The Roles of Nicotinic Acetylcholine Receptors in the Ventral Tegmental Area: Implications in Nicotine and Ethanol Addiction and Drug Intervention

by

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ABSTRACT

Tobacco and alcohol are the most commonly abused drugs worldwide. Many people smoke and drink together, but the mechanisms of this nicotine (NIC) –ethanol (EtOH) dependence are not fully known. EtOH has been shown to affect some nicotinic acetylcholine receptors (nAChRs), which potentially underlies NIC-EtOH codependence. Ventral Tegmental Area (VTA) dopamine (DA) and γ-aminobutyric acid (GABA) neurons express different nAChR subtypes, whose net activation results in enhancement of DA release in the Prefrontal Cortex (PFC) and Nucleus Accumbens (NAc). Enhancement of DA transmission in this mesocorticolimbic system is thought to lead to rewarding properties of EtOH and NIC, clarification of which is relevant to public health and clinical diseases. The aim of this study was to elucidate pharmacological mechanisms of action employed by both NIC and EtOH through nAChRs in VTA neurons by evaluating behavioral, network, synaptic and receptor functions therein. It was hypothesized that VTA GABA neurons are controlled by α7 nAChRs on presynaptic GLUergic terminals and α6 nAChRs on presynaptic GABAergic terminals. NIC and EtOH, via these nAChRs, modulate VTA GABA neuronal function. This modulation may underlie NIC and EtOH reward and reinforcement, while pharmacological manipulation of these nAChRs may be a therapeutic strategy to treat NIC or EtOH dependence. This data demonstrates that in VTA GABA neurons, α7 nAChRs on GLUergic terminals play a key role in the mediation of local NIC-induced firing increase. α6*-nAChRs on GABA terminals enhances presynaptic GABA release, and leads to greater inhibition to VTA GABA neurons, which results in an increase VTA DA neuron firing via a disinhibition mechanism. Genetic knockout of these nAChRs significantly prevents EtOH-induced animal conditioned place preference (CPP). Furthermore, levo-tetrahydropalmadine (*l*-THP), a compound purified from natural Chinese herbs, blocks nAChRs, prevents NIC-induced DA neuronal firing, and eliminates NIC CPP, suggesting it as a promising candidate in a new generation of interventions for smoking cessation. Improved understanding of underlying mechanisms and development of new drugs will increase the number of successful quitters each year and dramatically improve the quality of life for millions suffering from addiction, as well as those around them.

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LIST OF ABBREVIATIONS

Abbreviation//Symbol:	Definition:
α6*	α6 subunit containing
α-CTX	Alpha conotoxin
Ach	Acetylcholine
ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
APV	D-L 2-amino-5-phosphonopentanoic acid
$CaCl_2$	Calcium chloride
CNQX	6-cyano-23-dihydroxy-7-nitro-quinoxaline
CO_2	Carbon dioxide
CPA	Conditioned place aversion
CPP	Conditioned place preference
C_t	Cycle threshold
DA	Dopamine
DHβE	Dihydro-β-erythroidine
DIC	Differential Interference Contrast
eEPSC	Evoked excitatory post-synaptic current
EGTA	Ethylene glycol tetraacetic acid
eIPSC	Evoked inhibitory post-synaptic current
EtOH	Ethanol
FDA	U.S. Food and Drug Administration
FR	Fasciculus retroflexus
FSCV	Fast-scan cyclic voltammetry
GABA	γ-aminobutyric acid
GAD67	Glutamate decarboxylase
GAD-GFP	Glutamate decarboxylase green fluorescent protein

Abbreviation/Symbol	Definition:
GFP	Green fluorescent protein
GLU	Glutamate
HEPES	4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IACUC	Institutional animal care and use committee
Ih	Hyperpolarization-activated current
IP	Intraperitoneal
IR	Infrared
IV	Intravenous
KCL	Potassium chloride
K-glunconate	potassium gluconate
KH_2PO_4	Monopotassium phosphate
l-THP	levo-tetrahydropalmatine
LTP	Long-term potentiation
MEC	Mecamylamine
mEPSC	Miniature excitatory post-synaptic current
Mg-ATP	Adenosine 5'-triphosphate magnesium salt
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
mIPSC	Miniature inhibitory post-synaptic current
MLA	methyllycaconitine
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptors
NaCl	Sodium chloride
Na-GTP	Guanosine 5'-triphosphate sodium salt
NaH_2CO_3	Sodium bicarbonate
NaH_2PO_4	Monosodium phosphate

Abbreviation/Symbol	Definition:
NIC	Nicotine
NMDA	N-methyl-D-aspartate
NMDARs	NMDA receptors
O_2	Oxygen
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PPTg	Pedunculopontine tegmental nucleus
PTX	Picrotoxin
RMTg	Rostromedial tegmental nucleus
RT	Reverse transcription
SEM	Standard error of the mean
sEPSC	Spontaneous excitatory post-synaptic current
SH-EP1	Human epithelial cells
sIPSC	Spontaneous inhibitory post-synaptic current
SNc	Substantia nigra compacta
SNr	Substantia nigra reticulata
TH	Tyrosine hydroxylase
THP	Tetrahydropalmatine
THPBs	Tetrahydroprotoberberines
TTX	Tetrodotoxin
VTA	Ventral tegmental area
WT	Wild type

CHAPTER 1

INTRODUCTION

Background

Tobacco and alcohol are the most commonly abused drugs by humans. Nicotine (NIC) is the major contributor in the continuance of tobacco use (Benowitz, 1996a), while ethanol (EtOH) is the intoxicating agent in alcoholic drinks that can lead to abuse and dependence (Gilpin & Koob, 2008b). Tobacco and alcohol use are leading causes of preventable death in the United States (Danaei et al., 2009). Smoking tobacco, the leading cause of preventable death, is accountable for approximately 467,000 deaths per year, while alcohol contributes to another 90,000 (Danaei et al., 2009). The most common type of polydrug use is alcohol and tobacco taken in concert (Bien & Burge, 1990; Miller & Gold, 1998). The magnitude of tobacco smoking is extremely high among alcoholics (Grant, Hasin, Chou, Stinson, & Dawson, 2004) and is drastically higher than the rate in the general population (Batel, Pessione, Maitre, & Rueff, 1995; Walitzer & Dearing, 2013). Those who smoke are ten times more likely to be alcoholics than those who do not (DiFranza & Guerrera, 1990). Those who are not alcoholics have been more successful than their alcoholic counterparts in quitting smoking, 49% to 7% respectively (DiFranza & Guerrera, 1990). Although we know that the use of tobacco and alcohol together is prevalent, little is known about the mechanism of action when the two are used collectively. Clarification of these actions would be clinically useful in the treatment for the abuse of both tobacco and alcohol, as many requiring treatment for one also use the other.

Structure and Function of the Mesocorticolimbic Reward system

Projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), by way of the medial forebrain bundle, make up a vital component of the mesolimbic pathway (Fouriezos & Wise, 1976; Liebman & Butcher, 1973; Wise, 1998; Wise & Rompre, 1989). The rewarding effects of both NIC and EtOH have been linked to the mesocorticolimbic DA system (Mansvelder, Keath, & McGehee, 2002; Mansvelder & McGehee, 2000; R. A. Wise, 1996). Reward refers to any stimulus which evokes an approach response (N. M. White, 1989). An increase in DA in the NAc is thought to be vital for reward signaling.

This system has been connected to the rewarding effects of many abused drugs (Blackburn, Phillips, Jakubovic, & Fibiger, 1986a; D. L. McKinzie, Z. A. Rodd-Henricks, C. T. Dagon, J. M. Murphy, & W. J. McBride, 1999; Pierce & Kumaresan, 2006a; R. A. Wise, 1996; R. A. Wise & M. A. Bozarth, 1987). The VTA consists of three major types of neurons: DA, γ-aminobutyric acid (GABA), and glutamate neurons (**Fig. 1**). The most numerous are DA neurons. The second, GABA neurons, inhibit DA neurons in local circuitry and also project to other brain nuclei. Finally, there is a small population of glutamatergic neurons (Yamaguchi, Sheen, & Morales, 2007b), which can innervate both DA and GABA neurons. The NAc is part of the ventral forebrain and is segregated into two regions: the shell and the core. Of the two regions, the shell has been shown to be important for the rewarding effects (Lammel et al., 2012). The medial VTA seems to consist of the highest number of DA neurons innervating the NAc shell (Ikemoto, 2007).

Although the mesocorticolimbic DA system's involvement has been known to be critical for the rewarding effects of many drugs, such as morphine, phencyclidine and NIC also manifest DA independent mechanisms. The necessity of this DA system in the rewarding properties of benzodiazepines, barbiturates and caffeine is also questioned (Lammel et al., 2012; Sturgess et al., 2010; Vashchinkina et al., 2012). A rising hypothesis asserts that DA is not requisite for all rewarding effects of opiates, cannabis, cocaine and NIC. The idea that DA in the mesolimbic system is the only way by which reward occurs is perhaps too limiting.

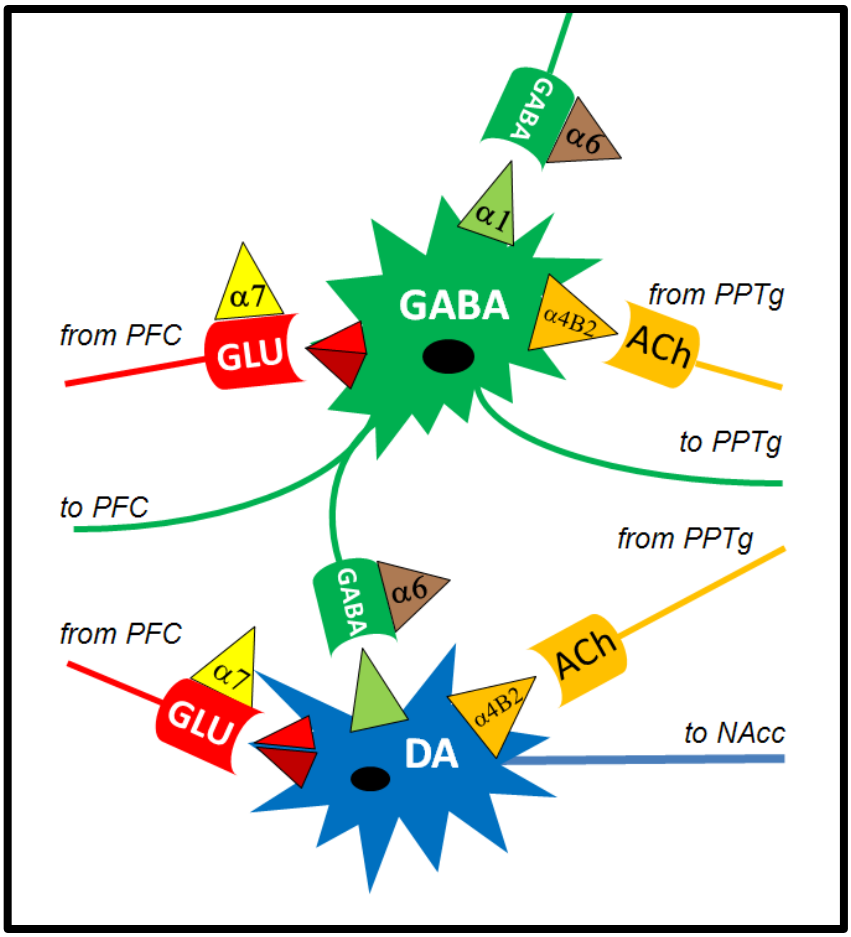


Figure 1 Theoretical framework of the VTA. DA neurons in the VTA project to the NAc via the medial forebrain bundle. VTA GABA neurons strongly inhibit VTA DA neurons via non-α1* GABA(A)Rs. They are also inhibited via α1 containing GABA(A)Rs postsynaptic to local circuit GABA neurons or reciprocal GABA input from the NAc. Activation of α6 nAChRs on GABA terminals to both GABA and DA neurons modulates GABA release. GLUergic input from the PFC activates AMPARs and NMDARs receptors on GABA neurons. PFC GLU neurons express α7 nAChRs which in the VTA are located on GLU terminals. Activation of α7 nAChRs enhances GLU release on both GABA and DA neurons. Cholinergic input from the PPTg excites GABA and DA (Garzon, Vaughan, Uhl, Kuhar, & Pickel, 1999; Kalivas, 1993).

Evidence supporting a lack of DA involvement in drug reinforcement, an observable strengthening in behavior (N. M. White, 1989), has been demonstrated in cocaine self-administration (Goeders & Smith, 1983), and conditioned place preference (CPP) (Mackey & van der Kooy, 1985; Spyraki, Fibiger, & Phillips, 1982). Additionally, there has been confirmation that GABA neurons in the mesolimbic pathway are involved in the rewarding properties of opiates (Margolis, Toy, Himmels, Morales, & Fields, 2012; Ting et al., 2013; Xi & Stein, 2002). It has been reported that $GABA_A$ receptors in the VTA could be a gating mechanism wherein opiate naïve animals utilize a DA independent system, while opiate dependent, and opiate withdrawn, animals utilize a DA dependent system (Laviolette, Gallegos, Henriksen, & van der Kooy, 2004). Following opiate exposure and withdrawal, VTA $GABA_A$ receptors change from acting in an inhibitory manner to an excitatory one. Moreover, high doses of the DA antagonist haloperidol neglected to stop the reinstatement of heroin seeking behavior, giving credence to a notion of a DA independent system, at least in the case of opiates (Di Chiara & North, 1992; Robinson & Berridge, 1993). Some assert that DA neurons are not exactly reward neurons, but instead may be pivotal for the initiation and reinstatement of drug use (Koob & Le Moal, 1997). These conflicting reports on the necessity of DA for the reinstatement of drug use shows that the role of DA is not fully understood. Therefore, DA transmission in the mesolimbic pathway may be important for the motivational effects of abused drugs in dependent animals, while other systems could be exploited when animals are naïve (Bechara & van der Kooy, 1992; Nader, 1994). These studies supply verification for the existence of DA independent mechanisms that also contribute to the reinforcing properties of drugs of abuse.

nAChRs in the Ventral Tegmental Area and Nicotine Addiction

NIC is thought to be the major contributor to the maintenance of tobacco use (Benowitz, 1996b). Recent studies show that rodents will self-administer both intravenous and oral NIC (Madsen et al., 2015; Renda & Nashmi, 2014). Rodents in behavioral studies have also shown an increased preference for NIC paired testing chambers (Biala & Budzynska, 2010a). Others

report that non-nicotinic agents contained in cigarettes are also reinforcing (Brennan et al., 2015). However, NIC replacement therapy is still the most studied, recommended and used form of treatment for smoking cessation (Heydari et al., 2014).

After inhalation, NIC is rapidly absorbed by the blood stream and within 10-19 seconds has entered the brain (Benowitz, 1996b). Human and animal studies show that NIC-induced stimulation of neurons in the mesolimbic DA system are central to the reinforcing effects of NIC use (Benowitz, 1996b). Accumulating lines of evidence demonstrate that nAChRs play critical roles in the mediation of NIC reward, dependence and addiction. NIC's rewarding and addictive properties appear to be mediated by DA neurons in the midbrain's VTA, which projects to limbic structures. DA antagonists in the VTA block NIC's addictive effects and cause cessation of self-administration in animal models, indicating that NIC must bind to receptors that cause action potentials and neurotransmitter release in DA neurons. GABAergic and GLUergic synaptic inputs to VTA DA neurons are modulated by different NIC acetylcholine receptor (nACHR) subtypes with distinct desensitization properties. Cholinergic inputs to the VTA originate in the habenula and project via the fasciculus retroflex (FR) as well as the pedunculopontine tegmental nucleus (PPTg). NIC can enhance GLUergic transmission while the nACHRs on GABA neurons are desensitized, thus shifting the balance of synaptic inputs to excitation. This desensitization silences endogenous cholinergic drive to the GABAergic inputs, which ultimately disinhibits DA neurons. Coordinated disinhibition and enhanced excitation likely contributes to prolonged increases in DA release and ultimately behavioral reinforcement.

Despite current knowledge of the deleterious effects of tobacco use, only six percent of the 35 million people who try to quit each year remain successful for more than a month (Volkow, 2006). Previous studies reveal that NIC is to blame for the highly addictive properties of tobacco products. NIC acts within the brain to increase DA levels in the reward circuits. Scientists have applied this knowledge in the development of gum, patches, and inhalers infused with NIC, which has been fairly successful in alleviation of some aspects of withdrawal. However, cravings may still persist. Further research is needed to determine NIC's actions on neurons in the brain's pleasure pathway, supposing that if we understand what neural substrates underlie NIC's effects,

we will be better equipped to design appropriate therapies and develop suitable medications for NIC addiction.

Ethanol Abuse and nAChRs

Neuronal nAChRs mediate cholinergic modulations in brain function through both pre- and post-synaptic mechanisms. Most nAChRs in the central nervous system are located on presynaptic terminals/boutons (Role & Berg, 1996; D. H. Taylor et al., 2013), where they modulate various neurotransmitter releases (Wonnacott, 1997), including acetylcholine (Ach) itself when nAChRs are activated (or desensitized). nAChRs are also expressed on neuronal somatodendrtic regions, where they presumably modulate neuronal excitability directly. Therefore, EtOH reward and dependence through nAChRs are involved in these pre- and post-synaptic mechanisms. On one hand, EtOH alters cholinergic modulations in neurotransmitter releases in the mesolimbic pathway. On the other hand, EtOH directly modulates nAChR expression, up or down regulation, and functions such as allosteric modulation, stabilization, desensitization or internalization (Dopico & Lovinger, 2009). Through these mechanisms, EtOH enhances mesolimbic DA signaling and consequently triggers reward and dependence. For example, systemic EtOH-induced DA release in the rat NAc. This DA release was completely abolished by nAChR antagonist, mecamylamine (Blomqvist, Engel, Nissbrandt, & Soderpalm, 1993). Interestingly, only perfused mecamylamine in the VTA, but not in the NAc, prevented the accumbal DA overflow after systemic EtOH (Blomqvist, Ericson, Engel, & Soderpalm, 1997). The voluntary EtOH self-administration demonstrated an increase in DAergic and cholinergic neurotransmission (Nestby et al., 1999), suggesting that VTA nAChRs may play an important role in mediating the mesolimbic activating and reinforcing properties of EtOH (Ericson, Blomqvist, Engel, & Soderpalm, 1998). During *in vitro* preparations, EtOH potently modulates nAChRs at low concentrations (100 μM–10 mM), suggesting nAChRs as potential targets for EtOH action (Nagata et al., 1996). In *Xenopus* oocytes, acute EtOH (75mM) potentiated ACh-induced current of α2β4, α4β4, α2β2, and α4β2 nAChRs, while lower concentrations of EtOH (20– 50 mM) inhibited NIC-induced current of α7 nAChRs and all concentrations of EtOH tested have no effect

on α3β2 or α3β4 nAChRs (Cardoso et al., 1999). In cultured cortical neurons, EtOH potentiated non-α7 nAChR- but inhibited α7 nAChR-mediated currents (Aistrup, Marszalec, & Narahashi, 1999). In brain slices (Brodie, Shefner, & Dunwiddie, 1990) or isolated neurons (Brodie & Appel, 1998b), EtOH excited VTA DAergic neurons and increased neuronal firing rate. Taken together, EtOH directly and/or indirectly modulates nAChR functions, which in turn alters mesolimbic function, and leads to reward and dependence.

Nicotine and Ethanol Reward Associated with the Ventral Tegmental Area

The effects of both NIC and EtOH involve the VTA (Koob & Volkow, 2010; Soderpalm, Ericson, Olausson, Blomqvist, & Engel, 2000). The direct excitatory effect of EtOH on neurons in the VTA has been observed (Brodie et al., 1990; Gallegos, Lee, Criado, Henriksen, & Steffensen, 1999); both *in vivo* and *in vitro* recordings have shown this effect (Brodie & Appel, 1998b; Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985). Rodents will self-administer NIC and EtOH individually into this region (Gatto, McBride, Murphy, Lumeng, & Li, 1994; Ikemoto, Qin, & Liu, 2006; Laviolette & van der Kooy, 2003c; Rodd et al., 2004; Rodd-Henricks, McKinzie, Crile, Murphy, & McBride, 2000) and synaptic plasticity has been demonstrated in the VTA in response to both substances (Saal, Dong, Bonci, & Malenka, 2003), giving further support to this theory. It is also well known that NIC binds to nAChRs throughout the brain. Rodents with NIC infusions into the VTA demonstrate conditioned place preference. However, similar infusions into areas dorsal or caudal to the VTA do not produce this preference, even if heavily populated by nicotinic receptors (Laviolette & van der Kooy, 2003a). This demonstrates that the VTA is essential for the rewarding behaviors of NIC. For the previously mentioned reasons, it is likely that the neural substrates underlying the co-use of NIC and EtOH depend on VTA neuronal activity.

Other midbrain tegmental regions are involved in the reinforcing characteristics of drugs such as opiates and NIC (Ikemoto et al., 2006; Yeomans, Mathur, & Tampakeras, 1993). Cholinergic receptors work together with neurons in the NAc, VTA, and PPTg to produce the rewarding effects of NIC (Lanca, Adamson, Coen, Chow, & Corrigall, 2000; Wise, 1998; Yeomans et al., 1993). As many drugs of abuse have demonstrated both rewarding and aversive

properties (Hunt & Amit, 1987; Lammel et al., 2012), it has been proposed that the VTA is involved in mediating both of these qualities in actions of NIC (Laviolette & van der Kooy, 2003a). The mediation of both NIC reward and aversion in the VTA could aid in explaining the cross-tolerance observed with NIC and EtOH interactions.

It is currently understood that the mesolimbic system, especially in the VTA, is involved in NIC-EtOH reward. There is, however, a question as to which type or subtype of receptor is most important and on which category of neuron they are found. The origin of long-term potentiation (LTP) induction in NAc DA neurons has been reported to be from presynaptic neurons (Nugent, Penick, & Kauer, 2007). GABA neurons play an integral role in the rewarding effects of drugs of abuse (Gallegos et al., 1999). In fact, stimulation of $GABA_A$ receptors is reinforcing (Ikemoto et al., 2006) and inhibition of GABA neurons in the VTA could lead to increased DA release in the NAc (S. H. Stobbs et al., 2004a). nAChRs can be found on postsynaptic, preterminal, and presynaptic segments of GABA neurons (Alkondon, Pereira, Cortes, Maelicke, & Albuquerque, 1997; Alkondon, Pereira, Eisenberg, & Albuquerque, 1999, 2000), and the reinforcing properties of EtOH is influenced by these receptors. These studies suggest GABA neurons in the VTA serve as an important locus for the modulation of the EtOH effects, possibly by nAChRs.

Nicotine and Ethanol interactions

Interactions between NIC and EtOH have been demonstrated in a number of experiments. Alteration of nAChRs in response to EtOH has been verified (Dohrman & Reiter, 2003). Mouse and rat studies have displayed cross-tolerance between EtOH and NIC (Biala & Budzynska, 2010b; Biala & Weglinska, 2004; Collins, Wilkins, Slobe, Cao, & Bullock, 1996; de Fiebre, Marks, & Collins, 1990). Additional testing has elucidated aspects of the interaction between NIC and EtOH on nAChRs. Nevertheless, much of what has been found is difficult to interpret and involves complex relationships. One such case involves locomotor stimulation in mice by EtOH, which was partially impeded by the non-selective non-competitive nAChR antagonist mecamylamine (MEC) (Soderpalm et al., 2000). Other studies show the ability of MEC to antagonize EtOH effects. Systemic EtOH induces DA release in the rat NAc and can be

blocked by MEC in the VTA but not the NAc (Soderpalm et al., 2000). The EtOH ingestion and preference in high EtOH-preferring rats was also decreased by MEC (Blomqvist, Ericson, Johnson, Engel, & Soderpalm, 1996; Ericson et al., 1998). Together this research confirms that EtOH affects are partially facilitated through nAChRs and suggests that these receptors as a likely candidate for sites of NIC-EtOH interaction.

It is known that the DAergic portion of the mesolimbic pathway is not the only contributor to the reinforcing effects of NIC and EtOH. Many neuron systems and receptor types have been implicated in the interaction involving NIC and EtOH. The serotonin (de Bruin et al., 2013), endogenous opioid (Drews & Zimmer, 2010), glutametergic (Proctor, Dobelis, Moritz, & Wu, 2011), and cholinergic (Lanca et al., 2000; Ribeiro-Carvalho et al., 2009) systems have been associated with NIC and EtOH interactions. Cholinergic receptors, especially nAChRs, have been implicated in this association for some time, but they are not the only mediators of the NIC EtOH interaction. Aside from nAChRs, endocannabinoid CB_1 receptors have been implicated in EtOH and NIC seeking (de Bruin et al., 2011), NIC-EtOH cross-sensitization (Biala & Budzynska, 2010b), and interactive effects of NIC and EtOH involved in passive avoidance learning (Alijanpour, Rezayof, & Zarrindast, 2011). Although nAChRs are not the sole agents involved in the NIC-EtOH interaction, they seem to have greater effects on this relationship than CB_1 receptors both in number and impact.

Rationale

The rationale for this study is predicated on the belief that advancement in the understanding of the brain mechanisms underlying the recreational use and abuse potential of drugs will pave the way for more effective treatment strategies that would save lives and resources throughout the world. Each year more people are enslaved by addiction. It can range from alcohol and illicit drugs to milder stimulants such as coffee or non-prescription drugs. Regardless of the drug the brain's chemistry is influenced by these chemicals, and often in a similar manner.

By understanding exactly how NIC and EtOH alter brain activity, we can determine more effective smoking cessation methods and alcohol abuse treatments. Improved treatment and quitting aids would increase the number of successful quitters each year and dramatically improve the quality of life for not only the millions of people who would be free of their addiction, but the lives of their children, friends, and loved ones who also may suffer from the secondary effects.

CHAPTER 2

NICOTINE ENHANCES THE EXCITABILITY OF GABA NEURONS IN THE VENTRAL TEGMENTAL AREA VIA ACTIVATION OF ALPHA 7 NICOTINIC RECEPTORS ON GLUTAMATE TERMINALS

Introduction

The alkaloid NIC is the major contributor to the maintenance of tobacco use (Benowitz, 1996b). The rewarding effects of NIC have been tied to the mesocorticolimbic DA system, which primarily consists of projections from the VTA to the NAc (D.L. McKinzie, Z.A. Rodd-Henricks, C.T. Dagon, J.M. Murphy, & W.J. McBride, 1999; Pierce & Kumaresan, 2006b). Activation and desensitization of the various nAChRs in the mesolimbic DA system may be crucial factors underlying the effects of NIC on the VTA (Mansvelder et al., 2002; Mansvelder & McGehee, 2000) and NAc (de Rover, Lodder, Kits, Schoffelmeer, & Brussaard, 2002).

The majority of endogenous cholinergic inputs into the VTA innervate GABA neurons (Fiorillo & Williams, 2000; Garzon et al., 1999). Both GABA and DA neurons can be activated by NIC (Mansvelder et al., 2002; Yin & French, 2000). GABA neurons express α4 and β2 subunits, which can be blocked by the non-selective, non-competitive antagonist mecamylamine (MEC) or the relatively more selective competitive antagonist dihydro-β-erythroidine (DHβE) (Mansvelder et al., 2002). The acute effects of NIC in the VTA predominantly affect GABA neurons. The most important nAChR subtypes associated with these cells (i.e., α4β2-containing nAChRs) desensitize rapidly after the action of NIC, leading to an excitation of DA neurons through removal of the inhibitory influence of GABA.

In the VTA, NIC also modulates glutamate (GLU) release. DA and GABA neurons in the VTA receive GLUergic input from the prefrontal cortex (PFC), providing the major excitatory control of the VTA and ultimately DA release in the NAc (Carr & Sesack, 2000c; Johnson, Seutin, & North, 1992; Kalivas, Churchill, & Klitenick, 1993; Sesack & Pickel, 1992; Suaud-Chagny, Chergui, Chouvet, & Gonon, 1992; Taber & Fibiger, 1995). NIC receptors on GLUergic terminals in the VTA are mainly homomeric α7 nAChRs located presynaptically (Mansvelder & McGehee,

2000), and its stimulation enhances the release of GLU on DA and GABA neurons (Girod, Barazangi, McGehee, & Role, 2000; McGehee, Heath, Gelber, Devay, & Role, 1995). NIC is present in the brain of smokers about 10-20 sec after absorption (Benowitz, 1988; Oldendorf, 1974), reaching blood concentrations between 250 and 500 nM during cigarette smoking (Henningfield, Stapleton, Benowitz, Grayson, & London, 1993). These chronic concentrations are sufficient to desensitize α4β2 nAChRs. Alpha-7 nAChRs, on the other hand, are less susceptible to the agonistic action of NIC and require higher doses to become desensitized, and recover more rapidly than other nAChRs (Fenster, Rains, Noerager, Quick, & Lester, 1997). In other words, during smoking, the NIC brain concentration is high enough to excite DA neurons.

There is a growing interest in studying the role of VTA GABA neurons in drug abuse, in particular NIC addiction. For example, a recent paper has shown that the concerted activation of VTA DA and GABA systems is necessary for the reinforcing actions of NIC (Tolu et al., 2013). NIC increases GABA neuron firing rates directly by acting on specific nAChR subtypes (Mansvelder & McGehee, 2000). However, the identification of GABA neurons has previously been based on electrophysiological criteria. Thus, the aim of our study is to evaluate the effects of NIC, as well as the expression of nAChR subtypes, on firing rate and GLU synaptic transmission in a homogeneous subpopulation of GABA neurons in the VTA of glutamic acid decarboxylase green fluorescent protein (GAD-GFP) mice, so that they can be evaluated unambiguously. We hypothesized that VTA GABA neurons would be excited by NIC via α4β2 nAChRs on cell bodies and α7 nAChRs on presynaptic GLUergic terminals. *In vivo* and *in vitro* studies were performed to establish the pharmacological mechanisms by which NIC affects VTA GABA neurons and the physiological and molecular relevance of this action.

Methods

Animal Subjects

Male CD-1 (white albino) mice were housed four to a cage from the time of weaning (P25), with *ad libitum* access to food and water. Their room temperature was controlled (22-25

^{O}C) and maintained on a reverse 12 hr light/dark cycle with lights ON from 8 PM to 8 AM. Animal care, maintenance and experimental procedures were in accordance with the Brigham Young University and Barrow Neurological Institute's Animal Research Committee and met or exceeded National Institutes of Health guidelines for the care and use of laboratory animals. Female CD-1 wild-type mice were bred with male CD-1 glutamate decarboxylase-67 (GAD67)-green fluorescent protein (GFP67) knock-in mice [GAD-GFP mice; (Tamamaki et al., 2003)]. The offspring from these crossings were heterozygous GAD-GFP mice. GAD-GFP+ offspring were distinguished from GAD GFP- offspring by gross inspection between PND1-7 with strong illumination and GFP optics. The GAD-GFP mice afforded us the ability to positively identify and record from GAD67-positive GABA neurons in the VTA of horizontal brain slices via fluorescence microscopy.

Single-unit Recordings in Anesthetized Mice

Extracellular potentials in Isoflurane (1%) anesthetized adult 30-40 g male GAD-GFP mice were recorded by a single 3.0 M NaCl filled micropipette (1-3 MΩ; 1-2 µm inside diameter), or by a single recording micropipette cemented 10-20 µm distal to a 4-barrel micropipette (20-60 MΩ resistance) for drug iontophoresis, and amplified and filtered with a MultiClamp 700A programmable amplifier (Axon Instruments, Union City, CA). Microelectrode assemblies were oriented into the VTA [from bregma: 5.6-6.5 posterior (P), 0.5-1.0 lateral (L), 7.0-8.5 ventral (V)] with a piezoelectric inchworm microdrive (Burleigh, Fishers, NY). Single-unit activity was filtered at 0.3-10 kHz (-3dB) and displayed on Tektronix 2200 digital oscilloscopes. In some experiments, square-wave constant current pulses (50-1000 µA; 0.15 msec duration; average frequency, 0.1Hz) were generated by an IsoFlex constant current isolation unit controlled by a MASTER-8 Pulse Generator (AMPI, Israel), or by computer. Extracellularly recorded action potentials (min 5:1 signal-to-noise ratio) were discriminated with a WPI-121 (Sarasota, Fl) spike analyzer and converted to computer-level pulses.

Characterization of VTA GABA Neurons in vivo

All neurons classified as VTA GABA neurons *in vivo* were located in the VTA, and met the criteria established in previous studies for spike waveform characteristics in rats (D.W. Allison et al., 2006; Steffensen, Svingos, Pickel, & Henriksen, 1998; S.H. Stobbs et al., 2004) and in mice (Allison et al., 2011b; Steffensen et al., 2010). Presumed VTA GABA neurons were characterized by short-duration (<200 μsec; measured at half-peak amplitude of the spike), initially negative-going, relatively fast-firing (10-80 Hz), non-bursting spikes. In some experiments, they were further identified by short latency (i.e., 2-5 msec) spike activation via single stimulation, and multiple spiking following high-frequency (10 pulses, 200 Hz) stimulation of the internal capsule (D.W. Allison et al., 2006; Lassen et al., 2007; Steffensen et al., 1998; S.H. Stobbs et al., 2004).

Analysis of Single-unit Recordings in vivo

Single-unit potentials, discriminated spikes, and stimulation events *in vivo* were captured by National Instrument's NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) and processed by customized National Instruments LabVIEW software in Macintosh-type computers. Potentials were digitized at 20 kHz and 12-bit voltage resolution. For single-unit activity, all spikes were captured by computer and time stamped. Spontaneous firing rates were determined on- and off-line from ratemeter records by rectangular integration over a 5 min epoch, typically 5 min before and at designated intervals after drug injection. Peri-stimulus and interval-spike histograms were generated off-line using IGOR Pro (WaveMetrics, Lake Oswego, OR).

Characterization of VTA GABA Neurons in vitro

In GAD-GFP knock-in mice (Tamamaki et al., 2003), GABA neurons were studied in horizontal brain slices with the aid of fluorescence microscopy. The VTA was visualized by first

locating the substantia nigra reticulata (SNr) in the horizontal slice preparation under low power (4X) fluorescence illumination. The SNr has a characteristic glow under low magnification with GFP fluorescence optics, likely due to dense GABA terminal innervation. Substantia nigra compacta (SNc) was then identified medial to SNr. GABA neurons in the VTA were studied by visualizing GAD+ neurons in an area medial to the glowing SNr, posterior to the fasciculus retroflexus and mammillothalamic tract, and anterior to the decussation of the superior cerebellar peduncle (Allison et al., 2011b; Steffensen et al., 2010). Neurons in the VTA of GAD-GFP mice that did not fluoresce but exhibited a non-cation specific inward rectifying current (I_h) in combination with relatively low input resistance and regular, slow spike activity were assumed to be DA neurons (Allison et al., 2011b; D.W. Allison et al., 2006; S.W. Johnson & R.A. North, 1992; Margolis, Lock, Hjelmstad, & Fields, 2006; Steffensen et al., 2010).

Drug Preparation and Administration

For iontophoretic experiments *in vivo*, NIC (NIC hydrogen tartrate salt; Sigma-Aldrich, St Louis, MO) or JN403 was dissolved in 1 mL of distilled water for 4-barrel micropipette studies (0.7 mM) or 1 M KCl for single-barrel microelectrode studies (1-2 MΩ; 0.7 mM) and ejected with positive current (+40 nA) with an Axon Instruments MultiClamp 700A amplifier in current clamp mode. For systemic experiments, NIC or the NMDA antagonist (+/-)-2-amino-5-phosphonovaleric acid (APV; Ascent Scientific) were dissolved in 0.9% saline and administered intravenously through an indwelling jugular catheter. The NIC antagonists mecamylamine hydrochloride (MEC; Sigma-Aldrich), dihydro-beta-erythroidine (DHBE; Sigma-Aldrich), methyllycaconitine (MLA; Ascent Scientific) were dissolved in 0.9% saline and administered intraperitoneally (IP).

Preparation of Brain Slices

GAD-GFP mice were anesthetized with Ketamine (60 mg/kg) and decapitated. The brains were quickly dissected and sectioned in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O_2 / 5% CO_2. This cutting solution consisted of (in mM): 220 Sucrose, 3 KCl,

1.25 NaH_2PO_4, 25 NaH_2CO_3, 12 $MgSO_4$, 10 Glucose, 0.2 $CaCl_2$, and 0.4 Ketamine. VTA targeted horizontal slices (210 μm thick) were immediately placed into an incubation chamber containing normal ACSF at 34-35°, bubbled with 95% O_2 / 5% CO_2 at 36° consisting of (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$, pH 7.3, and allowed to incubate for at least 45 minutes prior to being transferred to a recording chamber. Once transferred to a recording chamber with continuous normal ACSF flow (2.0 ml/min) maintained at 34-35° throughout the experiment, the slices were then allowed to settle for an additional 15 to 30 minutes before recordings begins. These incubation and settling periods allowed cells to recover and stabilize. Cells were visualized for patching with either Nikon Eclipse FN1 or E600FN microscopes in the transmitted de Sénarmont Differential Interference Contrast (DIC) / infrared (IR) configuration (IR-DIC).

Whole-cell Recordings in vitro

Electrodes pulled from borosilicate glass capillary tubes were filled with one of two types of pipette solutions. The pipette solution consisted of (in mM): 128 KCl, 20 NaCl, 0.3 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Mg-ATP, 0.25 Na-GTP and 4.5 QX314 (pH 7.3) to block sodium channels intracellularly. Series resistance (R_a), typically 10 to 20 MΩ, and input resistance (R_m), typically 300 to 400 MΩ, were continuously monitored with a 10 mV 20 msec voltage step delivered at 0.1 Hz throughout each experiment and only experiments that maintained stable R_a and R_m (less than 20 % change) were included in this study. Synaptic events were filtered at 2 kHz using an Axon Instruments Multiclamp 700B amplifier and digitized at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 and Igor Pro (Wavemetrics: Oswego, OR) software packages. VTA GABA neurons were identified as described above and patched first with a gigaohm seal and then the pipette was aspirated to break the membrane and achieve whole-cell recordings. To assure that spontaneous currents associated with relatively rapid VTA GABA neuron spiking would not confound the analysis of synaptic events we performed a special protocol that would assure block of sodium currents. Within 1-2 min after

going whole-cell, a voltage command waveform was administered in order to induce high-frequency sodium currents (>200 Hz for 500 msec) in VTA GABA neurons, which facilitated the block of sodium channels with the use-dependent blocker QX-314 from inside the cell (Steffensen et al., 2008). This protocol was repeated until sodium currents could no longer be elicited (typically only 2 trials blocked all sodium currents). Evoked and spontaneous EPSCs (sEPSCs) were recorded in the presence of 100 mM picrotoxin to block inhibitory synaptic transmission. Miniature EPSCs (mEPSCs) were isolated from spike-related events by addition of 0.5 μM TTX or 500 mM lidocaine. To evoke EPSCs (eEPSCs), cells were stimulated at 0.1 Hz with a stainless steel-platinum/iridium concentric bipolar stimulating electrode placed ~100-300 μm rostral to the recording electrode. Evoked EPSCs were inward at the holding potential of -70 mV and were completely blocked by 100 μM D-L 2-amino-5-phosphonopentanoic acid (APV) and 30 μM 6-cyano-23-dihydroxy-7-nitro-quinoxaline (CNQX). Evoked EPSCs amplitudes were calculated by taking the difference between the 1.0 msec window around the peak and the 5.0 msec baseline window immediately preceding the stimulation artifact. Spontaneous EPSC activity amplitude and frequency was calculated the same for both sEPSCs and mEPSCs; the average amplitude or frequency during a 2 min period 1 min following drug was normalized to the average amplitude or frequency from a 2 min window prior to drug.

Acutely Dissociated Neurons from the Mouse VTA

Single neurons were acutely dissociated from the VTA of 2-3-week-old mice (from Charles River) following procedures as previously described (J. Wu, George, et al., 2004b; K. Yang et al., 2009). Briefly, mice were anesthetized with isoflurane, and brain tissue was rapidly removed and immersed in cold (2-4°C) artificial cerebrospinal fluid (ACSF), which contained (in mM): 124 NaCl, 5 KCl, 24 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 2.4 $CaCl_2$, and 10 glucose; pH 7.4. From each mouse, two 400 μm coronal slices containing the VTA were cut using a vibratome (Vibratome 1000 Plus, St. Louis, MO). After cutting, the slices were continuously bubbled with 95% O_2 - 5% CO_2 at room temperature (22 ± 1°C) for at least 1 h in ACSF. Thereafter, the slices

were treated with papain (6 mg/ml, Sigma-Aldrich) at 33°C for 60 min in ACSF. After enzyme treatment, the slices were washed and transferred back to well-oxygenated ACSF for at least 15 min. The VTA was first identified using a stereomicroscope and then micro-punched out from the slices using a well-polished needle (0.5 mm diameter). Each punched piece was then separately transferred to a 35-mm culture dish filled with well-oxygenated standard extracellular solution, which contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES; pH 7.4 (with Tris-base). Each punched piece was then dissociated mechanically using a fire-polished micro-Pasteur pipette under an inverted microscope (Olympus IX-70, Lake Success, NY). The separated cells adhered to the bottom of the culture dish within 30 min. We used only VTA neurons that maintained their original morphological features of small- or medium-sized somata with 2-4 thin, primary dendritic processes.

Perforated Patch-clamp Whole-cell Recordings

Perforated-patch whole-cell recording techniques were employed (J. Wu, George, et al., 2004a; K. C. Yang, Jin, & Wu, 2009). Compared to performing conventional whole-cell recordings, this approach was crucial for obtaining stable nAChR responses from dissociated VTA neurons, presumably due to minimal perturbation of the intracellular environment. Pipettes (3-5 MΩ) used for perforated-patch recordings were filled with intracellular recording solution, which contained (in mM): 140 K-gluconate, 10 KCl, 5 MgCl2, and 10 HEPES; pH 7.2 (with Tris-OH) freshly supplemented before use with amphotericin B to 200 μg/ml from a 40 mg/ml DMSO stock. The liquid-junction potential was 14 mV and calculated using Clamplex 9.2 (Axon Instruments, Foster City, CA), and corrections were made for junction potentials post-hoc. After tight seal (>2 GΩ) formation, about 5-20 min was required for conversion to the perforated-patch mode, and an access resistance of <60 MΩ was accepted to start the experiments. Series resistance was not compensated in this study. Data were acquired at 10 kHz, filtered at 2 kHz, digitized on-line (Digidata 1322 series A/D board, Axon Instruments), and displayed and stored on a PC computer. Rapid application of drugs was performed using a computer-controlled "U-tube" system that allowed for complete exchange of solution surrounding the recorded cell within

30 msec. Periods of drug exposure are specified in the text and figures or legends. When ACh or choline, a precursor for local ACh formation, was used as a nAChR agonist, 1 μM atropine was routinely added to exclude actions mediated via endogenous muscarinic receptors, and this manipulation is known to have no clear effect on ACh-induced currents (J. Wu, George, et al., 2004a). All experiments were performed at room temperature. The GABAergic neurons were initially identified based on neuronal electrophysiological properties. Compared to DA neurons, GABAergic neurons have fast spontaneous action potential firing (>6 Hz), shorter action potential duration (the duration at 50% spike amplitude < 2 ms), no hyperpolarizing-induced current and insensitivity to DA. To further confirm these electrophysiologically identified GABAergic neurons, we delivered fluorescent dye (lucifer yellow; Sigma-Aldrich, St. Louis, MO; 1.0 mg/ml in the recording electrode) in some experiments. After conversion from perforated to conventional whole-cell recording mode, the dye was injected into the cytoplasm by a pulse (200 ms, 0.5 Hz) of hyperpolarizing current (1.0 nA) for 3 min. Labeled cells were visualized using epifluorescence microscopy and tyrosine hydroxylase (TH) staining post-hoc. In five cells tested, all electrophysiologically identified GABAergic neurons showed negative reaction for tyrosine hydroxylase staining.

Single-cell Quantitative RT-PCR

Following electrophysiological characterization, GAD-GFP VTA GABA neurons in mature mice were aspirated with gentle suction under visual observation, and were immediately added to a reverse transcription (RT) reaction mixture. The iScript cDNA synthesis kit (BioRad) was used for a total volume of 10 μl per reaction. Reactions were run at 25.0 ºC for 8 minutes, 42.0 ºC for 60 minutes, and 70 ºC for 15 minutes in a C1000 thermal cycler (BioRad). A preamplification round of multiplex polymerase chain reaction (PCR) was performed by adding diluted primers and iQ Supermix (BioRad) to the completed RT reaction, for a final volume of 30 μL. The reactions were cycled with a 95 ºC hot start step for 3 minutes, followed by 15 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC for 25 seconds. Samples (1.5 μl) of the initial multiplex

PCR was then used as substrate for each reaction in the subsequent real-time quantitative PCR. Real-time quantitative PCR using gene specific primers (TH forward primer: GGACAAGCTCAGGAACTATGC, TH reverse primer: GGTGTACGGGTCAAACTTCAC; 18s forward primer: GTGCATGGCCGTTCTTAGTTG, reverse 18s primer: GCCACTTGTCCCTCTAAGAAGTTG; α4 forward primer: TGGTCCTTGTCCGCTTTG, α4 reverse primer: CGTCATCATCTGGTTTTTCTCATC; α6 forward primer: TCACGGTGCATTTTGAATTG, α6 reverse primer: GGTCTCCATAATCTGGTTGACTTC; α7 forward primer: TTGCCAGTATCTCCCTCCAG, α7 reverse primer: CTTCTCATTCCTTTTGCCAG; β2 forward primer: CCTGAGGATTTCGACAATATGAAG, β2 reverse primer: GCATTGTTGTATAGAACCACATCTG; Connexin 36 forward primer: TGCAGCAGCACTCCACTATG, Connexin 36 reverse primer: ATGGTCTGCTCATCATCGTACAC; Invitrogen) with FAM-TAMRA TaqMan® probes (TH probe: CCCCATGTGGAATACACAGCGGAAGAG; 18s probe: TGGAGCGATTTGTCTGGTTAATTCCGATAAC; α4 probe: CTTGTCGATTGCTCAGCTCATTGATGT; α6 probe: CAATCACGCAACTGGCCAATGTG; α7 probe: ATGTACGCTGGTTCCCTTTTGATGTGC; β2 probe: TCCGACTCCCTTCTAAGCACATCTGGC; Connexin 36 probe: ATCCTGTTGACTGTGGTGGTGATCTTCC; Applied Biosystems) were performed using iQ Supermix (BioRad) with a CFX96 qPCR System (Bio-Rad). Samples were amplified in triplicate, together with a negative control for each subunit, which was an ACSF-only aspiration taken directly above the slice in an electrode that was previously placed in the slice . The amplification protocol was 95 ºC hot start for 3 minutes, followed by 50 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC for 25 seconds. Cycle threshold (C_t) values were calculated using CFX Manager software (BioRad). Amplification products from each sample were verified by gel electrophoresis using 4% agarose gels.

Statistical Analyses

The results for control and drug treatment groups were derived from calculations performed on VTA GABA neuron spontaneous firing rate. A two-tailed student's t test was invoked for comparison between control and treatment within subject. Comparison among individual means was made by Newman-Keuls post-hoc tests following ANOVA. Further analyses of saline vs. NIC dose-response data or other repeated measures were made by Duncan's new multiple-range test to determine the source of detected significance in the ANOVAs. The criterion of significance was set at $p<0.05$.

Results

Effects of Systemic Administration of Nicotine on VTA GABA Neuron Firing Rate In Vivo

In anesthetized (Isoflurane) mice, the average firing rate VTA GABA neurons recorded in GAD-GFP mice *in vivo* was 31.5 ± 2.5 Hz (n = 79), consistent with what we have reported previously in other studies in mice (Ludlow et al., 2009; Steffensen et al., 2010). Intravenous administration of freebase NIC (0.2 mg/kg) markedly enhanced the firing rate of VTA GABA neurons recorded in GAD-GFP mice *in vivo* (**Fig. 2A**). The rate of firing increased in about 20 sec and returned to baseline approximately 3-4 min after the first NIC injection. However, repeated administration of systemic 0.2 mg/kg NIC at 5 min intervals resulted in a marked tachyphylaxis of the activating effects of NIC on VTA GABA neuron firing rate. Indeed, the third and fourth injections of NIC resulted in a significant decrease in the activation of firing rate compared to the first injection (**Fig. 2B**; 1^{st} vs 3^{rd}: P = 0.01, $t_{(2,5)} = 3.3$; n = 6; 1^{st} vs 4^{th}: P = 0.005, $t_{(2,5)} = 4.8$; n = 6).

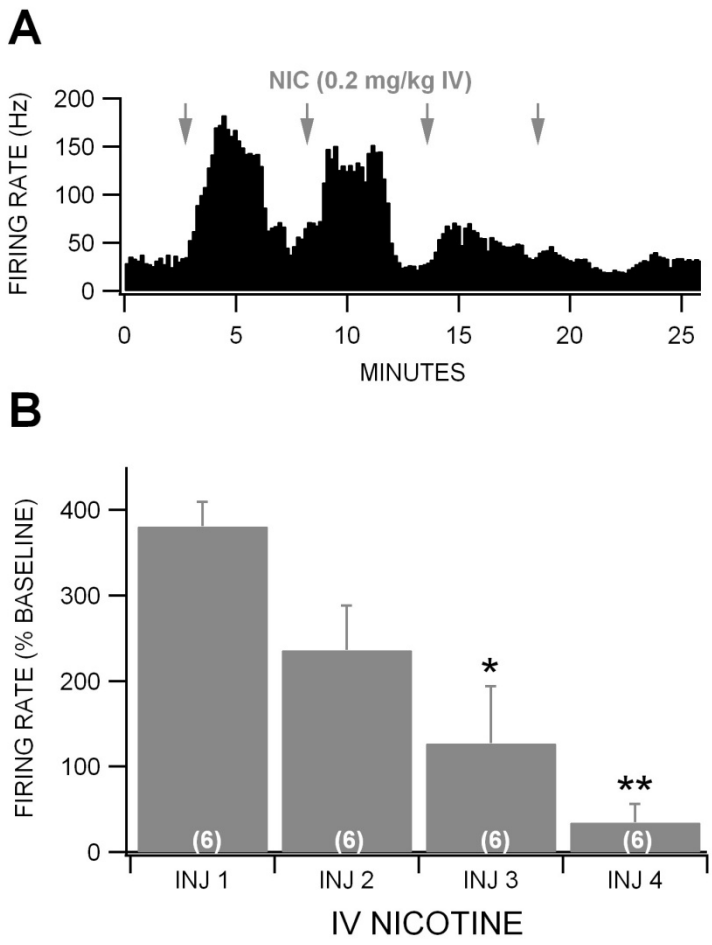


Figure 2 Effects of repeated systemic NIC exposure on GABA neuron firing rate. (A) This ratemeter record shows the firing rate of a representative VTA GABA neuron in a GAD-GFP mouse. Its baseline firing rate was approximately 30 Hz. Administration of intravenous (IV) NIC (0.2 mg/kg) markedly increased the firing rate of this neuron, but subsequent injections resulted in a diminished response. Each dose of NIC was administered at regular intervals of 5 min. (B) This graph summarizes the effects of IV NIC on repeated injections. Note the diminution in the systemic NIC activation of firing rate with repeated injections. Asterisks * and ** = significant reduction ($P < 0.05$) in NIC activation with repeated systemic NIC administration (N values).

Pharmacology of Local Nicotine Activation of VTA GABA Neuron Firing Rate In Vivo

In situ microelectrophoretic application of NIC (+40 nA) markedly enhanced the firing rate of VTA GABA neurons recorded in anesthetized GAD-GFP mice *in vivo* (330.7 ± 26.8 %; P = 5.6E-07, $t_{(2,54)}$ = 5.9; n = 55; **Fig. 3A,B**). Although a robust tachyphylaxis was observed with repeated systemic NIC injections (**Fig. 2**), it was surprising that there was no diminution of the NIC response with repeated, periodic (1.0 min ON, 1.0 min OFF) iontophoretic NIC current applications (**Fig. 3**). The ability to repeatedly activate VTA GABA neurons reliably with periodic NIC applications justified the utility of antagonists to determine which nAChR subtype might be involved in NIC activation of VTA GABA neuron firing rate. Accordingly, as α7 nAChRs are typically located on GLU terminals (Mansvelder & McGehee, 2000), we evaluated the effects of intraperitoneal administration of the non-selective, non-competitive antagonist mecamylamine (MEC), the α7 nAChR antagonist methyllycaconitine (MLA), and the NMDA GLU receptor antagonist aminophosphonovalerate (APV) at the same dose level (1 mg/kg) on NIC activation of VTA GABA neuron firing rate *in vivo*. While MEC had no significant effects (P > 0.05), MLA and APV significantly reduced NIC activation of VTA GABA neuron firing rate (MLA: P = 0.001, $t_{(2,9)}$ = 4.1; n = 10; APV: P = 0.04, $t_{(2,6)}$ = 2.3; n = 7; **Fig. 3A,B**), compared to an isovolumic injection of saline. Since MLA suppressed NIC activation of VTA GABA neuron firing rate, we evaluated the effects of local application of the α7 nAChR partial agonist JN403. Microelectrophoretic application of JN403 (+40 nA) significantly (444.0 ± 64.1%; P = 5.8E-05, $t_{(2,23)}$ = 4.8; n = 24) enhanced the firing rate of VTA GABA neurons. Intraperitoneal administration of MLA significantly reduced JN403-induced increase in VTA GABA neuron firing rate (P = 0.008, $t_{(2,4)}$ = 4.6; n = 5; **Fig. 3C,D**).

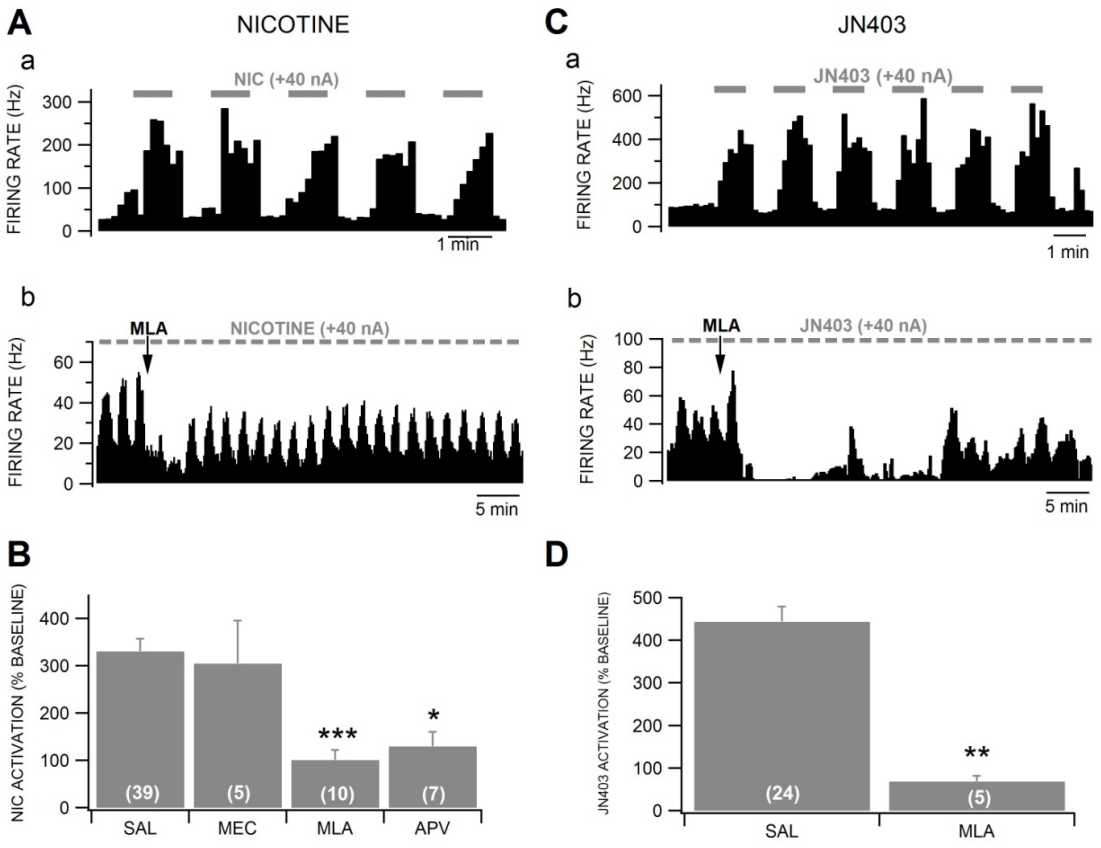
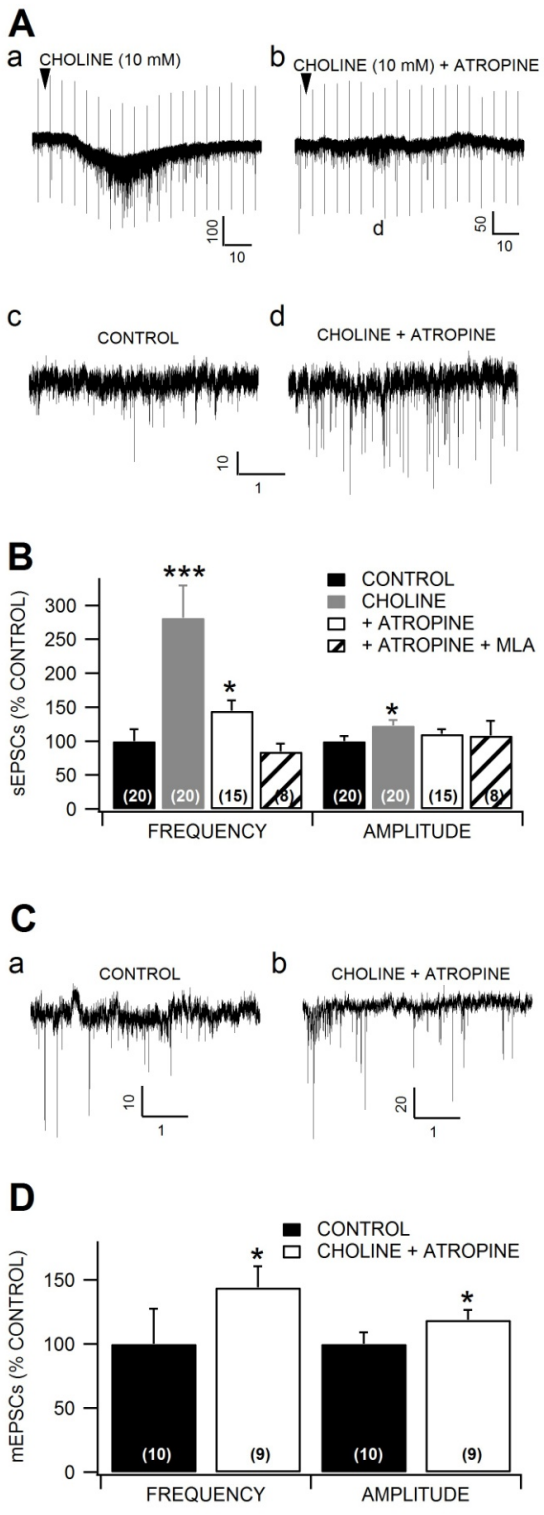


Figure 3 Pharmacology of NIC activation of VTA GABA neurons. (Aa) This ratemeter record shows the firing rate of a representative VTA GABA neuron in a GAD-GFP mouse and the robust activation by microelectrophoretic application of NIC. (Ab) NIC (0.2 mg/kg) activation of this neuron was markedly reduced by systemic administration of the $\alpha 7$ nAChR antagonist methyllycaconitine (MLA; 1 mg/kg). (B) While IP administration of the non-selective non-competitive antagonist mecamylamine (MEC; 1 mg/kg) had no significant effect, MLA significantly reduced NIC activation of VTA GABA neuron firing rate. In addition, IV administration of the selective NMDAR inhibitor APV (1 mg/kg) significantly reduced NIC activation of VTA GABA neuron firing rate. (Ca) This ratemeter record shows the firing rate of a representative VTA GABA neuron in a GAD-GFP mouse and the robust activation by microelectrophoretic application of the α7 nAChR partial agonist JN403. (Cb) JN403 (1 mg/kg) activation of this neuron was markedly reduced by systemic administration of MLA (1 mg/kg). (D) MLA significantly reduced JN403 activation of VTA GABA neuron firing rate. Asterisks *,**, and *** = significant reduction with P values of < 0.05, < 0.01, and < 0.001, respectively (N values).

Effects of α7 Nicotinic Receptor Agonist on Glutamatergic Synaptic Transmission to VTA GABA Neurons

Based on *in vivo* studies (**Fig. 2-3**), NIC markedly excites VTA GABA neurons, irrespective of the route of administration (i.e., systemic vs local). As the α7 nAChR antagonist MLA or the GLU antagonist APV suppressed NIC activation of VTA GABA neuron firing rate, we pursued *ex vivo* electrophysiology and pharmacology studies in the slice preparation to determine the mechanism of action of NIC effects on excitatory synaptic transmission to VTA GABA neurons. We hypothesized that NIC was acting on α7 nAChRs on GLU terminals enhancing the release of GLU to VTA GABA neurons. We tested the effects of the α7 nAChR agonist choline (10 mM) on VTA GABA neuron sEPSCs in the horizontal VTA slices. The average sEPSC frequency in VTA GABA neurons was 9.7 ± 1.7 Hz (n = 20) and the average amplitude was 20.3 ± 1.53 pA (n = 20). Superfusion of the slice with choline induced a slow inward current in VTA GABA neurons and markedly enhanced sEPSC frequency (**Fig. 4Aa**). However, as choline is also a muscarinic cholinergic agonist at this concentration level, we tested it in the presence of the muscarinic antagonist atropine (50 μM). The ability of choline-induced inward current was blocked by atropine (**Fig. 4Ab**), but its ability to enhance sEPSCs remained unchanged (Fig. 3Ac,d), albeit the enhancement was more transient than without atropine. Figure 3B summarizes the effects of choline on sEPSC frequency and amplitude. Choline alone significantly increased sEPSC frequency ($P = 0.001$, $t_{(2,19)} = 3.8$; n = 20) and amplitude ($P = 0.01$, $t_{(2,19)} = 2.8$; n = 20). Choline + atropine also significantly increased sEPSC frequency ($P = 0.01$, $t_{(2,14)} = 2.9$; n = 15) but not amplitude ($P = 0.16$, $t_{(2,14)} = 1.5$; n = 15). The effect elicited by choline + atropine was blocked by the α7 nAChR antagonist MLA (n = 8; **Fig. 4B**). Thus, action potential-dependent spontaneous GLU input to VTA GABA neurons in the slice preparation was modulated by activation of α7 nAChRs on GLU terminals, even when the muscarinic postsynaptic effects of choline were controlled. To further support our hypothesis that NIC enhances VTA GABA neuron firing rate *via* α7 nAChR-induced GLU release on GLUergic terminals, we tested the effects of choline + atropine on spontaneous miniature EPSCs (mEPSCs). The frequency of mEPSCs is a reliable measure of spontaneous GLU release, which we presumed would be sensitive to nAChR

agonist modulation. The mEPSCs were recorded in the presence of tetrodotoxin (TTX; 500 nM, which blocked action potential-dependent release of GLU, and picrotoxin (PTX; 100 mM), which blocked inhibitory synaptic transmission. The averaged mEPSC frequency in VTA GABA neurons was 5.5 ± 1.5 Hz (n = 10) and the averaged amplitude was 18.1 ± 1.6 pA (n = 10). Superfusion of the slice with choline + atropine enhanced mEPSC frequency (**Fig. 4Ca,b**). **Figure 4D** summarizes the effects of choline on mEPSC frequency and amplitude. Choline + atropine significantly increased mEPSC frequency ($P = 0.03$, $t_{(2,8)} = 2.6$; n = 9) and amplitude ($P = 0.04$, $t_{(2,8)} = 1.5$; n = 9), which was blocked by 0.5 μM MLA (n = 8). For the same reasons listed above, although nAChR-mediated GLU input to VTA GABA neurons from the PFC may not be spontaneously operational in the horizontal slice, it could still be evoked by electrical stimulation. Thus, we tested the effects of the selective α7 nAChR partial agonist JN403 on EPSCs evoked by local stimulation (i.e., eEPSCs), which would effectively activate GLU terminals on VTA GABA neurons irrespective of spontaneous action potential input. The average amplitude of eEPSCs in VTA GABA neurons was 56.1 ± 6.9 pA (n = 30) and the average paired-pulse response (50 msec interstimulus-interval) was 99.4 ± 5.65 % (n = 30). **Figure 5** shows the effects of NIC and JN403 on eEPSCs. While 1 μM NIC did not significantly alter eEPSC amplitudes ($P = 0.61$, $t_{(2,9)} = 0.53$; n = 10), superfusion of 1 μM JN403 significantly enhanced eEPSCs in VTA GABA neurons ($P = 0.02$, $t_{(2,11)} = 2.9$; n = 12).


A
a
CHOLINE (10 mM)
b
CHOLINE (10 mM) + ATROPINE
100
10
d
50
10
c
CONTROL
d
CHOLINE + ATROPINE
10
1
B
***
*
*
CONTROL
CHOLINE
+ ATROPINE
+ ATROPINE + MLA
sEPSCs (% CONTROL)
300
250
200
150
100
50
0
(20)
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FREQUENCY
AMPLITUDE
C
a
CONTROL
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CHOLINE + ATROPINE
10
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CHOLINE + ATROPINE
mEPSCs (% CONTROL)
150
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AMPLITUDE

Figure 4 Effects of the α7 nAChR agonist choline on spontaneous EPSCs in VTA GABA neurons. (Aa) Spontaneous EPSCs (sEPSCs) were recorded in VTA GABA neurons in the horizontal slice in the presence of 100 uM picrotoxin to block sIPSCs and the sodium channel blocker QX-314 in the pipette to block spikes. Superfusion of 10 mM choline to the slice induced a membrane depolarizing current and enhanced sEPSCs in this representative VTA GABA neuron. Vertical lines are capacitative currents from a membrane test performed every 10-s to monitor membrane input resistance. (Ab) Superfusion of choline in the presence of atropine (50 μM) did not evince an inward current but transiently enhanced sEPSCs in this representative VTA GABA neuron. (Ac,d) An expanded view of sEPSCs recorded before and after superfusion of choline. The trace in Ad is taken from the time denoted by the letter "d" in the trace in Ab. Calibration bars are in pA and seconds. (B) This graph summarizes the effects of choline alone, choline + atropine, and MLA (0.5 μM) on choline + atropine effects on sEPSC frequency and amplitude in VTA GABA neurons. Choline markedly enhanced sEPSC frequencies and amplitudes, while choline + atropine only increased sEPSC frequencies. (Ca,b) Representative recording of mEPSCs obtained from a VTA GABA neuron before and after choline + atropine. Note the small increase in sEPSC frequency. (D) This graph summarizes the effects of choline + atropine on mEPSCs in VTA GABA neurons. Choline significantly increased both mEPSC frequency and amplitude. Sample sizes are shown in parentheses.

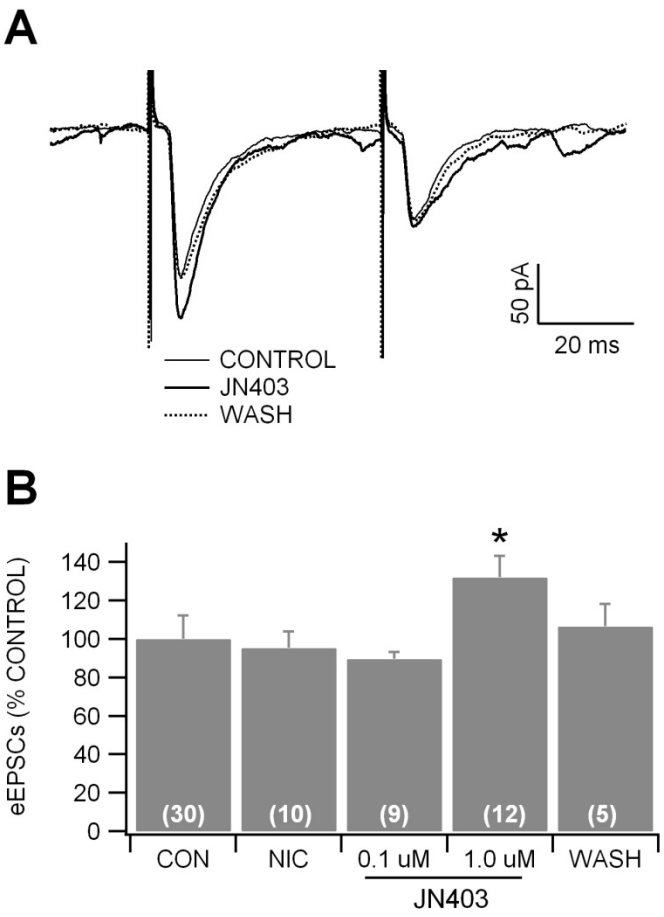

Figure 5 Effects of NIC and the α7 agonist JN403 on eEPSCs in VTA GABA neurons. (A) Representative recording of eEPSC amplitude obtained from VTA GABA neurons before, during, and after JN403 superfusion. (B) This graph summarizes the effects of NIC and JN403 on eEPSCs in VTA GABA neurons. Superfusion of JN403 significantly enhanced eEPSCs in VTA GABA neurons at the 1 uM concentration. Sample sizes are shown in parentheses. Asterisks * = significance level P<0.05 (N values).

Lack of α7 Nicotinic Receptor Function in Dissociated VTA GABA Neurons

In order to provide further evidence that NIC was acting on α7 nAChRs on GLU terminals, rather than on the somatodendritic area of VTA GABA neurons, we tested the effects of the α7 nAChR agonist choline on acutely-dissociated VTA GABA neurons. Under these conditions, dissociated single neurons have no presynaptic terminals/boutons (K. C. Yang et al., 2009). Thus, this single neuron model has only postsynaptic receptors. Under perforated whole-cell recording conditions in the voltage-clamp mode, bath-application of the α7 nAChR selective agonist choline (10 mM), via a U-tube, induced a small inward current at a holding potential of -60 mV (**Fig. 6Aa**) in only a few cells tested. In the 32 cells tested, from 20 mice, only 6 neurons (18.7%, Fig. 4B) exhibited choline responses with small amplitude of 10.2 ± 1.1 pA. Interestingly, ACh, GLU (1 mM), and GABA (0.1 mM) induced much larger inward current (**Fig. 6Ab-d**). When choline induced inward currents in this voltage-clamp model, it showed little effect on AP firing in current-clamp recording mode, unlike Ach, which markedly increased AP firing (**Fig. 6C**). These results suggest that α7 nAChRs are not predominantly expressed on the somatodendritic area of GABA neurons, and the activation of α7 nAChRs on postsynaptic GABAergic neurons has little effect on GABAergic neuron firing.

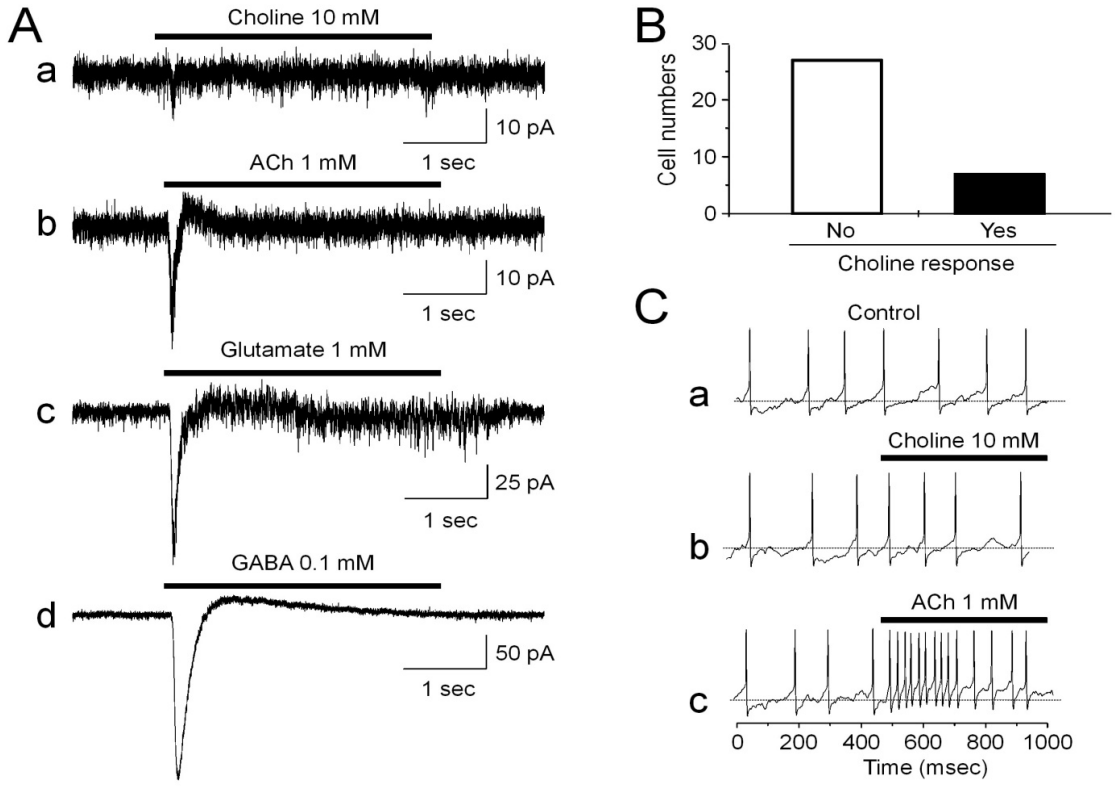

Figure 6 Lack of choline induced current in single GABAergic neurons acutely dissociated from mouse VTA. (A) Representative typical traces of choline-, ACh-, GLU- or GABA-induced inward current from a single VTA GABA neuron. Traces a-d were recorded from the same neuron. (B) This graph summarizes choline-induced responses from a total of 32 VTA GABA neurons tested. (C) Under current-clamp recording mode, choline did not show clear effect on GABA neuron firing, while in the same recorded neuron, ACh increased neuronal firing rate.

Lack of α7 Nicotinic Receptor Expression in VTA GABA Neurons

Performing quantitative real-time PCR on single VTA GABA neurons in GAD-GFP mice, we evaluated the expression of select nAChR mRNA transcripts. These cells were positively identified as GABA neurons, using fluorescence microscopy, due to their expression of GFP. While many VTA GABA neurons tested expressed α4 and β2 nAChR subunits as well as Cx36, a marker of VTA GABA neurons in GAD-GFP mice (D.W. Allison et al., 2006; Steffensen et al., 2010), few neurons expressed α6 subunits and none expressed α7 subunits (**Fig. 7**).

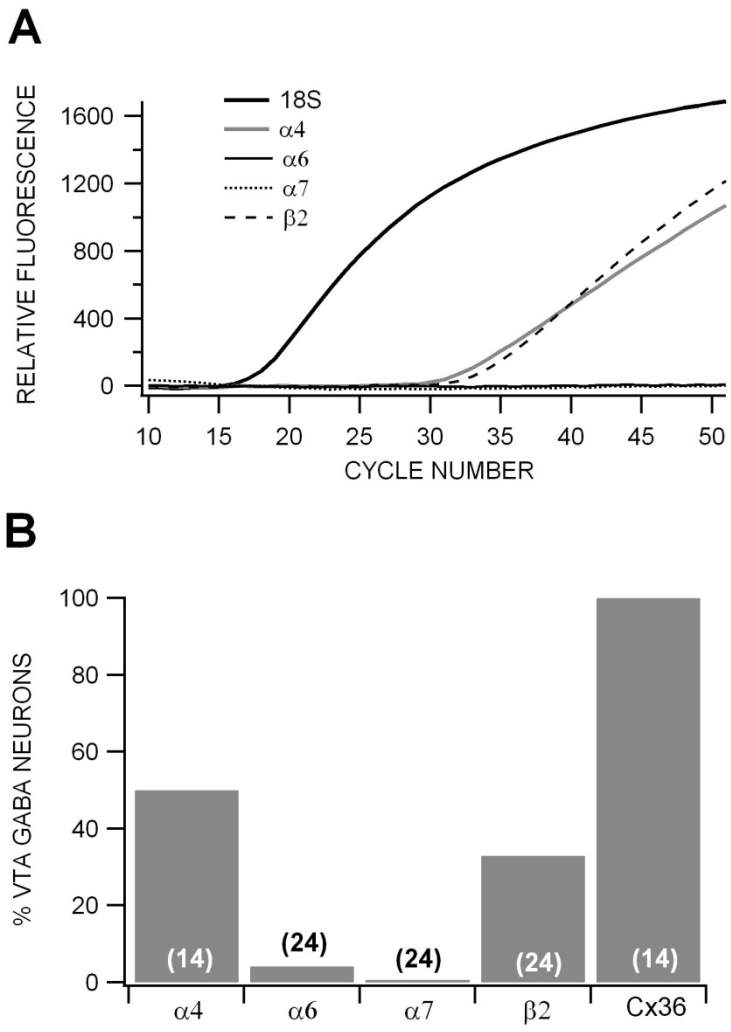

Figure 7 Single-cell real-time quantitative PCR of selected nAChR subunits in VTA GABA neurons from GAD-GFP mice. (A) This graph illustrates the expression of 18S and the nAChR subunits α4, α6, α7, and β2 in a representative VTA GABA neuron from a GAD-GFP mouse. Each plot is an average of triplicates. Note that the reference gene 18S was used to determine relative mRNA expression levels and ensure successful mRNA harvesting from each cell. In this representative cell, α4 and β2 nAChR subunits were expressed but the α6 and α7 subunits were not. (B) This graph illustrates the percent of VTA GABA neurons expressing α4, α6, α7, and β2 nAChR subunits in VTA GABA neurons from GAD-GFP mice. The expression of Cx36, a marker for GABA neurons in the VTA (D.W. Allison et al., 2006; Steffensen et al., 2010), is also shown for comparison. Sample sizes are shown in parentheses.

Discussion

We evaluated the activity of NIC on VTA GABA neuron firing rate *in vivo* to establish the pharmacological mechanisms involved in the acute effect elicited by NIC. Since systemic administration causes NIC circulation throughout the entire brain, NIC could be influencing neurons directly or indirectly through activation of nAChRs on afferents to the VTA, including GABA afferents to VTA GABA neurons from the NAc and ventral pallidum, as well as GLU inputs to VTA GABA neurons from the PFC that are sensitive to NIC (Carr & Sesack, 2000b; Geisler & Wise, 2008). In addition, there are putative ACh inputs from the habenula and pedunculopontine tegmental nucleus (Good & Lupica, 2009; Maskos, 2008). Thus, NIC systemic effects need to be evaluated in light of its possible net effects on GABA neurons and its afferents. However, since repeated administration of local NIC did not result in tachyphylaxis, one possible explanation is that a remote input to the VTA that is sensitive to NIC, in particular the PFC (D. Zhang et al., 2012), might be responsible for the acute tolerance to NIC activation by systemic NIC. Admittedly, NIC activation of DA neurons in the VTA is known to induce DA release locally, which could have some effect on nearby neurons (D. Zhang et al., 2012).

NIC activation, achieved with periodic *in situ* microelectrophoretic application, was found consistently and with profound magnitude, but did not diminish during current application or with repeated NIC exposures. Approximately 75% of VTA GABA neurons studied were activated by NIC in this manner. The ability of MLA to block NIC activation of VTA GABA neurons lends credence to the notion that α7 nAChRs play a crucial role in the activation of these neurons. The non-selective nAChR antagonist MEC had no effect on NIC activation of VTA GABA neuron firing rate, further supporting this proposition. Most importantly, local application of the potent α7 nAChR partial agonist JN403 (Feuerbach, Nozulak, Lingenhoehl, McAllister, & Hoyer, 2007) had consistent and robust excitatory effects on these neurons *in vivo* as well. Indeed, JN403 excited VTA GABA neurons in all neurons tested, without diminution in response with repeated administrations. Its excitatory effect was also greatly diminished with systemic application of MLA, providing further evidence that NIC excites VTA GABA neurons via α7 nAChRs.

Glutamate projections from the PFC innervate both DA and GABA neurons (Carr & Sesack, 2000a, 2000c) and often contain α7 nAChRs on GLUergic terminals (Jones & Wonnacott, 2004). Interestingly, α7 nAChRs were not associated with cholinergic synapses, consistent with their activation by a paracrine mode of ACh or choline delivery (Jones & Wonnacott, 2004). In order to evaluate the role of α7 nAChRs in local NIC activation of VTA GABA neurons, we tested the effects of the NMDA receptor antagonist APV. NIC activation was markedly reduced by systemic APV, suggesting that this increase in VTA GABA neuron firing rate was mediated by α7 nAChRs located on GLUergic terminals. Behavioral paradigms also support this electrophysiological evidence. MLA and APV have been shown to halt NIC's rewarding effects, when microinfused with NIC into the VTA, during a CPP procedure in rats (Laviolette and Kooy, 2003). As in our *in vivo* recordings in anesthetized mice, both α7 nAChR and NMDAR antagonists elicit similar outcomes.

Mansvelder *et al*. (Mansvelder et al., 2002) has reported increases in GABA neuron firing rate with NIC *in vitro*. There is some concern, however, that the neurons they studied were not GABA neurons, as they relied on electrophysiological criteria alone (i.e., Ih current—DA neurons being Ih^+ and GABA neurons being Ih^-), which have been shown more recently to not be universally valid (Margolis et al., 2006). Accordingly, we have also observed repeatedly that GAD^+ neurons in the VTA from GAD-GFP mice often evidence an appreciable Ih. Our *in vitro* data (**Fig. 4**) takes advantage of visually identified GABA neurons in GAD-GFP mice in slice recordings and acutely dissociated neurons to further support our understanding of NIC's mechanism of action in the VTA. Our *in vivo* findings suggested that NIC actions on α7 nAChRs on GLUergic innervations in the VTA enhanced the activity of VTA GABA neurons. Only a small percentage of dissociated VTA GABA neurons were sensitive to choline and none of the VTA GABA cells in GAD-GFP tested positive for α7 nAChR transcripts, supporting a presynaptic locus for α7 effects on GLU terminals to VTA GABA neurons. However, there is evidence that some VTA DA and GABA neurons express α7 nAChRs (Klink, Exaerde, Zoli, & Changeux, 2001). We have previously shown that the firing rate of VTA GABA neurons is strongly mediated by corticotegmental NMDAR-mediated GLU input to VTA GABA neurons (Steffensen et al., 1998).

Indeed, based on intracellular recordings *in vivo*, VTA GABA neuron activity is always preceded by a depolarizing current that is sensitive to NMDAR antagonists which abolishes their spiking. Thus, the activity of VTA GABA neurons appears to be driven strongly by GLUergic synaptic input, even at the high firing rates characteristic of these neurons *in vivo*.

Based on *in vivo* findings, we postulated that NIC was acting on α7 nAChRs to enhance GLU release on VTA GABA neurons. However, the synaptic mechanism of action of NIC on GLU synaptic transmission is best determined *in vitro*. Thus, we tested the effects of NIC and α7 nAChR agonists on spontaneous and evoked EPSCs in horizontal slice preparation. NIC did not alter sEPSC amplitude or frequency, perhaps because of complex temporospatial effects on multiple nAChRs serving VTA GABA neurons. Although choline increased sEPSC frequency in VTA GABA neurons in the slice preparation, this approach inherently possesses two limitations. First, the excitatory GLU input from the PFC that contributes significantly to VTA GABA neuron firing rate (Steffensen et al., 1998), and is modulated by α7 nAChRs, is likely severed in the slice, even when cut horizontally. Thus, the action potential-dependent events that compose sEPSCs may not represent PFC GLU input. Second, sEPSCs are likely composed of local-circuit GLU input to VTA GABA neurons (Yamaguchi, Sheen, & Morales, 2007a), as well as action potential-independent spontaneous GLU release. While GLU input from the PFC may not be spontaneously active on VTA GABA neurons in the slice preparation, action potential-independent spontaneous release of GLU would still be operational, albeit somewhat diluted by other GLU inputs that appear to be local circuit in origin. However, the α7 agonist JN403 increased eEPSC amplitude, providing further support that activation of α7 nAChRs on GLU terminals underlies NIC activation of VTA GABA neuron firing rate.

Dissociated neuron experiments showed very low percentages of VTA GABA neurons that responded to NIC agonists, although some responses are likely mediated through α7 nAChRs. We assert that these α7 nAChRs on postsynaptic somatodendritic sites may not play an important role in mediating the NIC-induced increase in VTA GABA neuron firing. First, the density of these somato α7 nAChRs appears to be very low. Second, these receptors, even though they may depolarize membrane potential, they desensitize very quickly at high NIC

concentrations. Finally, inhibition of ionotropic GLU receptors, such as produced by a NMDAR antagonist, can completely abolish this nicotinic effect.

In order to validate the physiological evidence supporting our hypothesis that NIC activation of VTA GABA neurons is through activation of α7 nAChRs on GLU terminals and not on VTA GABA neurons, we examined the expression of nAChR subunits in VTA GABA neurons from GAD GFP mice. A high percentage of VTA GABA neurons expressed α4β2, but only a small percentage expressed α6 and none of them expressed α7 nAChRs, providing molecular evidence in support of our physiological findings.

GLUergic innervation of both VTA GABA and DA neurons gives rise to the potential for the increase in firing of both major types of VTA neurons via pre-synaptic NIC-induced α7 nAChR activation (**Fig. 1**). This duel innervation would seem to result in a null effect when acute local NIC is present in the VTA. However, this is not the case, as some innervation of GLU neurons onto VTA GABA neurons may subsequently innervate other local VTA GABA neurons. This disinhibition mechanism would result in a net increase of VTA DA neuron firing, with these neurons projecting to the NAc. As discussed earlier, this DA neuron firing in the NAc is thought to be a signal for reward.

Conclusions

Our data suggest that α7 nAChRs are mainly expressed at GLUergic terminals onto GABA neurons, which likely play a key role in the mediation of local NIC-induced firing increase in these neurons. The combination of our *in vivo* and *in vitro* results, as well as the behavioral data of others, forms a strong justification for the previous affirmation. The results obtained in these experiments further illuminate the functional role of α7 nAChRs in the VTA. Since the experiments from this study were performed to determine the acute effect of NIC on VTA neurons, no direct conclusion can be obtained regarding the addictive activity of NIC after prolonged (chronic) use. Nevertheless, the obtained results elucidate the functional role of α7 nAChRs in GLUergic terminals, supporting the notion that ligands that modulate this nAChR subtype might be important for the development of therapies for NIC addiction

CHAPTER 3

ALPHA 6 SUBTYPE CONTAINING NICOTINIC RECEPTORS MEDIATE ETHANOL EFFECTS ON MESOLIMBIC NEURONS AND REWARD

Introduction

A major goal of basic research on drug addiction is to understand the neural basis of drug use and the pathological progression to dependence (Gilpin & Koob, 2008a). The compulsion to consume alcohol and NIC stems from their positive reinforcing properties, including their anxiolytic and euphoric effects, as well as their negative reinforcing properties, including aversive withdrawal symptoms that result from abstinence (Koob, Rassnick, Heinrichs, & Weiss, 1994). A large body of research has supported the idea that the rewarding effects of NIC, EtOH, and other psychoactive drugs are dependent on mesocorticolimbic DA-mediated neurotransmission (Blackburn, Phillips, Jakubovic, & Fibiger, 1986b; Koob, 1992; Wise, 2004; R.A. Wise, 1996; R.A. Wise & M.A. Bozarth, 1987). Current dogma maintains that DA neuron activation and release in the mesolimbic DA system is literally a scalar index of reward (Wise, 2008). The emerging view is that the dysregulated homeostasis that accompanies the development of drug addiction may result from experience-dependent neuroadaptations that hijack normal synaptic transmission in this system (Hyman & Malenka, 2001; Hyman, Malenka, & Nestler, 2006; Kauer & Malenka, 2007; Nugent & Kauer, 2008). The neuroadaptions that occur in this system in association with drug dependence, including alcohol and NIC, may result from plasticity associated with inhibitory GABA and/or excitatory GLU synaptic transmission in this system (Nugent & Kauer, 2008).

NIC has high affinity for nAChRs. Activation and desensitization of nAChRs in the mesolimbic DA system may be crucial factors underlying the effects of NIC on DA neurons in the midbrain VTA (Mansvelder et al., 2002; Mansvelder & McGehee, 2000) that project to limbic structures such as the NAc and PFC (de Rover et al., 2002)]. Neurons within the VTA express a wide variety of nAChRs (Wooltorton, Pidoplichko, Broide, & Dani, 2003), and NIC can activate both DA and GABA neurons (Mansvelder et al., 2002; Yin & French, 2000), although the majority of endogenous cholinergic inputs into the VTA appear to contact GABA rather than DA neurons (Fiorillo & Williams, 2000; Garzon et al., 1999). The majority of the neurons in the VTA express

α4 and β2 nAChR subunits, which can be blocked by the non-competitive, non-α7 antagonist MEC, or by the competitive antagonist DHβE (Mansvelder et al., 2002). It is the emerging view that the early, acute effects of NIC in the VTA predominantly affect GABA neurons, and that nAChRs that have been associated with these cells desensitize rapidly, leading to a long-lasting excitation of DA neurons through removal of the inhibitory influence of GABA (Mansvelder et al., 2002). In addition to the transient actions of NIC on DA and GABA neurons via α4β2 nAChRs, considerable evidence indicates that the actions of NIC within the VTA might be mediated by GLUergic transmission. Blockade of GLU NMDA receptors (NMDARs) and α7-nAChRs in the VTA reduces the increase in mesolimbic DA release that is induced by NIC (Schilstrom et al., 2000; Schilstrom, Svensson, Svensson, & Nomikos, 1998), and systemically- or locally-injected NMDA antagonists into the VTA can block the rewarding effects of NIC (Laviolette & van der Kooy, 2003b; Papp, Gruca, & Willner, 2002). We have recently shown that NIC activation of VTA GABA neuron activity is mediated by enhancement of GLU release via α7-nAChRs on GLU terminals (D.H. Taylor et al., 2013). In addition to α4β2 and homomeric α7-nAChRs, there is a considerable expression of heteromeric α6*-nAChRs (* denotes α6 subunits combined with other nAChR subunits) in the VTA. Alpha6*-nAChR subunits are functional in recombinant systems when paired with β subunits or hybrids of β subunits. Their mRNA levels are 16-fold higher than other subunits in the VTA (K. Yang et al., 2009). They have been implicated in DA transmission and NIC dependence (Drenan et al., 2010b; Drenan et al., 2008; Exley, Clements, Hartung, McIntosh, & Cragg, 2008; Gotti et al., 2010b; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009). We have recently shown that α6*-nAChRs are located on GABA terminals to VTA DA neurons and that their activation enhances GABA synaptic inhibition of VTA DA neurons (K. Yang et al., 2011a).

Unlike NIC, the molecular targets of alcohol effects in the brain are not well-defined. However, research over the past two decades has supported the direct involvement of protein receptors, in particular pentameric ligand-gated ion channels including GABA, glycine, GLU, Ach, and serotonin [for recent review see (Howard, Trudell, & Harris, 2014)]. Notwithstanding the lack of consensus regarding the molecular targets for EtOH, at the cellular/neurochemical level there

is confirmatory evidence to suggest that EtOH can alter excitatory amino acid GLU function (P. L. Hoffman & Tabakoff, 1993; P. Hoffman, Rabe, Moses, & Tabakoff, 1989; Lima-Landman & Albuquerque, 1989; D. M. Lovinger, G. White, & F. F. Weight, 1990; Lovinger, White, & Weight, 1989; D.M. Lovinger, G. White, & F.F. Weight, 1990; Roberto et al., 2004), that it can interact with inhibitory GABA subtype A receptor [GABA(A)R] complexes (Allan & Harris, 1986; Liljenquist & Engel, 1982; Mihic & Harris, 1996; Suzdak et al., 1986; Ticku, Lowrimor, & Lehoullier, 1986), that it can interact with neuromodulators such as neurosteriods (Brot, Akwa, Purdy, Koob, & Britton, 1996; Kumar, Fleming, & Morrow, 2004; Lambert, Peters, & Cottrell, 1987; Simmonds, 1991; Wieland, Belluzzi, Stein, & Lan, 1995), and that it influences several neuropeptide functions including somatostatin (G.R. Siggins, Nie, Schweitzer, Madamba, & Henriksen, 1996) and corticotropin releasing factor (Koob, Heinrichs, Menzaghi, Pich, & Britton, 1994). The prevailing dogma is that synaptic transmission is depressed by acute intoxicating doses of EtOH (Ariwodola et al., 2003; Berry & Pentreath, 1980; Bloom et al., 1984; Deitrich, Dunwiddie, Harris, & Erwin, 1989; Shefner, 1990; G. R. Siggins et al., 1987), which might result from either an attenuation of excitatory NMDA receptor-mediated GLU synaptic transmission (Lovinger et al., 1989; D.M. Lovinger et al., 1990; Nie, Yuan, Madamba, & Siggins, 1993; Roberto et al., 2004; G. White, Lovinger, & Weight, 1990) and/or an enhancement of inhibitory GABA(A) receptor-mediated synaptic transmission (Deitrich et al., 1989; Harris & Allan, 1989; Roberto, Madamba, Moore, Tallent, & Siggins, 2003). However, elucidation of the molecular targets for alcohol that influence GLU or GABA-mediated synaptic transmission has remained elusive. Notwithstanding, there are known interactions between EtOH and nACHRs [for review see (Davis & de Fiebre, 2006)]. For example, in animal studies, mice lacking the α7-nAChR subunit consume significantly less EtOH than WT controls (Kamens, Andersen, & Picciotto, 2010). Superfusion of NIC and EtOH to brain slices show synergistic effects on DA neuron firing rate, depending upon dose level of each drug (Clark & Little, 2004). Synergistic effects are also seen on DA release in the NAc when low-dose EtOH and NIC are injected into the midbrain VTA (Tizabi, Copeland, Louis, & Taylor, 2002). A possible target for interactive effects of NIC and EtOH is VTA GABA neurons. We have shown in multiple publications that VTA GABA neurons are inhibited by acute intoxicating EtOH *in vivo* at

low to high doses [0.25- 4.0 g/kg; (Diana et al., 2003; Ludlow et al., 2009; Steffensen et al., 2010; Steffensen et al., 2009; S. H. Stobbs et al., 2004b), and we have recently demonstrated their sensitivity to NIC (D.H. Taylor et al., 2013). As we have recently shown and that EtOH modulation of phasic DA release at terminals in the NAc is mediated by α6*-nAChRs (Schilaty et al., 2014), presumably via axoaxonic α6*-nAChRs on GABA terminals on DA terminals in the NAc, we postulated that EtOH and NIC effects on DA transmission in the mesolimbic system is mediated by interactive effects of NIC and EtOH on α6*-nAChRs on GABA terminals.

Methods

Animal Subjects

Black wild-type (WT) male C57BL/6 mice, α6 KO mice, and glutamate-decarboxylase-67 (GAD-67)-green fluorescent protein knock-in (CD-1) mice (Tamamaki et al., 2003) were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals were treated in strict accordance with the Brigham Young University and Barrow Neurological Institute's Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. The BYU and BNI IACUCs have reviewed and approved the procedures detailed herein. Once weaned at PND 21, all mice were housed in maximum groups of four and given ad libitum access to solid food and water and placed on a reverse light/dark cycle with lights ON from 8 PM to 8 AM.

Preparation of Brain Slices

All brain slice preparations were performed in P18-45 day old C57BL/6 and GAD-GFP knock-in mice. Brains were extracted via under isoflurane (5%) anesthesia and by intraperitoneal (IP) injection with ketamine (60 mg/kg). Upon extraction, the brain was glued onto a cutting stage. The brain was sectioned in ice-cold cutting solution (in mM: 220 Sucrose, 3 KCl, 1.25 NaH_2PO_4, 25 NaH_2CO_3, 12 $MgSO_4$, 10 Glucose, 0.2 $CaCl_2$, and 0.4 Ketamine) and was perfused with 95% O_2 / 5% CO_2. Horizontal slices (targeting the VTA were 210 μM thick; targeting the NAc were 400

μM thick) were placed in an incubation chamber containing artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$) perfused with 95% O_2 / 5% CO_2 for at least 30 minutes. After 30 minutes, brain slices were placed in a recording tissue chamber with ACSF continuously flowing at physiological temperature (36 °C).

Characterization of Neuron Types

GABA neurons were studied in GAD-GFP knock-in mice and C57BL/6 mice. In GAD-GFP knock-in mice, VTA GABA neurons were identified by a characteristic glow under fluorescence illumination. In C57BL/6 mice, VTA GABA neurons were characterized using a GABA spike command waveform [spikes at 200 Hz for 500msec; (Steffensen et al., 2008)] as GABA neurons will follow the command waveform. Neurons that did not fluoresce and/or exhibit a non-cation specific inward rectifying current (I_h) with low input resistance, not following the waveform are assumed to be DA neurons (D. W. Allison et al., 2006; Allison et al., 2011a; S. W. Johnson & R. A. North, 1992b; Margolis et al., 2006; Steffensen et al., 2011).

Whole-cell Recordings in vitro

Electrodes were pulled from borosilicate glass capillary tubes (1.5 mm o.d.; A-M Systems, Sequim, WA) and filled with a KCl pipette solutions [in mM: 128 KCl, 20 NaCl, 0.3 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.25 Na-GTP (pH 7.3)] for eIPSC studies. Pipettes having tip resistances of 2.5 - 5MΩ, and series resistances typically ranging from 7 to 15 MΩ were used. Voltage clamp recordings were filtered at 2 kHz while current-drive spikes were filtered at 10 kHz with an Axon Instruments Multiclamp 700B amplifier and digitized at 5 to 20 kHz respectively using an Axon 1440A digitizer. Axon Instruments pClamp ver10, Mini Analysis (Synaptsoft: Decatur, GA), and Igor Pro (Wavemetrics: Oswego, OR) software packages were utilized for data collection and analysis. eIPSCs were recorded in the presence of 50 μM APV and 30 μM CNQX or 3 mM kynurenic acid to block NMDA and AMPA mediated synaptic currents. Using a paired-pulse stimulus, the stimulation was adjusted to a half-max level in order to allow measurements of changes that increase or decrease eIPSC levels.

Cell-attached, Voltage-clamp Recording of Spike Activity in Brain Slices

Electrodes used for cell-attached, voltage-clamp spike studies of DA neurons were pulled from borosilicate glass capillaries (1.5 mm o.d.; A-M Systems, Sequim, WA) and then filled with 150 mM NaCl (2.5-5MΩ). Positive pressure was applied to the electrode when approaching the neuron under visual inspection. The electrode was pushed against the cell membrane and suction was applied to create a seal (10MΩ – 1GΩ) between the cell membrane and the recording pipette. Spontaneous spike activity was then recorded in voltage-clamp mode with an Axon Instruments Multiclamp 700B amplifier and sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. Neurons were voltage-clamped at 0 mV throughout the experiment. A stable baseline recording of firing activity was obtained for 5-10 minutes before adding any substances.

Acutely Dissociated Neurons from the Mouse VTA

Single neurons were acutely dissociated from the VTA of 2-3-week-old GAD GFP mice following procedures as previously described (Wu *et al*., 2004; Yang *et al*., 2009). Briefly, mice were anesthetized with isoflurane, and brain tissue was rapidly removed and immersed in cold (2-4°C) artificial cerebrospinal fluid (ACSF), which contained (in mM): 124 NaCl, 5 KCl, 24 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 2.4 $CaCl_2$, and 10 glucose; pH 7.4. From each mouse, two 400 mm coronal slices containing the VTA were cut using a vibratome (Vibratome 1000 Plus, St. Louis, MO). After cutting, the slices were continuously bubbled with 95% O_2 - 5% CO_2 at room temperature (22 ± 1°C) for at least 1 h in ACSF. Thereafter, the slices were treated with papain (6 mg/ml, Sigma-Aldrich) at 33°C for 60 min in ACSF. After enzyme treatment, the slices were washed and transferred back to well-oxygenated ACSF for at least 15 min. The VTA was first identified using a stereomicroscope and then micro-punched out from the slices using a well-polished needle (0.5 mm diameter). Each punched piece was then separately transferred to a 35-mm culture dish filled with well-oxygenated standard extracellular solution, which contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES; pH 7.4 (with Tris-base).

Each punched piece was then dissociated mechanically using a fire-polished micro-Pasteur pipette under an inverted microscope (Olympus IX-70, Lake Success, NY). The separated cells adhered to the bottom of the culture dish within 30 min. We used only VTA neurons that maintained their original morphological features of small- or medium-sized somata with 2-4 thin, primary dendritic processes and were GAD+.

Alpha Conotoxin MII Binding

Mechanically dissociated neurons from the VTA of embryonic day 13 to 15 mice expressing GFP driven by GAD67 production were allowed to settle onto 12 mm round poly-L-lysine, pre-coated coverglass (Fisher Scientific, Pittsburgh, PA) for 10 to 20 minutes before fixation in 4% freshly prepared paraformaldehyde in 1x PBS (pH 7.2) for 15 minutes at 4^{o} C. Biotinylated α-conotoxin MII binding protocol was adapted from Whiteacker et al., 2000. Briefly, fixed cells were incubated in 1x binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 15 minutes, and a second time for 5 minutes. Cells were then incubated in 0.5 nM biotinylated α-conotoxin MII in binding buffer supplemented with 5 mM EDTA, 5 mM EGTA, and 10 µg/ml of Aprotinin, Leupeptin trifluoroacetate and Pepstatin A for 2 hours. In control conditions, 50 nM non-biotinylated α-conotoxin MII was included to compete for α6-containing nAChR binding sites. Cells were subsequently washed with 1 x binding buffer for 30 seconds, washed again in 1 x binding buffer for 30 seconds at 0^{o} C. Cells were washed two more times in 0.1 x binding buffer (0.01% BSA) for 5 seconds at 0^{o} C. To quench endogenous peroxidases, cells were incubated in 0.3% H_2O_2 in 1x binding buffer for 10 minutes, followed by two brief washes in 1x binding buffer. A solution of horseradish peroxidase conjugated to streptavidin (SA:HRP; Vector Laboratories, Burlingame, CA) was diluted 1:300 in 1x binding buffer and applied to cells for 30 minutes. Unbound SA:HRP was removed by washing three times for 5 minutes with TNT wash buffer (0.15M NaCl, 0.1 M Tris-HCl, 0.05% Tween-20, pH 7.5). The cells were incubated in a solution of tyramide-conjugated Cy5 (PerkinElmer, Waltham, MA) diluted 1:50 in Amplification Diluent (PerkinElmer) for 15 minutes, then washed three times for five minutes with TNT wash buffer.

After a brief 1 x PBS wash, coverslips were mounted onto glass microscope slides (VWR, Randor, PA) using Prolong Gold mounting media (Life Technologies, Grand Island, NY). All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Conditioned Place Preference

The CPP apparatus (Med Associates, St. Albans, VT) consisted of two adjacent conditioning compartments (20 x 16 x 21 cm) separated by a manual guillotine-type door. One of the compartments was equipped with vertical striped acrylic walls and a steel mesh floor; the other was equipped with plain acrylic walls and a wire rod floor. Infrared photobeams monitored the animal's position in the apparatus and provided a measure of motor activity. First animals were habituated to the testing apparatus during a single 20 minute session with free access to both conditioning compartments. Animals were then subjected to two 20 minute pre-conditioning tests in order to determine any initial preference for one of the conditioning compartments. Each animal was then assigned to EtOH in the initially non-preferred compartment, and saline in the initially preferred compartment. Next, the animals underwent 20 minute conditioning sessions twice daily. Saline conditioning sessions were conducted in the morning and EtOH conditioning sessions were conducted in the afternoon. Following 4 sequential conditioning days, animals were tested for place preference by allowing free access to both conditioning compartments for 20 min. It is important to note that the CPP paradigm is a measure of drug reward, unlike a self-administration model, which measures reinforcement (Bardo & Bevins, 2000).

Drug Preparation and Administration

CNQX (30 μM; Abcam) and APV (50 μM; Abcam) were dissolved in distilled water and frozen until used. Kynurenic acid (3 mM; Sigma-Aldrich) was made fresh in distilled water and sonicated to get into solution. α-conotoxin MII (100 nM; provided by Michael McIntosh from the University of Utah) was prepared fresh by dissolving in distilled water. Atropine (10 μM;Sigma-Aldrich) and eticlopride (100 nM;Sigma-Aldrich) were dissolved in distilled water, while CGP55845 (10 μM;Abcam) required sonication to completely dissolve. Methylylcaconitine (10

nM) was prepared fresh in distilled water. While measuring eIPSCs, kynurenic acid or the combination of APV and CNQX was added to the ACSF perfused to the brain slice to block currents from AMPA and NMDA. Afterwards, an EtOH dose response (1 mM, 5 mM, 10 mM, 30 mM, and 50 mM; approximately 5 minutes at each dose) was administered to the ACSF to observe its effect on eIPSCs. In some experiments, only one dose of EtOH per slice was administered. In other experiments, CGP55845, atropine, and eticlopride was added to kynurenic acid/APV+CNQX to rule out GABA(B)R, D2R, and cholinergic muscarinic effects. After ensuring that EtOH had altered eIPSC peaks, the α6 nAChR antagonist, α-contoxin MII, was added before the EtOH dose response in order to see if it blocked EtOH's effects. In FSCV studies measuring DA release, methylylcaconitine (MLA), a α7-nAChR antagonist, was perfused to the brain slice preceding exposure to EtOH. In CPP studies, MEC (2 mg/kg) was used to block nAChRs, including α6*, as conotoxins (P1A and MII) are impermeable to the blood brain barrier.

Statistical Analyses

All results were presented as raw mean values and percent control ± SEM. Results between groups were compared using a two-tailed unpaired *t* test or ANOVA. Experiments relying on variance in time or current were analyzed using mixed models ANOVA with post hoc t-test at individual points. Statistical significance required ≥ 95% level of confidence ($P \leq 0.05$). CPP experiments used a post-hoc Tukey's Multiple Comparison Test to compare the groups. Analysis software included Microsoft Excel and Igor Pro (Wavemetrics, Oswego, OR). Significance levels were indicated on graphs with asterisks *,**,*** and correspond to significance levels $P<0.05$, 0.01 and 0.001, respectively. Figures were constructed with Igor Pro software.

Results

Interactions between acute ethanol and α6-nAChRs: Recombinant receptor studies*

Our first goal was to profile pharmacological features of interaction between EtOH and α6*-nAChRs. To accomplish this, we evaluated EtOH effects on the function of heterologously expressed α6*-nAChR-mediated currents in recombinant α6*-nAChRs expressed in an human

epithelia (SH-EP1) cell line. Although not as physiologically relevant as native systems, recombinant expression systems have several advantages including: 1) Better control over which subunits are being expressed (e.g., showing the difference between different combinations of subunits); 2) Better access to the cell in order to perform more accurate dose-response curves (e.g., less concern about the concentration of the drug becoming diluted as it diffuses into the tissue, etc.); 3) Better kinetic profiling of the responses (i.e., rise times, decay times, total current, peak amplitudes, desensitization rates); 4) Less artifact than with slices; 5) Simplified pharmacological analysis of the currents; and 6) Facilitation of the study of human nAChR subunits in an expression system. Furthermore, this expression system is extremely valuable as there are currently no available α6*-nAChRs agonists or direct α6*-nAChR-mediated effects in native neurons. Hence, incorporating this expression system was essential for a full understanding of EtOH effects on NIC currents.

Others have shown functional α6*-nAChRs using combinations of α6, α4, and β3 subunits. Therefore, we hypothesized that a combination would yield functional receptors to study EtOH effects on NIC currents. However, NIC currents are small, and interpretations regarding EtOH effects on α6 are put in question because of the addition of α4. Consequently, we established a functional cell line (SH-EP1) that stably expressed functional α6*-nAChRs including human α6, β4 and mutated β3 ($\beta3^{V9'V}$).

We evaluated the subunit profile necessary to optimize NIC currents and constructed dose-response curves for NIC alone and after EtOH superfusion. NIC was able to induce a typical dose response in NIC currents, indicating that the α6*-nAChRs in SH-EP1 cells were functional (**Fig. 8A)** and sensitive to the highly selective and potent α6*-nAChR antagonist, α-conotoxin P1A (α-CTX P1A) at a concentration of 1 nM (**Fig. 8B**). Through confirming that the α6*-nAChRs were appropriately responsive, we were able to test the effects of EtOH on these channels. We found that EtOH, at very low concentrations (1 mM) enhanced NIC currents in SH-EP1 cells (**Fig. 8C, D**), suggesting a potent and direct effect on α6*-nAChRs.

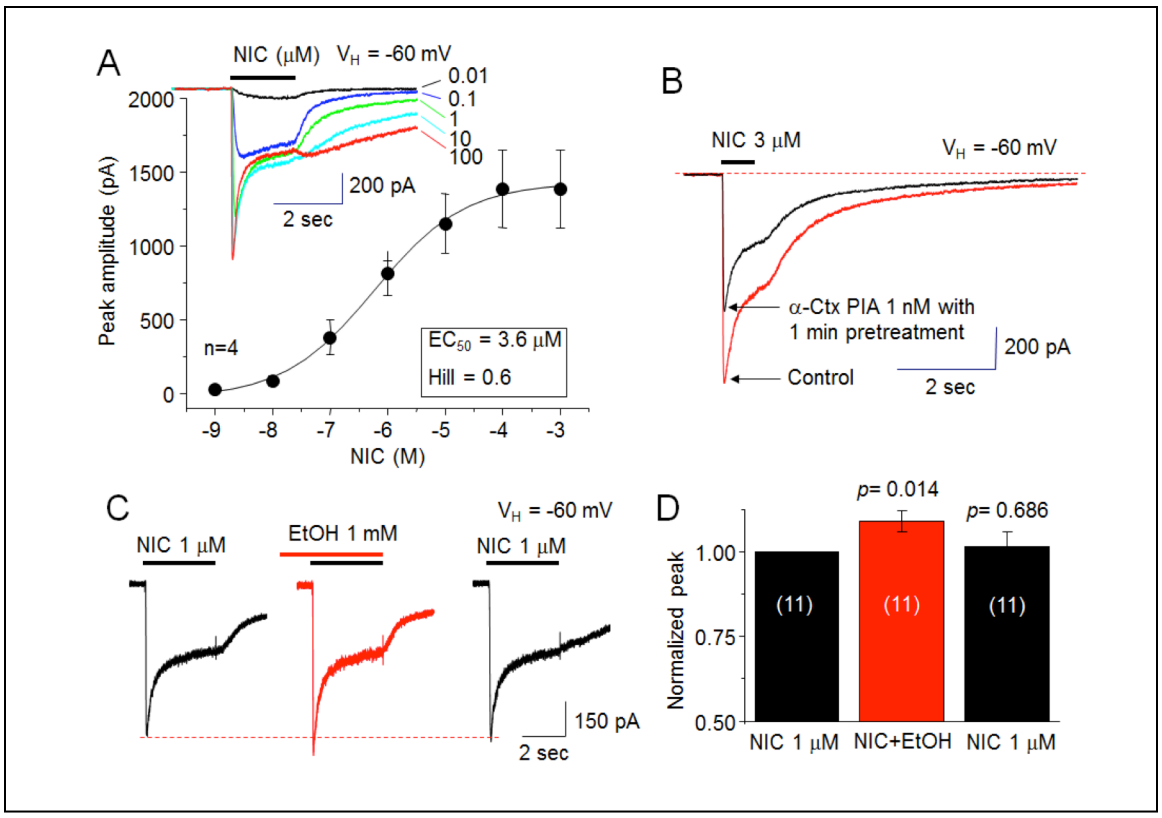

Figure 8 EtOH Enhances NIC Currents in SH-EP1 Cells Expressing α6*-nAChRs.
(A) NIC dose-response curve showed the functional α6*-nAChRs. Inset: Representative typical inward current responses induced by difference concentrations of NIC. (B) NIC-induced current was sensitive to very low concentration (1 nM) of selective α6-nAChR antagonist, α-Ctx P1A. (C) NIC-induced current was reversibly enhanced by co-application of NIC and EtOH (1 mM). (D) Bar graph summarizes a group of cells tested (indicated by number in each column) in C.

Acute EtOH's Effect on VTA Neuron Firing Rate via α6-nAChRs*

Next, we wanted to confirm that increased inhibitory input to GABA neurons would result in an inhibition of their firing rate. To accomplish this we decided to measure the effects of EtOH on firing rate in dissociated VTA GABA neurons. Using cell-attached, voltage-gated patch clamp studies, we were able to measure the firing rate of these GABA neurons. In our results, we found that EtOH (30 mM) caused a significant decrease in GABA firing rate (**Fig. 9**). Furthermore, this decrease in firing rate was prevented by the α6 nAChR antagonist, α-CTX P1A (10 nM; **Fig. 9**). These results provide further support to our hypothesis that EtOH is enhancing GABA inhibition to VTA GABA neurons EtOH and the role of α6*-nAChR in this process.

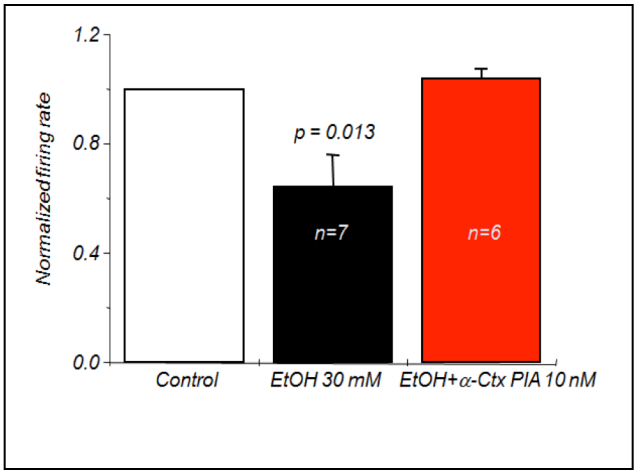

Figure 9 α-Ctx PIA Blocks the EtOH Effect on VTA GABA Neurons.
In dissociated VTA GABA neurons from a GAD-GFP mouse, EtOH (30 mM) significantly decreased firing rate. This decrease in firing rate was blocked by the α6-nAChR antagonist, α-Ctx PIA (10 nM).

The dogma is that local circuit GABA neurons inhibit DA neurons in the VTA. In order to link the changes we found in VTA GABA neurons to DA neurons, we evaluated the effects of EtOH on VTA DA neuron activity and DA release in the NAc. In addition, we examined the role α6*-nAChRs in this process. Through recording DA activity via firing rate we would be able to determine how EtOH may be acting on VTA GABA neurons to influence DA activity, and ultimately release, a common trait of addiction. In our firing rate studies, we performed cell-attached, voltage-clamped firing rate studies on VTA DA neurons in slice preparation from GAD-GFP mice. VTA DA neurons were identified as non-glowing cells and through their characteristically slow and regular firing rates. We found that low doses of EtOH (5 mM; $F_{(1,21)}$=12.58, p=0.002) inhibited VTA DA neuron activity, while high doses (50 mM; $F_{(1,15)}$=4.91, p=0.04 and 100 mM; $F_{(1,7)}$=9.98, p=0.02) tended to increase activity (**Fig. 10**). Furthermore, α-CTX MII was only able to block the effects of EtOH at the low dose level ($F_{(1,7)}$=0.70, p=0.43) , and did not appear to prevent the increase in firing rate associated with higher doses of EtOH (50 mM; $F_{(1,7)}$=6.39, p=0.04 and 100 mM; $F_{(1,8)}$=5.84, p=0.04).

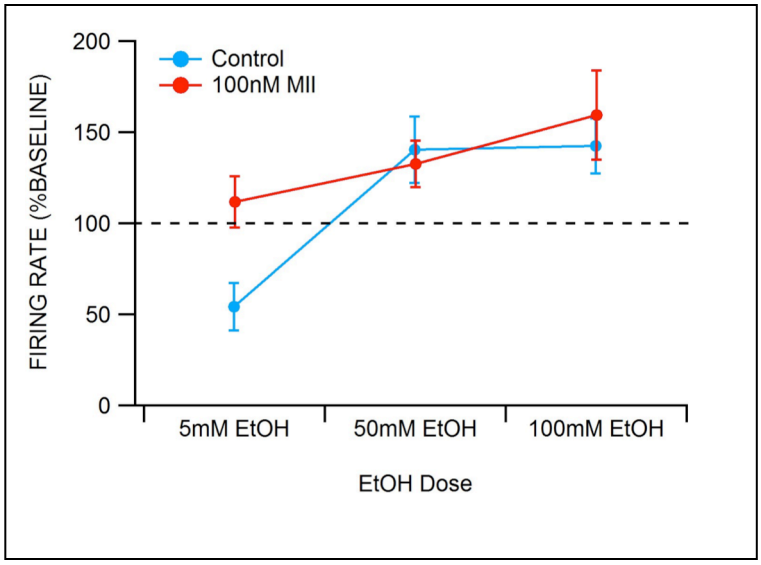

Figure 10 Effects of EtOH and α-CTX MII on VTA DA Neuron Firing Rate.
Using cell-attached mode, spontaneous spike activity was measured in horizontal slices of GAD-GFP mice. Low doses of EtOH inhibited DA activity while higher doses enhanced activity. α-CTX MII was able to block EtOH's low dose effects but not with higher doses.

Acute ethanol's effect on VTA GABA neurons on mIPSCs via α6-nAChRs: Dissociated neuron studies*

To increase our understanding of α6*-nAChRs involvement in alcohol addiction, we proposed to evaluate the effects of α6*-nAChR antagonists on EtOH effects on GABA-mediated synaptic responses in dissociated GABA neurons of the VTA. We set to determine this through studying GABA-mediated mIPSCs in dissociated, visually-identified VTA GABA neurons from GAD-GFP mice with fluorescent optics. Recently, it has been shown that dissociated DA neurons in the VTA have inhibitory synaptic boutons that contain α6*-nAChRs, and that ACh activation of these receptors leads to enhancement of inhibitory transmission (i.e., mIPSCs) that are GABA(A)R mediated (K. Yang et al.). The effects of nAChR antagonists on EtOH effects on mIPSCs, but not mEPSCs, can be studied in dissociated neurons.

Although dissociated neuron recordings suffer from the lack of physiological relevancy characteristic of intact circuits in the slice preparation or *in vivo*, its value in our proposed studies was three-fold: 1) Using the U-tube method, EtOH and NIC pharmacology can be accurately and easily studied; 2) While there are viable inhibitory somatic synaptic boutons on dissociated neurons, there are little or no dendritic excitatory synaptic boutons; and 3) Postsynaptic effects can be easily assessed with pharmacological blockers. For example, CGP55845 and atropine was included in the superfusate to assure GABA(A)R-mediated responses. To further confirm this we also superfused the GABA(A)R antagonist bicuculline (10 mM) at the end of some experiments to confirm that the mIPSCs were mediated by GABA.

In our studies, similar to what has been reported previously in dissociated DA neurons (K. Yang et al., 2011a), VTA GABA neurons evinced mIPSCs, which were enhanced by NIC (**Fig. 11A**). This enhancement could then be prevented by α-conotoxins (**Fig. 11B**).

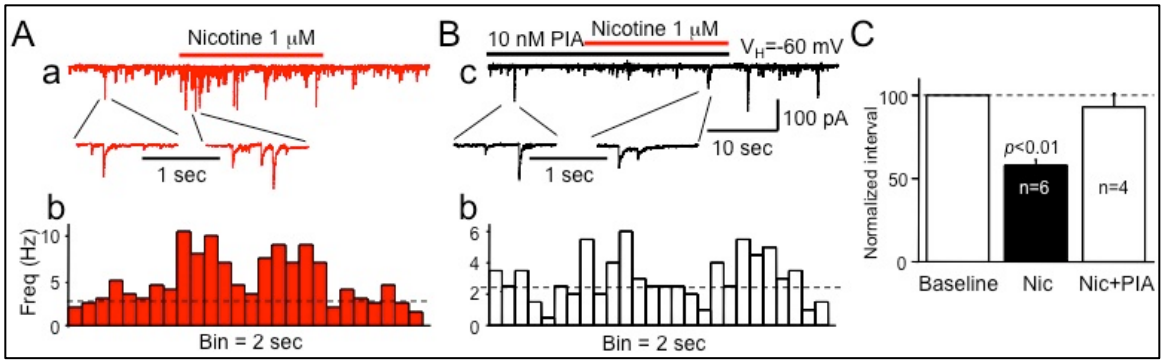

Figure 11 NIC Enhances mIPSCs in VTA GABA Neurons through α6*-nAChRs.
Bath-application of NIC (1 mM) significantly increased mIPSC frequency (A), and this effect was prevented by pretreatment with the α6*-nAChR antagonist, α-conotoxin P1A (10 nM, B). (C) Bar graph summarizes the pool data and suggests that NIC activates α6*-nAChRs on GABAergic presynaptic boutons and increases presynaptic GABA release.

Expounding upon our results from our recombinant system experiments, we found that EtOH enhanced both the frequency and amplitude of GABA mIPSCs at low doses, similar to NIC effects (**Fig. 12**). This is a critical finding, as it supports the literature demonstrating that EtOH enhances GABA release at physiologically relevant concentrations.

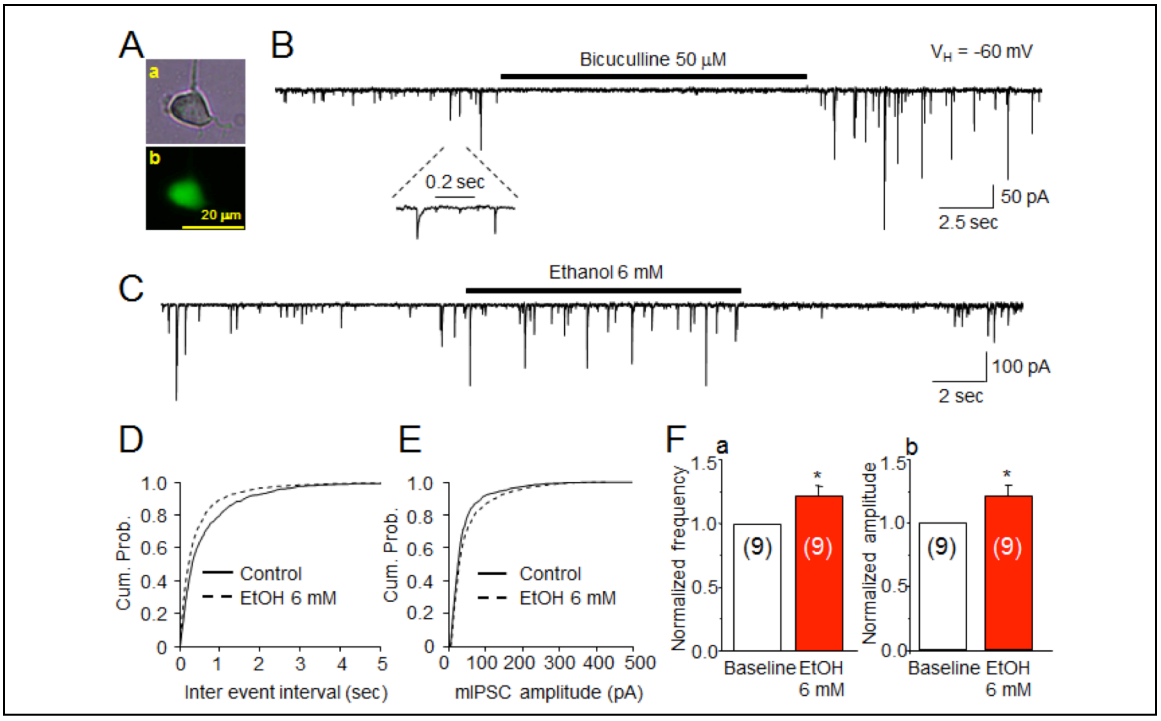

Figure 12 EtOH Enhances mIPSC Frequency and Amplitude in VTA GABA Neurons. (A) Images show an acutely-dissociated VTA neuron from a GAD-GFP mouse using phase-contrast (Aa) and GPF-filtered (Ab) modes of microscope. (B) A typical trace shows the spontaneous mIPSCs (in the presence of 0.5 µM TTX) since they are sensitive to a selective GABA(A)R blocker, bicuculline. (C) Bath exposure of 6 mM EtOH increased the frequency of spontaneous mIPSCs. Further analysis shows that 6 mM EtOH increases frequency (D) and amplitude (E) of the spontaneous mIPSCs. (F) Bar graphs summarize the effects of EtOH.

Of particular relevance to NIC and EtOH interactions, we found that the α6*-nAChR antagonist α-ctx P1A also blocked EtOH's enhancement of mIPSCs amplitude and frequency (**Fig. 13A, B**), suggesting that α6*-nAChRs on GABA terminals to VTA GABA neurons are an important link for EtOH and NIC interactions. Moreover, the enhancement of mIPSCs by EtOH in WT mice was not observed in α6 KO mice (**Fig. 13C, D**), providing further evidence that EtOH acts via presynaptic α6*-nAChRs to increase GABA release. These findings suggest that EtOH is enhancing GABA release at terminals and GABA responses postsynaptically.

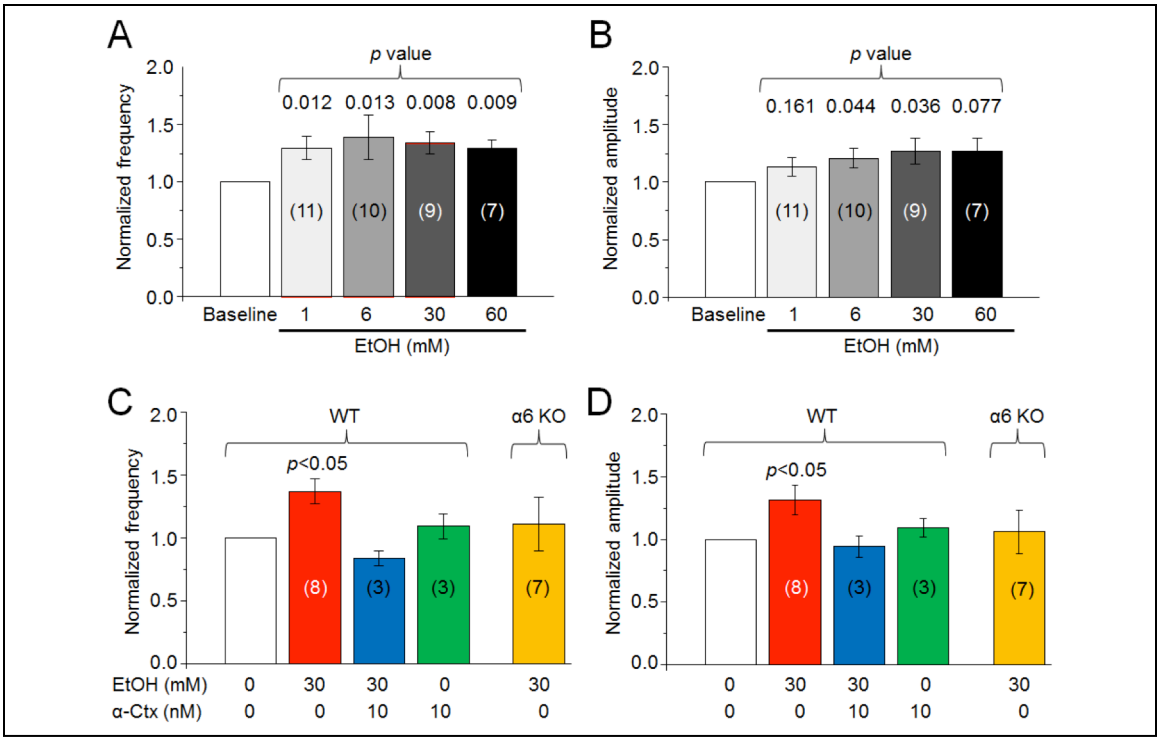

Figure 13 EtOH Enhancement of GABA mIPSCs to VTA GABA Neurons via α6*-nAChRs. Results here are shown at the 30 mM EtOH level. (A) EtOH dose response on mIPSC frequency. (B) EtOH dose response on mIPSC amplitude. (C) The α-conotoxin P1A (10 nM) did not affect mIPSC frequency, but following a 2 min treatment, it prevented EtOH (30 mM)-induced increase in mIPSC frequency in WT mice but not in α6 KO mice. (D) Similarly, P1A did not affect mIPSC frequency, but following a 2 min treatment, it prevented EtOH (30 mM)-induced increase in mIPSC frequency in WT mice but not in α6 KO mice Vertical bars represent means ± SEM.

Acute EtOH's Effect on VTA GABA Neuron Synaptic Transmission via α6-nAChRs: Slice Studies*

Next, for more physiological relevancy we evaluated the effects of EtOH in the slice preparation. In our slice studies, we utilized GAD-GFP mice to unequivocally identify VTA GABA neurons, similar to the dissociated neuron studies. Additionally, some studies were also performed in C57BL/6 mice, in case of possible EtOH sensitivity differences between the species. For example, GAD-GFP mice only express 50% of the GAD levels of WT mice. For these mice, we employed a variation of the conventional criteria to classify GABA neurons in C57BL/6 mice. Mainly, GABA neurons were subjected to our GABA spike command waveform [spikes at 200 Hz for 500 msec; (Steffensen et al., 2008)]. We have found that GABA neurons follow the command waveform while DA neurons do not. Additionally, GABA neuron spike activity is enhanced or not affected by DA (Lassen et al., 2007; S. H. Stobbs et al., 2004b) while DA neurons are known to be inhibited by DA or D2 agonists.

In order to test EtOH's effects on GABA-mediated responses and the role of α6*-nAChR, we performed synaptic evoked IPSC (eIPSC) studies. For these synaptic studies involving GABA(A)R responses, we included APV and CNQX to block excitatory synaptic transmission. In some experiments, we also included 10 mM CGP55845 to rule out GABA(B)R effects, 100 nM eticlopride to rule out D2R effects, and 10 mM atropine to rule out cholinergic muscarinic effects. This served as essential control conditions for nAChR studies in the slice. Notably, a recent study had shown interactions with EtOH and CNQX (Brickley, Farrant, Swanson, & Cull-Candy, 2001; Maccaferri & Dingledine, 2002). Thus, in some studies we used kynurenic acid (3 mM) to block GLU transmission in place of APV and CNQX.

While mIPSCs are best evaluated in dissociated neurons, eIPSCs in the slice preparation provide valuable support to mIPSC studies. Our studies showed that low-dose EtOH (1 and 5 mM) consistently enhanced VTA GABA neuron eIPSC amplitude (**Fig. 14A, C;** 1 mM*;* $F_{(1,13)}$=6.65, p=0.02 and 5 mM; $F_{(1,15)}$=8.16, p=0.01). This effect was blocked by α-conotoxin MII (α-CTX MII), indicating the involvement of α6*-nAChRs (**Fig. 14B, D;** 1 mM; $F_{(1,11)}$=0.16, p=0.691 and 5 mM; $F_{(1,11)}$=0.07, p=0.796**)**. However, results were inconsistent with higher doses of EtOH, which also resulted in inconclusive results for the effects of α-CTX MII on eIPSCs in the presence of EtOH.

Furthermore, this EtOH effect is likely acting on GABA terminals, as the paired-pulse ratio decreased, suggesting a presynaptic effect (**Fig. 14**).

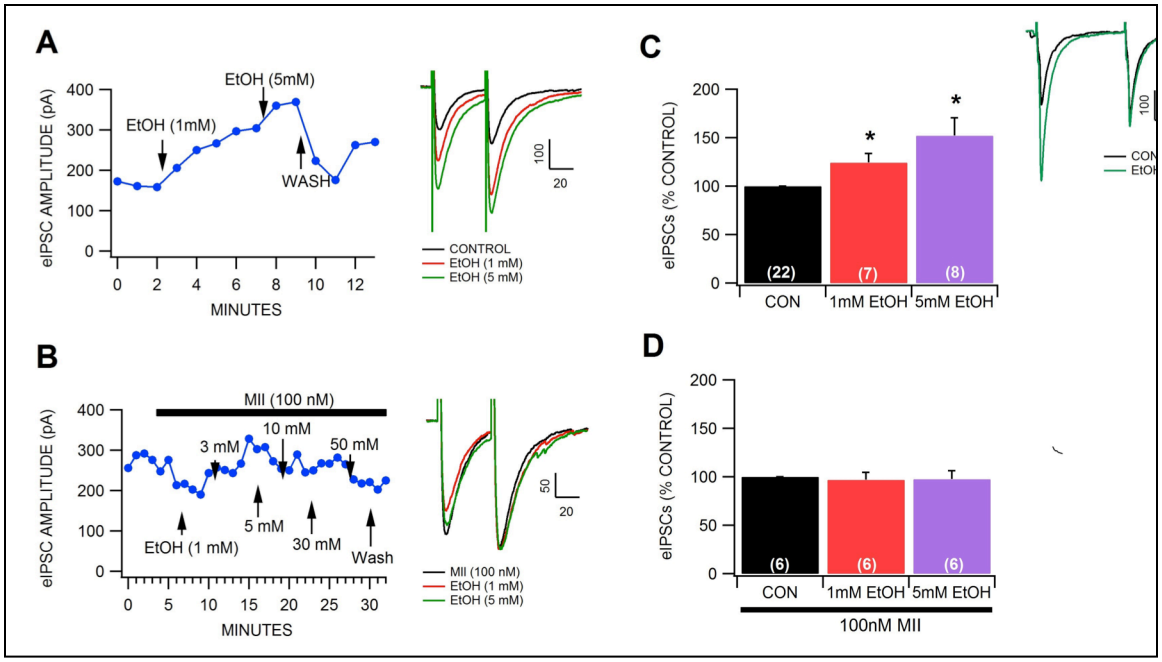

Figure 14 Effects of EtOH and α-CTX MII, on eIPSCs in VTA GABA Neurons. eIPSCs were recorded in VTA GABA neurons in the horizontal slice in the presence of APV/CNQX. EtOH enhanced eIPSCs at low doses (A), but this effect was blocked in the presence of α-CTX MII (B). (C) Bar graph summarizes the effects of EtOH (1 and 5 mM) and shows that α-CTX MII blocks this effect. Inset shows EtOH enhancement in kynurenic acid + eticlopride, atropine and CGP55845.

The Role of α6-nAChRs in Mediating EtOH Consumption and Reward*

Conditioned place preference is a procedure used by many labs to study reward. Given the changes in VTA GABA and DA neurons, including DA release in the NAc, that we observed in our EtOH experimental procedures, we attempted to use CPP to show how these neuronal alterations would manifest in affecting behavior in mice. We found that WT mice receiving EtOH injections (2 g/kg IP) showed a higher preference for the EtOH paired compartment compared to EtOH treated α6 KO (**Fig. 15**). Furthermore, to ensure that α6 KO did not have any other additional behavioral changes inherent to their genetic modification, we pretreated WT mice with mecamylamine (MEC; 2 mg/kg), a non-α7 nAChR antagonist. The mice treated with MEC showed an attenuated preference for the EtOH (2 g/kg) paired chamber. A one way ANOVA demonstrated significance between groups ($F_{(6,47)}$=5.676, p=0.0002), with Tukey's Multiple Comparison post-hoc analysis demonstrating significance in multiple groups (*= P<0.05, **= P<0.01).

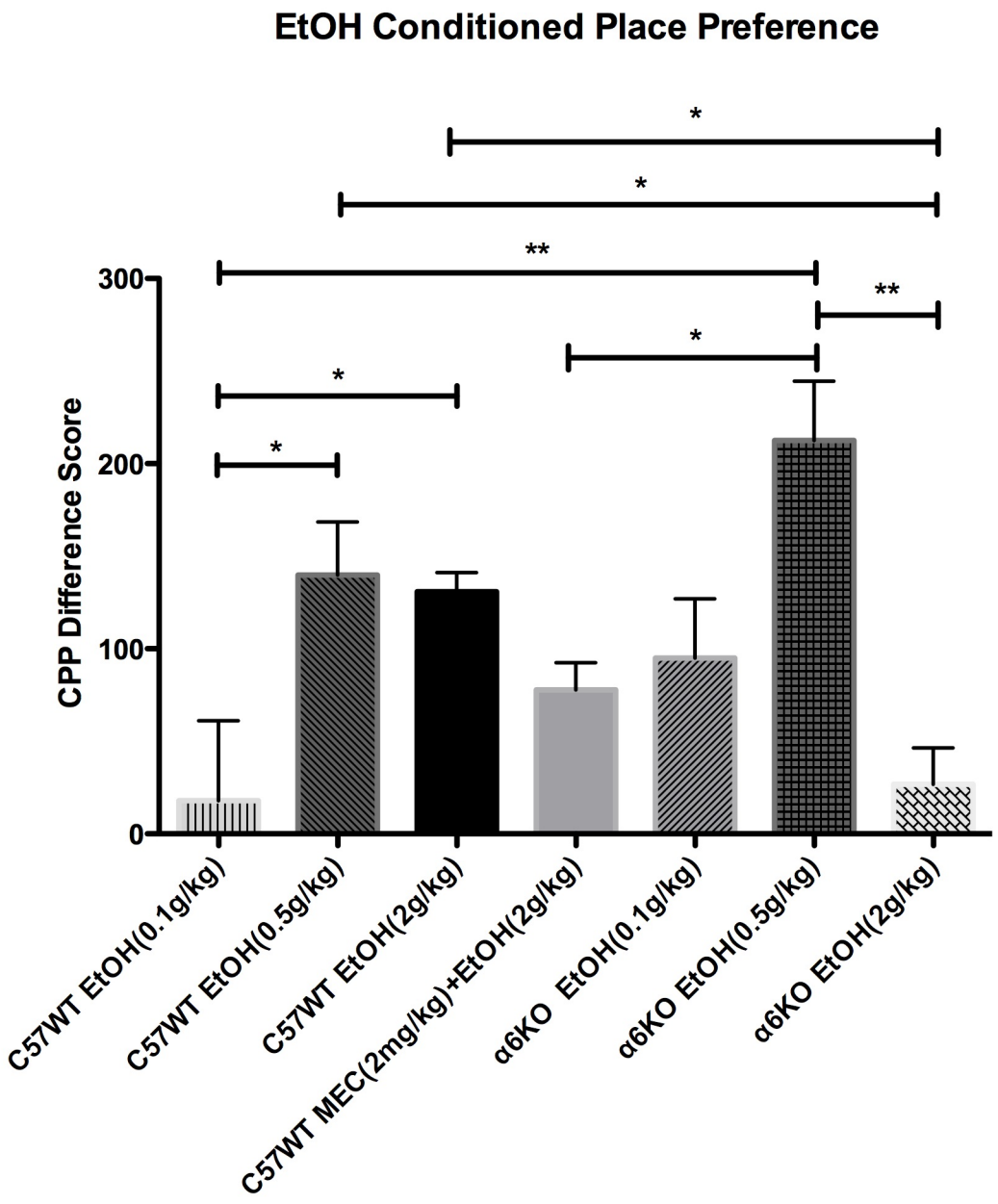

Figure 15 α6*-nAChR Mediate EtOH Consumption and Reward.
Results show WT mice have a 2g/kg EtOH preference, which is not exhibited with α6 KO mice and is attenuated in mice pretreated with MEC, a nonselective nAChR antagonist. However, EtOH at a lower dose (0.1 g/kg) is preferred by α6 KO mice, while WT mice exhibit no preference at this dose.

Discussion

In recombinant SH-EP1 cells, NIC currents in functional α6*-nAChRs showed a significant enhancement in the presence of EtOH. Our results confirmed the NIC and EtOH interaction that others have previously observed through population-based samples and general neuron activity in the VTA (Ford, McCracken, Davis, Ryabinin, & Grant, 2012; John et al., 2003; Tizabi et al., 2002). While previous research revealed a synergistic relationship between NIC and EtOH, we were able to outline that this mechanism of actions was likely working through nAChRs, specifically α6*-nAChRs. We found that α6*-nAChRs showed a potent response to low doses of NIC (1 μM) and EtOH (1 mM), which effect was blocked by α-CTX P1A suggesting α6*-nAChRs probable direct involvement in mediating addiction in these two drugs.

In order to increase our understanding of the role α6*-nAChRs in modulating alcoholism, we needed to study native GABA neurons. We found that NIC enhanced mIPSC frequency in dissociated VTA GABA neurons. A change in mIPSC frequency suggests a presynaptic response. As such, an increase in mIPSC frequency characterized greater inhibition to VTA GABA neurons. This effect was blocked by α6*-nAChR antagonists, suggesting that NIC enhances inhibition to VTA GABA neurons via α6*-nAChRs to modulate addictive changes. Low doses of EtOH (6 mM) also enhanced mIPSC frequency in dissociated VTA GABA neurons. Administration of bicuculline helped determine that this inhibitory input is a result from increased GABA release to VTA GABA neurons. Again, this increase in mIPSC frequency was blocked by α6*-nAChR antagonists and not seen in α6 KO mice, suggesting that EtOH also enhances GABA release on to VTA GABA neurons via α6*-nAChRs. These findings, mirroring NIC's effects on mISPC, further support the hypothesis that NIC and EtOH, at physiologically relevant levels, may be working through a shared mechanism.

Furthermore, an enhancement in mIPSC amplitude indicated that EtOH also had a postsynaptic effect on VTA GABA neurons. EtOH could be acting directly on GABA(A)Rs in addition to its presynaptic effect on GABA terminals via α6*-nAChRs to augment inhibition to VTA GABA neurons. As with frequency, the increase in mIPSC amplitude was blocked by α6*-nAChR antagonists and not observed in α6 KO mice too. Because α6*-nAChR antagonists prevented the

increase in mIPSCs, it is likely that this increased inhibition in VTA GABA neurons is dependent on GABA release through α6*-nAChR. However, as a postsynaptic effect exists, EtOH may have a dual role in enhancing GABA release and amplifying the subsequent response.

Although EtOH increases inhibition to VTA GABA neurons, it was necessary to determine if this increase was enough to alter VTA GABA neuron activity. Our results showed that EtOH markedly lowered firing rate of dissociated VTA GABA neurons. Again, as seen with our previous EtOH studies, this effect was blocked by α6*-nAChR antagonists. This demonstrates that EtOH decreases VTA GABA activity via α6*-nAChRs, signifying the potential role that α6*-nAChRs have in causing alcoholic addiction.

While our results supported a link between α6*-nAChRs and EtOH in the VTA in dissociated neurons, it was pivotal to strengthen and prove the role that α6*-nAChR have with EtOH in a more physiological setting. Using slice preparation as this resource, we found that low doses of EtOH (1 and 5 mM) enhanced eIPSC amplitudes in VTA GABA neurons. Additionally, this enhancement is blocked by α6*-nAChR antagonists, providing further support that presynaptic α6*-nAChRs on GABA terminals modulate GABA release which is sensitive to EtOH. This change in VTA GABA activity through α6*-nAChR could be the possible means by which EtOH addiction is developed.

However, our DA experiments showed somewhat of a discrepancy. Similar to many reports, we found that high doses of EtOH increased VTA DA firing rate (Brodie & Appel, 1998a). Moreover, we were also able to show that low doses of EtOH decreased VTA DA activity. Alpha6*-nAChR antagonists were able to block the effects of low dose EtOH, but were unable to do so in the higher doses. While there is evidence that α6*-nAChR may mediate changes in VTA DA activity, it appears likely that α6*-nAChR may only be involved in lower doses of EtOH.

While the effects of EtOH on GABA and DA may not coincide with what we expected to see based on our theoretical model of the VTA network, there could be some other workings to explain this discrepancy. It may be possible that the population of VTA GABA neurons we studied do not inhibit DA neurons in the VTA. The inhibitory input to DA may be from areas such as the rostromedial tegmental nucleus (RMTg) or ventral pallidum, as there are GABA neurons from the

RMTg that innervate the VTA. Another possibility is that EtOH may have an independent effect on VTA DA neurons. This may be plausible as high EtOH doses are unaffected by α6*-nAChR antagonist. In addition, our FSCV studies of DA release in the NAc do not contain VTA GABA neuron cell bodies. However, this is not very likely as α6*-nAChR antagonist block EtOH's effect on DA release, yet α6*-nAChR are not found on DA neurons. As we have hypothesized, α6*-nAChR are likely to be located on GABA terminals, which are present in the NAc, where they could synapse on DA terminals. As such, the VTA GABA neurons we studied might only synapse with DA neurons at their terminals, therefore not having an effect on DA activity, but only impacting DA release in the NAc. The latter theory is supported by our FSCV studies showing EtOH inhibition of DA release at terminals in the NAc being blocked by α6*-nAChR antagonists. Thus EtOH's mechanism of action is likely to be through α6*-nAChRs located on these VTA GABA terminals which synapse on DA terminals in the NAc.

Lastly, CPP studies showed that WT mice tend to spend more time in the EtOH (2 g/kg) paired compartment than α6 KO mice or mice treated with MEC, suggesting that α6*-nAChRs mediates some alcoholic behaviors in mice. It could be argued that EtOH CPP performed in this way could be influenced by EtOH withdrawal. We feel that this likelihood is low. In a CPP study in mice with low EtOH preference preformed during the withdrawal stage, and given a 4g/kg dose, there was no significant change in EtOH place preference (Chester & Coon, 2010). It is vital, as it shows that α6*-nAChRs are involved in process of addiction and its accompanying behaviors rather than the many physiological influences that EtOH can have on the brain other than addiction, such as ataxia. As WT mice exhibited more time in the EtOH compartment, it showed evidence that α6*-nAChRs are indeed involved in the process of alcohol addiction. The behavioral differences demonstrate that α6*-nAChRs allow EtOH to influence distinguishable reward-seeking behaviors caused by hijacking the mesolimbic DA system.

Conclusion

Taken together, our results show that EtOH is acting through α6*-nAChRs on GABA terminals to enhance GABA release. This enhancement of GABA release leads to greater

inhibition to VTA GABA neurons, which has the potential to influence VTA DA neurons and their release in the NAc. While further studies are necessary to outline the connection between VTA GABA neurons and VTA DA neurons to completely understand the hodology involved in EtOH addiction, our findings demonstrate a shared mechanism of alcohol and NIC co-dependence. As this indicates some specificity in EtOH's mode of action, we believe we have identified a mechanism of alcohol addiction that could help facilitate the development of future therapies/medications for the treatment of alcoholism and other addictions.

CHAPTER 4

THE NOVEL DRUG *l*-THP ATTENUATES THE REWARDING EFFECT OF NICOTINE IN C57BL/6 WILD TYPE MICE

Introduction

Cigarette smoking is a major public health problem, having been identified as the leading cause of preventable death in the world (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). Recently, it has been reported that about one-quarter of the world's population smokes (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). Smoking is currently responsible for the death of one in ten adults worldwide, about 5 million deaths each year, and this number will increase to 8 million by 2030 unless effective action is taken (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). Many smokers have great difficulty quitting, and many others are still becoming addicted, about 1200 per day in the U.S., due to NIC dependence. NIC, a major component of tobacco, is highly addictive. NIC usually acts on its target, nAChRs, to stimulate reward-associated circuits in the brain exerting its rewarding effects. Accumulating lines of evidence demonstrate that nAChRs play critical roles in the mediation of NIC reward, dependence and addiction.

Tetrahydroprotoberberines (THPBs), a series of active alkaloid compounds isolated from the Chinese medicinal herb *Corydalis ambigua* and various species of *Stephania*, have been clinically used as effective analgesic and sedative agents since ancient China (Bian et al., 1986; J. Mo et al., 2007; K. Yang, Jin, & Wu, 2007; Z. D. Zhang et al., 1986). Mounting evidence from biochemical, immunohistochemical, behavioral, electrophysiological, and pharmacological studies suggest that THPBs mainly exert their neuropharmacological effects through DA receptors with preferential affinity toward D1 receptors in the nigrostriatal and mesocorticolimbic DAergic pathways (Jin, Zhu, & Fu, 2002; J. Mo et al., 2007; Xu, Yu, Han, Chen, & Jin, 1989; K. Yang et al., 2007). However, different analogues of THPBs classified according to the hydroxyl group in their structures show distinct neuropharmacological profiles to DA receptors. The levo isomer of

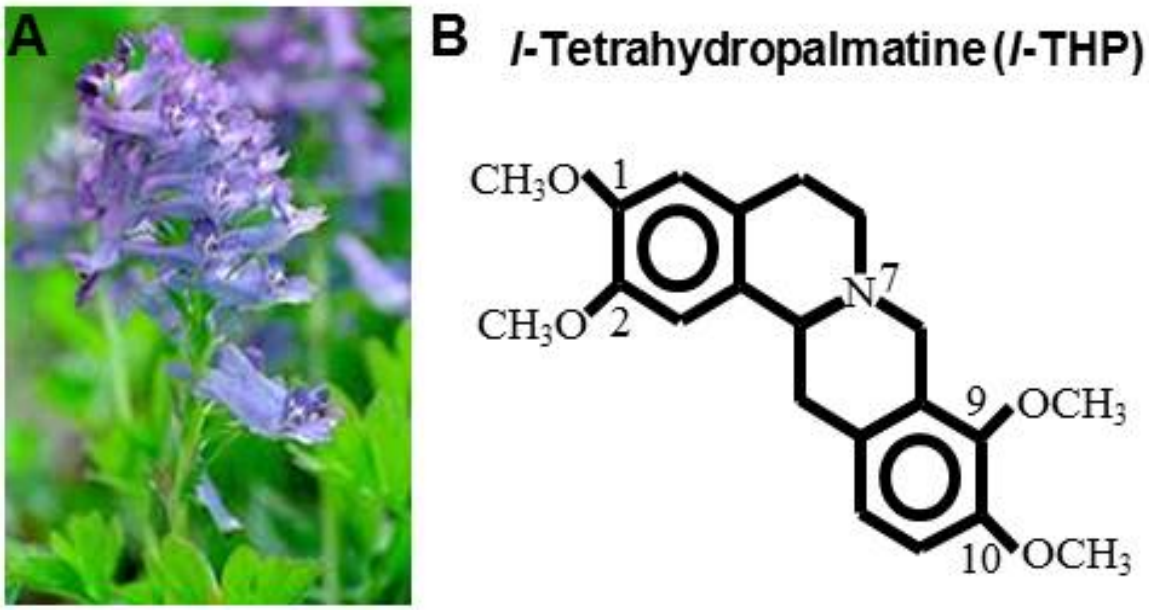

Figure 16 Levo-Tetrahydropalmatine. (A) A picture of the Chinese herb *Corydalis ambigua*. (B) The chemical structure of *l*-THP.

THP has been shown to be a major contributor to its therapeutic effects. This isomer, *l*-tetrahydropalmatine (*l*-THP), is the most extensively studied of the THPBs (**Fig. 16**), and has been used in clinical treatment for more than 50 years in China (Chao-Wu, Shuo, Hai-Qing, & Xiu-Mei, 2011; Chu, Jin, Friedman, & Zhen, 2008; Gao et al., 2011; Guo, Yu, Xing, Jin, & Zhou, 2002; Hu, Wu, & Jin, 1997; Jin et al., 2002; Mantsch et al., 2010; J. Mo et al., 2007; J. Mo et al., 2010; Y. Q. Mo, Jin, Chen, Jin, & Shi, 2005; Sun et al., 2009; C. Wang et al., 2010; C. Wu et al., 2010; J. Wu, Chen, & Jin, 1996; J. Wu & Jin, 1996, 1997; K. Yang et al., 2007). Considering that *l*-THP significantly modulates DA receptors, midbrain DA neuronal function and DA associated pathways/circuits, *l*-THP has been shown to have pharmacological effects on drug addiction (Chu *et al.*, 2008). For example, *l*-THP ameliorates morphine and heroin addiction (J. Wu, 2010; Z. Yang et al., 2008). In addition, *l*-THP attenuated cocaine self-administration and cocaine-induced reinstatement in rats, suggesting that *l*-THP has a potential role in the treatment of cocaine addiction (Lu et al., 2009; Mantsch et al., 2007; J. B. Wang & Mantsch, 2012). Therefore, clinical trials and animal studies have demonstrated that *l*-THP is a potential candidate for the treatment of drug abuse. However, whether *l*-THP can be used for NIC dependence or smoking cessation had previously remained unstudied.

Recent studies have demonstrated that dysfunction of mesocorticolimbic DArgic pathways are a critical etiology of drug addiction, even though much of the precise underlying pathogenesis is still unknown. Accumulating lines of evidence demonstrate that nAChRs play critical roles in the mediation of NIC reward, dependence and addiction. NIC's rewarding and addictive properties appear to be mediated by DA neurons in the midbrain's VTA, which projects to limbic structures. GABAergic and GLUergic synaptic inputs to VTA DA neurons are modulated by different NIC acetylcholine receptor (nACHR) subtypes with distinct desensitization properties. Cholinergic inputs to the VTA originate in the habenula and project via the fasciculus retroflex (FR) as well as the pedunculopontine tegmental nucleus (PPT). NIC can enhance GLUergic transmission while the nACHRs on GABA neurons are desensitized, thus shifting the balance of synaptic inputs to excitation. This desensitization silences endogenous cholinergic drive to the GABAergic inputs, which ultimately disinhibits DA neurons. Coordinated disinhibition and

enhanced excitation likely contributes to prolonged increases in DA release and ultimately behavioral reinforcement. Our hypothesis in this study was that the natural Chinese herb *l*-THP would significantly attenuate nAChR function as well as NIC induced actions, electrophysiologically and behaviorally.

Methods

Animal Subjects

Black wild-type (WT) male C57BL/6 mice were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals were treated in strict accordance with the Barrow Neurological Institute Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. The BNI IACUC has reviewed and approved the procedures detailed herein. Once weaned at PND 21, all mice were housed in maximum groups of five and given ad libitum access to solid food and water and placed on a light/dark cycle with lights ON from 6 AM to 6 PM.

Conditioned Place Preference

The conditioned place preference (CPP) apparatus (Med Associates, St. Albans, VT) consisted of two adjacent conditioning compartments (20 x 16 x 21 cm) separated by a manual guillotine-type door. One of the compartments was equipped with vertical striped acrylic walls and a steel mesh floor; the other was equipped with plain acrylic walls and a wire rod floor. Infrared photobeams monitored the animal's position in the apparatus and provided a measure of motor activity. First animals were habituated to the testing apparatus during a single 20 minute session with free access to both conditioning compartments. Animals were then subjected to two 20 minute pre-conditioning tests in order to determine any initial preference for one of the conditioning compartments. Each animal was then assigned to drug treatment (IP) in the initially non-preferred compartment, and saline in the initially preferred compartment. Next, the animals underwent 20 minute conditioning sessions twice daily. Saline conditioning sessions were

conducted in the morning and drug conditioning sessions were conducted in the afternoon. Following 4 sequential conditioning days, animals were tested for place preference by allowing free access to both conditioning compartments for 20 min.

Extracellular Single-unit Recordings in Anesthetized Mice

Extracellular single-unit recordings were performed in anesthetized mice as described previously (Gao et al., 2007; D. Zhang et al., 2012). Mice (at least 60 d old) were anesthetized with chloral hydrate (200 mg/kg, i.p., with supplemental doses administered via a lateral tail vein) and mounted in a stereotaxic instrument. Animal body temperature was maintained at 37°C by using a heating pad throughout the experiment. The skull was exposed and the wound margins were infiltrated with a 0.3% solution of mepivacaine hydrochloride. A glass microelectrode (5–10 M) filled with 2 M NaCl containing 2% pontamine sky blue dye was lowered through a small burr hole drilled above the VTA (3.0 mm anterior to the lambda, 0.5– 0.9 mm lateral to the midline, 6.5– 8.5 mm ventral to the cortical surface). VTA DA neurons were identified and recorded as described previously (Bunney, Aghajanian, & Roth, 1973; Gao et al., 2011; Gao et al., 2007; Grace & Bunney, 1980, 1983) based on evidence for positive/negative action potentials of long duration (2–5 ms), a firing rate of 1–10 Hz with a slow irregular or burst firing pattern, low pitch sound produced on an audio amplifier, and a duration of ≥1.1 ms from the start of the action potential to the negative trough (Ungless, Magill, & Bolam, 2004). Firing rates were collected on-line to a personal computer.

Preparation of Brain Slices

All brain slice preparations were performed in P18-45 day old C57BL/6 mice. Brains were extracted via under isoflurane (5%) anesthesia and by intraperitoneal (IP) injection with ketamine (60 mg/kg). Upon extraction, the brain was glued onto a cutting stage. The brain was sectioned in ice-cold cutting solution (in mM: 220 Sucrose, 3 KCl, 1.25 NaH_2PO_4, 25 NaH_2CO_3, 12 $MgSO_4$, 10 Glucose, 0.2 $CaCl_2$, and 0.4 Ketamine) and was perfused with 95% O_2 / 5% CO_2. Horizontal slices (targeting the VTA were 210 μM thick; targeting the NAc were 400 μM thick) were placed in

an incubation chamber containing artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$) perfused with 95% O_2 / 5% CO_2 for at least 30 minutes. After 30 minutes, brain slices were placed in a recording tissue chamber with ACSF continuously flowing at physiological temperature (36 °C).

Patch-clamp Recordings from VTA Slices

To prepare for conventional whole-cell recording, glass microelectrodes (GC-1.5; Narishige) were fashioned on a two-stage vertical pipette puller (P-830; Narishige), and the resistance of the electrode was 3–5 M when filled with internal solution. Cells were visualized under infrared-DIC (differential interference contrast) microscopy, and electrodes were positioned using a micromanipulator. After a tight seal (resulting in electrode resistance >2 GΩ) was formed between the electrode tip and the cell surface, suction was briefly applied until a whole-cell patch-clamp recording configuration was achieved (access resistance lower than 20 MΩ). Series resistance w as automatically compensated using a patch-clamp amplifier (Multiclamp 700A, Molecular Devices). Data acquisition and analysis were performed using a digitizer (DigiData 1332A, Molecular Devices) and pClamp 9.1 analysis software (Molecular Devices). Signals were filtered at 2 kHz and sampled at 10 kHz. All recordings were performed at 31 ± 1°C. Putative DA neurons were identified based on their pharmacological and physiological properties. Specifically, VTA DA neurons show the presence of prominent hyperpolarization-induced currents (I_h) and have firing rates that are increased in the presence of μ-opioid receptor ligand, DAMGO ([D-Ala^2, NMe-Phe^4, Gly^5-ol]-enkephalin) (S. W. Johnson & R. A. North, 1992a; Margolis, Hjelmstad, Bonci, & Fields, 2003). In the present study, all neurons from which data were collected and reported here exhibited profound I_h. After establishing the conventional whole-cell configuration, in some experiments, a florescent dye (biocytin 2 mg/ml) was delivered into recorded neurons at the end of the recording session, and immunohistochemical staining with an anti-tyrosine hydroxylase antibody was performed (Charpantier, Barneoud, Moser, Besnard, & Sgard, 1998; Charpantier, Besnard, Graham, & Sgard, 1999; Klink, Exaerde, et al., 2001; Le Novere, Corringer, &

Changeux, 2002; Sargent, 1993; K. Yang et al., 2009) post hoc to confirm DA neuronal phenotype.

For assessments of miniature EPSCs (mEPSCs), voltage-clamp recordings were done at a holding potential of 70 mV in the presence of the GABAA receptor blocker picrotoxin (100 M), D-APV (50 M), and the voltage-gated Na^+ channel blocker tetrodotoxin (TTX, 1 M). Miniature IPSCs were similarly recorded but in the presence of 10 M NBQX, 50 M D-APV and 1 M TTX.

Expression of Human Neuronal nAChRs in SH-EP1 Human Epithelial Cells

Heterologous expression of human α4β2-nAChRs has been previously described in detail (Eaton et al., 2003; J. Wu, Kuo, et al., 2004; J. Wu et al., 2006; Zhao et al., 2003). Briefly, human nAChR α4 and β2 subunits, subcloned into pcDNA3.1-zeocin or -hygromycin vectors, respectively, were introduced (Peng et al., 1999; Puchacz, Buisson, Bertrand, & Lukas, 1994) into native nAChR-null SH-EP1 cells (Lukas, Norman, & Lucero, 1993) to create the SH-EP1-hα4β2 cell line. Cells were maintained as low passage number (1–26 from our frozen stocks) cultures in medium augmented with 0.5 mg/mL zeocin and 0.4 mg/mL hygromycin and were passaged once weekly by splitting just-confluent cultures 1/10 to maintain cells in proliferative growth.

Patch-clamp Whole-cell Current Recordings and Data Acquisition in SH-EP1 Human Epithelial Cells

Conventional whole-cell current recordings were performed combined with a fast drug application system allowing for fast application and removal of drugs as previously described (J. Wu, Kuo, et al., 2004; J. Wu et al., 2006; Zhao et al., 2003). Briefly, transfected SH-EP1 cells were prepared in 35-mm culture dishes without poly(lysine) coating and then plated on the bottom of the dishes and later placed on the stage of an inverted microscope (Axiovert 200; Zeiss, Thornwood, NY, USA). Cells were perfused with standard external solution (2 mL/min). Glass microelectrodes with 3–5 MW resistance between the pipette and extracellular solution were used to form tight seals (> 2GW) on the surface of the cells, and standard whole-cell current recording

was initiated by suitable suction and then waiting for 5–10 min to allow for exchange of the pipette solution and the cytosol. Thereafter, recorded cells were lifted up gently from the bottom of the culture dishes, which allows for improved solution exchange and more accurate evaluation of differences in the kinetics of agonist-induced whole-cell currents (J. Wu et al., 2006). Before capacitance and resistance compensation, access resistance was measured and accepted for experiments if less than 20 MW. Whole-cell capacitance was minimized and series resistance was compensated routinely to 80%. Recorded cells were voltage clamped at a holding potential of) 60 mV, and inward currents induced by NIC were measured (Axopatch 200B amplifier; Molecular Devices, Sunnyvale, CA, USA). Current signals were typically filtered at 2 kHz, acquired at 10 kHz, and displayed and digitized on-line (Digidata 1440A series A/D board; Molecular Devices). Data acquisition and analyses were performed using PCLAMP10.0 (Molecular Devices), and results were plotted using ORIGIN 5.0 (OriginLab Corp., North Hampton, MA, USA). All experiments were performed at room temperature (22 ± 1°C).

Statistical Analyses

All results were presented as raw mean values and percent control ± SEM. Results between groups were compared using a two-tailed unpaired *t* test or ANOVA. Experiments relying on variance in time or current were analyzed using mixed models ANOVA with post hoc t-test at individual points. Statistical significance required ≥ 95% level of confidence (P≤0.05). CPP experiments used a post-hoc Tukey's Multiple Comparison Tests to compare the three groups. Significance levels were indicated on graphs with asterisks *,**,*** and correspond to significance levels P<0.05, 0.01 and 0.001, respectively.

Results

The Role of l-THP in Mediating NIC Reward

We evaluated the effect of *l*-THP on NIC CPP in C57BL/6 wild type (WT) mice. During pre-conditioning days, none of the animals used showed a significant preference for either side of the CPP apparatus. Following NIC (0.5 mg/kg) conditioning, C57BL/6 WT mice showed a

preference for the NIC paired chamber (**Fig. 17**). An IP injection of *l*-THP (10 mg/kg) prior to NIC (0.5mg/kg) attenuated this preference. Next, a group of mice, who had previously been conditioned to NIC (0.5 mg/kg) and showed an initial preference for the NIC paired chamber, were given *l*-THP (10 mg/kg) injections prior NIC (0.5 mg/kg) and immediately conditioned an additional time to the previously conditioned side of the chamber, where they were given NIC (0.5 mg/kg) alone. On the final test day, these mice no longer showed a preference for the previously preferred, NIC (0.5 mg/kg) paired chamber. Additionally, WT mice given NIC (1 mg/kg) injections (IP) showed a conditioned place aversion (CPA) to the NIC paired side of the chamber. A pre-injection of *l*-THP (10 mg/kg) did dot change the aversion to the NIC (1 mg/kg) paired side of the chamber. *l*-THP (10 mg/kg) alone also had an aversive effect. A one way ANOVA demonstrated significance between groups ($F_{(5,41)}$=5.905, p=0.0003), with Tukey's Multiple Comparison post-hoc analysis demonstrating significance in NIC (0.5mg/kg) *vs* NIC (1 mg/kg) ($p<0.05$), NIC (0.5mg/kg) *vs* *l*-THP (10 mg/kg) + NIC (1 mg/kg) ($p<0.05$) and NIC (0.5mg/kg) *vs* *l*-THP (10 mg/kg) ($p<0.05$).

The total number of photobeam breaks was recorded during each conditioning session in order to determinn differences in locomotor activity (**Fig. 18**). There was no significant difference between treatment groups in total number of beam breaks during conditioning sessions.

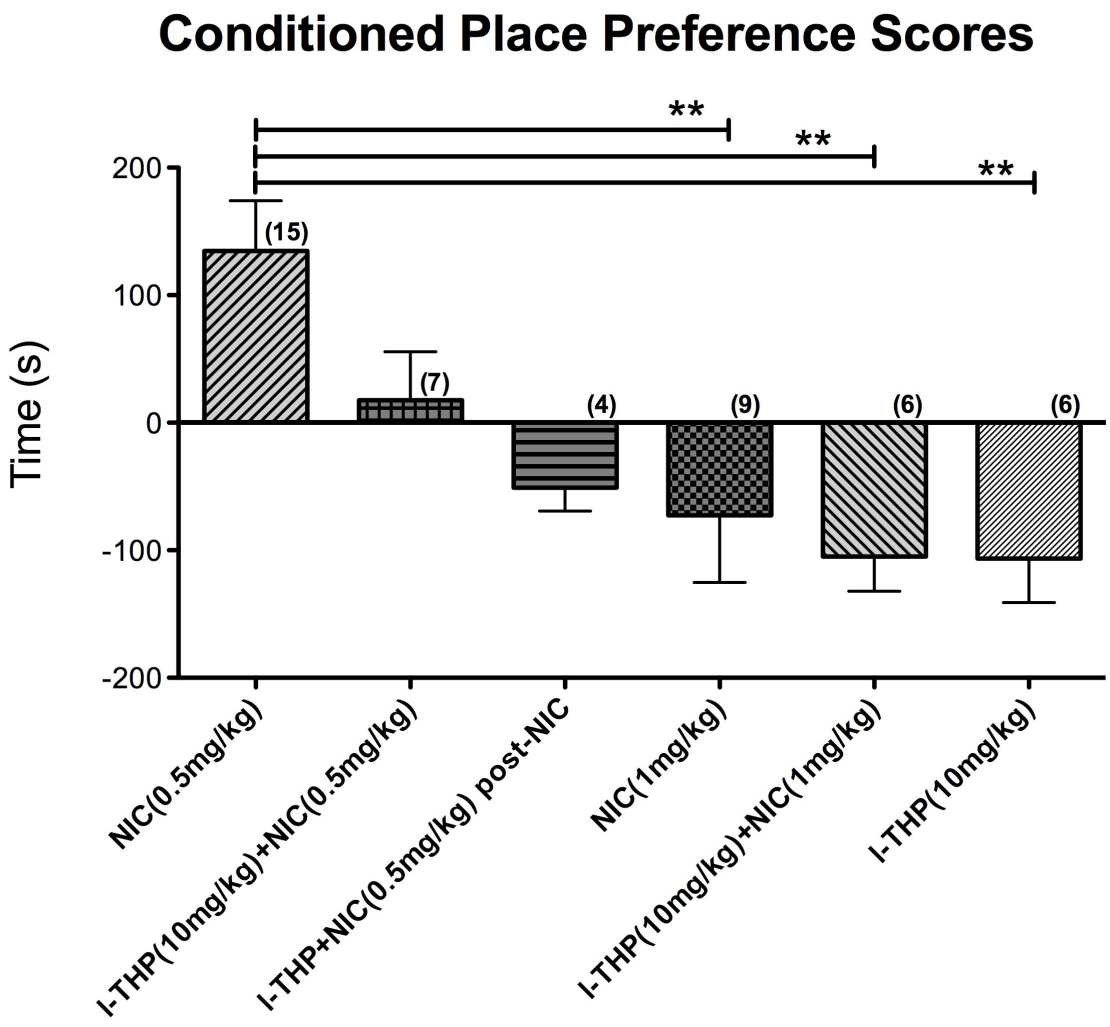

Figure 17 The effect of *l*-THP on CPP and CPA induced by NIC (0.5 mg/kg and 1 mg/kg IP respectively). *l*-THP was administered IP 5 minutes prior to NIC injection, except when administered alone. Sample sizes for each group are shown in parentheses above each bar. Data are presented in mean ± SE Asterisks **= significance level P<0.01.

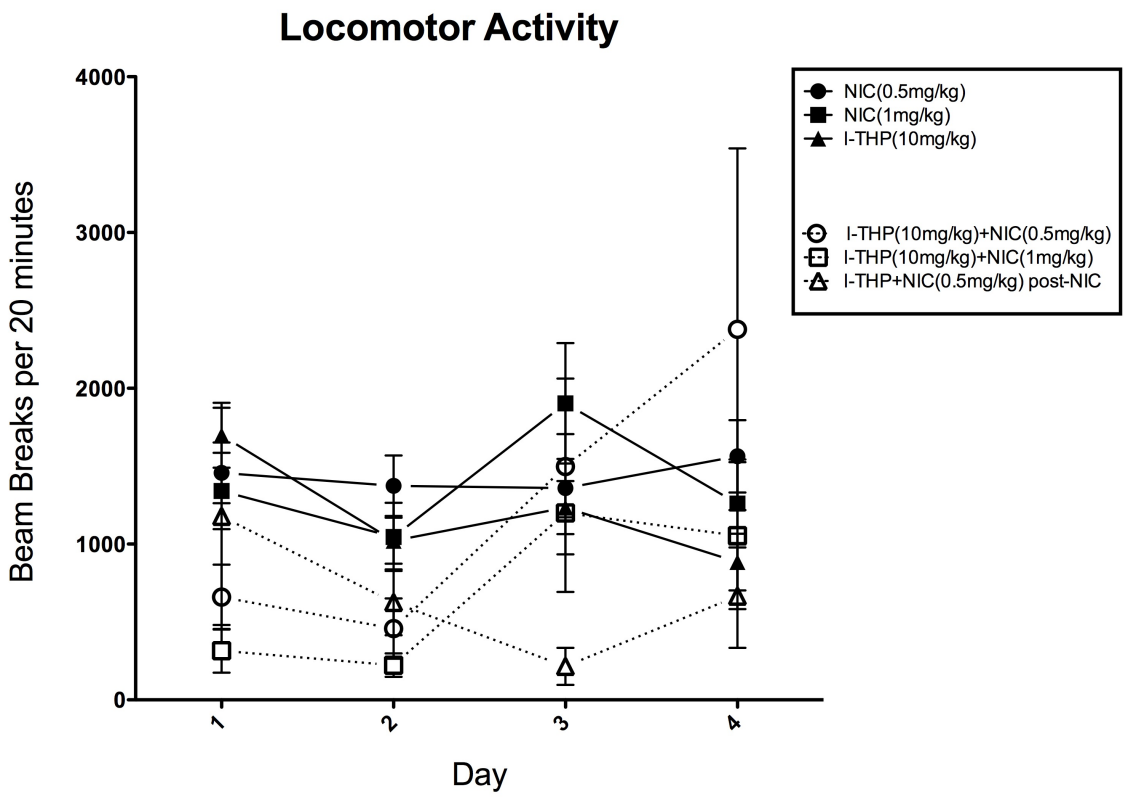

Figure 18 Locomotor activity during drug treatment conditioning sessions. Locomotor activity measured in total number of photobeam breaks per animal during 20 minute conditioning sessions in four consecutive days.

The Effect of Systemic l-THP on NIC Induced Firing Rate Increases in Single VTA DA Neurons

As our lab has previously reported that, in anesthetized (chloral hydrate) mice, systemic (IV) NIC (0.5 mg/kg) administration significantly increases firing rate (FR) in VTA DA neurons (D. Zhang et al., 2012). An example of this FR increase *in vivo* is shown in **Figure 19**. The rate of firing increased in about 30 sec and returned to baseline approximately 5-6 min after the NIC injection. Pre-treatment with *l*-THP (5 mg/kg, IV) 5 minutes before NIC (0.5 mg/kg) was able to block this NIC induced increase in DA firing (**Fig. 20B**). No significant increase in burst firing in the VTA DA neurons was recorded (**Fig. 20C**). Additionally, no FR or burst firing increase was seen with higher doses of NIC (1 mg/kg) (**Fig. 21B+C**).

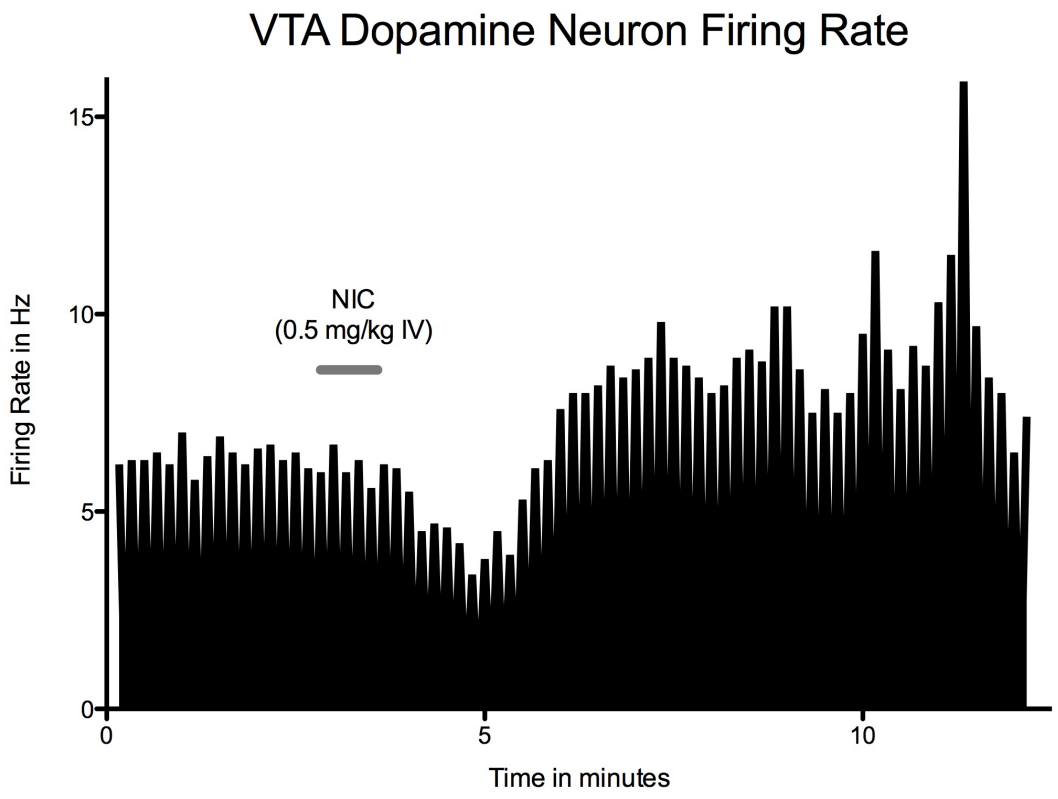

Figure 19 Representative example of the effect of systemic NIC (0.5 mg/kg IV) exposure on VTA DA neuron firing rate.

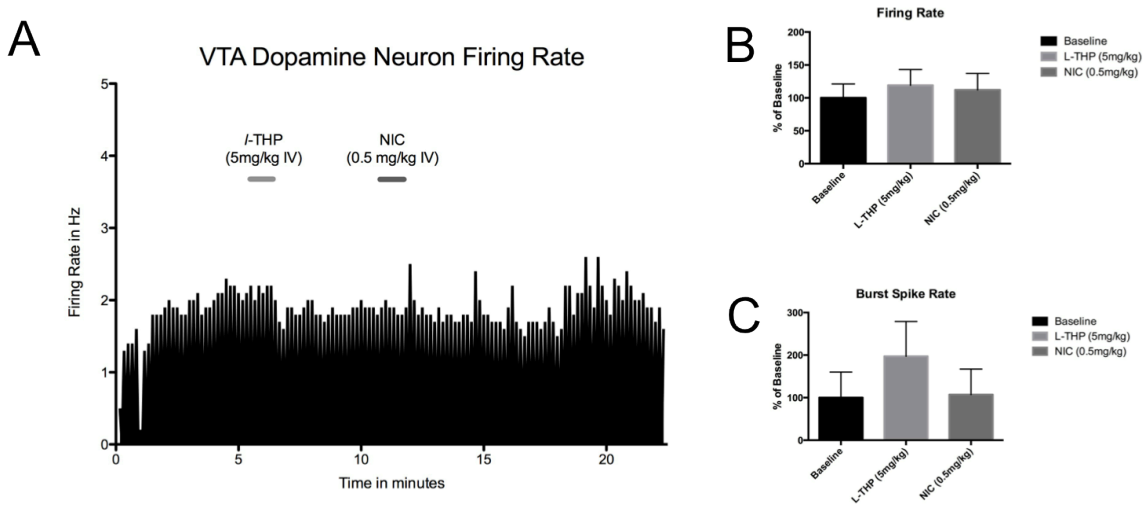

Figure 20 Effects of systemic NIC (0.5 mg/kg) exposure on DA neuron firing rate. (A) This record shows the firing rate of a representative VTA DA neuron in a C57BL/6 mouse. Its baseline firing rate was approximately 2 Hz. Administration of IV *l*-THP (5 mg/kg) 5 minutes prior to IV NIC (0.5 mg/kg) prevented NIC induced increase in firing rate normally seen in VTA DA neurons. (B) This graph summarizes the effects of IV *l*-THP and IV NIC on firing rate while (C) summarizes their effects on burst firing (*n*=5). No significant change was recorded.

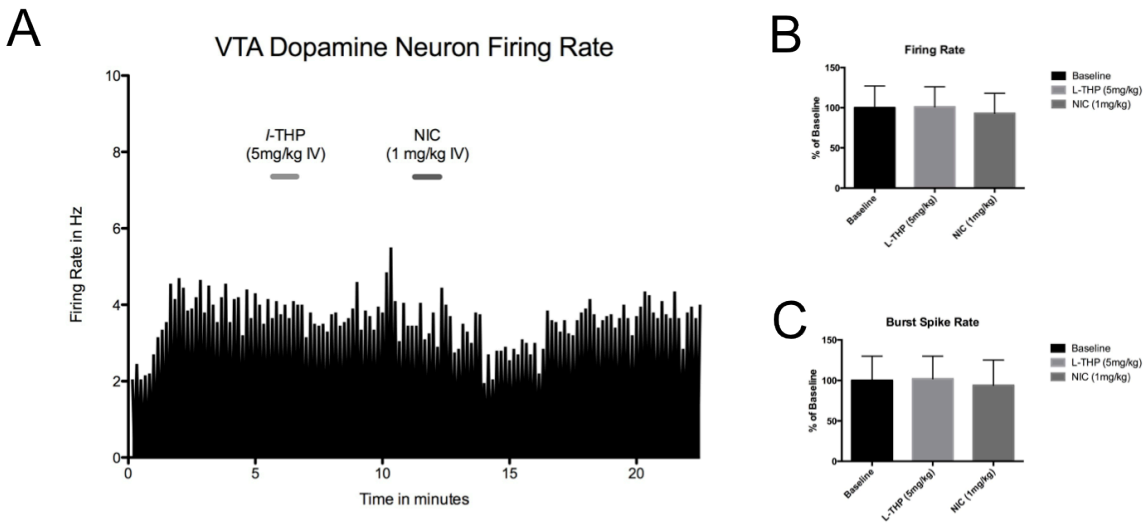

Figure 21 Effects of systemic NIC (1 mg/kg) exposure on DA neuron firing rate. (A) This record shows the firing rate of a representative VTA DA neuron in a C57BL/6 mouse. Its baseline firing rate was approximately 4 Hz. Administration of IV *l*-THP (5 mg/kg) 5 minutes prior to IV NIC (1 mg/kg) prevented NIC induced changes in firing rate. (B) This graph summarizes the effects of IV *l*-THP and IV NIC on firing rate while (C) summarizes their effects on burst firing (*n*=4). No significant change was recorded.

Acute l-THP's Effect on VTA DA Neurons mEPSCs: Slice Studies

In the slice, our study showed that miniature excitatory post-synaptic current (mEPSC) frequency, but not amplitude, was significantly increased by NIC (1 μM) (p<0.05) (**Fig. 22**). This increase in frequency was blocked when NIC was given with *l*-THP (30 μM).

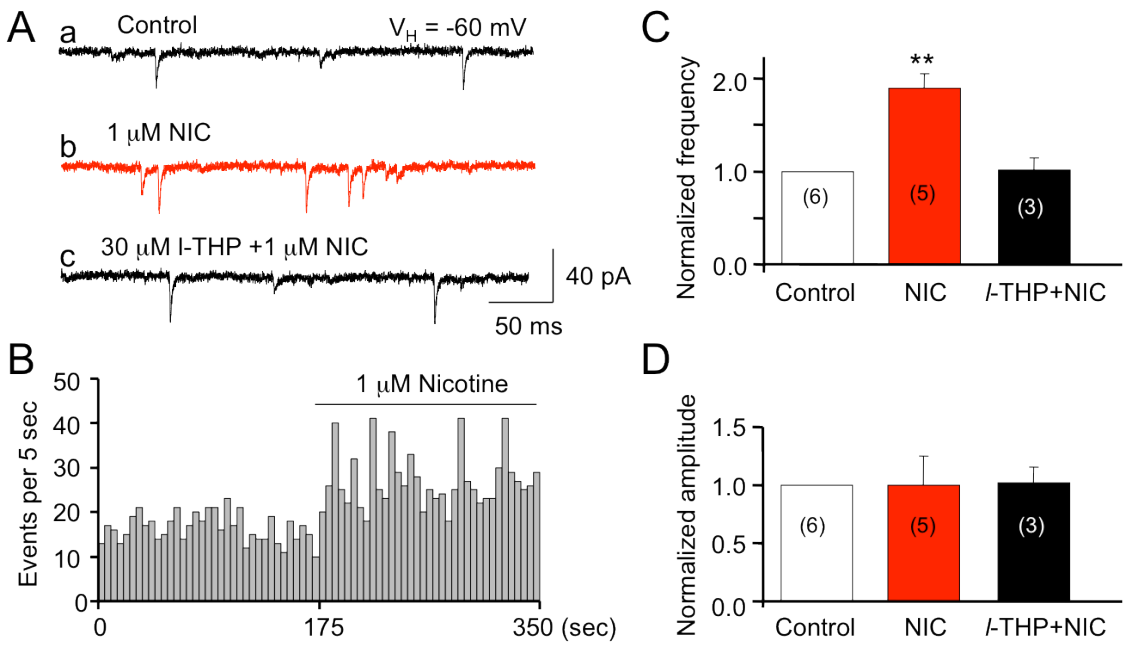

Figure 22 NIC enhances mEPSC frequency but not amplitude. (A) Typical trace from a VTA DA neuron in a C57BL/6 mouse slice during (Aa) control (Ab)1 μM NIC, and (Ac) 30 μM *l*-THP + 1 μM NIC treatment conditions. (B) Representative recording from a VTA DA neuron in a C57BL/6 mouse slice showing an increase in mEPSC frequency during 1 μM NIC treatment. (C) This graph summarizes the effects of NIC mEPSCs in VTA DA neurons. Superfusion of NIC significantly increased mEPSCs in VTA DA neurons at the 1 uM concentration. Sample sizes are shown in parentheses. Asterisks **= significance level P<0.01.

Effect of l-THP on Human α4β2 nAChR-mediated Currents

We evaluated the effect of *l*-THP on the function of heterologously expressed α4β2-nAChR-mediated currents in recombinant α4β2-nAChRs expressed in a human epithelia (SH-EP1) cell line. Although not as physiologically relevant as native systems, recombinant expression systems have several advantages including: 1) Better control over which subunits are being expressed (e.g., showing the difference between different combinations of subunits); 2) Better access to the cell in order to perform more accurate dose-response curves (e.g., less concern about the concentration of the drug becoming diluted as it diffuses into the tissue, etc.); 3) Better kinetic profiling of the responses (i.e., rise times, decay times, total current, peak amplitudes, desensitization rates); 4) Less artifact than with slices; 5) Simplified pharmacological analysis of the currents; and 6) Facilitation of the study of human nAChR subunits in an expression system. Experiments in recombinant α4β2 nAChRs in human epithelial (SH-EP1) cells *l*-THP showed a predominant, dose-dependent effect on conversion from peak to steady-state currents (**Fig. 23)**.

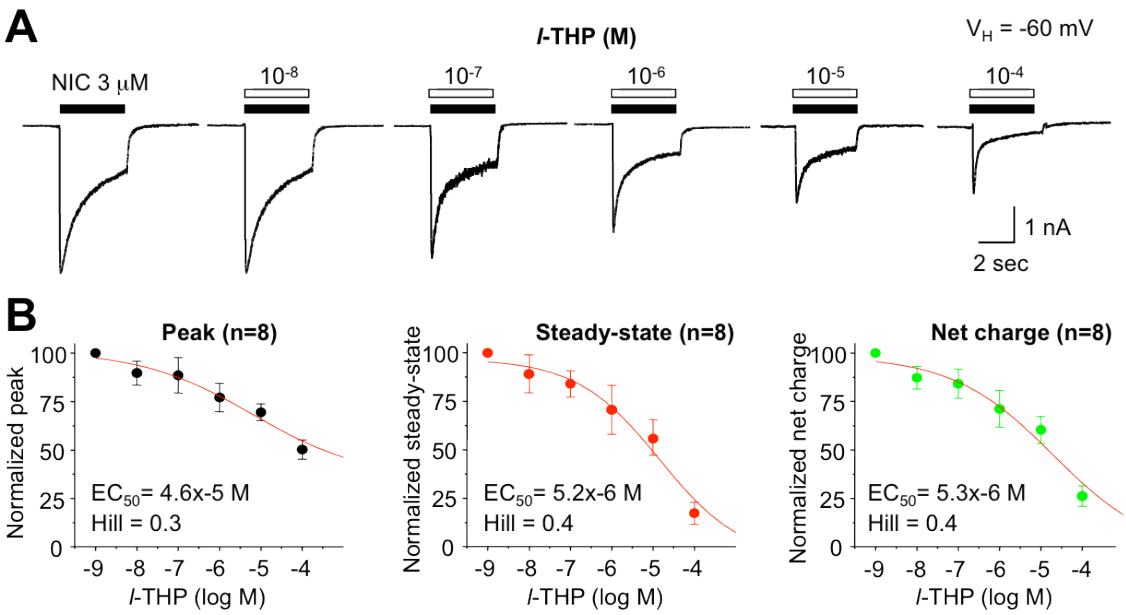

Figure 23 Effects of *l*-THP on human α4β2 nAChR-mediated currents. *l*-THP showed a predominant, dose-dependent effect on conversion from peak to steady-state currents, suggesting acceleration of acute desensitization.

Discussion

In this study we assessed the effects of *l*-THP on NIC induced electrophysiological and behavioral actions. It is generally thought that NIC acts on widely-distributed nAChRs in the brain and alters brain reward-associated circuits and pathways, thus contributing to NIC reinforcement and reward (Albuquerque, Pereira, Alkondon, & Rogers, 2009). We postulate that the basis for NIC reward and dependence exists at no less than three levels. At the receptor level, NIC activates and/or desensitizes different nAChR subtypes in brain reward centers (e.g., the VTA) and alters DA neuronal function. At the synaptic level, NIC acts on presynaptic nAChRs and modulates neurotransmitter release. At the neuronal network level, NIC alters neuronal circuits, signaling pathways and induces excitation of VTA DA neurons. We attempted to evaluate the effect of *l*-THP at all of these levels.

CPP was used as a tool in the examination of *l*-THP's ability to influence behavioral alterations induced by NIC. Significant place preference was established with a 0.5 mg/kg dose of NIC (**Fig. 17**). However, with *l*-THP (10mg/kg IP) injections preceding this dose of NIC mice, showed a reduction in the amount of time spent on the NIC (0.5 mg'kg) paired chamber. Furthermore, in an effort to more accurately mimic the use of *l*-THP as a therapeutic agent for smoking cessation, *l*-THP dramatically reduced time spent in the NIC (0.5 mg/kg) paired chamber when administered prior to NIC even after mice had been previously demonstrated a preference for the NIC (0.5 mg/kg) paired chamber. While these mice demonstrated a preference for a chamber paired with a 0.5 mg/kg dose of NIC, a higher dose (1 mg/kg) induced an aversive effect. Prior *l*-THP treatment exhibited no significant effect on this NIC (1 mg/kg) conditioned aversion.

Because *l*-THP is a known sedative, we were interested in the influence of this effect on NIC induced CPP and CPA. By tracking the locomotor activity of the animals in each treatment group we could identify it as a possible confound to place preference results. We found no significant difference in motor activity, as tracked by the number of beam breaks per drug conditioning session, between treatment groups in the study (**Fig. 18**).

Single neuron recordings in anesthetized mice help elucidate the effect of *l*-THP in NIC induced changes at the network level. Current ideology suggests that DA neuron firing in the midbrain's VTA projecting to limbic structures, I.e. the NAc, bear reward signal. As previously reported in detail, IV NIC (0.5 mg.kg) elicits a significant increase in VTA DA neuron FR (D. Zhang et al., 2012) (**Fig. 19**). However, in our study, we observed that *l*-THP (5 mg/kg IV) pretreatment prevented NIC induced increases in VTA DA neuron FR and burst firing at both the lower (0.5 mg/kg) (**Fig. 20B+C**) and higher (1 mg/kg) (**Fig. 21B+C**) doses of NIC. This blockade of VTA DA neuron firing seems consistent with the behavioral results in CPP animals. With NIC's induction of VTA DA firing damned, the reward signal is unable to be dispatched.

NIC induced modifications in synaptic signaling are also altered by *l*-THP. In VTA slices, mEPSC frequency, but not amplitude, was increased by 1 μM NIC, suggesting a presynaptic effect (**Fig. 22C**). This increase was blocked with co-application of 30 μM *l*-THP. It was not apparent from this data the exact mechanism of action utilized by *l*-THP in the blockade of NIC effects in VTA DA neurons in the slice. However, this increase in mEPSCs is another line of concurrent evidence for the efficacy of *l*-THP in the attenuation of NIC induced effects.

Lastly, in order to evaluate the effect of *l*-THP on NIC function at the level of the receptor, we utilized heterologously expressed α4β2-nAChR-mediated currents in recombinant α4β2-nAChRs expressed in a SH-EP1 cell line. This utilization lead to the observation of an antagonist effect of *l*-THP on transfected human α4β2-nAChR function (**Fig. 23**). NIC induced peak amplitude, steady state, and net charge are all reduced with increasing concentrations of *l*-THP. This novel finding, in conjunction with more physiologically relevant native systems, shows *l*-THP having an ability to block NIC induced receptor and synaptic functions. Nicotinic network and behavioral actuations are also prevented by *l*-THP.

Conclusion

NIC addiction is a complex disorder, which alters the function of the mesocorticolimbic DArgic pathway, of which the VTA is a crucial nucleus. We have discovered that *l*-THP's ability to mitigate NIC generated behavior, network, synapse and receptor functions. These new findings in

the neuropharmacology of *l*-THP suggest that the natural compound known as *l*-THP might be a candidate for developing the next generation of interventions for drug addiction in which nAChRs are involved. *l*-THP is also known to block D2 receptors (Jin et al., 2002; K. Yang et al., 2007) or activate cortical 5-HT receptors (Gao et al., 2010) which causes an increase of DA release from VTA to NAc. This “double target” feature of *l*-THP suggests that the *l*-THP is a promising candidate as a new generation of smoking cessation drugs.

CHAPTER 5

OVERALL DISCUSION OF DISSERTATION

Alcoholism and NIC addiction are chronic relapsing disorders with enormous societal impact. A major goal of addiction research is to characterize the critical neural substrates that are most sensitive to drugs of abuse, adapt in association with chronic consumption and drive subsequent drug-seeking behavior. The long-term objective of our research is to advance the understanding of the neural basis of NIC and EtOH reward and dependence. The rationale for this proposal is predicated on the belief that advancement in the understanding of the brain mechanisms underlying the recreational use and abuse potential of NIC and EtOH will pave the way for more effective treatment strategies that could reverse dependence on EtOH, thus saving lives and resources throughout the world.

Alcohol is one of the leading causes of preventable death in the United States and is accountable for approximately 90,000 deaths per year (Danaei et al., 2009), while NIC addiction is the leading cause of preventable death in the world (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). The rewarding effects of both NIC and EtOH, have been linked to the mesolimbic DA system (Wise & Rompre, 1989). An increase of DA levels in the NAc, especially the shell subregion, is thought to be vital for NIC and EtOH reward signaling (Wise & Rompre, 1989). However, NIC which has specific molecular targets, EtOH affects much wider neuronal functions including phospholipid membranes, various ion channels and receptors, synaptic and network functions, and intracellular signaling molecules. Thus far, the major targets mediating EtOH reward signaling and the precise mechanism activated by EtOH in the mesolimbic pathway (R. A. Wise, 1996) are still poorly understood. This gap in knowledge significantly inhibits our understanding of how alcohol reward and dependence occur, consequently resulting in a therapeutic barrier in the treatment of alcoholism. However, recently emerging data indicate an interaction between EtOH and nAChRs in both the VTA and NAc. EtOH likely regulates DA system function and responses to drug reinforcement through these nAChRs (Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001).

Therefore, these lines of evidence build a rationale that both NIC and EtOH may target neuronal similar nAChRs and increase mesolimbic pathway activity, leading to reward and dependence.

α6 nAChR subunits are not widely expressed in the brain, but are prevalent in midbrain DAergic regions associated with pleasure and reward (Klink, de Kerchove d'Exaerde, et al., 2001), suggesting that α6 containing nAChRs (α6*-nAChRs) could play critical roles in dependence attributed to NIC and EtOH (Shytle et al., 2002). Recently, α6 KO mice revealed that the α-conotoxin MII (α-CtxMII), derived from the *Conus magnus* marine snail, binds to α6*-nAChRs. In addition, the α4 subunit could play a role in reward when paired with the α6 subunit (Drenan et al., 2010a; Hendrickson, Gardner, & Tapper, 2011; Zhao-Shea et al., 2011). Studies using immunoprecipitation found that not only were α6 and β2 subunits expressed in the same receptors, but the β3 subunit was also found in most α6*-nAChR pentamers in mesolimbic and nigrostiatal DA pathways (Cui et al., 2003; Quik et al., 2000). β3 knockout mouse studies confirmed that this subunit plays a role in α-CtxMII binding (Cui et al., 2003; Gotti et al., 2009; Gotti et al., 2010a; Quik et al., 2005; Zoli et al., 2002), and this subunit may be involved in ion permeability and receptor location (Gotti & Clementi, 2004).

α-CtxMII administered in the VTA was able to reduce EtOH induced NAc DA release (Larsson, Jerlhag, Svensson, Soderpalm, & Engel, 2004) and locomotor activity (Jerlhag, Grotli, Luthman, Svensson, & Engel, 2006). α-CtxMII perfusion into the VTA also blocked recognition of EtOH associated cues (Lof et al., 2007) and voluntary EtOH drinking in rodents (Larsson et al., 2004). Genetic, electrophysiological, and pharmacological techniques have been employed to demonstrate functional α6*-nAChRs situated on GABA terminals innervating DA neurons in the VTA (K. Yang et al., 2011b). The combined data support α6 and β2 containing nAChRs are located on these terminals, however α4 subunits are not (K. Yang et al., 2011b). Therefore, the majority of α6*-nAChRs in the mesolimbic pathway are part of either an $\alpha6_{(1)}\alpha4_{(1)}\beta2_{(2)}\beta3_{(1)}$ or an $\alpha6_{(2)}\beta2_{(2)}\beta3_{(1)}$ heteromeric pentamer (Champtiaux et al., 2003; Charpantier et al., 1998; Cui et al., 2003; Quik & McIntosh, 2006; Salminen et al., 2005; Zoli et al., 2002) with the later located on VTA GABAergic boutons (K. Yang et al., 2011b); these receptors may have a significant role in the actions of NIC and EtOH in the VTA.

There is a growing interest in studying the role of VTA GABA neurons in drug abuse especially in the nAChR field as the majority of endogenous cholinergic inputs into the VTA innervate GABA neurons (Fiorillo & Williams, 2000; Garzon et al., 1999). The majority of the research here has been focused on the role of nAChRs on VTA GABA neurons and their effect of reward. The α7 nAChR study, in chapter 2, plays a significant role in this area as these receptors have been shown here to be located presynaptically on GLU terminals innervating VTA GABA neurons. This location is important in GABA neuron disinhibition and ultimately DA neuron firing in the NAc as we have previously shown that α6* nAChRs in the VTA are located on GABAergic synaptic boutons innervating DA neurons (K. Yang et al., 2011b). Therefore, GLU input, presumably from the PFC, has the ability to be a major influence on driving disinhibition of VTA DA through presynaptic α7 nAChRS and then presynaptic α6* nAChRs on GABA neuron terminals which innervate DA neurons. Since the experiments from the chapter 2 study were performed to determine the acute effect of NIC on VTA neurons, no direct conclusion can be obtained regarding the addictive activity of NIC after prolonged (chronic) use. However, we have seen a link in this proposed pathway in unpublished data in which once daily NIC injections (0.67 mg/kg IP) for 12 days showed nAChR transcript changes. Single-cell real-time quantitative PCR of selected nAChR subunits in VTA GABA neurons from GAD-GFP mice after 12 daily NIC injections showed both α6 and α7 nAChR subunit increases where none where found previously to NIC injections or in saline injected mice.

Recently, there have been three FDA approved medications for treating alcoholism; (1) Disulfiram, approved in 1954, is an acetaldehyde dehydrogenase inhibitor which improves alcohol symptoms such as headache, nausea, vomiting, weakness, mental confusion, or anxiety (Christensen, Moller, Ronsted, Angelo, & Johansson, 1991). (2) Naltrexone, available since 1994, is a competitive opioid receptor antagonist that works by decreasing the euphoric effects produced by alcohol (Volpicelli, Alterman, Hayashida, & O'Brien, 1992). (3) Acamprosate is a partial agonist of NMDA receptors and an antagonist of metabotropic glutamate receptors and is thought to act as an anti-craving medication by inhibiting GLU signaling (Mason, Goodman, Chabac, & Lehert, 2006). Unfortunately, only 20–30% of treated patients respond positively to

these drugs (Spanagel, 2009) and some of these drugs have shown serious negative side effects. Current FDA approved medications for smoking cessation are the antidepressant Bupropion and the nicotinic partial agonist Varenicline, both of which are often accompanied by strong negative side effects Thus, there is an urgent need to develop new drugs for the treatment alcoholism and NIC addiction. Recently, nAChR-associated ligands have been shown as potential candidates for alcoholism as well as NIC. For example, pharmacotherapeutic targeting nAChRs such as cytisine, sazetidine A, lobeline mecamylamine, PF-4575180 and CP-601932 are the new strategies to treat alcohol dependence including in reducing voluntary alcohol consumption or modulate alcohol drinking behavior in animal models and humans. Because α6 nAChR subunits are not widely expressed in the brain, but are prevalent in midbrain DAergic regions side effects with a drug that worked selectively on α6* nAChRs could not only be effective (Brunzell, 2012) but also have less side effects.

We have also shown here that *l*-THP possess unique neuropharmacological actions on DA neurons in the mesocorticolimbic DAergic pathway. By understanding more fully the circuits involved in both NIC and EtOH addiction, we are better able to test pharmacological interventions with a translational potential. This study with *l*-THP shows the practical application of the knowledge gained from the previous chapters. Experiments in recombinant α4β2 nAChRs in SH-EP1 cells *l*-THP showed a predominant, dose-dependent effect on conversion from peak to steady-state currents, suggesting acceleration of acute desensitization. As the effect of *l*-THP in slice (increase in mEPSCs) studies are currently underway in which the effect of *l*-THP is being tested on NIC currents in SH-EP1 cells with α6* nAChRs. As α6* nAChRs are located presynaptically in the VTA more often than α4β2 nAChRs. If *l*-THP also displays a similar effect on NIC currents in SH-EP1 cells with α6* nAChRs as it does with α4β2 nAChRs, *l*-THP might be a candidate for developing the next generation of interventions for both alcoholism and NIC addiction.

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