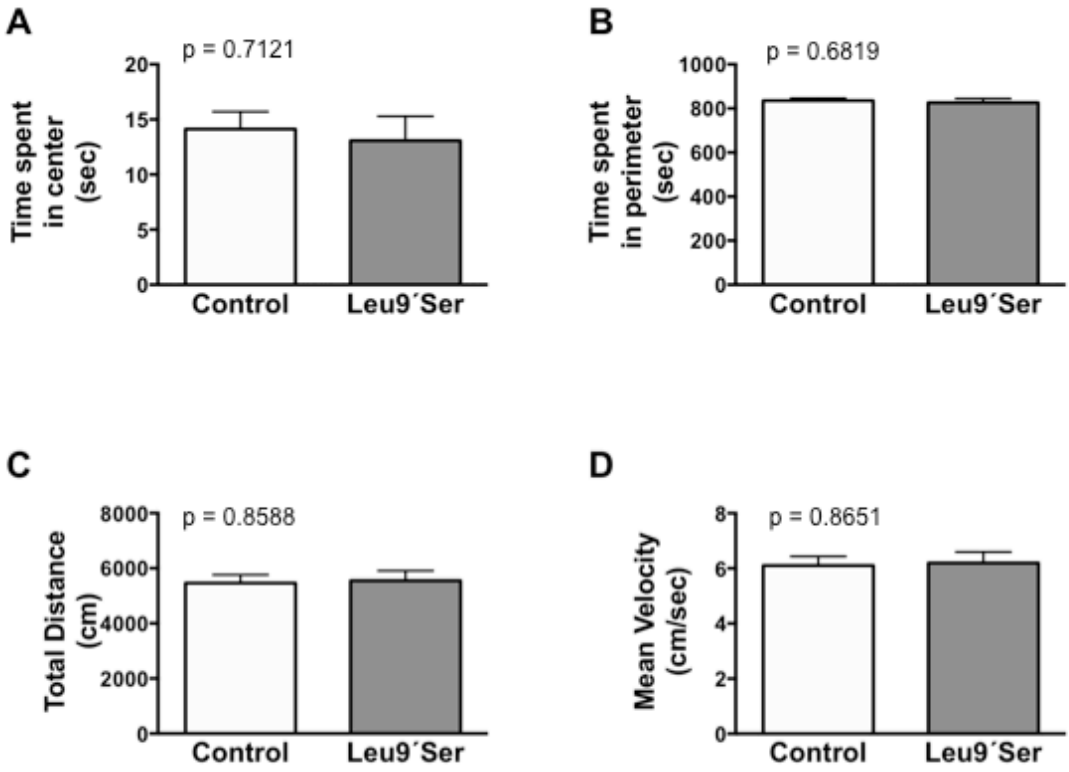

Figure III-1. Expression of the Leu9´Ser α4 nAChR subunit does not hinder locomotor activity. A) Total amount of seconds control (n = 5) or Gad2^{VTA}: Leu9´Ser (n = 5) mice spend in the center of the open field arena. B) Time spent in the periphery C) Total distance mice moved throughout the test, in centimeters. D) Mean velocity of mice, calculated as centimeters per second. Error bars are s.e.m.

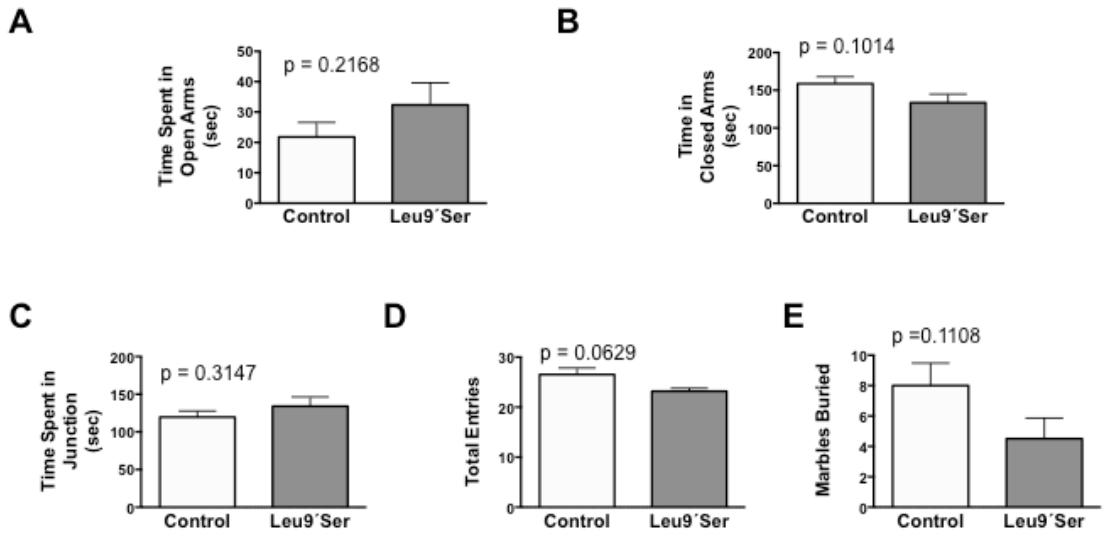

Figure III-2. Gad2VTA: Leu9´Ser mice do not exhibit any overt changes in anxiety-like behavior compared to control mice. A) Total time in seconds $Gad2^{VTA}$: Leu9´Ser (n = 11) and control mice (n = 16) spent in the Open Arms of the Elevated Plus Maze. B) Total time in seconds spent in the Closed Arms of the Elevated Plus Maze. C) Total time spent in the junction. D) Total entries into either closed or open arms. E) Number of marbles buried by control (n = 6) and $Gad2^{VTA}$: Leu9´Ser (n = 8) mice. Error bars are s.e.m.

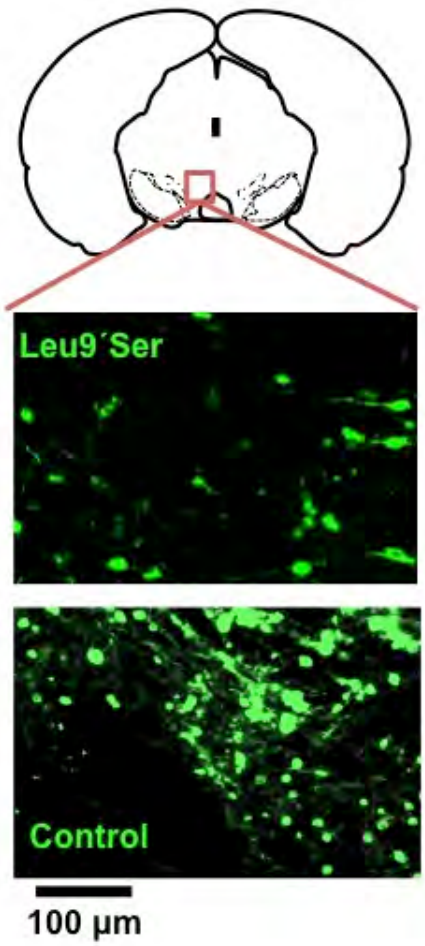

Figure III-3. Verifying viral-mediated gene expression in mouse brains. Depiction of a coronal section from mice used in anxiety tests. Representative pictographs illustrate YFP signal from (top) $Gad2^{VTA}$: Leu9´Ser and (bottom) control mice.

CHAPTER IV.

DISCUSSION

Chronic nicotine exposure incites a multitude of compensatory modifications within the brain to facilitate persistent nicotine use. One of these neuroadaptations is the upregulation of nAChRs, the ligand-gated cation channel that nicotine binds onto to incite nicotine tolerance. Behavioral studies using nicotine-withdrawn mice observed changes in consumption behavior, reward sensitivity and anxiety (Irvine et al., 2001; Hilario et al., 2012; Renda et al., 2014). Of importance, chronic nicotine leads to the upregulation of α4 nAChR subunits, a subunit critical in nicotine-reward behaviors, (Flores et al., 1992; Tapper et al., 2004; Pons et al., 2008; McGranahan et al., 2011) particularly in midbrain GABAergic neurons (Nashmi et al., 2007; Xiao et al., 2009; Renda and Nashmi, 2014). However, it is not clear whether upregulated α4* nAChR are acted upon, for increased receptor binding could reflect the binding of nAChR in a high affinity yet desensitized conformation (Quick and Lester, 2002; Picciotto et al., 2008). Several studies suggest that chronic nicotine treatment brings forth increased nAChR activation, (Wonnacott, 1990; Vallejo et al., 2005; Srinivasan et al., 2011) yet the role of activation of α4* nAChR on VTA GABAergic neurons in nicotine dependence behaviors is unclear.

To gain insight into how VTA GABAergic-specific upregulation of α4* nAChR contributes to behaviors involved in nicotine dependence we developed a Cre-regulated, viral-mediated technique to selectively express an α4-YFP nAChR subunit containing a 9´ leucine to serine point mutation in the TM2 domain (Leu 9´ Ser) (Labarca et al., 1995; 2001). Mice with mutations at the 9´ position of TM2 in an nAChR alpha subunit are hypersensitive to agonist, and have been

used to understand the contributions of a nAChR subunit to neuronal activity and overall behavior (Labarca et al., 2001; Fonck et al., 2003, 2005; Orb et al., 2004; Tapper et al., 2004, 2007; Drenan et al., 2008; Drenan and Lester, 2012). The VTA contains a heterogenous population of neurons that when activated can elicit either rewarding or aversive behavior (Tsai et al., 2009; Lammel et al., 2012, 2015; Gunyadin et al., 2014). Nicotine exhibits an 'inverted U' dose response, in that different concentrations trigger opposite behaviors (Picciotto, 2008), providing a challenge in developing effective therapies for smoking cessation. Therefore, understanding how nicotine affects the GABAergic subset of VTA neurons would help further discern the contributions of this subpopulation to nicotine dependence.

We hypothesized that direct activation of VTA GABAergic neurons through upregulated α4* nAChRs would elicit aversion, based on previous studies that activated VTA GABAergic neurons through optogenetic stimulation (van Zessen et al., 2012, Tan et al., 2012). Sub-threshold doses of nicotine sufficient to activate VTA GABAergic neurons that expressed functional Leu9´Ser α4* nAChRs induced nicotine hypolocomotor activity and increased nicotine reward sensitivity (Chapter II). Previous studies observed in mice withdrawn from chronic nicotine exhibited increased nicotine place preference and consumed more nicotine-laced water which coincided with increases in nAChR binding and α4* nAChR expression (Hilario et al., 2012; Renda and Nashmi, 2014). Our data imply direct activation of upregulated α4* nAChR VTA GABAergic neurons increased the rewarding properties of nicotine during a period of withdrawal.

Furthermore, the overexpression of α4* nAChRs had no effect on anxiety-like behaviors often associated with nicotine withdrawal.

The technique described within this thesis provides a novel method into studying the contributions of α4* nAChRs in a cell-specific manner. This is the first time to my knowledge that overexpressing a nAChR subunit in adult mice expressing endogenous α4 nAChR subunits has been done. One advantage of this technique over transgenic and knockout mice is the ability to bypass developmental issues brought forth by the manipulation of the α4 nAChR subunit (Muller et al., 1999; Picciotto and Wickman, 1998; Champtiaux and Changeux, 2004). Although the technique holds tremendous potential, several technical issues must be addressed to appropriately interpret these results. These issues include finding a reliable marker to identify VTA GABAergic neurons, quantifying GABAergic neurons expressing α4* Leu 9′ Ser nAChRs as well as ensuring that the α4* Leu 9′ Ser nAChRs are expressed within the pVTA.

VTA GABAergic neurons make up 20-35% of the total VTA population (Margolis et al., 2006; Yamaguchi et al., 2007; Nair-Roberts et al., 2008; Chieng et al., 2011). The Gad2-Cre mouse driver line, previously used to target GABAergic neurons (Tan et al., 2012; Taniguchi et al., 2011), was used in these studies to ensure the greatest amount of VTA GABAergic neurons were infected. Glutamic acid decarboxylase (Gad) is the enzyme involved in the production of GABA and comes in two isoforms, Gad1 and Gad2 (Gad 67 and Gad 65, respectively) (Soghomonian and Martin, 1998). Cre expression was restricted to the majority of GABAergic cells in the Gad2-Cre driver line and colocalized with 90% of

Gad1^{+} cells (Taniguchi et al., 2011). Indeed, the Gad2-Cre mouse driver line restricted Leu9´Ser α4* nAChRs expression to non-DA, GAD1/2 neurons (Chapter II).

It has recently come to light that mouse Cre-driver lines may not confer cell-specificity within the VTA (Lammel et al., 2015; Stuber et al., 2015) due to the heterogeneous nature of the VTA. For example, there are small populations of TH^{+}VGluT2^{+} that make up a small fraction of the VTA (Yamaguchi et al., 2015), although studies using a VGluT2-Cre driver line suggest the percentage of VTA glutamatergic neurons is higher than expected (Hnasko et al., 2010; Tecuapetla et al., 2010). VTA TH^{+}Gad1/2^{+} neurons also have been identified that send inhibitory projections to the LHb (Stamatakis et al., 2013), but later defined as GABAergic (Stuber et al., 2015). The TH^{+}Gad1/2^{+} positive neurons found within the VTA released GABA but not DA (Stamatakis et al., 2013); therefore, if TH^{+}GAD^{+} neurons expressed Leu9´Ser α4-YFP* nAChRs in the Gad2-Cre driver mice, GABA but not DA would likely be released. Although these observations occurred in a different mouse driver line, this does not necessarily confirm that the Gad2-Cre driver line expresses nAChRs exclusively in GABAergic neurons. Notably, astrocytes can express Cre in the Gad2-Cre driver line (Taniguchi et al., 2011). Although not widely studied, nicotine can induce increases in intracellular Ca^{++} through activating α4* nAChRs on astrocytes (Oikawa et al., 2005). Not much is known about how non-neuronal cells contribute to VTA function and nicotine reward, and further studies would reveal another layer of complexity within the VTA circuitry.

The number of cells infected may not properly reflect the number of cells that actually express GABA. Cre-driver mouse lines were used to identify subpopulations of neurons that express a certain gene (Taniguichi et al., 2011). However, the presence of transcript does not necessarily indicate protein expression as observed in TH-Cre mice (Stamatakis et al., 2013; Yamaguchi et al., 2015). Further tests to measure protein production or transmitter release should follow these studies to ensure the appropriate subpopulation is assessed. Cre-driver lines may provide an overestimate of the percent of neurons releasing a particular neurotransmitter, for low levels of Cre recombinase may be necessary to initiate homologous recombination and the expression of Cre-restricted genes. Although the cell population may not produce enough GABA for vesicular release, the production of Cre recombinase would distinguish it as a GABA producing cell. This would increase the number of false positives and not appropriately identify the subpopulation's function. The Gad2-Cre driver line was generated through inserting the Cre coding cassette into the Gad2 gene, so it is unlikely that Gad2 expression differ from Cre expression (Taniguichi et al., 2011). Like all genetically engineered mice, Cre-driver mouse lines are susceptible to developmental issues that affect adult behavior. Transgenic ChAT-Cre mice demonstrated severe cognitive deficits compared to their control line due to overexpression of vescicular acetylcholine transporter (Kolisnyk et al., 2013).. One of the objectives of performing the experiments in Chapter III was to identify differences in baseline behaviors that could ripple into other behavioral assays. In my hands, I did not observe any overt differences in anxiety-like behavior

(Chapter III). However, these observations may be premature and more tests need to be performed to confidently conclude that the Gad2-Cre driver line has no deficits in the behaviors performed in this thesis.

Production of the Leu9´Ser α4 nAChR subunit is driven by the constitutive promoter EF1a. This strategy allows for the robust expression of the Leu9´Ser α4 nAChR in order to drive the incorporation of the subunit into a nAChR. Overexpressing the nAChR subunit may override protein degradation mechanisms that would prevent the Leu9´Ser α4 nAChR from being expressed. However, the continuous expression of Leu9´Ser α4 nAChR subunits may not reflect endogenous nAChR overexpression instigated by nicotine or allow for transcriptional regulation. Furthermore, overexpression may initiate unforeseen issues, such as non-physiological nAChR combinations (subtypes), cell death and ectopic expression of nAChRs in a neuron.

Expression of “gain-of-function” nAChRs can be lethal in mice homozygous for the mutant nAChR (Labarca et al., 2001; Orr-Urtreger et al., 2000). Studies done in mice expressing the Leu9´Ser α4 nAChR suggest that the number of Leu9´Ser α4 nAChR subunits within a nAChR decreases overall viability (Labarca et al., 2001; Fonck et al., 2003). However, it is hypothesized the overall percentage of nAChRs containing 2 or more Leu9´Ser α4 nAChR subunits is ~4% (Fonck et al., 2003). This implies that there are additional regulatory mechanisms regulating nAChR subunit integration into a nAChR. The baseline electrophysiological characteristics recorded from Gad2^{VTA}: Leu9´Ser brain slices exhibit similar properties found in VTA GABAergic neurons, suggesting Leu9´Ser α4 nAChR

subunit expression does not perturb overall function (Chapter II). Preliminary studies using a construct containing a Cre-restricted, unmutated α4-YFP nAChR subunit in TH-Cre driver mouse line suggests that overexpression of this subunit does not affect cell viability. Follow-up studies quantifying overall number of VTA GABAergic neurons in Gad2-Cre Leu9´Ser and Gad2-Cre α4 mice would further distinguish whether the behavioral changes are due to viable GABAergic neurons expressing the Leu9´Ser -YFP subunit and not to the lack of GABAergic cells. TUNEL staining or other cell death assay would further determine the overall cytotoxic properties of the Leu9´Ser -YFP nAChR subunit.

Overexpression could lead to the formation and expression of non-physiological α4* nAChR subtypes. *Xenopus* oocytes expressing nAChRs Leu9´Ser α4 nAChRs subunits and wildtype β2 subunits were sensitive to choline, a byproduct of nicotine metabolism that activates α7 nAChRs, at physiological concentrations (Alkondon et al., 1997; Labarca et al., 2001). This could affect studies that require subsequent nicotine administration, such as nicotine tolerance and nicotine CPP. The same study measured the effects of choline in neuronal progenitor cells from mice heterozygous for the Leu9´Ser α4 nAChR subunit and observed smaller currents activated by choline than seen in cells from wildtype mice (Labarca et al., 2001). Furthermore, the presence of WT α4 nAChRs may prevent choline-mediated activation in my system, as it is unknown whether choline sensitivity is dependent on the number of Leu9´Ser α4 nAChR subunits in a nAChR. Studies in *Xenopus* oocytes reported that Leu9´Ser α4* nAChRs exhibited a decrease in the rate of desensitization (Revah et al., 1995; Labarca et

al., 1995). This may ultimately affect the baseline activity of nicotine-mediated GABAergic activation that may not reflect the characteristics of nAChRs in the $(\alpha4)_2(\beta2)_3$ conformation. More thorough analysis in measuring the rate of desensitization of VTA GABAergic neurons expressing Leu9´Ser -YFP nAChRs through biophysical techniques would address these issues.

Neuronal nAChRs can be found both on the soma as well as presynaptic dendrites and terminal boutons (Laviolette and van der Kooy, 2004; Gotti and Clementi, 2004; Changeux, 2010). Chronic nicotine induces an increase of $\alpha4$-YFP* nAChRs particularly in the dendrites over the soma of primary neuronal cell cultures (Nashmi et al., 2004) suggesting that upregulation preferentially occurs in certain areas of the neuron. Overexpression of the Leu9´Ser $\alpha4$ nAChR subunit can promote the expression of non-physiological Leu9´Ser $\alpha4$* nAChRs expressed throughout the neuron. Activation of Leu9´Ser $\alpha4$* nAChRs in GABAergic terminals located in the NAc could lead to the inactivation of inhibitory inputs and incite DA transmitter release which may explain reward signaling despite low number of TH+/c-Fos+ cells within the VTA (Chapter 2), which may or may not be caused by upregulation of nAChRs. Although the goal of these studies was to uncover possible roles of $\alpha4$* nAChR-mediated activation in VTA GABAergic neurons, overexpression may mask the subtle contributions nAChRs have on overall neuronal membrane potential.

Overall increase of available $\alpha4$ nAChR subunits could shift the overall dose response independent of the “gain-of-function” mutation. The amount of $\alpha4\beta2$* nAChRs expressed is dependent on the internal pool of $\beta2$ nAChR subunits

contained in the endoplasmic recticulum that is regulated by proteasome degradation. Indeed, upregulation of $\alpha4\beta2$* expression is dependent on the reduction of $\beta2$ nAChR degradation (Darrow et al., 2005; Rezvani et al., 2009; Govind et al., 2012). Furthermore, it is thought that nicotine acts as a chaperone to initiate the formation of high-sensitivity nAChRs, so despite increasing the potential pool these nAChRs are regulated by the presence of nicotine (Srinivasan et al., 2010). It is possible that overexpression of $\alpha4$-YFP subunits could lead to the formation $(\alpha4)_3(\beta2)_2$ nAChRs. Nicotinic receptors in this stoichiometry are considered low sensitivity (Nelson et al., 2003). Therefore, overexpression would result in the expression of low-sensitivity nAChRs, which is contrary to what was observed with c-Fos immunoreactivity and whole-cell patch clamp recordings (Chapter II). Furthermore, *Xenopus* oocyte studies indicate that at least one Leu9´Ser nAChR variant is sufficient for increasing sensitivity to agonist (Labarca et al., 1995). Therefore, the possibility that increasing nAChR expression leads to the abundance of low sensitivity $(\alpha4)_3(\beta2)_2$ is bypassed by using the Leu9´Ser nAChR variant. In fact, more Leu9´Ser $\alpha4$* nAChRs further helps to selectively activate VTA GABAergic neurons. Regardless, expressing a non-mutated, Cre restricted $\alpha4$ nAChR subunit would address potential shifts in the dose response based on increased receptor number or altered subunit composition and serve as a physiological control rather than ChR2-YFP. Radioligand binding studies could also assess whether an increase of $\alpha4$ nAChR expression in VTA GABAergic neurons leads to an increase of the number of $\alpha4$* nAChRs. To further mitigate concerns due to overexpression, using a moderate

expressing promoter to drive Leu9´Ser -YFP expression (to reflect endogenous expression levels and upregulation of α4* nAChRs) would be preferable to the strong promoter used here. Refinement of this approach, by using a construct producing more modest levels of expression, using a Cre-dependent WT (L9´L) α4 receptor as control, and more riogorous quantitation of the number of cells infected in each animal will greatly improve the ability to draw conclusions from studies such as those presented here.

Because this is first time this technique has been implemented, detection of YFP fluorescence in VTA GABAergic neurons was deemed sufficient to validate that animals had hypersentive receptors and were appropriate to examine for changes in behavior. Injections were aimed towards the pVTA, due to previous studies suggesting that various drugs of abuse, including nicotine, act in a sub-region specific manner to induce reward (Zangen et al., 2002; Rodd et al., 2005; Ikemoto et al., 2006; Zhao-Shea et al., 2011). The intent of these studies was to investigate how selective activation of α4* nAChRs affects VTA GABAergic neurons and downstream behaviors. Although the AAV2 serotype is thought to exhibit minimal spread, animals with YFP expression throughout the whole VTA were included in data analysis. The VTA is a relatively small region and often YFP expression was found both in the aVTA as well as the pVTA. Mice that did not exhibit detectable YFP fluorescence were classified as “sham” injections and excluded from the study. Mice that were injected in areas outside of the VTA such as the substantia nigra were included only if there was YFP expression in the VTA; otherwise, the mouse was excluded from further behavioral studies.

These criteria, although minimal, serve as a foundation for future studies to improve upon this technique. In future, it should be possible to distinguish the contributions of the aVTA from the pVTA. GABAergic neurons within each subregion can elicit vastly different responses to drugs such as ethanol (Guan et al., 2012). Therefore, expression of “gain-of-function” nAChRs in both GABAergic neuronal subpopulations may obfuscate interpretations. However, these differences in the optogenetic studies and my studies could be explained by innervation of these particular subregions. Quantifying how many YFP neurons are expressed in the pVTA versus the aVTA, and correlating these numbers with the behavioral responses of individual animals would provide insight to this question.

Additional experiments in quantifying expression would further support the efficacy of this technique. Although one study utilizing Cre-driver mice has indicated small populations can induce behavioral changes (Brown et al., 2012), it would be useful to quantify the number of GABAergic neurons needed to increase nicotine reward sensitivity. Several caveats in the system have precluded this assessment, which are discussed below along with suggestions on how to address these issues.

The identification of GABAergic neurons within the VTA is not trivial. The VTA is a heterogenous population that has been undercharacterized until recent tools were developed to delve into the neural circuitry (Lammel et al., 2015). These tools highlighted previously neglected differences observed with the criteria established for identifying different VTA subpopulations. Although antibodies are

available to detect GABAergic markers, few quantification studies were done utilizing immunohistochemistry, and markers used to identifiy cortical GABAergic neurons may not overlap with VTA GABAergic neurons (Olsen and Nestler, 2007). Mouse studies that quantify VTA GABAergic neurons use markers expressed on presynaptic terminals assumed to synapse primarily on GABAergic neurons, which may highlight a subset of VTA GABAergic neurons (Van Zessen et al., 2012; Tan et al., 2012), or Gad67-GFP mice (Chieng et al., 2012, Taylor et al., 2014). Cre recombinase is driven by the Gad65 promoter and could be used as an additional marker for GABAergic neurons. However, in my hands, I have not been able to detect Cre recombinase expression using immunofluorescence. Previous studies have verified the expression of Cre recombinase in the Gad65-Cre mouse driver line, so Cre recombinase is expressed at levels sufficient to activate floxed inverted constructs but not high enough to detect through immunofluorescence (Taniguichi et al., 2012). Previous VTA GABAergic quantification studies utilized in situ hybridization (Yamaguchi et al., 2007; Nair-Roberts et al., 2008), which can be coupled with fluorescence detection. Overall, improving techniques in detecting VTA GABAergic neurons would help with appropriate quantification of Leu9´Ser infected neurons.

Tracking Leu9´Ser α4 nAChR subunit expression via the YFP fused to the intracellular loop provides a direct method to track subunit expression and improves on previous studies that relied on lentiviruses and radioligand binding to verify α4 expression (Maskos et al., 2005; Pons et al., 2008; Exley et al., 2011). In my hands, when detected, Leu9´Ser expression was similar to what is

observed in mice expressing α4* nAChRs tagged with YFP (α4-YFP) (Nashmi et al., 2007; Renda et al., 2014). However, detecting YFP fluorescence has been troublesome at times and others have reported struggling to detect YFP fluorescence from α4-YFP mutant subunits despite observing substantial effects with nicotine (R. Loring, personal communication).

One explanation for poor YFP detection is amount of YFP protein expressed is insufficient for detection through standard fluorescent microscopy. The expression of one nAChR subunit containing the Leu9´Ser mutation was sufficient to drastically increase the EC50 of an overall nAChR compared to non-mutated nAChRs in *Xenopus* oocytes (Labarca et al., 1995). Therefore, strong YFP fluorescence may not be detected in cells despite a significant change in behavior. Our microscopy equipment is not equipped to detect fluorescence at single receptor resolution, and using equipment with higher resolution may help with locating YFP expression. Detection and quantification of α4-YFP was done using the α4-YFP expressing mice in conjunction with advance microscopy techniques, in that YFP expression was abundant (Nashmi et al., 2007, papers discussing the technique). Furthermore, YFP emission is prone to diffusing in the brain, leading to poor fluorescence signal (cite). Other studies using the α4 nAChR-YFP mice have resorted to immunostaining for YFP and detecting with the Alexa 594 fluorophore (Bailey et al., 2013). In my studies, immnunostaining for YFP using an Alexa 488 fluorophore improved detection (Chapter 2) and future studies could follow suit, albeit reducing the convenience the fluorescent protein directly attached to the nAChR subunit. An HA tag was also inserted into

the M3-M4 nAChR intracellular loop and serve as an alternative marker of exogenous α4 nAChRs either through immunofluorescence or western blot (Nashmi et al., 2004).

In contrast with previous studies, I used adeno associated virus (AAV), a class of small icosahedral parvoviruses, to mediate gene delivery. AAV is ideal to use in non-dividing cells such as neurons for long-term gene expression (Lentz et al., 2012; Wu et al., 2010). Yet, one of the limiting factors of AAV is the size of the packaged genome containing the transgene as well as other elements to induce and improve gene expression. AAV packaged with genomes exceeding 5.0 kb were sheared at the 5´ end of the genome resulting in the reduction of expression efficiency (Wu et al., 2010). Although I observed an increased current in YFP+ neurons with nicotine, the overall size of the expression cassette was 5.8 kb (Chapter II). Therefore, expression of YFP could be reduced, attributed to technicalities due to viral packaging. Several options are available to overcome issues due to viral packaging. Decreasing the genome size by reducing the size of the promoter or other regulatory elements without sacrificing expression would address this issue (Choi et al., 2014). Changing the serotype of the adenovirus to AAV5, which is reported to have better expression in neurons and can package genomes up to 8.9 kb, could considerably improve transgene expression (Allocca et al., 2008). Switching viral vectors to lentivirus would allow for genomes up to 8 kb to be packaged into a viral particle (Lentz et al., 2012). Furthermore, altering the design of the construct to take into account neurons that are infected but do not express Cre recombinase, as was done in

Tolu et al., 2012, would further help assess the percentage of cells infected with virus and provide a better measure of infection efficiency.

In light of this, YFP fluorescence may not fully account for the amount of Leu9´Ser α4* nAChRs expressed within the brain. In fact, measuring Leu9´Ser expression through YFP fluorescence may be underestimating overall expression. This has serious implications for results in Chapter II using YFP as an indicator of Leu9´Ser-α4*-expressing neurons and as a tracer. Although YFP fluorescence is detected in the LHb in Gad2^{VTA}: Leu9´Ser mice, this should not be interpreted as the only projection. It is entirely possible that Leu9´Ser α4* nAChRs are expressed on VTA GABAergic projections to the NAc and the PFC, but the low amount of Leu9´Ser necessary to activate these regions may be below the limit of detection. A cursory analysis of Gad65 and Gad67-Cre mice brains infected with doubly floxed ChR2-YFP in the VTA identified a greater amount of YFP fluorescence in the LHb compared to the NAc and the PFC (Taylor et al., 2014), suggesting a substantial population of VTA GABAergic neurons project to the LHb. The amount of projections to the LHb may explain why Leu9´Ser -YFP projections were detected in the LHb over PFC and NAc (Chapter II).

One may also question whether the Leu9´Ser α4 nAChR subunit is expressed solely in VTA GABAergic neurons if YFP detection is unreliable. Expression of the Leu9´Ser α4 nAChR subunit at low levels in DAergic neurons would explain why increased reward was observed in Gad2VTA: Leu9´Ser mice. Preliminary studies in HEK293 cells indicate co-transfection of Cre Recombinase is

necessary for Leu9´Ser -YFP expression. If Cre Recombinase was not necessary, YFP expression should be detected in cells not transfected with Cre Recombinase. Furthermore, if expression of the Leu9´Ser α4 nAChR subunit in DAergic neurons did not result in excitotoxicity, then I hypothesize that 0.09 mg/kg nicotine would induce more TH+ C-Fos+ neurons in Gad2^{VTA}: Leu9´Ser mice based on previous studies utilizing mice expressing hypersensitive α4* nAChR subunits (Hendrickson et al., 2010; Zhao-Shea et al., 2012). Although the mutation may be different, the overall result is the expression of "gain-of-function" α4* nAChRs. Improving detection of the Leu9´Ser α4 nAChR subunit would further address this issue.

Because of variable expression of the YFP tag and the difficulty to detect GABAergic neurons, it has been difficult to quantify VTA GABAergic neurons that express the Leu9´Ser subunit through immunoreactivity in brain tissue. The method developed to quantify α4-YFP expression in VTA GABAergic neurons developed by Nashmi and colleagues (2007) would assist in YFP detection. Using alternative markers or additional antibodies to boost fluorescence signal may also aid in detection. However, quantification through brain slices can increase the number of false positives. As an alternative, Fluorescence Activated Cell Sorting (FACS) could assess the percent of VTA cells expressing the Leu9´Ser subunit through YFP or HA fluorescence. FACS has been successfully utilized to analyze neuronal cell populations (Guez-Barber et al., 2011, 2012; Schwarz, 2015). This technique sorts a heterogenous population into homogenous groups based on size and fluorescent expression. Even using

one fluorescent marker such as HA would provide a HA+ and HA- homogenous population that can be analyzed by fluorescence intensity further analyzed for differences in gene expression. One major drawback is the large number of cells needed to do this analysis and therefore the large number of animals needed to generate a sufficient amount of data. Related to this drawback, it would be difficult to analyze small regional subpopulations if the whole VTA is extracted for analysis. However, FACS would provide a better way to identify potential genetic markers within a heterogenous population such as the VTA, including potential VTA GABAergic markers. Coupling of laser-capture microscopy for sample collection and gene analysis methods refined for assessment of very small samples (e.g., single-cell PCR) would also improve the functional characterization of VTA subregions.

Expressing the Leu9´Ser nAChR in Gad2-Cre, α4 null mouse in order to rescue α4 nAChR expression may improve YFP detection by allowing the Leu9´Ser - YFP subunit to be incorporated into the receptor without competion from endogenous α4 expression. This approach also address the potential concerns about exogenous nAChRs expressed due to the abundance of α4 nAChRs discussed earlier. This approach improves upon the studies done by Maskos et al., 2005 and Pons et al., 2008 by further studying how cell-type selective rescue affects behavior. Indeed, previous studies re-expressing the β2 nAChR subunit specifically in DAergic or GABAergic of β2 KO mice have found that expression of the subunit is important for shaping burst firing activity in DAergic neurons (Tolu et al., 2012). Knocking out α4 nAChR subunits specifically in DAergic

neurons decreased nicotine reward but not overall reward, implicating the α4 nAChR subunit as important in nicotine reward behavior (McGranahan et al., 2012). However, similar to what is observed in DAergic neurons of Leu9´Ser knock-in mice, exclusively re-expressing the Leu9´Ser nAChR variant may reduce the viability of GABAergic neurons. It is possible that endogenous α4 expressed in the Gad2-Cre mice could be buffering the cytotoxic effects brought forth by the Leu9´Ser subunit (Fonck et al., 2003). A separate control group of mice expressing the non-mutated α4-YFP nAChR subunit in VTA GABAergic neurons would further determine whether the effects observed with nicotine are due to re-expressing α4 nAChRs or to the "gain-of-function" variant. This would be an excellent experiment to compliment the observations in the previous chapters and provide more evidence on how VTA GABAergic neurons, through α4* nAChRs, affect behavior.

Studies utilizing mice containing a leucine to alanine point mutation in the TM2 (Leu9´Ala), have been used to further implicate the importance of the α4 nAChR subunit in nicotine reward (Tapper et al., 2004, 2007; Zhao-Shea et al., 2008). The Leu9´Ala α4 nAChR knock-in mouse line does not exhibit any developmental defects, unlike the Leu9´Ser α4 nAChR mouse. Furthermore, concentration response studies in *Xenopus* oocytes expressing muscle nAChR subunits indicate the Leu9´Ser nAChR confers higher sensitivity to agonist than Leu9´Ala nAChRs (Revah et al, 1995). It may be beneficial to use this mutation in my viral-mediated gene overexpression system, especially in the proposed rescue experiments, in order to circumvent the possibility of reduction of cell

viability and provide a moderate level of nicotine sensitivity. Gad2-Cre mice infected with a similarly designed Leu9´Ala virus exhibited changes in locomotor activity but not in CPP nor anxiety. I hypothesize endogenous α4 nAChRs are masking the Leu9´Ala "gain-of-function" properties. The Leu9´Ala mutation has not been characterized in a subunit receptor manner, and the number of mutated subunits is important for the overall sensitivity of a nAChR, then the addition of endogenous α4 within a Leu9´Ala α4 nAChR could possibly reduce the overall nAChR sensitivity.

As mentioned in the discussion section in Chapter III, one of the limitations of the study was the low sample size in several of the experiments. The danger of interpreting data from low sample groups or small effect sizes is the conflation of the actual observed effect. These inadequately powered studies are susceptible to type II errors or false negatives. Errors of this type fail to reject the null hypothesis (the hypothesis that the effect observed is due to chance) so non-significant results due to this error may be statistically significant with increasing sample size. In other words, type II errors prematurely accept the null hypothesis, hence the term 'false negative'. Low powered studies are implicated with other biases, such as changes in effect magnitude depending on the analytical test used ('vibration of effects'), publication bias and selective data analysis (Ioannidis et al., 2008; Button et al., 2013). Increasing the sample size in several of the studies would definitely address this issue, but more consideration into experimental design must be done beforehand.

Animal studies are limited by time, availability and cost. Determining a sample size beforehand with *a priori* power analysis would provide a minimum number of subjects needed to observe an effect that could possibly reject the null hypothesis (Button et al., 2015). The term “power” refers to probability that the null hypothesis is rejected and is an indicator of the ability of a test to properly assess for the desired effect. Variables such as effect size, the probability of false positives and negatives occurring also factor into the power analysis. Determining effect size depends on prior research. Studies infecting Leu9´Ser α4* nAChRs into VTA GABAergic neurons had not been performed before, but the data from the present experiments can be used as the framework for establishing sample size for future studies. However, additional factors, such as percentage of GABAergic neurons expressing in Leu9´Ser α4* nAChRs could affect the effect size, and proper assessment of this need to be done before further experiments are to be done. Understanding this may help factor into the effect size needed for appropriate power analysis.

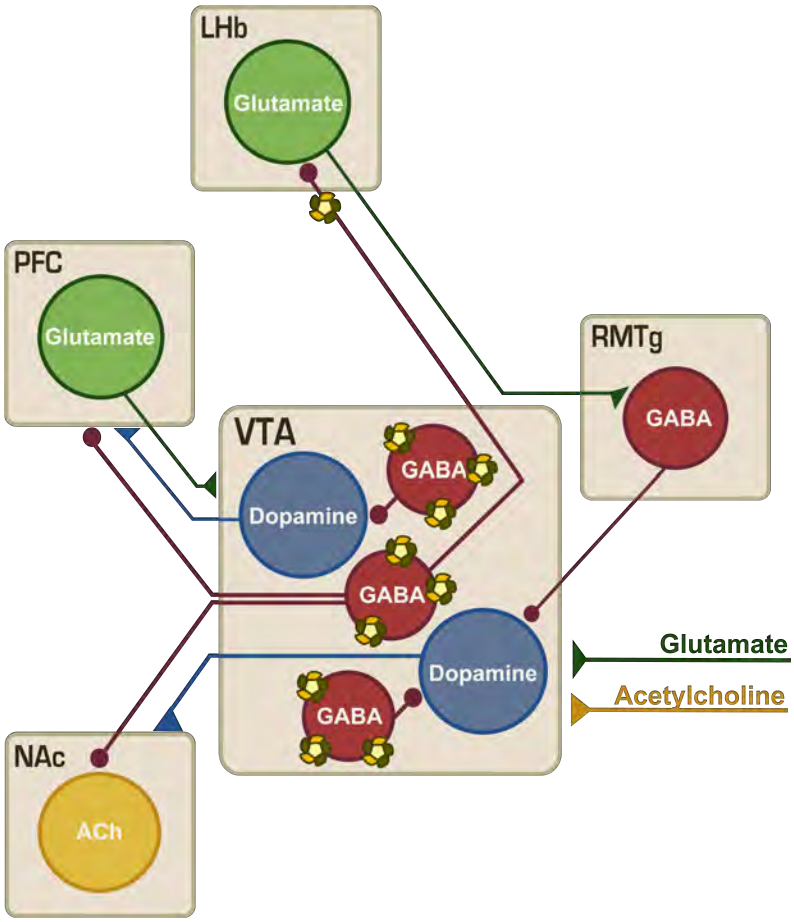

Fig IV-I. Upregulation of α4* nAChRs alters multiple VTA GABAergic subpopulations in the mesocorticolimbic dopaminergic pathway to increase nicotine reward. A simplified schematic of the VTA neuronal subpopulations, illustrating the projections of the DAergic neurons forming the mesocorticolimbic DAergic pathway along with GABAergic projection neurons and interneurons. The flower-like symbols indicate α4* nAChRs upregulated with chronic nicotine.

IV.A. The role of functional VTA GABAergic α4 nAChRs in reward*

Nicotine acts upon the brain, particularly through the VTA DAergic neurons, to promote continued and compulsive consumption (Benowitz, 1996, 1999; Biasi and Dani, 2011). Although much has been done to assess how nicotine activates nAChRs to modulate VTA DAergic activity, not much is known about how nicotine acts upon VTA GABAergic neurons. The observation that selective

activation of upregulated α4* nAChRs on VTA GABAergic neurons by nicotine is sufficient to increase nicotine reward sensitivity is a curious one because it contradicts previous findings done in VTA GABAergic neurons. The current hypothesis of how VTA GABAergic neurons are involved in the VTA reward microcircuitry is though inhibition, either by directly hypopolarizing VTA GABAeric neurons, indirect silencing from other regions such as the RMTg, or inactivating nAChRs on VTA GABAergic by chronic nicotine (Johnson and North, 1992a; Mansvelder et al., 2002; Jhou et al., 2009). My results do not necessarily refute the possibility of inhibition of GABAergic neurons contributes to reward signaling, as we observed a trend for place preference in Gad2^{VTA}: Leu9´Ser when blocking α4* nAChRs with DHβE (Chapter II). However, there may be other mechanisms in which VTA GABAergic neurons augment nicotine reward through upregulated α4* nAChRs.

Many studies have shown that cues associated with nicotine use can facilitate relapse. Cues paired with nicotine intake can reinstate nicotine-seeking behaviors (Liu et al., 2006, Patterson and Markou 1999, Donny et al., 1999). Previous studies in rodents observe increased VTA GABAergic neuron activity during reward anticipation (Steffensen et al., 2001; Cohen et al., 2012). Increasing activation of VTA GABAergic neurons through the overexpression of Leu9´Ser α4* nAChRs may strengthen anticipation behavior by elevating GABAergic neuronal activity with 0.09 mg/kg nicotine during the training session of CPP. This would somewhat explain the increased time spent in the nicotine-paired chamber with Gad2^{VTA}: Leu9´Ser mice (Chapter II). High-affinity nAChRs

in VTA GABAergic as well as DAergic neurons have been shown to be necessary in shaping the firing activity of DAergic neurons with nicotine exposure (Tolu et al., 2012). Increasing nAChR activation on GABAergic neurons may further shape the firing activity of the DAergic neurons by increasing the amount of spikes within a burst. This parameter has been implicated in higher DA released into the NAc, with more spikes indicating more DA released (Tolu et al., 2012). Further studies measuring DA release in the VTA in Gad2^{VTA}: Leu9´Ser mice with 0.09 mg/kg nicotine would provide insight into this question.

However, previous studies have observed abolished sucrose consumption and avoidance with optical activation of VTA GABAergic neurons (Van Zessen et al., 2012; Tan et al., 2012). However, it is unclear whether ChR2-YFP expression was restricted to the VTA (Tan et al., 2012). Although the coordinates indicated that the pVTA was targeted, there was no attempt to distinguish between aVTA from pVTA. There is evidence suggesting that posterior VTA GABAergic neurons are different from anterior VTA GABAergic neurons (Guan et al., 2012). Therefore, the overall activation of both regions may result in one behavior as opposed to the activation of a discrete part of the VTA. Therefore, it is plausible that selective activation of GABAergic neurons in the pVTA can elicit one behavior while overall activation of the VTA results in another behavior. These behavioral differences could explain the 'Inverted U' response observed with different doses of nicotine (Picciotto, 2008).

Further going into the regional differences in the VTA, GABAergic transmission to DAergic neurons in one region could have opposing effects on overall behavior.

It is thought that DAergic neurons that project to the prefrontal cortex reside in the medial VTA while DAergic neurons that project to the NAc reside in the lateral VTA (Lammel et al., 2012). These populations exhibit different electrophysiological characteristics (Lammel et al., 2014). DAergic neurons that project to the PFC are thought to be anxiogenic (Gundayin et al., 2014), so activation of GABAergic interneurons that inhibit PFC-projecting DAergic neurons may decrease activation. However, whether nAChRs are upregulated on GABAergic neurons in a region-specific manner remains to be determined.

GABAergic neurons in the VTA project to other regions in the brain involved in reward (Taylor et al., 2014) (Fig IV-1). Gad2^{VTA}: Leu9´Ser mice expressed Leu9´Ser α4 nAChR projections in the LHb (Chapter II), a brain area associated with aversion (Hong et al., 2011; Lammel et al., 2012). Activating inhibitory projections from the VTA to the LHb induced reward through an indirect feedback loop that disinhibits DAergic neurons (Stamatakis et al., 2013). It is possible that α4* nAChR upregulation occurs in neurons that project to the LHb to lead to increased reward sensitivity. I detected Leu9´Ser α4-YFP* nAChR fluorescence in the LHb of Gad2VTA: Leu9´Ser mice (Chapter II). Although signaling through the proposed meso-habenular GABAergic loop may be insufficient to account for the strong increase in nicotine reward sensitivity observed in Gad2^{VTA}: Leu9´Ser mice. Signaling for VTA DA disinhibition could be watered down and ultimately lost due to the number of brain regions necessary and the multiple inputs each region receives from other brain regions. If anything, upregulation of nAChRs in

VTA GABAergic neurons projecting to the VTA helps promote but does not drive increased reward sensitivity.

VTA GABAergic neurons project to other regions involved in reward, such as the NAc (Taylor et al., 2014) (Fig IV-1). Activation of VTA GABAergic neurons synapsing onto cholinergic interneurons of the NAc leads to increased associative learning (Brown et al., 2012). Conditioned place preference is a conditioning assay to train a subject to associate a chamber with a stimulus. In this assay, Gad2^{VTA}: Leu9´Ser exhibited a strong place preference to the chamber that was paired with 0.09 mg/kg nicotine, the dose shown to activate VTA GABAergic neurons (Chapter II). Upregulation of α4* nAChRs VTA GABAergic neurons may strengthen nicotine associated cues such as the location where nicotine was administered through inhibition NAc cholinergic interneurons. However, I was unable to detect α4* nAChRs in the NAc, possibly due to cholinergic interneurons being sparsely expressed in the striatum, therefore unable to confirm projections in this region (Chapter II) (Tepper and Bolam, 2004). Furthermore, the amount of VTA GABAergic neurons projecting to the NAc appears to be small compared to the LHb (Taylor et al., 2014). Preliminary studies probing for choline acetyltransterase (ChAT) immunoreactivity were unsuccessful in my hands, but control experiments assessing antibody efficacy would identify if the Gad2-Cre driver line has a ChAT deficiency.. Although optogenetic studies suggest that cholinergic interneurons are important for cocaine-mediated reward (Witten et al., 2010), a small

population of neurons is unlikely to be the major driver of nicotine reward sensitivity.

VTA GABAergic projections containing the Leu9´Se α4 nAChR subunit were not detected in the PFC (Chapter II). However, this assessment is limited by the resolution of the YFP tag on the Leu9´Ser subunit as discussed above. In fact, it is thought that putative VTA GABAergic projections to the PFC are responsible for associative learning. It is suggested that information from the VTA to the PFC is transmitted immediately through GABA or glutamate and then modulated by DA, due to the slow modulatory behavior of DA and the expression of extrasynaptic DA receptors in the PFC (Kim et al., 2010). The increase of high-affinity nAChRs on these VTA GABA projection neurons to the PFC would improve GABA transmission and possibly heighten associative learning.

Activation of Leu9´Ser α4* nAChRs on VTA GABAergic neurons may alter glutamatergic transmission to increase nicotine reward. The LDTg has been shown to be important for DAergic firing (Lodge and Grace, 2006) and provides a mixture of glutamatergic, GABAergic and cholinergic inputs to the VTA (Omelchenko and Sesack, 2005, 2006). It is possible the release of acetylcholine, glutamate and GABA work in synchrony to activate VTA DAergic neurons. VTA GABAergic neurons are activated by acetylcholine released by the LDTg to increase burst activity in DAergic neurons (Tolu et al., 2012), while glutamate and acetylcholine activate DAergic neurons. Inducing burst activity in DAergic neurons may result in increased the AMPA/NMDA ratio in glutamatergic synapses and facilitating glutamatergic transmission (Saal et al., 2003).

Therefore, increasing activation of VTA GABAergic neurons through Leu9´Ser α4* nAChRs may further increase bursting activity, and further strengthen glutamatergic synapses on DAergic neurons, resulting in increased NAc DA concentrations.

Overall, increased nicotine reward instigated by activating Leu9´Ser α4* nAChRs on VTA GABAergic neurons was induced by targeting as many pVTA GABAergic neurons as possible. Being able to parse out how much a GABAergic neuronal subpopulation contributes to this outcome would provide a better understanding of how nicotine changes the VTA to encourage continued nicotine use. Future studies looking at VTA GABAergic projections using of retrograde viral vector containing the Leu9´Ser α4 nAChR construct injected into the LHb, NAc or PFC would highlight the subset of VTA GABAergic neurons that provide input to the injected region. This method would provide a better idea of the cytoarchitecture of the VTA, especially the organization of GABAergic neurons that project to a specific brain region. Using 0.09 mg/kg nicotine to selective activate specific VTA GABAergic subpopulations based on projection region would dissect what percentage of VTA GABAergic neurons project to a particular region and the contribution of these particular neurons to increased nicotine reward. However, this generalized approach may reflect what occurs with chronic nicotine exposure. High-affinity nAChRs could possibly be upregulated on multiple VTA GABAergic subpopulations and the activation of these multiple pathways via nicotine promotes nicotine reward. One way to define this phenomenon is the dysregulation of separate VTA GABAergic pathways. The other way is to

suggest that upregulation of α4* nAChRs on multiple VTA GABAergic pathways produces an additive effect resulting in the enhancement of the rewarding properties of nicotine.

IV.B. The role of increased expression of VTA GABAegic α4 nAChRs in anxiety*

Chapter III focuses on anxiety-like behaviors in an attempt to determine whether Leu9´Ser α4 nAChR subunit overexpression in VTA GABAergic neurons changes baseline anxiety levels or contributes to nicotine withdrawal symptoms. The rationale is as follows: smokers that eventually relapse exhibit intense negative affect feelings such as anxiety during nicotine withdrawal (Piasecki et al., 2000). The VTA contains the necessary circuitry to induce negative affect either through directly activating VTA GABAergic neurons (van Zessen et al., 2012; Tan et al., 2012) or by activating DAergic neurons that project to the PFC (Gunaydin et al., 2014). Increased expression of Leu9´Ser α4* nAChRs can offset the normal VTA microcircuitry and induce anxiety-like behaviors through these anxiogenic pathways. For example, heterozygous Leu9´Ser knock-in mice exhibit increased baseline anxiety-like behaviors (Labarca et al., 2001). Despite the decrease of overall DAergic neurons in the Leu9´Ser knock-in mice, one can argue that the hypodopaminergic state in these mice reflects what occurs with increased functional expression of α4* nAChRs of VTA GABAergic neurons. To test the hypothesis that overexpression of Leu9´Ser α4* nAChRs on VTA GABAergic neurons can promote anxiety-like behaviors, I assessed $Gad2^{VTA}$: Leu9´Ser mice and control mice with three anxiety-like tests. There were no

differences in Gad2^{VTA}: Leu9´Ser behavioral activity compared to control, either locomotor or anxiety-related (Chapter III). There is a non-significant trend for Gad2^{VTA}: Leu9´Ser mice to exhibit anxiolytic behavior (Chapter III). Although I provide evidence supporting functional Leu9´Ser α4-YFP* nAChR are expressed in Gad2^{VTA}: Leu9´Ser (Chapter II), appropriate controls need to be done to confirm these observations. Activation of α4* nAChRs in chronic treated animals alleviates anxiety during withdrawal (Yohn et al., 2014). However, other factors involved in anxiety modulated by chronic nicotine, such as CRF, may contribute to the nicotine withdrawal symptoms (Oliver et al., 2007; Grieder et al., 2014; Zhao-Shea et al., 2015).

Nicotine induces locomotor activity changes in drug naïve mice (Marks et al., 1983; Tapper et al., 2007). However, the specific change differs from different strains of mice, as the C57BL/6, DBA and BALB mouse strain exhibited dose dependent hypolocomotor activity, while low doses of nicotine induced hyperlocomotor activity in C3H mice (Marks et al., 1983a; Schwartz and Kellar, 1985). The Gad2-Cre driver line was generated in the 129/Sv strain and was backcrossed to C57BL/6 at least twice. Although subsequent backcrossing needs to be done to generate congenic mice, the Gad2-Cre strain exhibits hypolocomotion with 0.5 mg/kg nicotine, consistent with C57BL/6J mice (Marks et al., 1983a). An acute injection of low doses of nicotine shown to selectively activate VTA GABAergic neurons drastically decreased locomotor activity in Gad2^{VTA}: Leu9´Ser mice but not control mice (Chapter II). This effect was not due to a deficit in locomotor activity due to nAChR subunit overexpression, for there

was no significant difference in locomotor activity between Gad2^{VTA}: Leu9´Ser and control mice (Chapter III). Hypolocomotor activity in Gad2^{VTA}: Leu9´Ser mice could be due to the anxiogenic properties of low doses of nicotine (Irvine et al., 2001; Biala and Budzynska, 2006). Gad2^{VTA}: Leu9´Ser but not control mice are prostrate on the cage floor within one minute of receiving an s.c. injection of 0.09 mg/kg nicotine, with no overt movements other than breathing. The instantaneous response to nicotine administration indicates that nicotine affects locomotor activity rather than affective behavior. The hypolocomotor effect of nicotine may have no relevance to CPP because mice receive nicotine during the training period, where they are confined to a small chamber that does not allow much room for movement (Chapter II). If anything, it may induce a negative association for the chamber, but ultimately does not deter the mouse from spending more time in the chamber post-training.

Regardless of whether the hypolocomotor activity result from anxiety or deficits in locomotor activity, Gad2^{VTA}: Leu9´Ser mice exhibited tolerance to nicotine over the span of 7 days as indicated by total locomotor activity recovering back to baseline (Chapter II), which coincides with the development of tolerance to the anxiogenic effects of nicotine 7 days after nicotine treatment (Biala and Budzynska, 2006). These data suggest that activating VTA GABAergic neurons through α4* nAChRs is sufficient in inducing hypolocomotor activity and initiating tolerance. It is hypothesized that chronic nicotine exposure increases α4* on VTA GABAergic neurons that inhibit pathways involved in motor suppression (Tapper et al., 2007). On the other hand, intermittent exposure of nicotine

induced the functional upregulation of nAChRs in VTA DAergic neurons. This observation coincides with the sensitization of locomotor activity, suggesting that sustained locomotor sensitization is due to increased nAChR activation on DAergic neurons (Baker et al., 2013). These two models may represent two separate stages of drug dependence, with the upregulation of α4* nAChR receptors in VTA GABAergic neurons preceding nAChR upregulation of VTA DAergic neurons. Selectively inhibiting VTA GABAergic neurons either through optogenetic or chemogenetic techniques prior to nicotine administration would further test the hypothesis that VTA GABAergic transmission causes hypolocomotor activity and ultimately incites nicotine tolerance.

Therefore, selectively activating VTA GABAergic neurons in Gad2^{VTA}: Leu9´Ser mice would not help in assessing anxiety-like behaviors because drug naïve Gad2^{VTA}: Leu9´Ser mice exhibit hypolocomotor activity immediately after nicotine administration (Chapter 2). Anxiety tests are relatively short and the half-life of nicotine in a mouse is around 6 minutes (Petersen et al., 1984; Bourin and Hascoët, 2003; Matta et al., 2007; Walf and Frye, 2007; Brooks and Dunnett, 2009), so administering nicotine to Gad2^{VTA}: Leu9´Ser mice prior to testing for anxiety-like behaviors would severely impact results. Additionally, waiting for the mice to recover would not allow for the assessment of direct activation of VTA GABAergic neurons by nicotine.

Nicotinic receptor antagonists, such as mecamylamine and DHβE, have been used to precipitate withdrawal in chronic nicotine treated rodents (Hildebrand et al., 1997; Damaj et al., 2003, Salas et al., 2004, 2009; Zhao-Shea et al., 2012,

2014). Administering nicotinic receptor antagonists to nicotine naïve Gad2^{VTA}: Leu9´Ser mice to measure somatic or affective withdrawal symptoms would highlight a subpopulation within the VTA involved in nicotine withdrawal symptoms. There are several possible outcomes to this proposed experiment, and several important controls should be included. The emergence of withdrawal symptoms in Gad2^{VTA}: Leu9´Ser mice with nicotinic receptor antagonists would coincide with increased anxiety-like behaviors exhibited in the α4 KO mouse (Ross et al., 2000) and suggest activation of VTA GABAergic neurons dampens the intensity of withdrawal symptoms. Alternatively, inactivating Leu9´Ser α4* nAChRs may lead to the decrease of anxiogenic behaviors through decreasing GABA transmission to other regions that provide glutamatergic input, such as the PFC (Fig. IV-1). Glutamate transmission from the PFC increased DA activity (Murase et al., 1996) while restoring NMDA receptor expression in VTA DAergic neurons ameliorated anxiety-like behaviors brought on by fear conditioning (Zweifel et al., 2011). These data indicate that glutamatergic activation of DAergic neurons can alleviate anxiety, and that inhibiting inhibitory afferents to these areas may result in disinhibition and the alleviation of anxiety. Using a known anxiogenic or anxiolytic drug during the anxiety-like test would also confirm the face validity of the anxiety-like tests and provide an appropriate control to compare the effects observed in the experimental groups.

Recent research indicates nAChRs in the habenulo-peduncular pathway are involved in nicotine withdrawal symptoms, framing the IPN as a prime nucleus for mediating both somatic and affective symptoms (Salas et al., 2004, 2009; Zhao-

Shea et al., 2013, 2015). Infusion of nAChR antagonist for α4β2 or α6β2 in the medial habenula (MHb) was sufficient to alleviate anxiety-like symptoms in mice withdrawn from nicotine, which coincided with increased expression of α4α6* nAChRs in the MHb (Pang et al., in review). Knock out mice lacking specific subunits such as α2, α5 and β4 KO did not exhibit either spontaneous or precipitated nicotine mediated withdrawal symptoms (Salas et al., 2004, 2009). Interestingly enough, a connection between the VTA and the IPN has been recently observed, as CRF from VTA DAergic neurons is thought to activate CRF1 receptors in the IPN responsible for the anxiolytic effects signaled through the IPN (Grieder et al., 2014; Zhao-Shea et al., 2015). It is possible that α4* nAChR upregulation reward signaling and anxiety signaling are two different pathways and the reward pathway feeds into the anxiety pathway.

IV.C. Future Directions

The Leu9´Ser α4-YFP nAChR gene expression system is a useful tool to allow for the cell-specific assessment of α4* nAChRs within a diverse neuronal brain region. This technique tests the sufficiency of the α4 nAChR subunit within a certain component of the neuronal circuitry. As described above, experiments detecting and quantifying the expression of the Leu9´Ser α4 nAChR subunit would further strengthen the observations made with this technique. Several components of the system, such as the Cre-driver mouse line, also could be improved upon. The current mouse driver lines available do not provide enough resolution to distinguish subpopulations within a particular group of neurons,

especially in the VTA. It would be important to understand how these subpopulations could contribute to reward signaling in order to develop a comprehensive understanding of the workings of the VTA and better develop treatments that specifically target the subpopulation of cells that are responsible for the addictive properties of nicotine without upsetting the other neural connections that could decrease treatment efficacy. Advanced recombinase regulated gene expression systems are currently under development that could further restrict expression of a gene based on Boolean logic (Fenno et al., 2014). This opens a whole avenue of honing into a specific neuronal subpopulation. Utilizing a neuron specific promoter such as synapsin can also reduce false positives by restricting expression exclusively in neurons (Kügler et a., 2003). These new developments in viral-mediated gene expression would refine our understanding of addiction- and withdrawal-relevant circuits in the brain.

How does increasing α4 nAChR expression affect DAergic neuronal activation, and how does selectively activating VTA GABAergic neurons through α4* nAChRs modify DAergic activity? Answering these two questions would provide a better idea behind the increase in nicotine reward sensitivity. Chronic nicotine exposure decreased nicotine-mediated activation of VTA DAergic neurons while VTA GABAergic neurons exhibited increased activation with chronic nicotine exposure (Nashmi et al., 2007), suggesting that activation of upregulated α4* nAChRs in GABAergic neurons reduced activation of DAergic neurons by nicotine. Although c-Fos studies suggest that DAergic neurons are not activated with 0.09 mg/kg nicotine (Chapter II), electrophysiological studies on DAergic

neurons from Gad2^{VTA}: Leu9´Ser brain slices would: 1) further confirm that low concentrations of nicotine do not activate DAergic neurons and more importantly 2) determine if Leu9´Ser α4* VTA GABAergic interneurons decrease DAergic firing frequency upon application of a low does of nicotine. The second experiment would further reveal what VTA GABAergic neuronal subpopulation contributes to increased nicotine reward sensitivity and further suggest that increasing α4* nAChR activation contributes to the shaping of DAergic burst firing activity (Tolu et al., 2014). Measuring NAc DA concentrations through fast-cyclic voltammetry would provide evidence to further suggest increased reward behavior observed in CPP is due to elevated DA concentrations in the NAc.

Are the Leu9´Ser α4* nAChRs on VTA GABAergic neurons hypersensitive? Throughout this thesis, I refrained from referring to Leu9´Ser α4 nAChRs expressed via this system as hypersensitive because the appropriate tests were not done to confirm hypersensitivity. The electrophysiological and immunohistochemical studies only demonstrate that the nAChRs expressed in VTA GABAergic neurons are “gain-of-function”, in that a single dose of nicotine increased current or c-Fos immunoreactivity over control samples (Chapter II). To confirm hypersensitivity, either voltage clamp recordings or c-Fos immunostaining in response to a series of nicotine doses would be needed. Three nicotine doses were tested in the CPP assays, but these studies do not show what occurs at the molecular and cellular level (Chapter II). Additional doses, especially lower doses would further assess the hypersensitive nature of the Leu9´Ser α4* nAChR in the Gad2^{VTA}: Leu9´Ser model.

The dose of 0.09 mg/kg nicotine was derived from previous studies (McGeehan and Olive, 2003; Brunzell et al., 2009) that elicited CPP in C57BL/6 mice. One may ask why 0.09 mg/kg dose was insufficient to induce reward in Gad2-Cre mice that are assumed to be on a C57BL/6 background or why 0.5 mg/kg nicotine failed to elicit CPP in control or $Gad2^{VTA}$: Leu9´Ser mice (Chapter II). It is possible the Gad2-Cre driver line has 129/Sv strain properties that may confer nicotine insensitivity. Additionally, variation in handling and administration can factor into overall CPP expression (Matta et al., 2007). Assessing the expression of nAChRs in the Gad2-Cre line versus a C57BL/6 line would confirm the nAChR profile and determine if Leu9´Ser α4 expression is compensating for low α4 nAChR expression. To complement these studies, overexpressing non-mutant α4-YFP nAChR subunits in Gad2-Cre mice address this issue rather than ChR2-YFP.

Human genome studies have implicated rs1044396 C single nucleotide polymorphism in the *CHRNA4* gene in increasing the hedonistic effects of smoking, the exacerbation of negative affective behaviors as well as the likelihood to develop nicotine dependence (Feng et al., 2004; Markett et al., 2011; Tsai et al., 2012; Lazary et al., 2014). Studies in *Xenopus* oocytes injected with α4 receptors with different exon 5 haplotypes observed nicotine activation of cells expressing the rs1044396 C allele at lower nicotine concentrations compared to the T allele (Winterer et al., 2011). Designing a viral construct containing the appropriate single nucleotide polymorphism, similar to the α5

D398N experiments of Morel and colleagues could provide molecular insight into the mutation (Morel et al., 2013). Infusing viral particles into either Gad2-Cre or α4 KO mice specifically in the VTA, IPN or MHb would also further help determine how these *CHRNA4* variants alter overall receptor function.

Nicotine is known to be co-abused with other drugs of abuse, such as alcohol. Alcohol does not have a well-defined target to act upon in order to signal reward. However, α4* nAChRs have been shown to be important for the rewarding properties of alcohol (Henderickson et al., 2010). It is thought that alcohol acts through indirect and allosteric methods to induce nAChR activation and induce reward. It could be that increasing α4* nAChR expression on VTA GABAergic neurons can also increase sensitivity to alcohol reward. Other drugs of abuse, such as cocaine, appear to act on mechanisms independent of nAChRs (McGranahan et al., 2011), but it is possible that α4* nAChR upregulation can potentiate the response to low levels of cocaine and induce reward. Using Gad2^{VTA}: Leu9´Ser mice during the appropriate reward assays with these drugs would help elucidate this part.

A disproportionate amount of smokers also have a neuropsychiatric disorder, (Annamalai et al., 2015). This is an issue when dealing with smoking cessation, as complications can arise from other medications used in concurrence with smoking cessation. Increasing cholinergic activation through inhibiting neurotransmitter breakdown has been shown to play a role in eliciting manic behaviors (Janowsky 1978, Risch 1981). Acting upon nAChRs either with antagonists or partial agonist have yielded promising effects in treating the

symptoms associated with depression and anxiety (Rolema et al., 2009; Patterson et al., 2009; Turner et al 2010; Yohn et al., 2014). It is hypothesized that aberrant DA activity, particularly in the VTA, can lead to various psychiatric disorders such as schizophrenia and depression (Lodge and Grace, 2011). Smoking may provide an avenue for neuropsychiatric patients to regulate DAergic activity, because a smoker can titrate the amount of nicotine they receive through the number of puffs from a cigarette they take. Indeed, studies assessing the effects of smoking history in reward responsiveness in depressed individuals indicated that depressed individuals without a history of smoking performed worst than those in control, while depressed individuals with a history of smoking exhibited reward responsiveness similar to control (Janes et al., 2015). These data imply that smoking at least in depressed individuals is a method of therapy to counteract the negative state. Further studies into understanding how VTA GABAergic specific α4* nAChR upregulation affects circuits involved with reward and aversion may provide valuable insight into novel therapies to treat neuropsychiatric disorders.

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