ASSESSMENT OF ETHANOL AND NICOTINE INTERACTIONS IN THE RAT MODEL: PHARMACOTHERAPEUTICS, ADOLESCENCE, AND THE MESOLIMBIC SYSTEM

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DEDICATION

This dissertation is dedicated to my wife Abby Waeiss, and to my parents Bob and Linda Waeiss. I cannot thank you enough for your endless patience and always believing in me, especially when I failed to do so. Your unconditional love and support were integral in completing this body of work.

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Robert Aaron Waeiss

ASSESSMENT OF ETHANOL AND NICOTINE INTERACTIONS IN THE RAT MODEL: PHARMACOTHERAPEUTICS, ADOLESCENCE, AND THE MESOLIMBIC SYSTEM

Alcohol use disorder (AUD) and nicotine dependence often result in serious health problems and are top contributors to preventable deaths worldwide. Co-addiction to alcohol and nicotine is the most common form of polysubstance abuse. Epidemiological studies indicate that more than 80% of individuals diagnosed with AUD concurrently use nicotine. The prevalence of alcohol and nicotine comorbidity may stem from interconnected mechanisms underlying these disorders. A better understanding of how these drugs interact and the biological basis behind the high comorbidity rates could generate key targets for the development of more effective treatments for AUD and nicotine dependence.

The following experiments utilized four similar overall groups consisting of vehicle, ethanol (EtOH), nicotine (NIC), and EtOH+NIC. Chapter Two investigated the efficacy of naltrexone and varenicline, the pharmacological ‘gold standards’ for treating AUD and nicotine dependence, on voluntary drug intake by rats selectively bred for high EtOH drinking. The results indicated that the standard treatments for AUD and nicotine dependence were effective at reducing consumption of the targeted reinforcer but neither reduced EtOH+NIC co-use/abuse. Chapter Three examined the effects of peri-adolescent EtOH drinking

on the ability of NIC infused into the posterior ventral tegmental area (pVTA) to stimulate dopamine release within the nucleus accumbens (NAc) shell during adulthood. The results suggest a cross-sensitization to NIC produced by peri-adolescent EtOH consumption demonstrated by a leftward and upward shift in the dose response curve. Chapter Four investigated the effects of intra-pVTA infusions on NAc shell neurochemistry, EtOH reward within the NAc shell, and the role of brain-derived neurotrophic factor (BDNF) on EtOH reward within that region. The data indicated that only EtOH+NIC significantly increased glutamate, dopamine, and BDNF in the NAc shell. Repeated pretreatment with EtOH+NIC also enhanced EtOH reward in the NAc shell and BDNF infusions were sufficient to recapitulate these findings. Collectively, the data indicate that concurrent exposure to EtOH and NIC results in unique neuroadaptations that promote future drug use. The failure to develop effective pharmacotherapeutics for AUD or nicotine dependence could be associated with examining potential targets in models that fail to reflect the impact of polydrug exposure.

William A. Truitt, Ph.D., Chair

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LIST OF ABBREVIATIONS

3BC	3-bottle choice
5-HT_3	serotonin-3 receptor
AAALAC	Association for the Assessment and Accreditation of Laboratory Animal Care
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
ADE	alcohol deprivation effect
AIE	adolescent intermittent ethanol
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	anterior posterior
AUD	alcohol use disorder
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
$CaCl_2$	calcium chloride
CeA	central nucleus of the amygdala
ChAT	choline acetyltransferase
CNS	central nervous system
COMT	catechol-O-methyltransferase
CR	calretinin
DA	dopamine
DAT	dopamine transporter
DMSO	dimethyl sulfoxide
DRN	dorsal raphe nucleus
DS	dorsal striatum
DV	dorsal ventral
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMIT	electrolytic microinfusion transducer
ERK	extracellular signal-regulated kinase
EtOH	ethanol

FDA	Food and Drug Administration
g	gram
G proteins	GTP-binding proteins
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glia-derived neurotrophic factor
GPBC	G protein-binding cluster
H_2O_2	hydrogen peroxide
HCl	hydrochloride
HPC	hippocampus
HPLC	high performance liquid chromatography
ICSA	intracranial self-administration
IgG	Immunoglobulin G
IICR	IP_3-induced calcium release
i.p.	intraperitoneal
IP_3	inositol trisphosphate
IR	immunoreactive
i.v.	intravenous
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
LDT	lateral dorsal tegmentum
LGIC	ligand-gated ion channel
LTP	long-term potentiation
MAO	monoamine oxidase
MeOH	methanol
mg	milligram
mg%	milligram percent
$MgCl_2$	magnesium chloride
micro-micro	microinjection-microdialysis
ML	medial lateral
ml	milliliter
mm	millimeter

mM	millimolar
MSN	medium spiny neuron
N	normal
$Na_2HPO_4 \bullet 7H2O$	sodium phosphate dibasic heptahydrate
NAc	nucleus accumbens
NaCl	sodium chloride
nAChR	nicotinic acetylcholine receptor
Nal	naltrexone
NDE	nicotine deprivation effect
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NIDA	National Institute on Drug Abuse
NIC	nicotine
nl	nanoliter
nM	nanomole
NMDA	N-methyl-D-aspartate
NRT	nicotine replacement therapy
P	alcohol-preferring rat
$p75^{NTR}$	75 kDa neurotrophin receptor
PBS	phosphate buffered saline
PFA	paraformaldehyde
PFC	prefrontal cortex
PND	post-natal day
PPT	pedunculopontine tegmentum
PV	parvalbumin
r-bPiDI	1,10-*bis*(3-methyl-5,6- dihydropyridin-1(2*H*)-yl) decane dihydrochloride
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SN	substantia nigra
SOM	somatostatin
TH	tyrosine hydroxylase

TrkB	tropomyosin receptor kinase B
µl	microliter
µm	micrometer
µM	micromolar
v/v	volume/volume
Var	varenicline
VTA	ventral tegmental area
VGluT2	vesicular glutamate transporter 2
VMAT	vesicular monoamine transporter
VP	ventral pallidum

Chapter One: Introduction

Clinical significance

Alcohol and nicotine abuse result in numerous serious health problems and account for a large proportion of preventable deaths worldwide. The devastating impact on physical health from alcohol and nicotine abuse includes increased risk for many different types of cancer, liver disease, heart disease and lung disease (Grucza and Bierut 2006). There are over 42 million individuals in the United States that smoke and almost 20 million that abuse alcohol (Agaku et al. 2014; Falk et al. 2006). Past research has shown that more than 80% of those with alcohol use disorder (AUD) also exhibit comorbid use of nicotine in comparison to 34% of non-alcoholics (John et al. 2003a, b). Likewise, individuals suffering from nicotine dependence are 10 times more likely to be diagnosed with AUD in their lifetime (DiFranza and Guerrera 1990).

Research has revealed that the amount of nicotine ingested by an individual is positively correlated with AUD as well as with larger quantities of alcohol intake and number of drinking sessions when compared to non-smoking alcoholics (Daeppen et al. 2000; Gulliver et al. 1995). Additionally, the severity of AUD has also been positively correlated to a number of other elements that include urge to smoke, years smoked, number of attempts to quit smoking and age an individual began smoking (John et al. 2003a, b). Alternatively, spontaneous smoking cessation in individuals that are alcohol and nicotine dependent is seven times lower than those not abusing alcohol (DiFranza and

Guerrera 1990). The intensity of dependence on nicotine has been associated with more severe bouts of relapse in those with AUD. It has also been established that individuals with AUD who stop smoking have a better chance of remaining abstinent from alcohol than those who continue to smoke (Daeppen et al. 2000; Gulliver et al. 1995). This agrees with more recent research that indicates when an individual trying to abstain abuses both alcohol and nicotine there is a significantly worse clinical outcome than those who use one or the other (Lajtha and Sershen 2010; Grant et al. 2004).

Moreover, early initiation of alcohol consumption is associated with greater risk of a diagnosis of AUD in adulthood (Dawson et al. 2008). Alcohol drinking during adolescence has consistently been found to occur in greater than 80% of individuals before 18 years of age (Johnston et al. 2004). Furthermore, the adolescent brain appears to be more susceptible to the effects of binge alcohol exposure than the mature brain (Crews et al. 2016). Relevant to the present report, adolescent binge drinking enhances the likelihood of using nicotine products during adolescence as well as during adulthood (Best et al. 2000). In contrast, individuals that do not engage in regular binge drinking sessions have significantly lower rates of smoking during adulthood (Bobo and Husten 2000).

Together, addiction to alcohol and nicotine occurs in a greater number of individuals than any other drug of abuse (Grant et al. 2004). It has been suggested that the prevalence of alcohol and nicotine comorbidity may stem from interconnected mechanisms underlying these particular disorders (Grucza and Bierut 2006). Additionally, developmental perturbations induced by adolescent

drinking could partly explain the increased rates of AUD and nicotine dependence during adulthood (Bates and Labouvie 1997). Elucidating how these drugs interact and the neurobiological mechanisms behind the frequency of co-abuse is necessary in order to develop effective treatments and preventive measures in the future.

Mesocorticolimbic circuitry and signaling

There is evidence for dysregulation of neurochemical mechanisms in brain reward regions that act through respective receptor systems to mediate the reinforcing effects of these drugs and subsequent neuroadaptations (Falk et al. 2006; Vengeliene et al. 2008; Deehan et al. 2015; Hauser et al. 2014). An immense amount of research has been carried out on the neurobiology of the mesocorticolimbic dopamine (DA) pathway and how drugs of abuse highjack this system resulting in detrimental behaviors. A clear description of the dopaminergic system under normal physiological conditions provides a basis of comparison for subsequent neuroadaptations and altered behaviors following drug exposure.

DA is the most abundant catecholamine neurotransmitter in the brain and is produced by mesencephalic neurons of the substantia nigra (SN) and ventral tegmental area (VTA; Baik 2013). DA is synthesized through the enzymatic actions of tyrosine hydroxylase, which is the rate-limiting step, followed by amino acid decarboxylase. DA is then removed from the cytoplasm and packaged into synaptic vesicles by vesicular monoamine transporters (VMATs) using a proton electrochemical gradient (Howell and Kimmel 2008). Following the release of DA

into the synaptic cleft, signaling is inactivated through a combination of reuptake and metabolism. DA is removed from the extracellular space by transporter proteins that are part of the sodium:neurotransmitter symporter family. This is primarily carried out by the DA transporter (DAT). Next, DA is either repackaged into vesicles for re-release or broken down and eliminated by catechol-O-methyltransferase (COMT) or monoamine oxidase (MAO; Meyer and Quenzer 2005).

DA signaling occurs through its binding to seven transmembrane domain G-protein coupled receptors resulting in the activation of downstream signaling cascades (Beaulieu and Gainetdinov 2011). Five different DA receptors have been identified (D1R – D5R) and are divided into two groups based on structure and pharmacological properties. D1-like receptors (D1R and D5R) mediate excitatory neurotransmission by stimulating adenylyl cyclase and increasing intracellular cAMP. D2-like receptors (D2R – D4R) mediate inhibitory neurotransmission via inhibition of adenylyl cyclase (Beaulieu et al. 2015). One important feature of D2Rs is its function as an autoreceptor. D2-like receptors are often located presynaptically and dampen excitability as well as DA synthesis and packaging (Beaulieu et al. 2015). The distinct functional characteristics and localization of each DA receptor subtype allow dynamic regulation of several physiological processes.

Midbrain DA neurons have been associated with increasingly complex functions and connectivity following continued delineation of neuronal subtypes that have unique molecular, anatomical, and electrophysiological features

(Ikemoto 2010; Sesack and Grace 2010; Lammel et al. 2014). Interspersed γ-aminobutyric acid (GABA) and glutamatergic subpopulations within the midbrain DA system have added to the complexity of functions associated with DA neuron activity. Studies indicate this DA activity is linked to signaling of salience, reward, aversion, novelty and motivation (Garris et al. 1999; Pierce and Kumaresan 2006; Berridge 2007; Hikida et al. 2010; Sesack and Grace 2010; Koob and Volkow 2010; Schultz 2013; Lammel et al. 2014).

A number of different brain regions and circuits have been identified that play a role in drug reward and reinforcement. These often involve the mesocortical, mesostriatal and mesolimbic DA pathways. Specifically, the mesolimbic DA system is made up of the dopaminergic cells bodies in the VTA that project into the nucleus accumbens (NAc) as one piece in a series of parallel integrated circuits that engage several other critical brain regions (Figure 1; Ikemoto 2010; Volkow et al. 2011). Mesocortical (VTA projections to frontal cortex) and mesostriatal (SN/VTA projections to the dorsal striatum) DA pathways have been implicated in various aspects of drug related behaviors (e.g. drug-seeking, withdrawal, relapse; Koob and Volkow 2010; Volkow et al. 2011).

The following background will predominantly cover VTA to NAc projections as this is of particular relevance to Chapters Three and Four. Focus on this projection is pertinent due to the different rates of development, neurotransmitters released, reuptake mechanisms, and plasticity of the separate DA projections associated with discrete functional roles and behaviors (McCutcheon et al. 2012; Spear 2000).

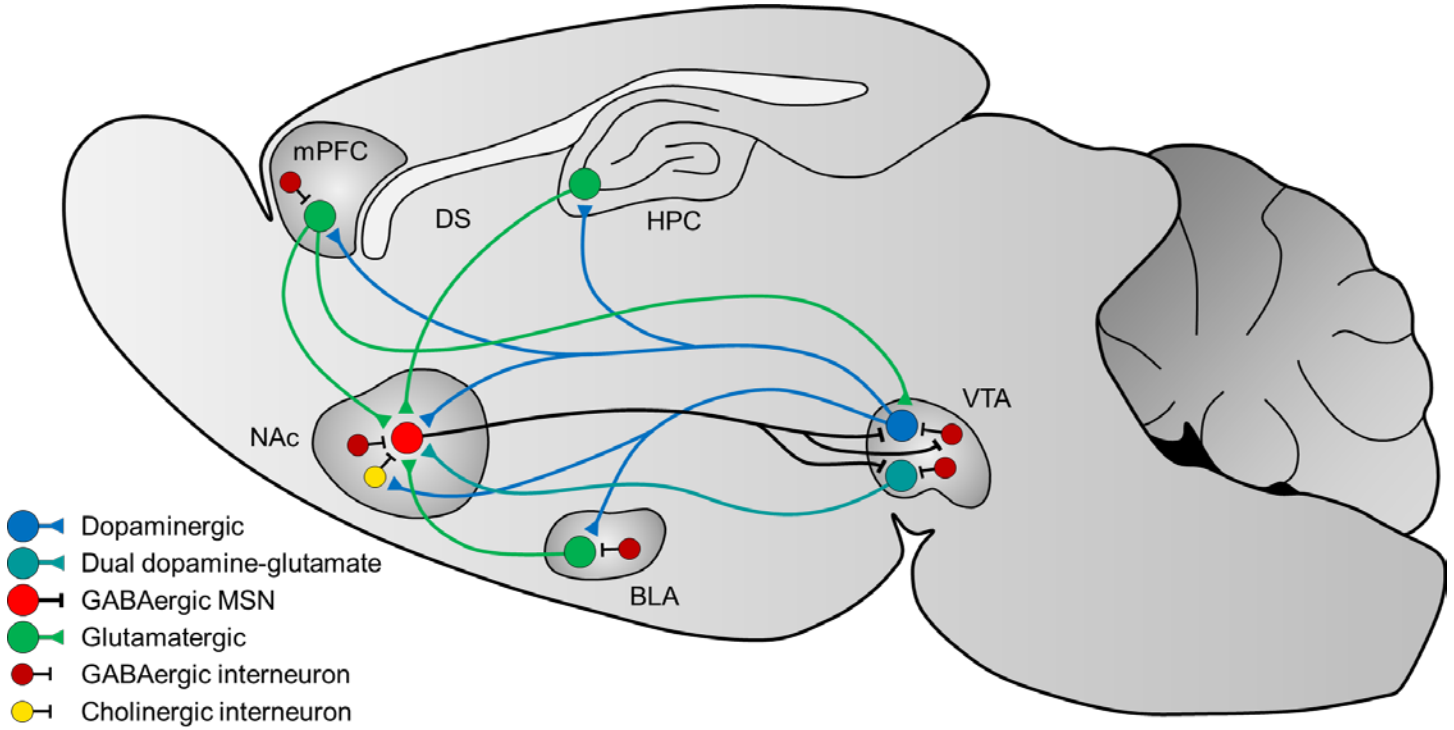

Figure 1. Simplified diagram of the ventral tegmental area (VTA) and nucleus accumbens (NAc) reward circuit. The illustration depicts key dopaminergic, glutamatergic, and GABAergic connections between the VTA and NAc. The primary reward circuit includes projections from the VTA to the NAc, which release dopamine in response to reward stimuli. GABAergic projections from the NAc to the VTA occur through a direct pathway mediated by D1-type medium spiny neurons (MSNs) that innervate the VTA or the indirect pathway mediated by D2-type MSNs which innervate the VTA via GABAergic neurons in the ventral pallidum (not shown). The NAc receives dense glutamatergic inputs from the hippocampus (HPC) and basolateral amygdala (BLA), and medial prefrontal cortex (mPFC). These glutamatergic inputs control aspects of reward-related perception and memory. Additional details of the NAc and VTA microcircuitry are shown in Figures 2 and 3. DS, dorsal striatum.

The NAc composes a portion of the ventral striatum and is positioned as a region where cortical afferent systems under dopaminergic modulation are integrated with motivational inputs from limbic structures to regulate goal-directed behavior. There are two major subdivisions of the NAc. The core region surrounds the anterior commissure while the shell portion is composed of the ventral and medial areas (Ikemoto 2010; Sesack and Grace 2010). The NAc is made up of more than 90% medium spiny neurons (MSNs; Sesack and Grace 2010; Russo and Nestler 2013). MSNs are inhibitory cells that primarily signal via GABA. The majority of MSNs can be identified through the expression of high levels of either D1R or D2R (See Figure 2; Russo and Nestler 2013). The remaining portion is composed of local GABAergic interneurons that are identified by expression of calretinin, parvalbumin, somatostatin, or calretinin in addition to a small population of cholinergic interneurons (Figure 2). NAc inhibitory efferents project to the ventral pallidum (VP), SN, hypothalamus, brain stem and VTA (Russo and Nestler 2013; Sesack and Grace 2010). Strong glutamatergic projections from the prefrontal cortex (PFC), hippocampus, and amygdala innervate the NAc along with DA, glutamate, and GABA projections from the VTA. Finally, the NAc also receives serotonergic and noradrenergic inputs from the dorsal raphe nucleus (DRN) and locus coeruleus, respectively (Sesack and Grace 2010).

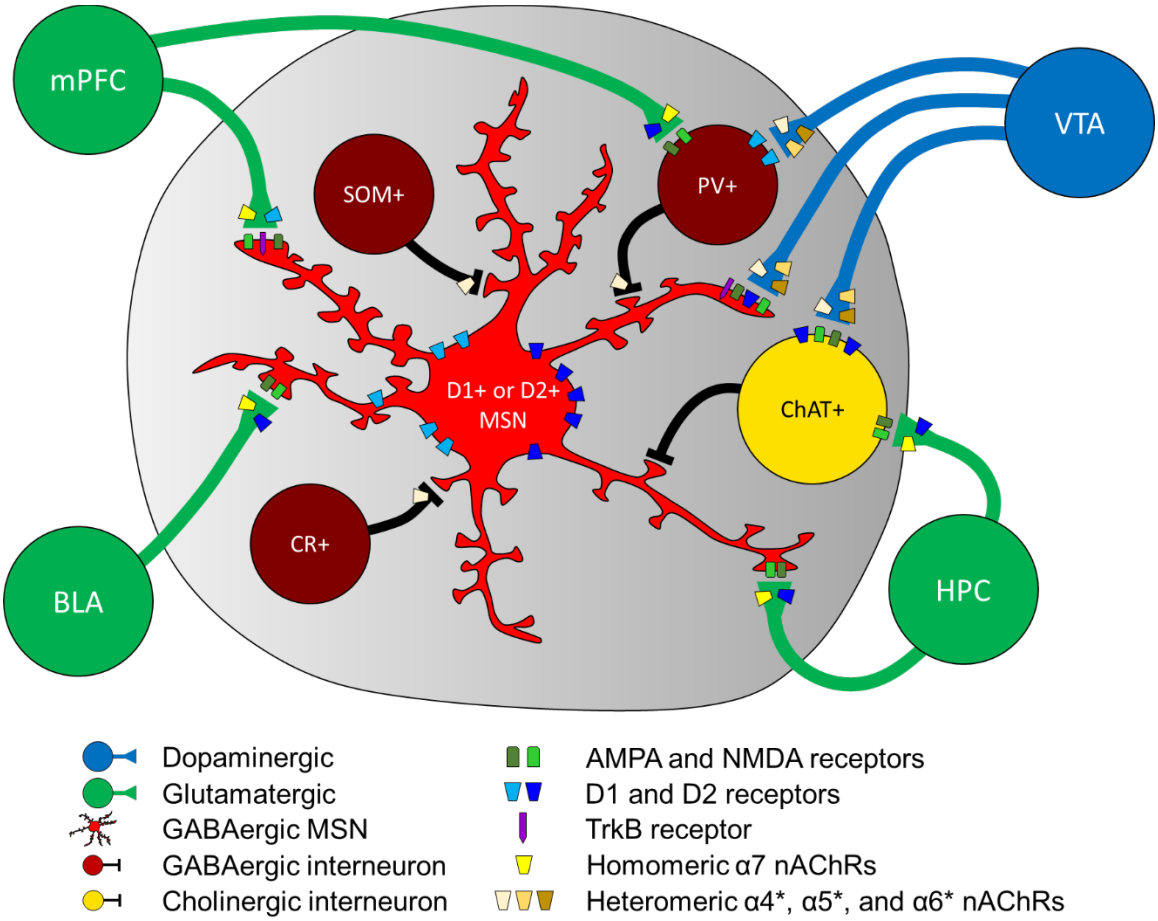

Figure 2. Local microcircuitry of the NAc. Simplified schematic of presynaptic inputs onto D1- and D2-type GABAergic medium spiny neurons (MSNs) as well as interneurons within the NAc. Striatal GABAergic interneurons express somatostatin (SOM), calretinin (CR), parvalbumin (PV), or choline acetyltransferase (ChAT). Glutamatergic inputs from the medial prefrontal cortex (mPFC), hippocampus (HPC), and basolateral amygdala (BLA) provide excitatory signals to GABAergic MSN projection neurons. Excitatory inputs also synapse directly onto the GABAergic and cholinergic interneurons that modulate MSN activity. D1- and D2-type MSNs also receive signals from VTA DA neurons.

VTA neurons have been shown to be composed of a heterogeneous mixture with approximately 65% being dopaminergic, 30% GABAergic, and 5% glutamatergic. Small subsets of these neurons have also been reported to co-release functionally significant amounts of glutamate and/or GABA from DA neurons (Yetnikoff et al. 2014; Lammel et al. 2014). In addition to the previously

mentioned DA VTA efferents, significant innervations also target the hippocampus, central amygdala, basolateral amygdala, olfactory tubercle, VP, and motor cortices (Figure 1; Ikemoto 2010; Yetnikoff et al. 2014). Primary afferents of the VTA include orexinergic and glutamatergic neurons from the lateral hypothalamus. Additional excitatory inputs come from the PFC and lateral dorsal tegmentum while the extended amygdala sends both glutamatergic and GABAergic projections that synapse directly on DA neurons (Figure 3). Lateral habenula inputs into the VTA are also glutamatergic but synapse onto GABA interneurons, which control aspects of reward-related perception and memory. (Russo and Nestler 2013; Yetnikoff et al. 2014).

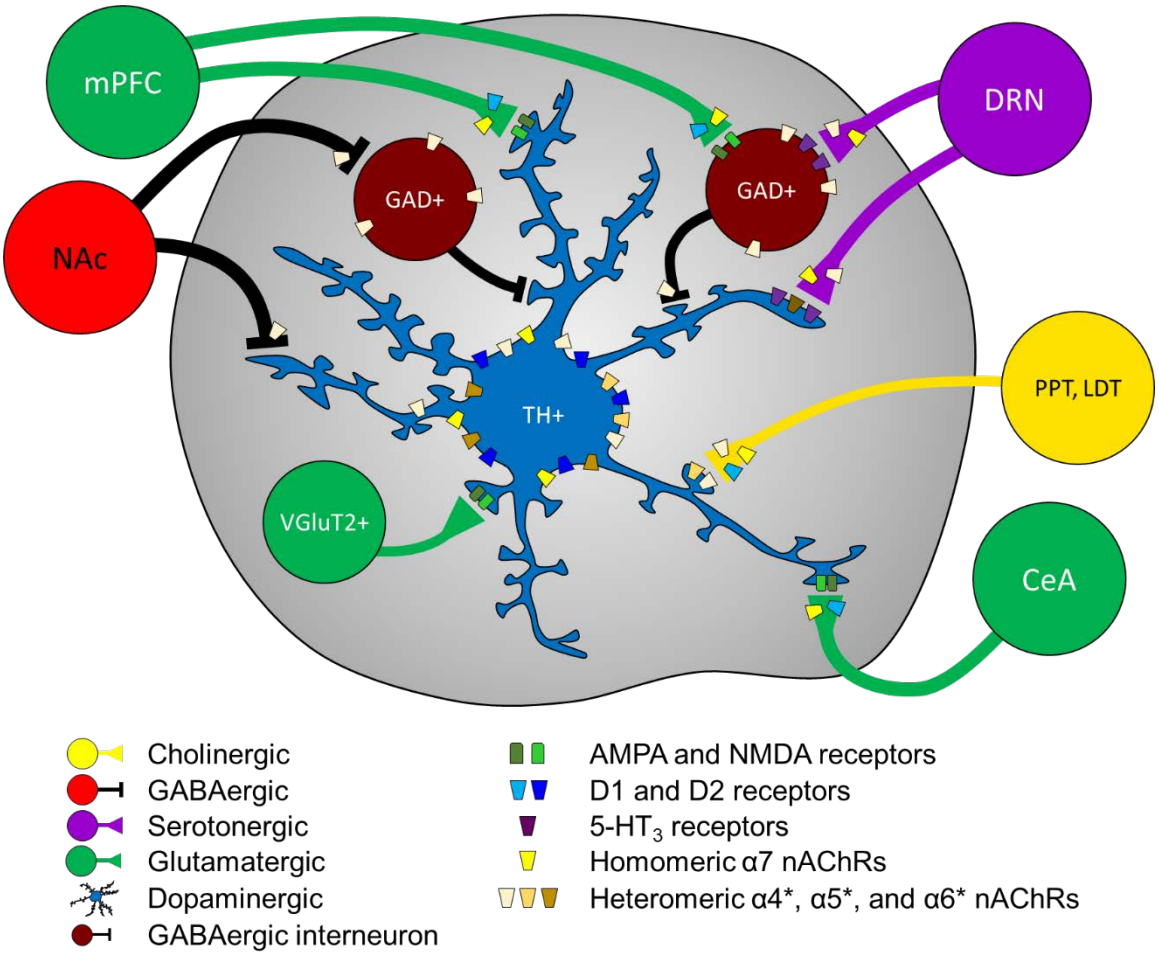

Figure 3. Local microcircuitry of the VTA. Simplified diagram of presynaptic inputs onto VTA DA neurons expressing tyrosine hydroxylase (TH) and local GABAergic (expressing glutamic acid decarboxylase, GAD) and glutamatergic (expressing vesicular glutamate transporter 2, VGluT2) neurons within the VTA. Glutamatergic neurons from the mPFC and central nucleus of the amygdala (CeA) as well as local glutamate neurons synapse directly onto VTA DA neurons. DA neurons also receive direct serotonergic input from the dorsal raphe nucleus (DRN) and cholinergic inputs from the pedunculopontine tegmentum (PPT) and lateral dorsal tegmentum (LDT), which modulate DA release and promotes reward. Inhibitory projections from the NAc to the VTA innervate both VTA DA neurons as well as local GABAergic interneurons.

Neuropharmacology of alcohol and nicotine

The mesocorticolimbic DA pathway (Figure 1) described above is known to be altered following alcohol and nicotine exposure. The rewarding and

reinforcing properties of alcohol and nicotine are well known to be associated with increased DA release within the NAc (Di Chiara and Imperato 1988; Wise and Rompre 1989). Furthermore, the associated reinforcing effects of pharmacologically relevant levels of alcohol and nicotine within the posterior VTA (pVTA) has been established (Exley et al. 2011; Hauser et al. 2014; Rodd-Henricks et al. 2000; Truitt et al. 2014). The pVTA is a neural target where alcohol and nicotine are self-administered and interact to enhance DA release in the NAc shell (Tizabi et al. 2002, 2007; Rodd et al. 2010; Corrigall et al. 1994). It has been suggested that the elevated NAc DA release induced by alcohol and nicotine could be through both independent actions as well as through shared neurotransmitter systems.

Alcohol has been demonstrated to have a number of pharmacological targets within the central nervous system (CNS). In addition to the well-established interaction with its metabolic enzyme alcohol dehydrogenase (ADH), alcohol exerts rapid and acute effects on protein function that involves both excitatory and inhibitory synaptic processes. Alcohol concentrations from 5 – 20 mM are considered physiological relevant and approximate the legal intoxication range of many countries (Abrahao et al. 2017). Within this range, alcohol directly alters the function of numerous receptors and ion channels. Therefore, the following background will focus on targets of alcohol that likely interact with the systems and circuits affected by nicotine. The function of N-methyl-D-aspartate (NMDA) receptors are inhibited by alcohol in a dose-dependent manner. The impact of alcohol on NMDA receptors was also specific to subunit composition

with NR1/NR2A or NR1/NR2B identified as more sensitive to the inhibitory effects of alcohol (Lovinger et al. 1989; Allgaier 2002).

Additionally, alcohol was demonstrated to enhance activity of both $GABA_A$ and glycine receptors (GlyRs; Mihic 1999). Previous research has established the activation of neuronal nicotinic acetylcholine receptors (nAChRs), particularly within the VTA, is one convergent site of action behind the effects of alcohol and nicotine (De Biasi and Dani 2011; Doyon et al. 2013). These receptors, as well as $GABA_A$ and GlyRs, are part of the Cys-loop ligand-gated ion channel (LGIC) superfamily and are composed of five subunits. The subunits assemble in various combinations to make up specific subtypes with unique channel properties, desensitization, and agonist affinity (Doyon et al. 2013). The nAChR subunits assemble into hetero- or homopentamers that are expressed on DA, glutamate, and GABA neurons at the somatic, perisomatic, and presynaptic levels within the VTA (Figure 3; Morel et al. 2018). Alcohol potentiates nAChR function in a subtype dependent manner. Enhanced activity by alcohol has been demonstrated for α2β4*, α4β4*, α2β2*, and α4β2* nAChRs (asterisk indicates other potential nAChR subunits). The impact of alcohol on these nAChRs is thought to occur through stabilization of the receptor open state and inhibition activity-induced receptor desensitization (Aistrup et al. 1999; Marszalec et al. 1999). Additionally, function of α3β2* and α3β4* nAChRs are not affected by alcohol. The α4β2* nAChR is the most abundant within the VTA, is highly sensitive to ACh and nicotine, and is expressed on DA and GABA neurons (Hendrickson et al. 2013). The α7-homomeric nAChR has been found to

desensitize at a slower rate under physiological conditions due to a lower affinity for ACh and is predominately localized to glutamatergic terminals from the PFC that synapse on DA neurons (Figure 3). Interestingly, alcohol has repeatedly been shown to inhibit α7 nAChR activity (Yu et al. 1996; Aistrup et al. 1999; Cardoso et al. 1999; Spanagel 2009). This effect, however, could be due to an inherently fast desensitization rate, suggesting alcohol induced inhibition results in enhanced desensitization (Hendrickson et al. 2013). Previous work has demonstrated both of these nAChR subtypes contribute to increased DA release by alcohol and nicotine (Morel et al. 2018; Hauser et al. 2019).

Another member of the Cys-loop superfamily, the serotonin 3 receptor (5-hydroxytryptamine, 5-HT, 5-HT_3), has been implicated as a convergent site of action for alcohol and nicotine (Lovinger and White 1991; Breitinger et al. 2001; Hauser et al. 2014b). Alcohol exposure has been shown to enhance 5-HT_3 receptor activation and DA release within the NAc, while nicotine antagonizes the action of 5-HT at 5-HT_3 receptors (Campbell et al. 1996; Gurley et al. 1998; Breitinger et al. 2001; Rodd et al. 2010). The opposing actions of simultaneous alcohol and nicotine on 5-HT_3 receptors have yet to be studied.

Importantly, it has also been demonstrated that voluntary responding for alcohol failed to change after almost complete denervation of the NAc and the mesolimbic DA system (Rassnick et al. 1993). This suggests there are DA-independent neurochemical systems that contribute critically to mediating the reinforcing actions of alcohol and nicotine. Of particular importance to Chapter Four, are studies that have been demonstrated alterations in glutamate signaling.

The involvement of glutamate has been implicated in several drugs of abuse including alcohol and nicotine (Gipson et al. 2013; Berglind et al. 2009; Campbell et al. 2009; Wang et al. 2008). Specifically, glutamate signaling in the medial PFC (mPFC) has been shown to play a significant role in both alcohol and nicotine addiction. Grüsser and colleagues (2004) demonstrated that individuals with AUD exhibit greater neuronal activity in the mPFC than control participants when presented alcohol related cues. This heightened activity may support increased attention to drug and alcohol cues that can trigger drug seeking. There is also evidence that there is a reduction of glial cells in the prefrontal cortex of both humans with AUD and the alcohol-preferring (P) rat compared to controls (Miguel-Hidalgo 2009). This is notable due to extracellular glutamate levels being strongly governed by glial glutamate transporters. The reduction of mPFC glial cells suggests an enhanced susceptibility to dysregulation of glutamate homeostasis within a region implicated in drug-seeking and relapse (Grüsser et al. 2004). Additionally, glutamate activity in the mPFC has been shown to increase following chronic nicotine self-administration as well as upregulate both NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Wang et al. 2007; Wang et al. 2008). Several studies have demonstrated that experience with alcohol increases AMPA mediated synaptic transmission and can induce a long-term potentiation (LTP) of glutamatergic synaptic afferents onto VTA DA neurons (Stuber et al. 2008; Oliva and Wanat 2016). This has also been associated with increased NMDA receptor expression as well as elevated phosphorylation of GluN2B receptor subtype (Pascual et al.

2009). Additional research found increased levels of NR1 and GluR1 subunits, indicating higher levels of both NMDA and AMPA receptors, following long-term exposure to alcohol (Ortiz et al. 1995).

Collectively, DA release by alcohol and nicotine exposure may be partly driven by increased responsivity to reward stimuli via excitatory afferents to the VTA. The research described above also suggests alcohol and nicotine interact at specific sites, in addition to independent actions, to increase DA release within the NAc through both nAChRs and 5-HT_3 receptors.

Pharmacotherapeutics

The United States Food and Drug Administration (FDA) currently has seven approved first-line pharmacotherapies for smoking cessation. These include five types of nicotine replacement therapies (NRT), sustained release bupropion, and varenicline (Jorenby et al. 2006; Hurt et al. 2018). Varenicline binds with high affinity to α4β2* nAChRs as a partial agonist. This binding is hypothesized to reduce both the rewarding effects of nicotine as well as withdrawal symptoms that occur upon smoking cessation (Rollema et al. 2007). However, the selectivity of varenicline at higher concentrations is reduced with known activity at α3β2*, α6β2*, and α3β4* nAChRs. Clinical trials have pointed to varenicline as the most effective monotherapy at increasing likelihood of smoking abstinence over both bupropion and nicotine replacement products (Gonzales et al. 2006; Jorenby et al. 2006; Anthenelli et al. 2016). These results indicated varenicline abstinence rates were approximately 23% after one year compared to

bupropion at 15% and placebo at 10%. However, the vast majority of these varenicline treated individuals will ultimately relapse (Volkow and Skolnick 2012). Despite successful clinical trials, it is clear that varenicline has limited efficacy in the long-term.

There are even fewer FDA approved first-line pharmacological treatments for AUD in the United States. These medications include acamprosate, disulfiram, and naltrexone (Soyka and Müller 2017). It is worth noting, no single drug is considered widely successful in all individuals with AUD. This has been partly attributed to the widespread pharmacological actions of alcohol that change as alcohol use progresses from social or acute exposure to chronic abuse (Vengeliene et al. 2008; Soyka and Müller 2017). Naltrexone initially demonstrated moderate efficacy compared to the other available treatments for AUD. Naltrexone is a non-specific opioid antagonist that was originally approved for opioid dependence. Subsequent research indicated naltrexone treatment was associated with reduced alcohol craving as well as lower relapse rates. Unfortunately, as with other pharmacotherapeutics, additional investigations found that naltrexone is actually more effective in reducing the number of binge drinking sessions rather than maintaining abstinence (Garbutt et al. 2005).

Taken together, it becomes clear the pharmacotherapeutic treatment options for AUD or nicotine dependence are marginal at best. Naltrexone and varenicline were developed through preclinical and clinical studies that contributed significantly to the basic understanding of addiction neurobiology. Nevertheless, extensive investigations that focus exclusively on alcohol or

nicotine intake have likely contributed to the development of these pharmacotherapeutics with limited effectiveness. Major gaps in knowledge still exist in the realm of polysubstance abuse, which is undeniably the case with alcohol and nicotine given the known comorbidity rates.

Therefore, preclinical studies need to treat AUD and nicotine dependence as a polysubstance use disorder and provide simultaneous drug access in order to identify neuroadaptations unique to the comorbid state. A co-exposure model will increase the likelihood of detecting vital targets and support the development of more efficacious therapeutics. Chapter Two aims to test the efficacy of naltrexone or varenicline on concurrent alcohol and nicotine intake by rats selectively bred for high alcohol drinking. Chapter Three examined the effects of peri-adolescent alcohol drinking on the ability of nicotine infused into the pVTA to stimulate DA release within the NAc shell during adulthood. Finally, Chapter Four aimed to determine the effects of alcohol and nicotine infusions within the pVTA on NAc shell neurochemistry, alcohol reward within the NAc shell, and the role of brain-derived neurotrophic factor (BDNF) on alcohol reward within that region. Collectively, these series of studies hope to shed light on some neurobiological factors that may partially contribute to alcohol and nicotine comorbidity and the future development of novel medications to treat addiction.

Chapter Two: Therapeutic challenges for concurrent ethanol and nicotine consumption: naltrexone and varenicline fail to alter simultaneous ethanol and nicotine intake by female alcohol-preferring (P) rats

It is well established that simultaneous alcohol and nicotine consumption occurs in the majority of individuals with alcohol use disorder (AUD). Despite evidence that ethanol (EtOH) and nicotine (NIC) co-use produces unique neuroadaptations, preclinical research regularly focuses on the effects of these pharmacotherapeutics on a single reinforcer. Therefore, the goal of the present chapter was to investigate the effectiveness of the pharmacological 'gold standards' for treating AUD and nicotine dependence under conditions of concurrent drug intake. Varenicline (Var) is used to assist in the cessation of nicotine use, while naltrexone (Nal) is the standard treatment for AUD. The current experiments examined the effects of Var and Nal on EtOH, NIC, or EtOH+NIC intake. Animals were randomly assigned to one of four drinking conditions of 24-hour access to a three-bottle choice paradigm, one of which always contained water. Drinking conditions were water only, 0.07 and 0.14 mg/mL NIC (NIC only), 15% and 30% EtOH (EtOH only), or 15% and 30% EtOH with 0.14 mg/mL NIC (EtOH+NIC). The effects of Var (0, 1, or 2 mg/kg) or Nal (0, 1, or 10 mg/kg) injections on maintenance and relapse consumption were determined during four consecutive days. Var reduced maintenance and relapse NIC intake but had no effect on EtOH or EtOH+NIC drinking. Conversely, Nal reduced EtOH maintenance and relapse drinking, but had no effect on NIC or

EtOH+NIC drinking. The results indicate the standard pharmacological treatments for nicotine dependence and AUD were effective at reducing consumption of the targeted reinforcer but neither reduced EtOH+NIC co-use/abuse. These findings suggest that co-abuse may promote unique neuroadaptations that require models of polysubstance abuse to develop pharmacotherapeutics to treat AUD and nicotine dependence.

Introduction

Alcohol use disorder (AUD) and nicotine dependence cause, contribute to, and exacerbate many serious health problems and are among the top contributors to preventable deaths occurring worldwide (Grucza and Bierut 2006). Past research has shown that more than 80% of those afflicted with AUD also exhibit comorbid use of nicotine in comparison to 34% of non-alcoholics (John et al. 2003a, b). Likewise, individuals suffering from nicotine dependence are 10 times more likely to be diagnosed with AUD in their lifetime (DiFranza and Guerrera 1990).

The amount of nicotine use is positively correlated with the rate of AUD, and smoking enhances alcohol consumption in AUD individuals compared with non-smoking AUD individuals (Daeppen et al. 2000; Gulliver et al. 1995). Additionally, the severity of AUD has also been positively correlated to a number of other elements including urge to smoke, years smoked, number of attempts to quit smoking, and age an individual began smoking (John et al. 2003a, b). The intensity of nicotine dependence has been associated with exacerbated bouts of

relapse in alcoholics (DiFranza and Guerrera 1990). AUD individuals who concurrently stop smoking have a better chance of remaining abstinent than those who continue to smoke (Daeppen et al. 2000; Gulliver et al. 1995). Preclinical research has consistently reported that nicotine can potentiate EtOH-seeking, and drug-seeking is enhanced in rats simultaneously co-administering EtOH and nicotine (Lê et al. 2014; Hauser et al. 2012a, b). These preclinical findings are paralleled in the human literature that indicate individuals concurrently diagnosed with AUD and nicotine dependence have significantly worse clinical outcomes than those who are only diagnosed with AUD or nicotine dependence (Lajtha and Sershen 2010; Grant et al. 2004).

There are no reports of humans injecting nicotine. In contrast, the use of oral spitless products (e.g., snus, a moist powder tobacco packet in which the byproduct is swallowed) has increased in the USA in the last 20 years. In 2011, Americans spent over 340 million dollars on snus products (Delnevo et al. 2012). In Sweden and Norway, the average annual intake of snus approaches the annual combined budgets of NIAAA and NIDA at 1.3 billion dollars (Digard et al. 2009).

The mixing of alcohol and nicotine for oral consumption is evident throughout most of the USA and the rest of the world. In 2003, the creation of the Nicotini was considered one of the top 10 Ideas of the Year by the New York Times Magazine (NYTM 2003). Nicotine-infused alcohols appear to have established a foothold in high-end bars throughout the US (Martell 2014). In Europe, there are established brands of nicotine-infused alcohols as well as

nicotine-infused bitters and salt for the construction of cocktails. According to European standards, there are no known biological consequences of infusing nicotine into alcoholic solutions. Therefore, recently developed animal models of concurrent alcohol and nicotine oral consumption are ecologically valid, and that validity is increasing in relevance (Hauser et al. 2012b; Kasten et al. 2016).

The United States Food and Drug Administration (FDA) approved varenicline (Var) for smoking cessation in 2006 (Jorenby et al. 2006; Hurt et al. 2018). Var is the most effective monotherapy at increasing the likelihood of smoking abstinence (Gonzales et al. 2006; Jorenby et al. 2006). Pharmacological analyses have indicated multiple sites of action by Var that may mediate the effects on smoking cessation. Var exhibits the strongest affinity for the α4β2 nicotinic acetylcholine receptor (nAChR), where it acts as a partial agonist. Var interacting with this receptor has been postulated to be the mechanism for clinical efficacy. Var is also a less potent full agonist at α7 and α3β4 nAChRs (Rollema et al. 2007; Grady et al. 2010) and a potent full agonist of 5-HT_3 receptors (Lummis et al. 2011). It is important to note that nicotine has a higher affinity for the 5-HT_3 receptors than all cholinergic receptors (Gurley and Lanthorn 1998), suggesting maximally efficacious pharmacotherapies for nicotine dependence are likely to involve an interaction with this system as well.

Preliminary clinical data indicated that Var treatment of nicotine-dependent individuals was associated with a decrease in alcohol consumption (Erwin and Slaton 2014). Research conducted with animals and humans have suggested a role for nAChRs in alcohol use disorder (Blomqvist et al. 1993; Chi and de Wit

2003). Activation of the mesocorticolimbic dopamine system by alcohol and the resulting rewarding effects have been consistently shown to involve central nAChR stimulation (Blomqvist et al. 1993; Soderpalm et al. 2000). A more complete examination of decreased alcohol consumption during Var treatment indicated that it was able to decrease alcohol craving (de Bejczy et al. 2015). Conversely, it is important to note that Var failed to decrease overall alcohol drinking compared with placebo-treated individuals (de Bejczy et al. 2015).

Naltrexone (Nal), a nonselective opioid receptor antagonist, has been shown to reduce the reinforcing effects of alcohol as well as cravings associated with alcohol use (Volpicelli et al. 1992; O'Malley et al. 1996; Soyka and Muller 2017). However, these studies also demonstrate the efficacy of Nal is limited. Overall, the efficacy of Nal for the treatment of AUD is equivocal. Nal is effective at treating AUD in a subset of patients (Gueorguieva et al. 2010) while in other patient populations, Nal has very limited effects (c.f., Petrakis et al. 2012). Nal efficacy for the treatment of AUD is further complicated by data reporting only 1 in 10 AUD patients are actually prescribed relevant pharmacotherapy (Thompson et al. 2017). In addition, Nal suffers from low patient adherence (Thompson et al. 2017).

Although nicotine (NIC) has little or no action on opioid receptors, the utility of using Nal as a treatment for nicotine dependence has been examined. Clinical trials investigating Nal and smoking cessation have demonstrated minor short-term increases in abstinence rates that diminish over time (Covey et al.

1999; King et al. 2012) or no significant difference from placebo (Wong et al. 1999).

Together, AUD and nicotine dependence are the most common comorbid addiction diagnosis (Grant et al. 2004). It has been suggested that the prevalence of AUD and nicotine dependence comorbidity may stem from interconnected mechanisms underlying these particular disorders (Grucza and Bierut 2006). Preclinical studies indicate that the co-abuse of EtOH+NIC can produce unique CNS changes not observed with abuse of either drug separately (Deehan et al. 2015), and combinations of EtOH and NIC can have synergistic CNS rewarding effects (Truitt et al. 2015).

A better understanding of how efficacious therapeutic drugs are during the distinct condition of concurrent consumption is necessary in order to develop better treatments and preventive measures. Research commonly focuses on the effects of pharmacotherapeutics on a single reinforcer despite mounting preclinical evidence that chronic consumption of EtOH and NIC produces unique neuroadaptations that lessen the efficacy of pharmacotherapies designed to treat AUD or nicotine dependence. Recent data examining the effects of Var on concurrent intravenous (i.v.) NIC and oral EtOH has indicated no reduction in self-administration (Maggio et al. 2018a). Therefore, the current experiments examined the effects of Var or Nal on EtOH, NIC, or EtOH+NIC consumption to test the hypothesis that these compounds would be less effective in reducing NIC and EtOH intake, respectively, when co-abused compared with when abused individually.

Materials and methods

Subjects

Adult female alcohol-preferring (P) rats from the 74th generation weighing 250–300 g at the start of the experiment were used. Female animals were utilized in the present study due to the long-term nature of the experiments and ability to maintain stable body weights over time. Epidemiological studies also indicate the number of females diagnosed with AUD is increasing in addition to experiencing heightened susceptibility to ethanol injury compared with males (Ceylan-Isik et al. 2010). Additionally, despite fewer females regularly using NIC products, studies show lower quit rates than males (Smith et al. 2017; Wetter et al. 1999). These differences are among the growing evidence of sex dimorphism in response to EtOH and NIC as well as pharmacotherapies and highlight the importance of examining females in models of co-abuse. Previous research indicated that EtOH intake of female P rats was not significantly altered by the estrus cycle (McKinzie et al. 1998). Rats were maintained on a 12-hour reverse light-dark cycle with lights off at 0900. Food and water were available *ad libitum* throughout the experiment. The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council,

Institute for Laboratory Animal Research, Division on Earth and Life Sciences 2011). The total number of rats (n = 198) used in the current experiments were as follows: Var on water consumption (n = 18, 6/group), Var on EtOH consumption (n = 26, 8–9/group), Var on NIC consumption (n = 23, 7–8/group), Var on EtOH+NIC (n = 27, 9/group), Nal on water consumption (n = 21, 7/group), Nal on EtOH consumption (n = 25, 7–8/group), Nal on NIC consumption (n = 29, 9–10/group), Nal on EtOH+NIC consumption (n = 29, 9–10/group).

Chemical agents and vehicle

Ethyl alcohol (190 proof; McCormick Distilling Co., Weston, MO, USA) was diluted to 15% and 30% with distilled water for oral EtOH consumption. Nicotine HCl was purchased from Sigma (St. Louis, MO, USA). NIC concentrations of 0.07 or 0.14 mg/mL were calculated based on the salt and were added to a solution of 0.0125% saccharin. This was done only for the NIC-alone condition. The EtOH+NIC solutions consisted of 15 or 30% EtOH and 0.14 mg/mL NIC without saccharin added to the solution. Rats will readily consume NIC solutions at the concentrations employed (about 4.5–5 mg/kg/day). We observe equivalent levels of NIC self-administration between rats self-administering EtOH+NIC solution and NIC, if saccharin (0.0125%) is added to the NIC solutions (Hauser et al. 2012b). This is reflected in equivalent blood NIC and cotinine levels in these two groups (Hauser et al. 2012b). Moreover, previous research from our group has found no significant differences in neurochemistry or locomotor activity between animals allowed to consume saccharin and water

controls (Deehan et al. 2015; Melendez et al. 2002, 2004) and was therefore not included in the present study.

Varenicline tartrate (Sigma) was dissolved in 3% DMSO. Treatment with 2.5–3 mg/kg Var results in locomotor deficits, decreased food intake, and other nonspecific actions (Ortiz et al. 2012; O'Connor et al. 2010; Rollema et al. 2007). Concentrations of Var used in the current experiments were 0, 1, or 2 mg/kg (i.p.). Naltrexone HCl was obtained from Sigma and dissolved in saline. Nal was given in doses of 0, 1, or 10 mg/kg (s.c.). Doses for Nal were determined by previous studies carried out with adolescent and adult P rats demonstrating as much as 20 mg/kg was necessary to decrease EtOH intake while having no impact on water or food consumption (Dhaher et al. 2012; Sable et al. 2006).

Effects of varenicline and naltrexone on EtOH, NIC, and EtOH+NIC maintenance and relapse consumption

Rats were randomly assigned to one of four three-bottle choice drinking conditions illustrated in Figure 4, top panel. These consisted of (1) water only, (2) EtOH only with concurrent access to 15 and 30% EtOH and water, (3) NIC only with 0.07 and 0.14 mg/mL in 0.0125% saccharin solutions and water, or (4) EtOH+NIC with 15 and 30% EtOH, each containing 0.14 mg/mL NIC, and water. The EtOH-only, NIC only, and EtOH+NIC groups were given access to the three bottles throughout the experiments of two reinforcing solutions and one water. Despite the development of effective voluntary oral NIC self-administration models (Hauser et al. 2012b; Deehan et al. 2015), i.v. NIC is commonly

employed in an attempt to parallel rapid increases in blood NIC levels produced by smoking, which is not observed during the use of chewing tobacco (Benowitz 1988). However, oral tobacco products, such as snus, produce the same rapid increase in blood NIC levels as observed in smokers. There are no significant differences between smokers and first-time snus users in blood NIC levels during the initial 20-min period (Digard et al. 2013). Additionally, there are a number of limitations inherent in i.v. administration that include the need for food restriction, surgery, catheter patency, and overcoming aversion during the initial test sessions. The duration of the present study therefore required the use of an oral consumption model to examine Var or Nal following chronic drug intake.

The experimental timeline of the present study is visually represented in the bottom panel of Figure 4. Rats were given 24-hour free-choice access to their assigned solutions for 8 weeks prior to drug testing. Rats were then assigned to two overall groups of Var treated or Nal treated. Var or Nal was administered daily for four consecutive days. Four days of treatment was chosen to allow sufficient time for any additive drug effects, indication of altered efficacy, or behavioral changes to be readily apparent. After the initial Var or Nal treatment period, all rats were allowed 14–20 days of free choice access to the assigned solutions. All rats were then deprived for 2 weeks of EtOH, NIC, or EtOH+NIC with water constantly available to all rats. Two weeks of deprivation results in an increased amount of EtOH consumption during the initial period of re-exposure (Rodd et al. 2003, 2009; Rodd-Henricks et al. 2001, 2002a, b; Spanagel et al. 1996; Spanagel and Zieglgansberger 1997; Toalston et al. 2008). Similarly, a 2-

week deprivation from NIC self-administration results in an increased amount of NIC consumed during re-exposure (Hauser et al. 2012b). The alcohol deprivation effect (ADE) and nicotine deprivation effect (NDE) are valid animal models of drug relapse (Rodd et al. 2003, 2009). Prior to re-exposure to the previously assigned solution group, rats were again treated with Var or Nal. The doses of Var and Nal were counterbalanced from the initial treatment with respect to past treatment. Specifically, the same subjects used in the Var experiment during the maintenance test were also used during the relapse test for Var. Similarly, for the Nal experiments, the same rats were used for the maintenance and relapse tests. For example, rats treated with 1 mg/kg Nal during the maintenance period were segmented into three groups during the relapse period and received 0, 1, or 10 mg/kg Nal. Var or Nal treatment occurred during the initial 4 days of re-exposure. Additionally, baseline means for maintenance intake were calculated from the last 3 days before Var or Nal treatment and the final three drinking days prior to deprivation for the relapse data.

Solution preference was determined for each subject consuming EtOH and/or NIC daily. The preference of solution consumed has previously been used to examine the reward valence of each solution (Rodd et al. 2003, 2009). With multiple concentrations, statistical analysis of solution preference is complex. It is common to use standard parametric statistics despite preference violating the “independence of measure” assumption of ANOVA/multiple regression analysis and invalidates these statistical tests. Therefore, non-parametric analysis is appropriate. The Kuiper analysis is sensitive to cyclic variations and can be

modified to have collection error estimate included in the analysis. However, the general unfamiliarity with this test and the length of the presentation of the data have resulted in preference not being reported for the current study.

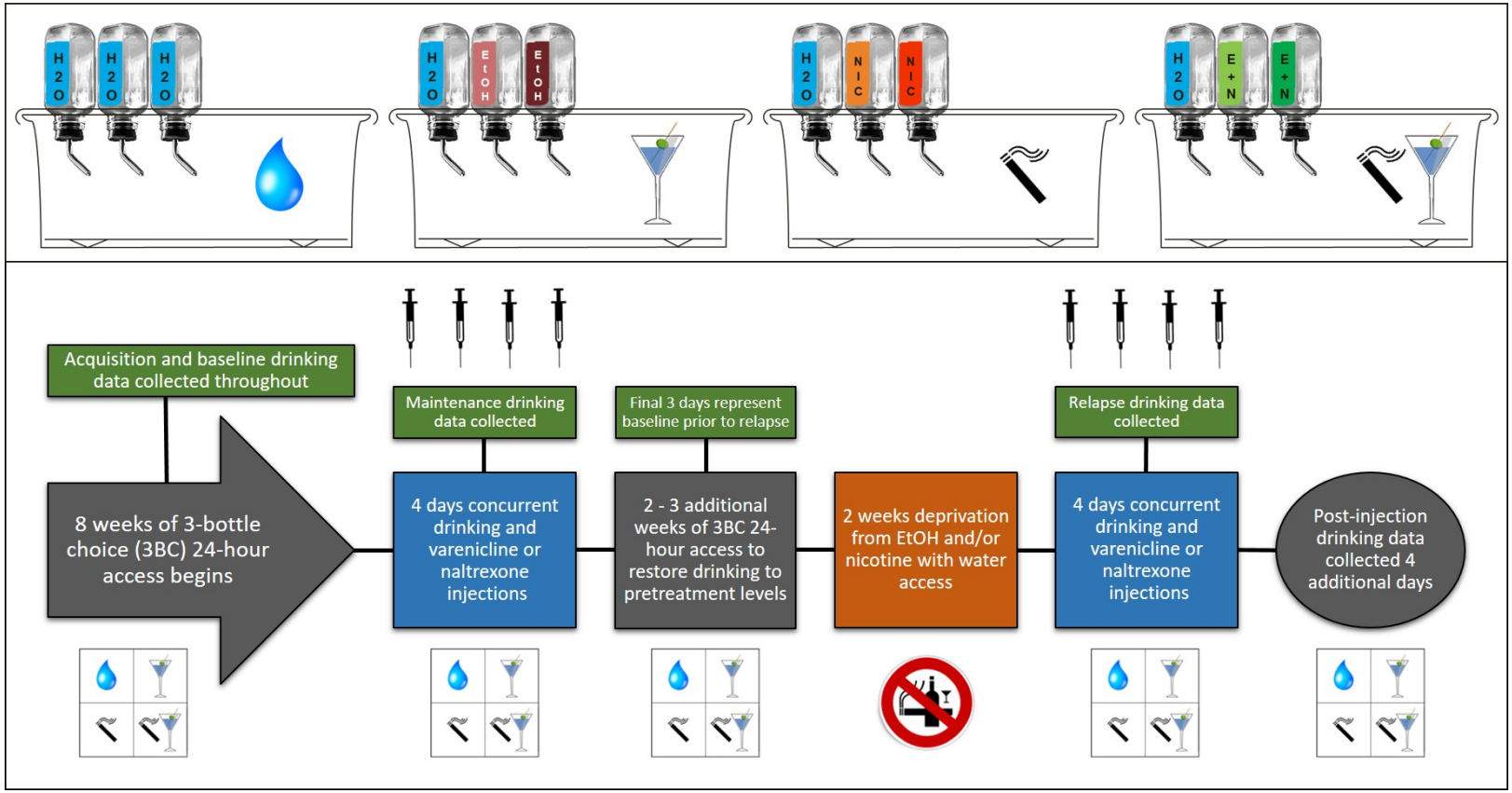

Figure 4. Illustration of the 3-bottle choice (3BC) paradigm, drinking conditions, and experimental timeline. Top panel: Depicts the 3BC paradigm and 4 drinking conditions consisting of (1) water only, (2) EtOH only with concurrent access to 15 and 30% EtOH and water, (3) NIC only with 0.07 and 0.14 mg/mL in 0.0125% saccharin solutions and water, or (4) EtOH+NIC with 15 and 30% EtOH, each containing 0.14 mg/mL NIC, and water. Bottom panel: Experimental timeline for the present study to determine the effects of Var (0, 1, or 2 mg/kg) or Nal (0, 1, or 10 mg/kg) injections on maintenance and relapse consumption determined during 4 consecutive treatment days.

Results

Effects of varenicline and naltrexone on water consumption

A repeated measure ANOVA was performed on the average daily water intake (g/kg) of the “water-only” group during “maintenance” consumption in animals treated with Var (Figure 5, top panel) or Nal (Figure 5, bottom panel). The analysis revealed that there was a significant Day × Dose interaction term ($F_{16,168} = 5.488$; $p < 0.001$). The interaction term was decomposed by holding “Day” constant and performing individual ANOVAs for each time point. The ANOVAs indicated that there were significant “Dose” differences during the four injection days and the first day post-injection. Post hoc comparisons (Tukey’s b) indicated that consumption of rats in the “water-only” group administered 2 mg/kg Var was reduced compared with saline and 1 mg/kg Var during the four injection days. Conversely, water consumption was enhanced in the 2 mg/kg Var group during the first post-injection day (Figure 5, top panel). In contrast, Nal treatment did not alter water consumption (Figure 6, bottom panel; Day, Day × Dose, Dose; p values > 0.05).

Water animals were never deprived but were treated identically to the other groups. Therefore, there was a water condition to parallel the other “relapse” groups that received a second round of four injection days and received Var or Nal to identify potential nonspecific drug treatment effects (data not shown). In the Var rats, there was a similar Day × Dose interaction term ($F_{16,168} = 3.892$; $p = 0.005$), and post hoc comparisons indicated that 2 mg/kg Var reduced water consumption during all four injection days without a post-injection rebound

observed. Again, there was no effect of Nal on water consumption during the "relapse" period (all *p* values > 0.05).

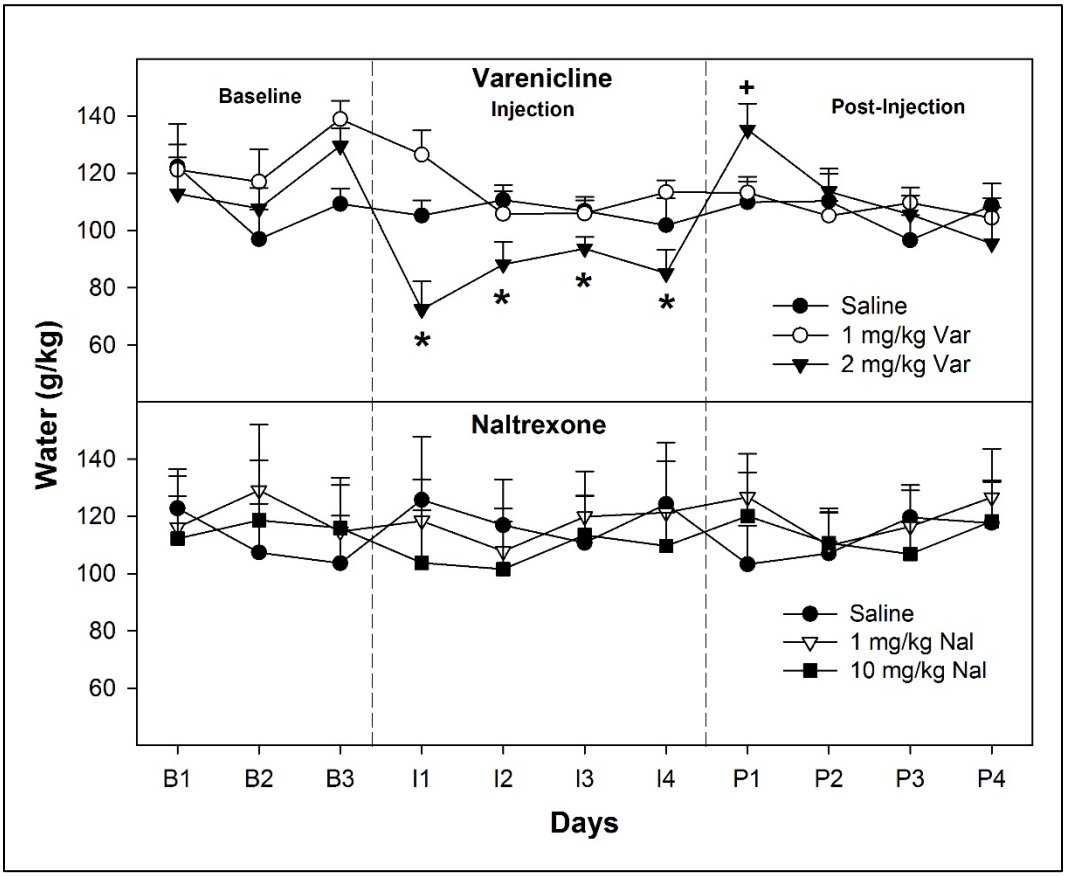

Figure 5. Effects of varenicline or naltrexone on water maintenance. Mean (+ SEM) consumption of water in P rats treated with varenicline (top panel) or naltrexone (bottom panel) during maintenance consumption. Asterisk (*) indicates treatment with 2 mg/kg varenicline reduced water consumption compared with saline treated and 1 mg/kg Val–treated rats. Plus symbol (+) indicates rats previously treated with 2 mg/kg varenicline consumed more water than rats previously treated with saline or 1 mg/kg.

Effects of varenicline and naltrexone on EtOH consumption

During maintenance testing, Var had no effect on EtOH consumption (Figure 6, top panel). Statistically, there was no effect of Day, Dose, or Day × Dose interaction (*p* values > 0.05). In contrast, Nal reduced EtOH consumption during maintenance testing (Figure 6, bottom panel; Day × Dose interaction,

$F_{16,288} = 8.586$, $p < 0.001$). Performing ANOVAs on individual days revealed significant effects of Dose on all four injection days ($F_{2,22}$ values > 11.404, p values < 0.001). Post hoc comparisons (Tukey's b) indicated that during the first and fourth injection days, rats treated with 1 or 10 mg/kg Nal consumed less EtOH than saline-treated rats, and the 1 mg/kg Nal group consumed more EtOH than the 10 mg/kg Nal group. EtOH intake recovered to baseline on the first post-injection day.

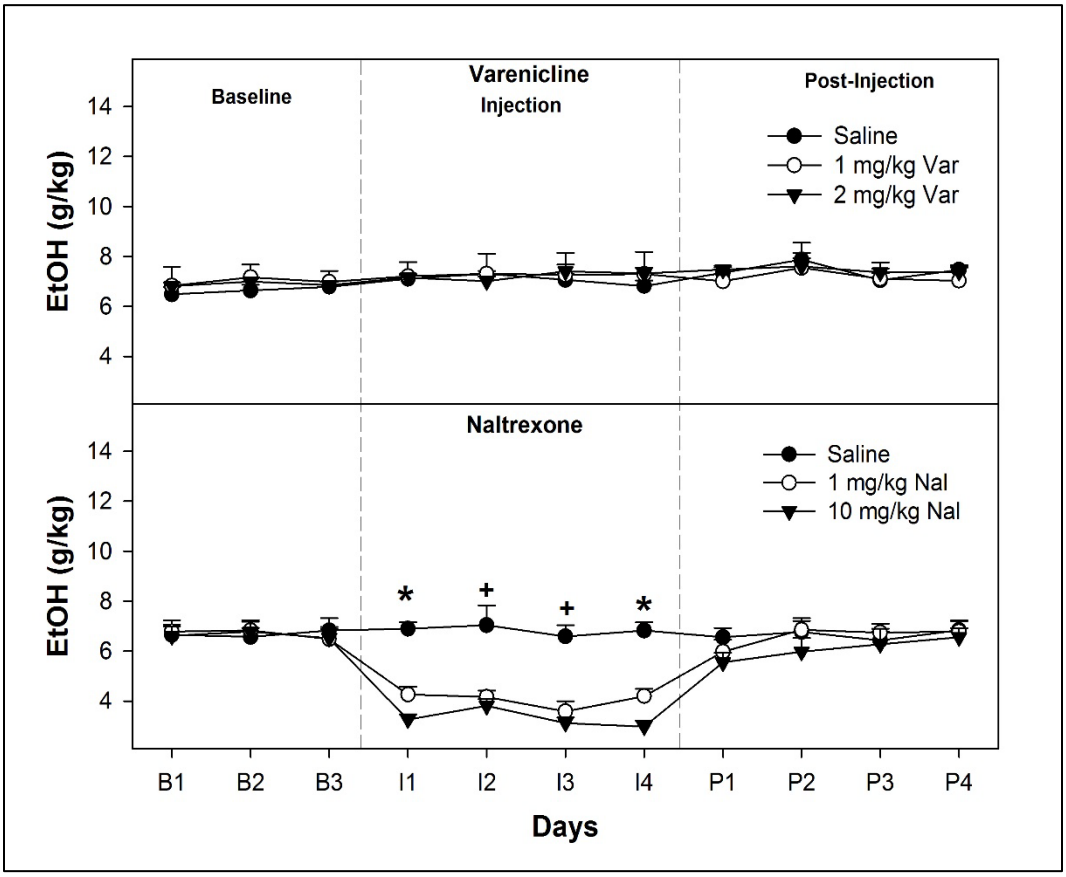

Figure 6. Effects of varenicline or naltrexone on EtOH maintenance. Mean (+ SEM) consumption of EtOH in P rats treated with varenicline (top panel) or naltrexone (bottom panel) during maintenance consumption. Asterisk (*) indicates treatment with 1 or 10 mg/kg naltrexone reduced EtOH consumption compared with saline-treated rats, and 1 and 10 mg/kg groups differ from each other. Plus symbol (+) indicates treatment with 1 or 10 mg/kg naltrexone reduced EtOH consumption compared with saline treated rats.

During EtOH relapse test conditions, Var failed to alter EtOH consumption (Figure 7, top panel). Statistically, there was a significant effect of Day ($F_{8,184}$ = 191.65, $p < 0.001$), but no effect of Dose or Day × Dose interaction (p values > 0.05). These findings forced the analysis to examine the one significant factor (Day), and within-subject comparisons (two-tailed t tests) indicated that EtOH consumption was elevated during the first and second re-exposure day ($p <$ 0.001). In contrast, Nal inhibited the expression of the alcohol deprivation effect (relapse drinking; Day × Dose interaction, $F_{16,176} = 7.756$, $p < 0.001$). Decomposing the significant interaction term by holding Day constant (ANOVAs performed on each day) revealed significant effects of Dose ($F_{2,22}$ values > 9.493, p values < 0.01) on the first three re-exposure days. On the first and second re-exposure days, post hoc comparisons (Tukey's b) revealed that all groups were different from each other. During the third re-exposure day, saline-treated and 1 mg/kg Nal–treated rats were significantly different from the 10 mg/kg Nal group. The significant interaction term was also reduced by holding Dose constant, and within-subject (Day) ANOVAs were performed for each Dose group. The analyses revealed that there was a significant "Day" effect in each Dose group (p values < 0.005). In saline-treated rats, within-subject contrasts, two-tailed t tests, revealed that EtOH consumption during the first and second re-exposure days was elevated compared with each baseline intake days (p values < 0.005). In rats treated with 1 mg/kg Nal, there was a significant increase in EtOH consumption only during the first re-exposure day compared with each baseline day ($p < 0.01$). Treatment with 10 mg/kg Nal not only blocked the expression of

the alcohol deprivation effect but also reduced EtOH consumption compared with baseline for the first and second re-exposure days (*p* values < 0.05).

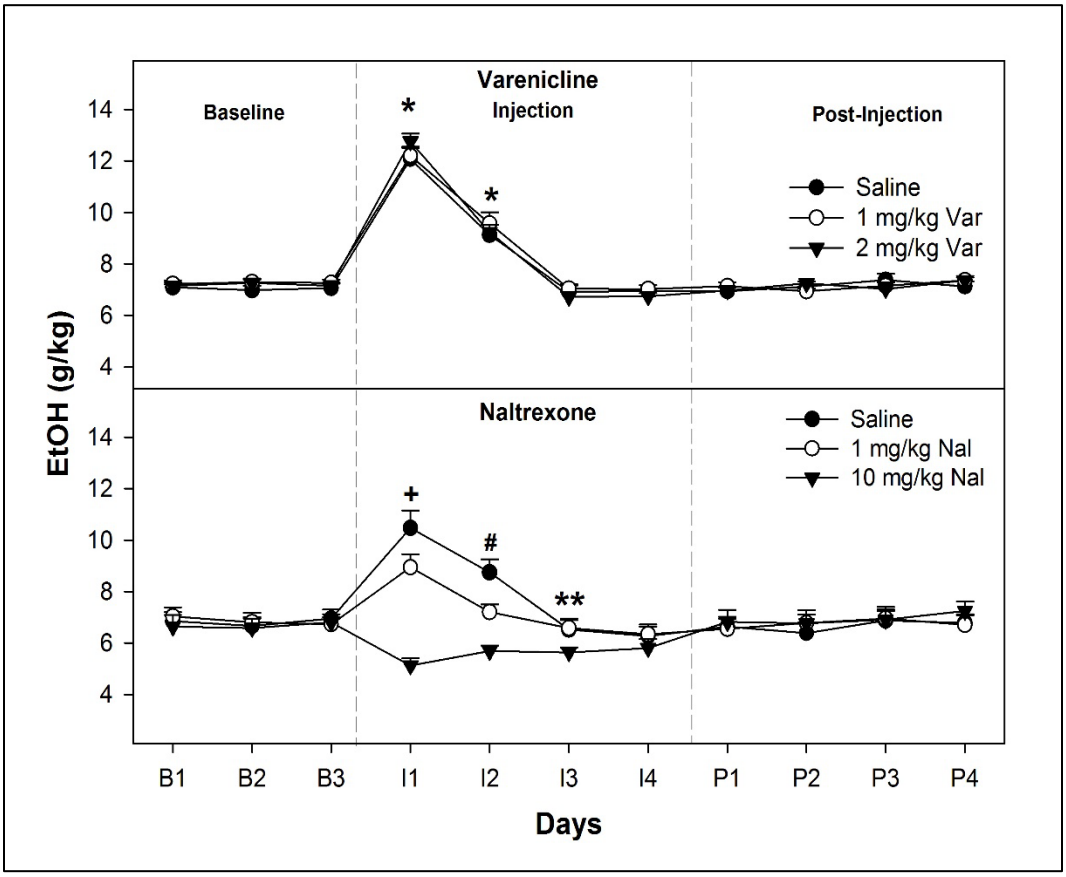

Figure 7. Effects of varenicline or naltrexone on EtOH relapse. Mean (+ SEM) consumption of EtOH in P rats treated with varenicline (top panel) or naltrexone (bottom panel) during relapse consumption following a 2-week period of forced abstinence. Baseline means are the final three drinking days prior to the deprivation. Asterisk (*) indicates all varenicline-treated rats consumed more EtOH than baseline intake levels. Plus symbol (+) indicates saline and 1 mg/kg naltrexone–treated rats consumed more EtOH than baseline intake levels, all treatment groups differ from each other, and rats treated with 10 mg/kg naltrexone consumed less EtOH than baseline intake levels. Number sign (#) indicates saline-treated rats consumed more EtOH than baseline intake levels, and all groups are different from each other. Double asterisk (**) indicate EtOH consumption in the 10 mg/kg naltrexone–treated rats is less than that observed in the saline or 1 mg/kg naltrexone–treated rats.

Effects of varenicline and naltrexone on NIC consumption

Var reduced oral NIC consumption during maintenance test conditions (Figure 8, top panel; Day × Dose interaction, $F_{16,160} = 7.264$, $p < 0.001$). Decomposing the interaction term by examining the effect of Dose on each individual day revealed significant “Dose” differences on the four injection days, and during the second and fourth post-injection days ($F_{2,20}$ values > 3.774, p values < 0.05). During the four injection days, treatment with 2 mg/kg Var significantly reduced NIC consumption compared with the saline and 1 mg/kg Var groups. Following the termination of Var treatment, NIC consumption was significantly increased during days 2 and 4 of post-injection in the 2 mg/kg Var group compared with saline-treated rats. Nal treatment had no effect on oral NIC consumption (Figure 8, bottom panel). Statistically, there was no effect of Day, Dose, or Day × Dose interaction (p values > 0.05).

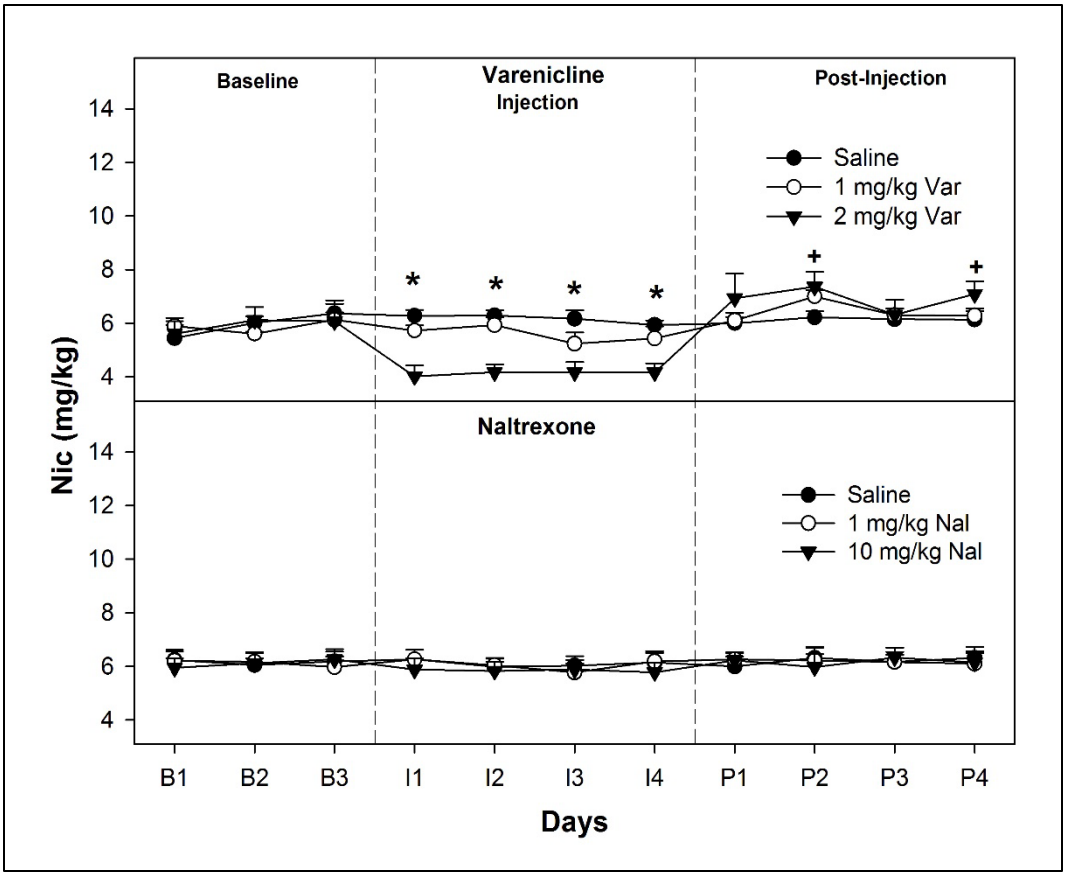

Figure 8. Effects of varenicline or naltrexone on NIC maintenance. Mean (+ SEM) consumption of NIC in P rats treated with varenicline (top panel) or naltrexone (bottom panel) during maintenance consumption. Asterisk (*) indicates 2 mg/kg varenicline reduced NIC consumption compared with saline and 1 mg/kg varenicline groups. Plus symbol (+) indicates rats previously treated with 2 mg/kg varenicline consumed more NIC that saline controls.

A period of forced abstinence resulted in increased oral NIC consumption during the initial period of re-exposure (Figure 9). Var did alter relapse NIC intake (Figure 9, top panel, Day × Dose interaction, $F = 7.064$, $p < 0.001$). Decomposing the interaction term by holding the factor of Day constant revealed significant effect of Dose during the first three re-exposure days (injection days 1–3; $F_{2,20}$ values > 3.774, *p* values < 0.05). Post hoc comparisons (Tukey's b) indicated that, during the first 3 days of NIC re-exposure, rats treated with 2 mg/kg Var

consumed less NIC than the saline-treated and 1 mg/kg Var–treated rats. Alternatively, the significant interaction term was reduced by holding Dose constant and performing repeated measure ANOVAs across Day for each individual treatment group. Rats treated with saline and 1 mg/kg Var consumed significantly more NIC during the first and second re-exposure days compared with baseline (one-way ANOVA, p values < 0.001; two-tailed t tests, p values < 0.032). In contrast, rats treated with 2 mg/kg Var exhibited no significant alteration in NIC consumption across the test period ($p = 0.378$). In rats treated with Nal, there was no effect of Dose or a Day × Dose interaction (p values > 0.05), but there was a significant effect of Day ($F_{8,208} = 247.68$, $p < 0.0001$). Examining the amount of NIC consumed across days in all treated groups (Figure 9) revealed that during the first and second re-exposure periods, the amount of NIC consumed was significantly higher than the three baseline days (two-tailed t tests, p values < 0.001).

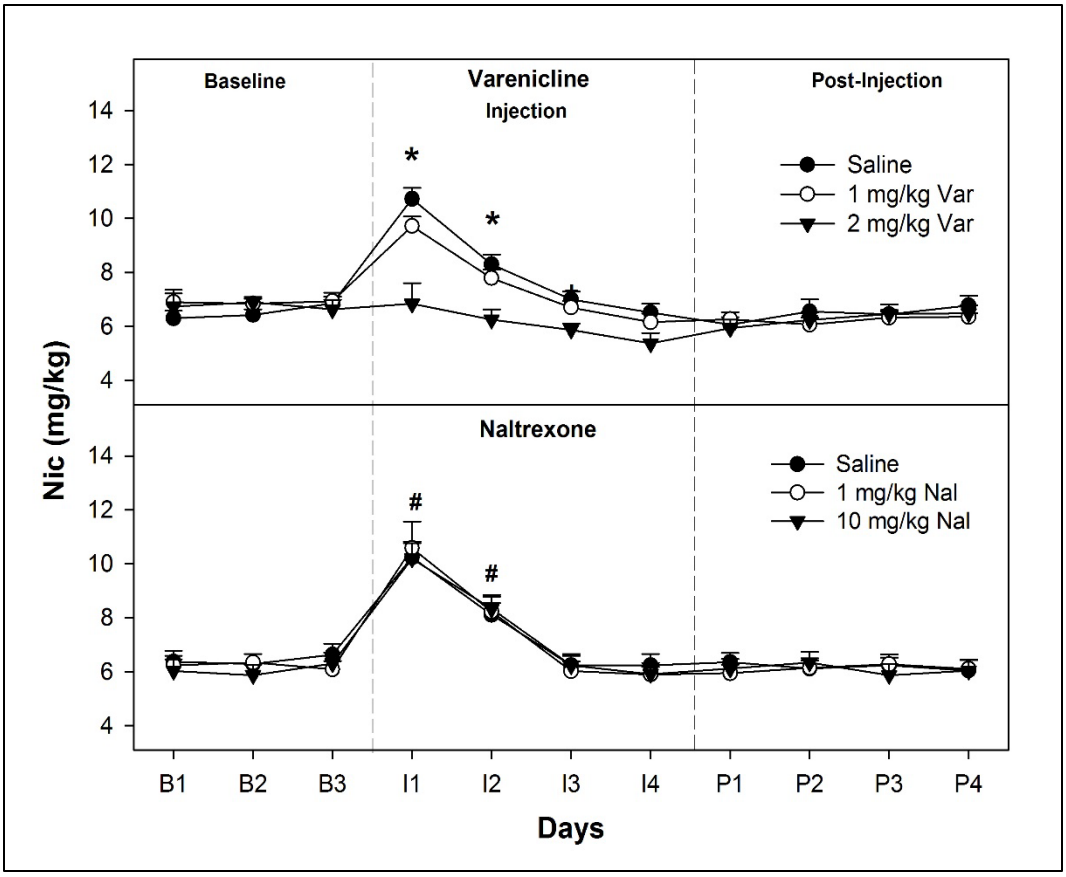

Figure 9. Effects of varenicline or naltrexone on NIC relapse. Mean (+ SEM) consumption of NIC in P rats treated with varenicline (top panel) or naltrexone (bottom panel) during relapse consumption following a 2-week period of forced abstinence. Baseline means are the final three drinking days prior to the deprivation. Asterisk (*) indicates saline and 1 mg/kg varenicline groups are significantly higher from baseline and differ from the 2 mg/kg group. Plus symbol (+) indicates saline and 1 mg/kg varenicline groups are different from the 2 mg/kg group. Number sign (#) indicates all naltrexone-treated rats consumed more NIC than baseline intake levels.

Effects of varenicline and naltrexone on EtOH+NIC consumption

In general, Var and Nal failed to alter the maintenance consumption of either EtOH or NIC when given concurrently. In Var-treated rats, there were no effects of Day, Dose, or Day × Dose interaction term (*p* values > 0.05; Figure 10) for NIC or EtOH consumption. Similarly, Nal treatment had no effect on co-administration of EtOH+NIC (*p* values > 0.05; Figure 11).

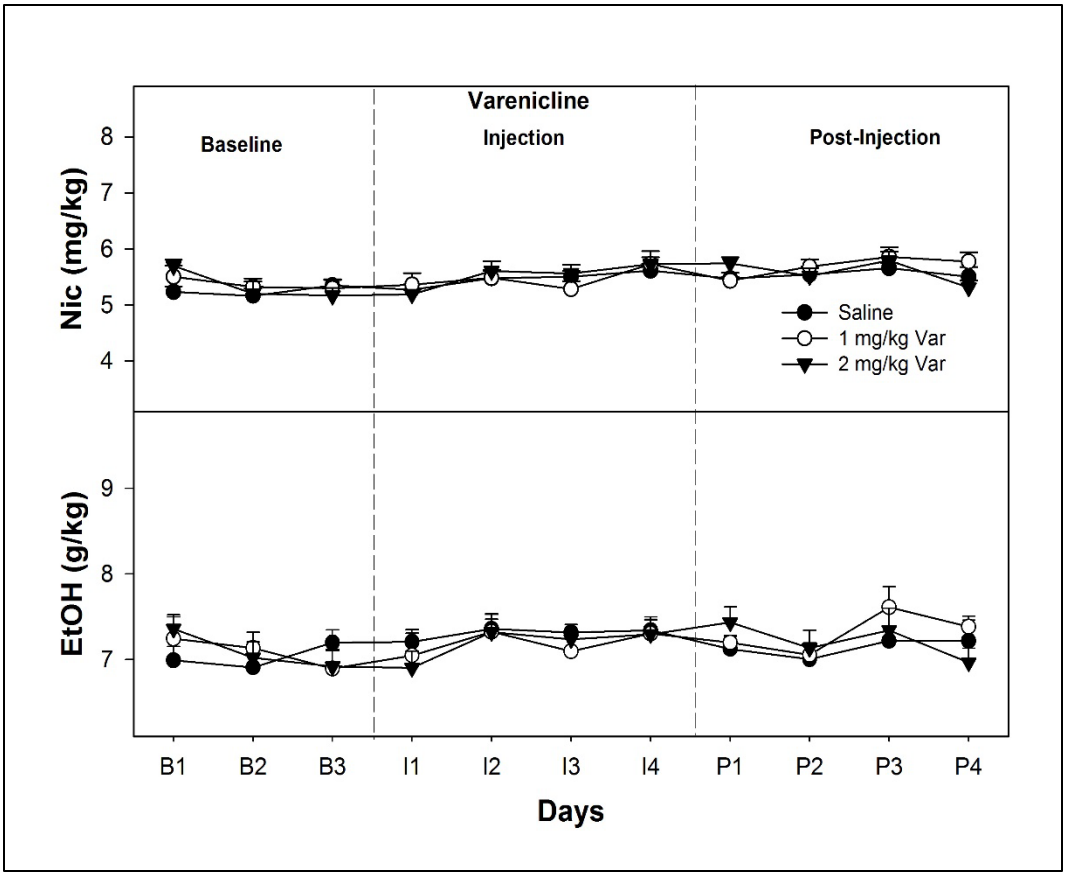

Figure 10. Effects of varenicline on concurrent EtOH and NIC maintenance. Mean (+SEM) consumption of concurrently available NIC (top panel) and EtOH (bottom panel) in P rats treated with varenicline during maintenance consumption. Overall, the data indicate varenicline was ineffective at reducing EtOH+NIC consumption during maintenance drinking.

A period of forced EtOH+NIC abstinence produced a prolonged increase in EtOH+NIC consumption that was not altered by Var (Figure 12) or Nal (Figure 13) treatment. In Var-treated rats, there was an effect of Day for both NIC and EtOH intake ($F_{8,192}$ = 239.228 or 208.321, *p* values < 0.001), but no effect of Dose, or Day × Dose interaction term (*p* values > 0.05). Examining the effect of Day for both EtOH and NIC consumed for all Var rats revealed that intake for NIC and EtOH was increased during the first four re-exposure days (two-tailed t tests, *p* values < 0.001). In Nal-treated rats, there was an effect of Day for both NIC

and EtOH intake ($F_{8,208}$ = 219.795 or 175.024, *p* values < 0.001), but no effect of Dose or Day × Dose interaction term (*p* values > 0.05). Defining the effect of Day was performed by contrasting the overall average intake for a re-exposure day to that observed during the third baseline day with two-tailed t tests. The analyses indicated that NIC consumption was elevated during the first four re-exposure days (*p* values < 0.001), and EtOH consumption was elevated for the first five re-exposure days which included the first post-injection day (*p* values < 0.01).

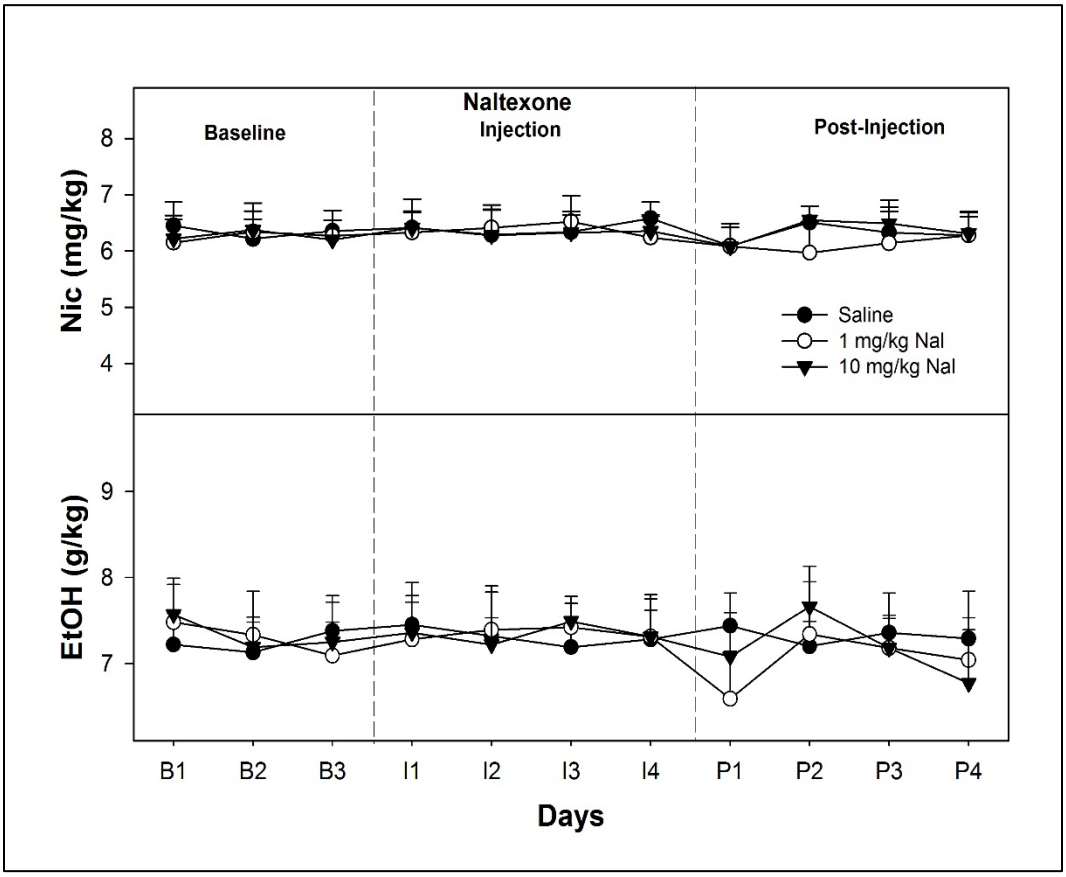

Figure 11. Effects of naltrexone on concurrent EtOH and NIC maintenance. Mean (+SEM) consumption of concurrently available NIC (top panel) and EtOH (bottom panel) in P rats treated with naltrexone during maintenance consumption. Overall, the data indicate naltrexone was ineffective at reducing EtOH+NIC consumption during maintenance drinking.

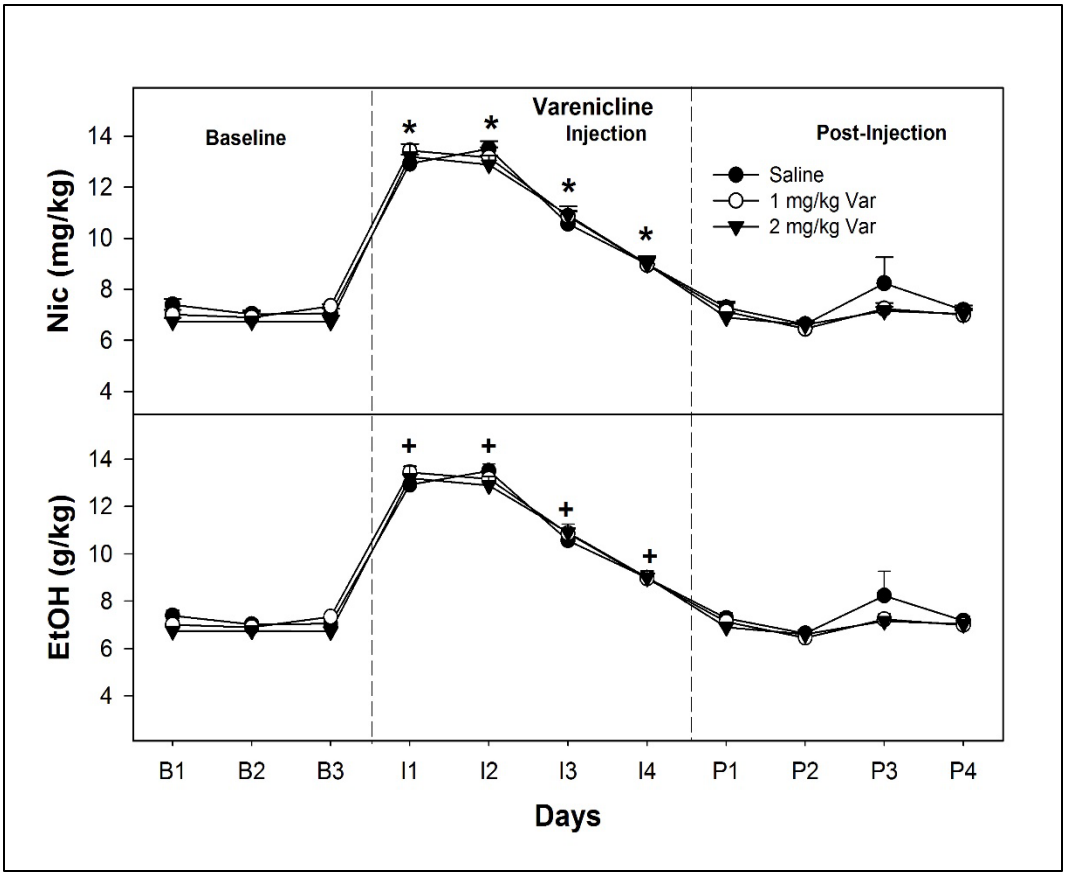

Figure 12. Effects of varenicline on concurrent EtOH and NIC relapse. Mean (+ SEM) consumption of concurrently available NIC (top panel) and EtOH (bottom panel) in P rats treated with varenicline following a 2-week period of forced abstinence. Baseline means are the final three drinking days prior to the deprivation. Overall, the data indicate varenicline was ineffective at reducing EtOH+NIC consumption during relapse drinking. Asterisk (*) indicates NIC consumption was higher in all groups compared with baseline intake. Plus symbol (+) indicates EtOH consumption was higher in all groups compared with baseline intake.

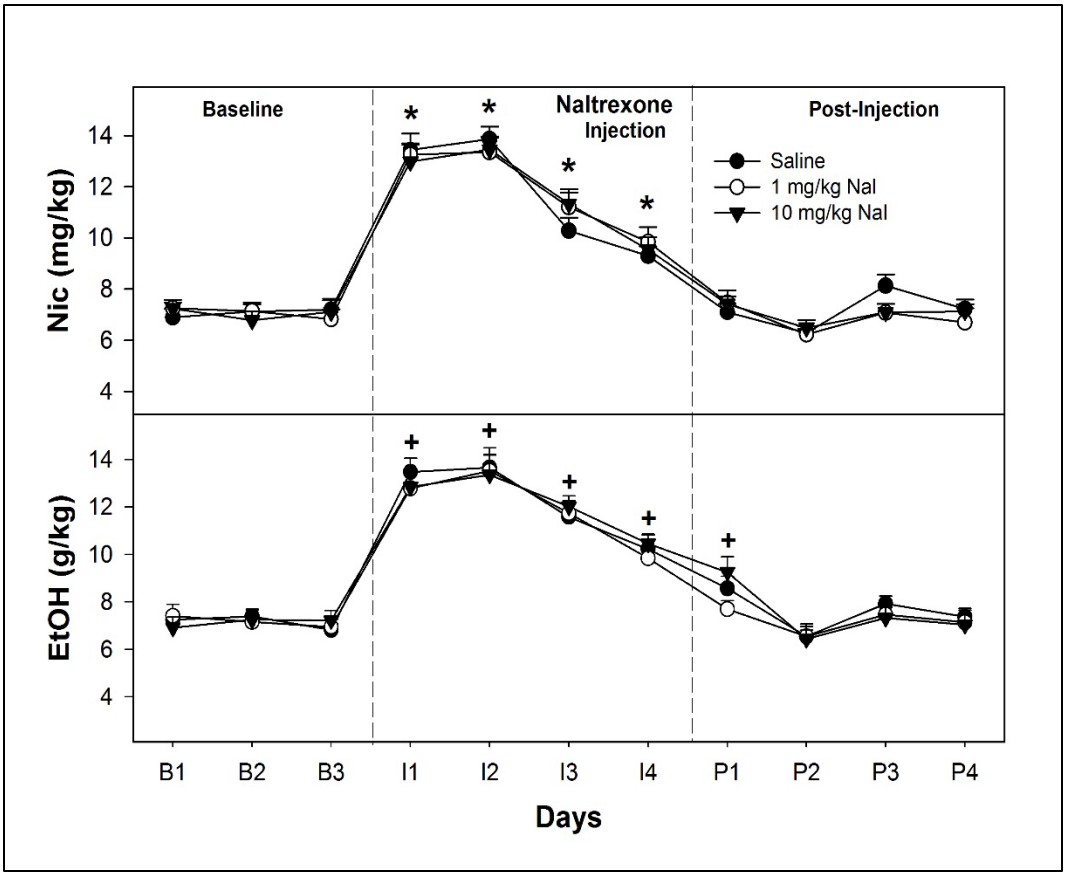

Figure 13. Effects of naltrexone on concurrent EtOH and NIC relapse. Mean (+ SEM) consumption of concurrently available NIC (top panel) and EtOH (bottom panel) in P rats treated with naltrexone following a 2-week period of forced abstinence. Baseline means are the final three drinking days prior to the deprivation. Overall, the data indicate naltrexone was ineffective at reducing EtOH+NIC consumption during relapse drinking. Asterisk (*) indicates NIC consumption was higher in all groups compared with baseline intake. Plus symbol (+) indicates EtOH consumption was higher in all groups compared with baseline intake.

Discussion

Overall, the data indicate the two prototypical pharmacotherapeutics for AUD and nicotine dependence were efficacious at partially reducing the ongoing consumption of the corresponding drug of abuse (Figures 6, 7, 8, and 9). In contrast, Var and Nal had no effect on reducing the intake of the non-indicated

drug of abuse (e.g., Nal on NIC intake; Figures 6, 7, 8, and 9) or on any aspect of EtOH+NIC co-use (Figures 10, 11, 12, and 13).

The current experiments examined relapse using the ADE model and a similar NDE model. ADE has been postulated to mimic the increase in alcohol consumption observed following periods of abstinence in humans and animals (Rodd et al. 2003). The ADE model is associated with adaptations in the posterior ventral tegmental area (pVTA) that enhance EtOH reward (Rodd et al. 2005). The NDE has not been examined extensively in the literature since standard i.v. NIC self-administration precludes the testing of chronic nicotine self-administration due to the limited duration of catheter patency. The current data sets indicate that under 24 hour free-choice oral drinking conditions, NIC intake is enhanced following a period of deprivation (Figures 9, 12, and 13). However, studies have not examined NIC reinforcement in the pVTA following a period of deprivation to determine if neuroadaptations have developed that would augment the reinforcing properties of NIC. The lack of basic research addressing NDE mechanisms supports the need to expand on the present study and identify such changes to understand relevant nicotine consummatory behaviors. Similar to past research, relapse EtOH+NIC consumption was significantly greater and prolonged than that in rats consuming EtOH or NIC alone (compare Figures 12 and 13 with Figures 7 and 9; Hauser et al. 2012b). It is likely that EtOH+NIC co-abuse may augment neuroadaptations produced by periods of drug abstinence or produce unique neuroadaptations not observed following intake of EtOH or NIC (Deehan et al. 2015).

The present data sets indicate that Var can reduce ongoing and relapse NIC intake (Figures 8 and 9). It is important to note that rats treated with the higher dose of Var consumed significant amounts of NIC during the 24-hour periods of NIC re-exposure (Figure 9). Nal had no effect on NIC relapse drinking and all treated groups displayed similar amounts of NIC intake. The data indicate that Nal, but not Var, can reduce EtOH relapse drinking (Figure 7). The efficacy of Nal to reduce EtOH drinking is altered during relapse conditions. The lower dose of Nal (1 mg/kg) produced a similar reduction in EtOH consumption during ongoing EtOH consumption testing as the higher dose (10 mg/kg; Figure 6). However, during relapse testing, rats administered 1 mg/kg Nal displayed a blunted ADE that was significantly more than drinking baseline and 10 mg/kg Nal (Figure 7). Previous research indicated that the efficacy of Nal to reduce EtOH self-administration is decreased under relapse conditions (Dhaher et al. 2012) and the ADE is associated with alterations in the mesolimbic opioid system (Breese et al. 2005). In rats with a history of EtOH+NIC co-abuse, Nal and Var failed to alter relapse co-administration of the two compounds (Figures 12 and 13), suggesting some unique CNS neuronal changes may have occurred with co-abuse that is not found with the individual abuse of EtOH or NIC.

Previous research indicated that treatment with Var significantly reduced NIC self-administration from mean baseline levels during maintenance and relapse phases of drug intake (Funk et al. 2016; O'Connor et al. 2010; Scuppa et al. 2015). Additional reports support these results but utilized doses > 2 mg/kg Var and therefore, nonspecific motor inhibitory effects cannot be excluded

(George et al. 2011; Maggio et al. 2018a). Although 2 mg/kg Var modestly decreased water intake in the “water-only” condition (Figure 5), it is important to note that preference for water and overall fluid consumption in the other three groups was not altered and remained over 100 g/kg/day. This suggests the potential nonspecific effects of Var at this dose on water consumption were minor and counteracted in the presence of reinforcers.

EtOH drinking was found to be unaltered following treatment with Var consistent with recent reports (Funk et al. 2016; Maggio et al. 2018a, b; Randall et al. 2015; Scuppa et al. 2015). However, some studies examining Var and EtOH intake appear to conflict with the present findings. For example, treatment with 2 mg/kg Var reduced EtOH self-administration to ~ 1.5 g/kg/2-hour session following scheduled access and repeated cycles of deprivation (Froehlich et al. 2016, 2017; Czachowski et al. 2018). A number of factors contribute to these differences. The current experiments examined the effects of Var following more than 8 weeks of continuous access to EtOH versus 4 weeks of 2 h/day limited access. The extended EtOH access period could have allowed time to establish lasting adaptations that occur following chronic drinking that decreased the effectiveness of Var compared with animals with less EtOH experience. The previous study notes the importance of maintaining sufficient levels of Var in the blood in order for treatment to remain effective. The current data indicate that administration of 2 mg/kg Var reduces water consumption for a 24-hour period (Figure 5). Therefore, despite the inhibitory gustatory actions of the high dose of Var for 24 hours, there was no effect on EtOH consumption.

Two studies applying limited but free access to EtOH have also shown some effectiveness of Var in reducing EtOH self-administration (Holgate et al. 2017; Steensland et al. 2007). A significant reduction in EtOH drinking following Var treatment was found at 2 hours, but not at 4 hours, during a 4-hour drinking in the dark paradigm but was not evident at the conclusion of the session (Holgate et al. 2017). The significant decrease at 2 hours could be attributed to the small amount of EtOH being consumed under this study design (blood alcohol concentrations were relatively low with an average of 24 ± 5 mg%; Holgate et al. 2017). This level of EtOH self-administration is likely affected more by Var treatment, in agreement with their previous work, than animals in the present study consuming approximately 6.5 g/kg/day (Steensland et al. 2007).

The current experiments support previous evidence demonstrating the ability of Nal to reduce EtOH drinking (Dhaher et al. 2012; Froehlich et al. 2016; Lê et al. 2014; Steensland et al. 2007). Additionally, results from this study indicated no significant changes to NIC intake when treated with Nal (Lê et al. 2014). This agrees with earlier work involving both animal models and clinical trials. Research examining the impact of only naltrexone on smoking cessation demonstrated very limited (Covey et al. 1999) or no effect at all (Wong et al. 1999). Opioid antagonists are known to enhance nicotine withdrawal in nicotine-dependent animals (Malin et al. 1993, 1996; Biala et al. 2005). For this reason, clinical trials investigating naltrexone and smoking cessation may involve augmentation of treatment with nicotine replacement therapy (NRT) to alleviate some NIC withdrawal symptoms (O'Malley et al. 2006; Toll et al. 2010). A meta-

analysis carried out on naltrexone and smoking cessation that included eight separate trials and 1213 participants concluded that there was no positive effect of naltrexone alone or in combination with NRT on short-term or long-term smoking abstinence (David et al. 2014).

The reality of AUD is that it is not a single drug addiction disease. Most cases of AUD are a polysubstance syndrome and are thus much more complicated than those arising from the use of alcohol alone. Ignoring the polysubstance characteristic of AUD limits our knowledge of the disease and greatly reduces the ability to establish functional treatments. Preclinical studies clearly indicated that acute exposure to EtOH and NIC results in unique responses that are not observed following an acute exposure to EtOH or NIC. The pVTA is a site where pharmacologically relevant levels of both NIC and EtOH can produce reinforcing effects (Exley et al. 2011; Hauser et al. 2014; Truitt et al. 2015; Rodd-Henricks et al. 2000). The pVTA is a site that regulates oral EtOH (Hodge et al. 1993; Rodd et al. 2010) and i.v. NIC (Corrigall et al. 1994) self-administration and is a site where EtOH and NIC can interact synergistically (Truitt et al. 2015). Intra-VTA injections of NIC can enhance dopamine release in the nucleus accumbens (NAc) shell produced by systemic administration of EtOH (Tizabi et al. 2002). Rats will self-administer EtOH and NIC directly into the pVTA in a synergistic manner. Furthermore, equivalent microinjections of EtOH+NIC directly into the pVTA result in significant alterations to gene expression in the NAc shell, which includes *bdnf*, *gdnf*, and *homer2* than either EtOH or NIC alone (Truitt et al. 2015).

Chronic EtOH+NIC co-use/abuse also results in unique neuroadaptations throughout the brain. The oral EtOH+NIC model of self-administration results in equivalent consumption of EtOH and NIC in the solo- and poly-drug exposure groups (Hauser et al. 2012b). In the NAc shell, chronic EtOH+NIC self-administration results in enhancement of NIC reward that is not observed in rats consuming equivalent amounts of EtOH or NIC (Deehan et al. 2015). In the medial prefrontal cortex, EtOH+NIC co-use/abuse resulted in a threefold increase in basal glutamate extracellular levels while comparable consumption of EtOH or NIC had no effect (Deehan et al. 2015). The unique neuroadaptations of chronic EtOH+NIC self-administration could be the biological basis for the pharmacological results reported herein.

The present study indicated there was no effect of Var or Nal treatment on EtOH+NIC maintenance or relapse self-administration. The significant reductions of EtOH consumption by Nal treatment and NIC intake by Var treatment are no longer evident when both reinforcers are presented together. This is consistent with preclinical research from our lab and others demonstrating that acute and chronic exposure to EtOH+NIC results in neuronal alterations specific to co-administration that are not observed with either drug alone (Clark and Little 2004; Deehan et al. 2015; Lê et al. 2014; Tizabi et al. 2002, 2007; Truitt et al. 2015). Similar to the current data set, in P rats concurrently self-administering i.v. NIC and oral EtOH, Var and other smoking cessation agents failed to alter EtOH or NIC consumption (Maggio et al. 2018a). The failure of Var and Nal, as well as the specific α6β2* antagonist r-bPiDI, to alter concurrent oral EtOH and i.v. NIC self-

administration has been replicated in a novel model that increases intake levels of both drugs (Maggio et al. 2018b). Therefore, consistent preclinical data has indicated that Var is not effective at reducing EtOH+NIC co-administration.

Despite the disproportionately high rate of EtOH and NIC comorbid abuse, the prevailing pharmacotherapeutic strategy has been to develop treatments that target EtOH or NIC use as separate conditions. This approach has resulted in an inadequate number of approved pharmacological treatment options that, overall, have only demonstrated limited success. For example, a number of meta-analyses revealed that Nal does not increase abstinence rates or decrease the risk of relapse to heavy alcohol drinking. However, the delay to initiation of drinking was increased as well as the total number of days abstinent prior to relapse (Maisel et al. 2013; Jonas et al. 2014; Donoghue et al. 2015). Furthermore, despite FDA approval for Var in 2006, overall smoking cessation rates at the population level in the USA have not risen in over two decades (Zhu et al. 2012). This appears to contradict expected results following dispensing of more than 2.1 million Var prescriptions per year and numerous randomized controlled trials demonstrating the effectiveness of Var (Gonzales et al. 2006; Jorenby et al. 2006; Bolliger et al. 2011; Zhu et al. 2012; Baker et al. 2016; Motschman et al. 2016). These outcomes could in part be explained by the ability of Var clinical trials to recruit nicotine-dependent individuals into abstinence but fail to prevent instances of relapse over placebo-treated smokers (Agboola et al. 2015). Examination of AUDs and nicotine dependence separately in preclinical research and during clinical trials has resulted in approved therapeutics of Nal

and Var with relapse rates at greater than one third of patients at a 6-month follow-up and up to 75% at 1 year, respectively (Jorenby et al. 2006; Volpicelli et al. 1997).

The current study demonstrates the failure of Var or Nal to reduce EtOH+NIC consumption. However, potential limitations to the above model should be considered when evaluating these findings. First, future studies should examine the impact of Var or Nal treatment on EtOH+NIC intake when presented separately in the three-bottle choice drinking paradigm, rather than in a combined solution. This would provide further insight into the effect of Var or Nal on their respective reinforcers. When presented together, potential reductions in just EtOH or NIC intake during treatment would be highlighted that otherwise could be obscured with the combined solution paradigm. Second, increasing the number of Var or Nal treatment days would provide additional time for the blood levels of each therapeutic to stabilize. Finally, the inclusion of measurements taken at specific time points during 24-hour access would be beneficial. A better representation of drinking behavior during Var or Nal treatment could be determined with data collection points at 2, 4, and 6 hours post-treatment.

These results indicate that the standard pharmacological treatments for nicotine dependence or AUD were effective at reducing the consumption of a single targeted drug but neither reduced the amount of EtOH+NIC consumed. Overall, the findings suggest that failures to develop successful treatment for comorbid AUD and nicotine dependence may result from limitations in models used to develop such treatments, specifically through a failure to address the

unique neuroadaptations produced by co-use of these drugs. Continued refinement of the preclinical co-abuse model described herein will be invaluable in the pursuit of assessing future pharmacotherapies for the treatment of heavy alcohol drinking smokers.

Chapter Three: Peri-adolescent, but not adult, alcohol consumption increases sensitivity and dopaminergic response to nicotine during adulthood: alterations in α7 nicotinic acetylcholine receptor expression

An important aspect of alcohol and nicotine co-use/abuse is the age of onset. Early initiation of alcohol drinking has been linked to increased risk for alcohol use disorder (AUD) during adulthood. Additionally, adolescent alcohol drinking is associated with increased adulthood use of tobacco, cannabis, and other drugs of abuse. There is little preclinical research examining the consequences of adolescent alcohol consumption and the response to other drugs of abuse during adulthood. Therefore, this chapter aimed to investigate how peri-adolescent ethanol (EtOH) drinking affects the response to nicotine during adulthood. Previously, our laboratory demonstrated that nicotine microinjected directly into the posterior ventral tegmental area (pVTA) stimulates dopamine (DA) release in the nucleus accumbens (NAc) shell. The α7 nicotinic acetylcholine receptor (nAChR) is a potent regulator of dopaminergic activity in the pVTA. Specifically, the current experiments examined the effects of peri-adolescent EtOH drinking on the ability of intra-pVTA nicotine to stimulate DA release during adulthood and alterations in α7 nAChR expression within the pVTA. Alcohol-preferring (P) female rats consumed EtOH and/or water during adolescence (post-natal day [PND] 30–60) or adulthood (PND 90–120). Thirty days following removal of EtOH, subjects received microinjections of 1 µM, 10 µM, or 50 µM nicotine into the pVTA concurrently with microdialysis for

extracellular DA in the NAc shell. Brains were harvested from an additional cohort after PND 90 for quantification of α7 nAChR within the pVTA. The results indicated that only adolescent EtOH consumption produced a leftward and upward shift in the dose response curve for nicotine to stimulate DA release in the NAc shell. Investigation of α7 nAChR expression within the pVTA revealed a significant increase in animals that consumed EtOH during adolescence compared to naïve animals. The data suggest that peri-adolescent EtOH consumption produced cross-sensitization to the effects of nicotine during adulthood. The interaction between adolescent EtOH consumption and inflated adult risk for drug dependency could be predicated, at least in part, upon alterations in α7 nAChR expression within the mesolimbic reward pathway.

Introduction

The prevalence of alcohol (ethanol, EtOH) drinking in adolescence is high in the United States with 80-90% having consumed EtOH before graduating high school (Johnston et al. 2004). This includes 58% of 12^{th} graders reporting the use of EtOH within the past year and 28% of which engaged in binge drinking within the previous 2 weeks (i.e. > 5 consecutive drinks per drinking episode; Miech et al. 2016; Johnston et al. 2004). Epidemiological data suggest that early initiation of EtOH consumption is associated with a greater risk of developing an alcohol use disorder (AUD) during adulthood (Dawson et al. 2008). Additionally, adolescent EtOH drinking has been linked to increased adulthood use of opioids, cannabis, and other drugs of abuse (Anthony and Petronis 1995). Specific to nicotine, adolescent binge drinking enhances the likelihood of smoking during

adolescence by 88% as well as during adulthood (Best et al. 2000). Conversely, individuals who do not engage in binge drinking have lower rates of smoking during adolescence and adulthood (Bobo and Husten 2000).

Adolescence is a gradual period of transition characterized by widespread neuronal remodeling eventually resulting in the mature adult brain (Spear 2000). Studies of the adolescent brain have consistently demonstrated that numerous regions continue to undergo significant development including the refinement of several neurotransmitter systems. Maturation of cortical and limbic regions during adolescence is marked by excess production of neuronal networks later followed by extensive pruning (Spear 2000; Brenhouse and Andersen 2011).

Dopamine (DA) neurons of the ventral tegmental area (VTA) project to the dorsal striatum, nucleus accumbens (NAc; ventral striatum), amygdala, and prefrontal cortex (PFC; Figure 1). The number of connections, activation of these projections, and firing rate of DA neurons in the VTA reaches its peak during peri-adolescence (i.e. 10 – 19 years old in humans; McCutcheon et al. 2012; Marinelli and McCutcheon 2014). Moreover, studies in both humans and animals found that mesolimbic and mesocortical DA projections have a heightened sensitivity to reward-related events in the peri-adolescent brain (Hoogendam et al. 2013). Thus, the reorganization and subsequent pruning of connections within this system during adolescence is associated with maturation of the reward pathway with altered excitatory afferents into the VTA. Glutamatergic inputs from the PFC to the VTA are pruned during adolescence, which is associated with a reduction in glutamate receptors (Brenhouse and Andersen 2011; Spear and

Swartzwelder 2014). Relevant to the current study, α7 nicotinic acetylcholine receptors (nAChR) are located presynaptically on glutamatergic inputs into the VTA, on GABA interneurons in the VTA, and on VTA DA neurons (Jones and Wonnacot 2004).

Alcohol drinking during adolescence affects development through alterations to ongoing neuronal remodeling processes (Spear and Swartzwelder 2014). Lasting alterations found within the mesolimbic system of the adult brain resulting from adolescent EtOH exposure involves (i) reduced cholinergic regulation, (ii) elevated DA stimulation and release, as well as (iii) epigenetic reprogramming (Vetreno et al. 2014; Sahr et al. 2004; Toalston et al. 2014; Kyzar et al. 2019). A number of studies have investigated how these changes affect the response to ethanol (EtOH) in adulthood. For example, animals given voluntary access to EtOH throughout adolescence were able to acquire operant self-administration of EtOH faster and resisted extinction of self-administration compared to animals without prior EtOH exposure (Gass et al. 2014; Rodd-Henricks et al. 2002a, 2002b; Toalston et al. 2015). Adolescent EtOH drinking was also shown to enhance sensitivity to EtOH reward within the posterior VTA (Toalston et al. 2014). This was in addition to increased stimulated DA release within the NAc shell by either peripheral EtOH administration or direct VTA infusions of EtOH during adulthood (Sahr et al. 2004; Toalston et al. 2014).

Research continues to provide evidence demonstrating the influence of adolescent EtOH drinking on long-term EtOH abuse susceptibility. However, there is little preclinical research examining the consequences of adolescent

EtOH consumption on responses to other drugs of abuse during adulthood. Specifically, a single study was found to have examined the effect of adolescent EtOH exposure with intragastric administration of up to 5 g/kg three times per day followed by nicotine self-administration in adulthood (Boutros et al. 2016). The limited research in this area provides the opportunity to investigate the impact of voluntary adolescent EtOH exposure on the response to nicotine within the mesolimbic system during adulthood. Additionally, lasting expression changes and the potential involvement of interaction sites for EtOH and nicotine can be explored.

Previous research has established the activation of nAChRs found on DA neurons in the VTA is the principal mechanism behind the psychoactive effects of nicotine (De Biasi and Dani 2011; Doyon et al. 2013). Evidence indicates the expression levels of nAChRs are significantly altered within the VTA following adolescent EtOH exposure and contribute to increased DA release by EtOH and nicotine (Morel et al. 2018; Hauser et al. 2019). These long-lasting changes within the mesolimbic circuitry likely alter reward processing and response providing some biological explanation for an increased adult risk for nicotine use. Overall, evidence demonstrates significant perturbations in both the dopaminergic and cholinergic systems following adolescent EtOH exposure (Vetreno et al. 2018).

The present study aimed to determine the long-term effects of voluntary peri-adolescent EtOH consumption on the action of nicotine within the mesolimbic DA system during adulthood. The first experiment determined

whether a history of adolescent EtOH drinking produced persistent neuroadaptations in VTA DA neurons. This was tested by examining the ability of intra-VTA nicotine to stimulate DA release in the NAc shell. This was followed by an examination of α7 nAChR levels within the posterior VTA as an indicator of significant and lasting changes to the cholinergic system, following adolescent EtOH consumption.

Materials and methods

Subjects

Female adolescent alcohol-preferring (P) rats from the 79th—85th generations at Indiana University School of Medicine (IUSM; Indianapolis, IN) were utilized in the current study. In order to build on previously published work, as well as the ability to maintain body weight throughout the duration of the study, the following experiments were carried out only in female rats (Ding et al. 2009; Toalston et al. 2014, 2015; Hauser et al. 2019; Waeiss et al. 2019). The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All research protocols were approved by the IUSM Institutional Animal Care and Use Committee and followed the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (NRCC 2011). Importantly, research indicates the number of females diagnosed with AUD has increased 85% over the past 10 years as well as being more susceptible to EtOH induced neurodegeneration, liver injury, and cancer (Ceylan-Isik et al. 2010; Peltier et al.

2019). These significant differences provide evidence of a dimorphic response to EtOH and highlight the necessity of examining female rats in the following experiments.

Peri-adolescent and adult EtOH consumption procedure

The peri-adolescent EtOH drinking model utilized in the present study was carried out in a similar fashion to those published previously (Toalston et al. 2014, 2015; Waeiss et al. 2019). Beginning on PND 28, P female pups were double-housed in plastic shoebox cages. Rats were maintained on a 12-hour reverse light cycle with lights off at 0900. Food and water were available *ad libitum* to all animals throughout the experiment. Ethyl alcohol (95%; McCormick Distilling Co., Weston, MO) was diluted to 15% and 30% (v/v). Rats were provided 24-hour access to water and both EtOH concentrations in the 3-bottle choice (3BC) paradigm beginning on PND 30 until PND 60. A second cohort of P rats, housed under identical conditions, did not receive access to EtOH during this period. Fluid weights were recorded daily while body weights were measured every other day. After PND 60, all animals remained pair housed in plastic shoebox cages without access to EtOH through at least PND 90.

A third cohort of adult female P rats underwent the same drinking procedure. Briefly, beginning at PND 90 the animals were double-housed in plastic shoebox cages. At this time, animals were provided continuous access to water, 15%, and 30% EtOH (v/v) through PND 120. All adult animals were then maintained in plastic shoebox cages while pair housed until at least PND 150. It

would be redundant to have both naïve adolescent and adult water drinking control groups since the animals would have the same experience. Therefore, the total number of animals in the experiments was reduced by utilizing a single water control group.

Microinjection-Microdialysis protocol

The microinjection-microdialysis (micro-micro) procedure was carried out as described previously (Ding et al. 2009, Toalston et al. 2014; Deehan et al. 2018). Given the global effects of adolescent EtOH drinking, the micro-micro procedure allows specific investigations of whether EtOH induced neuroadaptations in the pVTA modulate the activity and DA response to nicotine within the mesolimbic system. Following PND 90 for the adolescent cohorts or PND 150 for the adult cohort, rats were stereotaxically implanted with two ipsilateral guide cannulas in the right hemisphere. While under isoflurane anesthesia, a 22-guage microinjection cannula (Plastics One, Inc., Roanoke, VA) was aimed 1.0 mm above the posterior ventral tegmental area (pVTA). An 18-guage microdialysis cannula was also implanted 3.0 mm above the NAc shell. The coordinates for the pVTA were AP -5.6 mm, ML +2.1 mm, DV -8.0 mm and those for the NAc shell were +1.5 mm, ML +2.0 mm, DV -5.3 mm (Paxinos and Watson 1998). Both cannulas were implanted at a 10° angle and protected with stylets until the micro-micro stages of these experiments. Following surgery, animals were single housed in shoebox cages and allowed at least one week of

recovery. During this time, the animals were habituated to the experimental housing and handled daily.

Loop-style microdialysis probes were constructed as previously described (Engleman et al. 2000; Ding et al. 2009; Toalston et al. 2014). Probes were manufactured with an active length of 2.0 mm from regenerated cellulose Spectra/Por (Spectrum Laboratories, Inc., Rancho Dominguez, CA) hollow fiber tubing with an inner diameter of 200 µm and molecular weight cut-off of 13 kDa. One day prior to testing, animals were placed under isoflurane anesthesia and the microdialysis probes were inserted into the NAc shell by extending 3.0 mm below the guide cannula. The micro-micro procedure was carried out the following day.

Experiments were performed in awake freely moving animals. Subjects were placed in the experimental chambers and the microdialysis probes were connected to a Harvard pump (Harvard Apparatus, Holliston, MA) used to continuously perfuse the NAc shell with artificial cerebrospinal fluid (aCSF) at a rate of 1 µL/minute throughout the experiment. Microdialysis aCSF was composed of 140.0 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 2.0 mM Na_2HPO_4 • 7H2O, and 1.0 mM $MgCl_2$ with a pH 7.2 to 7.4. Following a 90-minute washout period, samples were collected in 20-minute intervals beginning with five baseline samples.

Next, rats received a single microinjection challenge directly into the pVTA of either aCSF, 1 µM nicotine, 10 µM nicotine, or 50 µM nicotine solutions. Nicotine HCl (Sigma-Aldrich, St. Louis, MO) concentrations were calculated

based on the salt-form. Nicotine concentrations known to be reinforcing and stimulate DA release were selected based on previous intracranial self-administration (ICSA) and micro-micro studies within the pVTA (Hauser et al. 2014a, b; Truitt et al. 2015; Deehan et al. 2018). Importantly, the in vivo concentration of nicotine within the pVTA following microinjection is not known but is expected to be lower than that emitted due to local metabolism and diffusion during the session. Although intra-pVTA injections produce distinct spatial and temporal gradients not experienced in smokers, nicotine concentrations likely approach what was observed in previous experiments investigating peripheral treatment or self-administration in P rats (27 ng/ml; Hauser et al. 2012b; Katner et al. 2015). Passive microinjections were carried out with an electrolytic microinfusion transducer (EMIT) system as described previously (Gatto et al. 1994; Rodd-Henricks et al. 2000; Ding et al. 2009). Briefly, subjects received 30 pulse injections over a 10-minute period designed to simulate average intracranial self-administration (ICSA) levels (Rodd-Henricks et al. 2000; Toalston et al 2014). Each infusion injected 100 nl of solution over 5 seconds and was followed by a 15-second timeout period for a total of 3 µl over the 10-minute infusion session. After the microinjection challenge, six additional 20-minute samples of dialysate samples were collected into tubes containing 5 µl of 0.1 N perchloric acid. All samples were immediately frozen on dry ice and stored at -80 °C until analysis for DA content with high performance liquid chromatography (HPLC).

The present microdialysis study utilized 85 female P rats. Subjects were randomly assigned to one of four microinjection conditions with n = 8–9/group of naïve, n = 7–9/group adolescent EtOH drinkers, and n = 4–6/group of adult EtOH drinkers.

Dialysate analysis

DA was analyzed using a reversed phase HPLC system with electrochemical detection (Engleman et al. 2000; Ding et al. 2009; Toalston et al. 2014). Samples were loaded into a 10 µl loop and injected onto an analytical column (BDS Hypersil C18, 3 µm, 150 mm x 2.1 mm, Thermo Fisher Scientific, Waltham, MA). Mobile phase consisted of 0.1 mM EDTA, 8 mM KCl, 50 mM phosphoric acid, 100 mg/L OSA, and 10% MeOH with a pH of 6.0. DA detection occurred with a glassy-carbon electrode and an amperometric detector with the oxidation potential set at 350 mV and sensitivity of 100 pA/V (Decade II EC Detector, Antec Scientific, Netherlands). Signal analysis was carried out with a ChromePerfect (Justice Innovations, Inc., Palo Alto, CA) data station. DA content was then determined through comparison to a standard curve generated from 1 nM, 5 nM, and 10 nM DA solutions.

Histology

Once the experiments were completed, subjects were euthanized and a solution of 1% bromophenol blue was infused into each microdialysis probe and microinjection cannula. Brains were removed, immediately frozen on dry ice, and

stored at -80 °C. Forty-micron sections were then collected with a cryostat microtome for verification of microdialysis probe and microinjection placements.

Immunohistochemistry

On PND 90, naïve rats (n = 6) and adolescent EtOH drinkers (n = 7) were deeply anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS. Perfused brains were then removed and post-fixed overnight in 4% PFA at 4 °C. Next, brains were transferred to 30% sucrose in PBS and stored at 4 °C until processed. Serial coronal sections were collected at a thickness of 30 µm using a freezing microtome, immediately placed in a cryoprotectant solution, and stored at -20 °C until processing. Based on results from the micro-micro experiment demonstrating a similar response between adult EtOH drinkers and naïve rats, adult EtOH drinkers were not included in the immunohistochemical analysis to reduce the total number of animals used in the present study.

Immunohistochemistry was carried out as described previously (Johnson et al. 2015). Free-floating sections containing the VTA were initially washed with PBS for 30 minutes at room temperature prior to incubation in 1% H_2O_2 in PBS for 20 minutes. Sections were then washed in PBS for 10 minutes and PBS with 0.3% Triton X-100 (PBST) for an additional 20 minutes. Sections were then incubated overnight for 12-16 hours in PBST containing mouse anti-α7 nAChR subunit monoclonal antibody (1:100; Catalog # 836701, BioLegend, San Diego, CA) at room temperature. Following a 30-minute wash in PBST, sections were

incubated in biotinylated horse anti-mouse IgG secondary antibody (1:500; Catalog # BA-2000, Vector Laboratories, Burlinghame, CA) for 2 hours. Sections were again washed in PBST for 30 minutes and incubated for 1.5 hours with an avidin–biotin–horseradish peroxidase complex provided in a standard Vector Elite kit (1:1000; Catalog # PK-6100, Vector Laboratories). The resulting peroxidase complexes were visualized by exposure for 10 minutes to a chromogen solution containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO) to produce a dark brown reaction product for α7 nAChR subunit staining. Repeated washing in 0.1 M PB was used to terminate the reaction. Sections were then mounted on clean glass slides, dried overnight, dehydrated, and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA).

Due to known concerns with antibodies directed toward α7 nAChRs, steps were taken to investigate specificity (Garg and Loring 2017). An experiment to determine non-specific binding was carried out on separate tissue sections by applying the above-mentioned procedure and substituting the primary antibody with an isotype control (IgG1). Another round of immunohistochemistry was conducted with the omission of the primary antibody to determine the contribution of other components to background staining. These experiments (data not shown), as well as those conducted by other research groups, have determined there was relatively little non-specific binding and likely would not influence the analysis (Dominguez del Toro et al. 1994; Mielke and Mealing 2009; Garg and Loring 2017). Additionally, Western blotting was conducted with a different α7

nAChR antibody as a complementary technique to increase confidence in the immunohistochemical findings. Finally, orthogonal data recently published by our group indicate similar alterations to α7 nAChRs following EtOH exposure with TaqMan low-density array (TLDA) cards for real-time PCR (Hauser et al. 2019).

Sections were analyzed by an observer blind to the treatment conditions and the number of α7 nAChR subunit-immunoreactive (α7-IR) cells were counted. Only tissue sections containing the pVTA, located between -5.2 mm bregma and -6.2 mm bregma, were included in the analysis (Paxinos and Watson, 1998). Overall, four to seven sections per brain were analyzed. The total number of cells counted per section was divided by the total volume of each section. Total volumes from the sections counted for the pVTA were calculated by multiplying the sum of area, determined with ImageJ analysis software (NIH, Bethesda, MD), by a depth of 19 µm. Data are presented as number of cells per mm^3.

Protein quantification, SDS-PAGE, and Western blotting

On PND 90, naïve rats (n = 6) and adolescent EtOH drinkers (n = 6) were deeply anesthetized with isoflurane and rapidly decapitated. Brains were quickly removed, flash frozen in isopentane on dry ice, and stored at -80 °C until processed. Serial coronal sections were then collected at a thickness of 300 µm with a freezing microtome. Micro-punches containing the pVTA were obtained using a 1 mm diameter Harris micro-punch (Electron Microscopy Sciences, Hatfield, PA) as described previously (McBride et al. 2009). The tissue was

immediately homogenized in 150 µl of ice-cold N-PER lysis buffer supplemented with Halt Protease inhibitor cocktail (Thermo Scientific, Waltham, MA). At 4 °C, samples were incubated in the lysis buffer on a nutator for 2 hours followed by centrifugation at 10,000 x g for 20 minutes. Supernatants were removed and protein concentrations were measured by the bicinchoninic acid assay (Pierce, Rockford, IL). Protein concentrations were determined with 10 µl of lysate and 200 µl of working reagent at an absorbance of 570 nM with a microplate reader (Bio-Rad, Hercules, CA). All samples were analyzed in duplicates and absorbance values averaged. Adult EtOH drinkers were not included based on neurochemical results from the micro-micro experiment.

Approximately 10 µg of processed tissue protein was loaded per lane and separated on 7.5% polyacrylamide denaturing gels (Bio-Rad, Hercules, CA). This was followed by transfer to nitrocellulose membranes (Amersham, Pittsburgh, PA). Membranes were blocked with 5% milk in TBST buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl; 0.1% Tween 20) overnight at 4 °C. Next, membranes were incubated for 2 hours at room temperature with rabbit anti-α7 nAChR subunit (1:10,000; ab10096, Abcam, Cambridge, UK) and mouse anti-GAPDH (1:100,000; Catalog # MA5-15738, Thermo Scientific, Waltham, MA). Secondary antibodies were HRP conjugated goat anti-mouse (1:20,000; Catalog # 31430, Invitrogen, Carlsbad, CA) or HRP-conjugated goat anti-rabbit (1:20,000; ab205718, Abcam, Cambridge, UK). Protein band signals were visualized by adding chemiluminescent HRP substrate reagent (Millipore, Billerica, MA). Films

were scanned and densitometry was performed with ImageJ software (NIH, Bethesda, MD).

The second α7 nAChR antibody, ab10096, was also scrutinized for specificity. First, ab10096 was preincubated with a synthetic blocking peptide (ab101467, Abcam, Cambridge, UK) corresponding to a region of the human α7 nAChR subunit, and the ab10096 antigen, in a ratio of 1:5 overnight at 4 °C prior to immunoblotting. Next, in order to exclude the possibility of non-specific interactions with other antigens, a comparison was made using liver tissue, which has been determined to express negligible levels of α7 nAChR (Mielke and Mealing 2009; Fagerberg et al. 2014). Finally, omitting ab10096 from the Western blotting procedure was also carried out to substantiate specificity. In each of the three control experiments described above, bands at the expected molecular weight were faint or not detected.

Statistical analysis

The average EtOH intake of adolescent drinkers and adult drinkers was calculated based on the last 10 sessions of the 30-day drinking procedure. Contrasting differences between adolescent and adult EtOH consumption was examined with an overall mixed factor ANOVA with between subject factor of 'Age' and a within subject factor of 'Day'. Individual "Day' differences were determined with an unpaired Student's *t*-test ($p < 0.05$). Microdialysis data are expressed as a percentage of basal DA values to correct for subject-to-subject variability (Engleman et al. 2000). Basal dialysate DA values were calculated as

the mean of three baseline samples collected prior to the challenge microinjection. The effects of pVTA nicotine microinjections on extracellular DA were analyzed using a Time × Drinking Condition × Nicotine mixed analysis of variance (ANOVA). Post-hoc comparisons used to determine significance were Tukey's b for between group differences or Student's two-tailed *t* for within group differences. Immunohistochemical data were analyzed with the mean number of α7-IR cells per mm^3 calculated from the naïve treatment group and adolescent EtOH drinkers. These values were then compared using a univariate ANOVA ($p < 0.05$). Western blot densitometric signals were normalized to GAPDH. Analysis of signals were then also compared using a univariate ANOVA ($p < 0.05$).

Results

Mean EtOH intake analysis

Average daily EtOH intake over the final 10 drinking days were 10.2 ± 0.6 g/kg/day for adolescent drinkers and 10.6 ± 1.2 g/kg/day for adult drinkers (Figure 14). Analysis revealed that the average EtOH consumption was not significantly different between adolescent drinkers and adult drinkers ($F_{1,53} = 0.47$, $p = 0.50$).

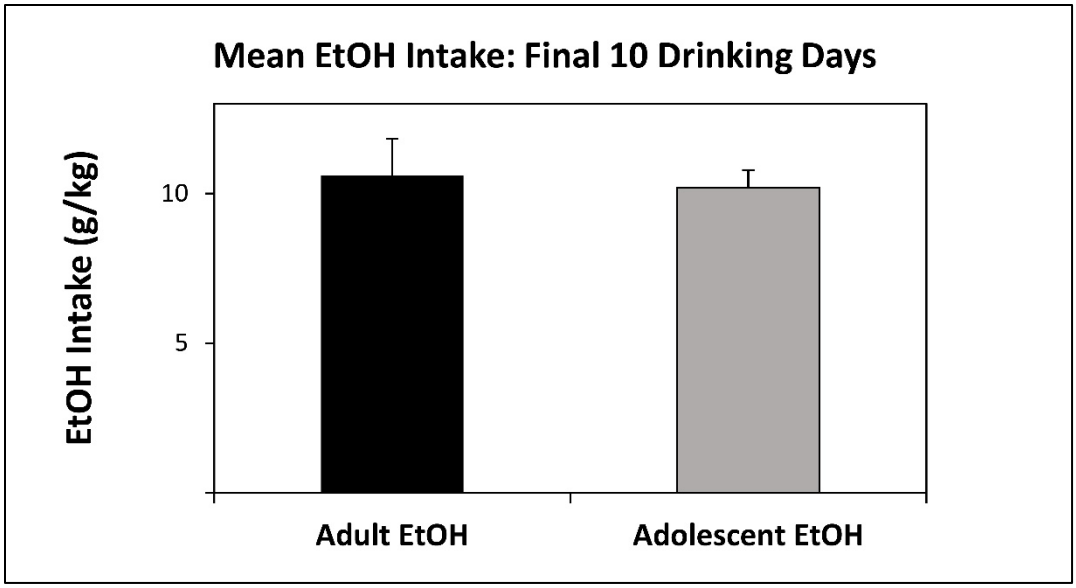

Figure 14. Average daily EtOH consumption for all animals given free-choice access to 15% and 30% EtOH during adolescence (PND 30–60; n = 32) or adulthood (PND 90–120; n = 24). Intake values are calculated as grams per kilogram per day and averaged into a block of the final 10 days. All data shown are mean +SEM.

Dopamine microinjection-microdialysis analysis

The mean DA levels in dialysate samples from the NAc shell were 1.22 ± 0.12 nM for the naïve group, 1.28 ± 0.11 nM for adolescent drinkers, and 1.17 ± 0.18 nM for adult drinkers. The basal DA levels in dialysis samples are strongly influenced by the rate of recovery through the microdialysis probe. An accurate assessment of extracellular DA levels with microdialysis can only be determined by a quantitative method such as with the No-Net-Flux protocol.

The overall analysis that included individual time points revealed a significant Time × Drinking History × Nicotine interaction term ($F_{48,584} = 1.65$; $p = 0.005$; Figure 15). The 3-way interaction term was initially decomposed by holding Time constant. A significant Drinking History × Nicotine interaction was found for both the first ($F_{6,73} = 2.84$; $p = 0.015$) and second ($F_{6,73} = 4.00$; $p =$

0.002) samples collected following nicotine microinjection. To determine the effect of Drinking History on the ability of nicotine microinjected into the pVTA to stimulate DA release in the NAc shell at each time point was derived by reducing the significant 2-way interaction term by holding Nicotine constant. There were no significant group differences during the first and second sample period following treatment with aCSF into the pVTA ($F_{2,18} < 1.69$; *p* values > 0.21). In animals treated with 1 μM, 10 μM, or 50 μM nicotine there were significant group differences during the first and second sample period following microinjection ($F_{2,18}$ values > 7.24; *p* values < 0.01). Tukey's b post-hoc comparisons revealed that all nicotine concentrations and both sample time points, adolescent EtOH consuming rats had significantly higher levels of DA than adult EtOH drinkers or naïve rats.

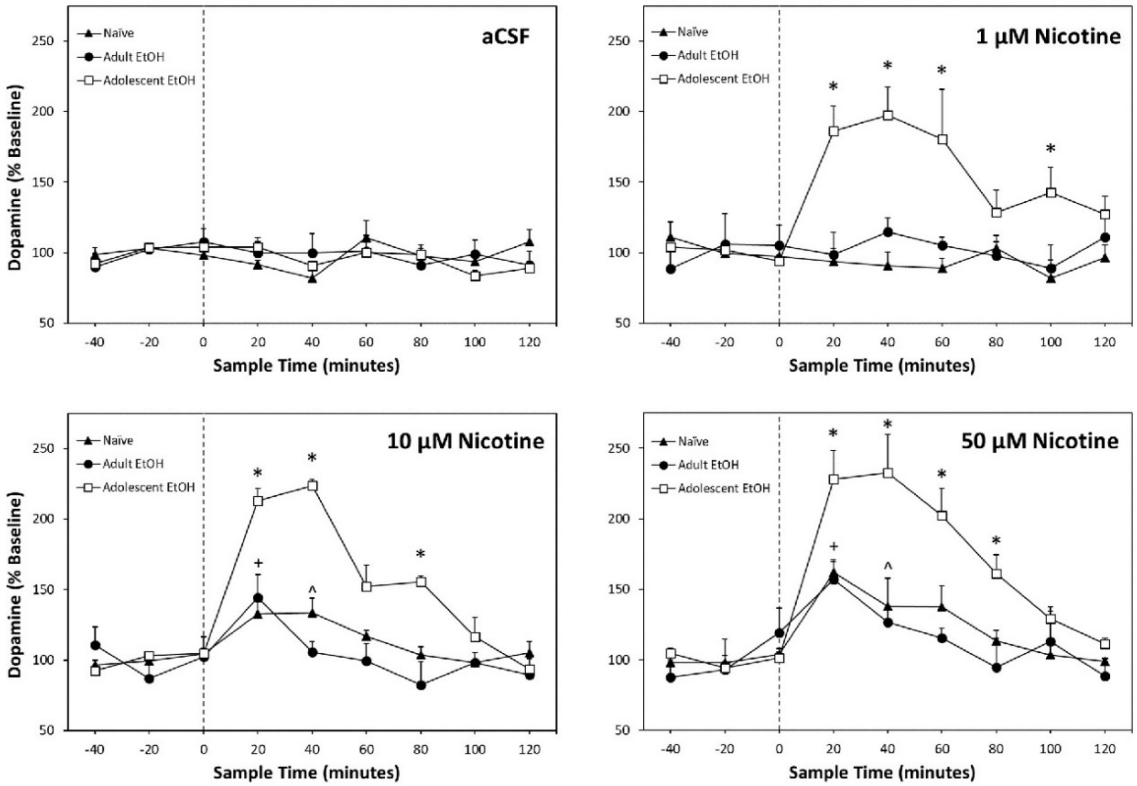

Figure 15. Average change in DA of rats microinjected with nicotine. Mean (+SEM) percentage change in extracellular DA in the NAc shell of adult rats microinjected with 0, 1, 10, or 50 µM nicotine directly into the pVTA previously allowed to consume EtOH during adolescence (Adolescent EtOH; n = 7–9/group), during adulthood (Adult EtOH; n = 4–6/group), or water only (Naïve; n = 8–9/group). Asterisk (*) indicates significantly greater DA levels in Adolescent EtOH rats compared to Naïve, Adult EtOH, and respective baseline DA at the specified time points. Plus sign (+) indicates Naïve and Adult EtOH extracellular DA was significantly higher than respective baseline levels. Carrot (^) indicates Naïve DA levels were significantly increased over baseline.

The overall 3-way interaction term was also reduced by holding Drinking History constant. For the naïve animals, individual ANOVAs revealed a significant effect of nicotine concentration during the first three samples following microinjection ($F_{3,28}$ values > 2.99; p values < 0.05; Figure 15). During the first and second samples following pVTA microinjection, Tukey's b post-hoc indicated DA levels in the NAc shell were significantly greater in naïve rats administered 10 and 50 µM nicotine compared to aCSF. During the third sample, DA levels remained significantly elevated in the NAc shell following administration of 50 µM nicotine compared to animals receiving 1 µM nicotine. Within-subject examination of individual nicotine concentrations revealed a significant effect of 50 µM nicotine ($F_{8,56} = 6.19$; $p < 0.001$). Post hoc comparisons indicated DA levels of the first post microinjection sample were significantly higher ($t_7 = 5.71$, $p = 0.001$) at 162% of baseline.

Adult EtOH drinkers also exhibited a significant effect of nicotine concentration during the first sample following microinjection ($F_{3,17} = 5.96$; $p = 0.006$; Figure 15). Adult drinkers receiving 50 µM nicotine into the pVTA produced significantly elevated DA levels in the NAc shell compared to those receiving 1 µM nicotine or aCSF. Within-subject analysis of individual nicotine concentrations revealed a significant effect of 10 µM ($F_{8,24} = 2.52$; $p = 0.038$) and 50 µM nicotine ($F_{8,40} = 2.23$; $p = 0.046$). Post hoc comparisons indicated DA levels of the first sample following 10 µM nicotine microinjection were significantly higher than baseline ($t_3 = 3.35$, $p = 0.044$). Additionally, microinjection of 50 µM

nicotine resulted in significantly increased extracellular DA during the first sample to 157% compared to baseline ($t_5 = 3.01$, $p = 0.027$).

Analyses of adolescent EtOH drinkers revealed a significant effect of nicotine concentration during all six samples collected following nicotine treatment ($F_{3,28}$ values > 3.24; p values < 0.05; Figure 15). Specifically, 1 µM, 10 µM, and 50 µM nicotine produced significantly higher DA levels during the first and second samples post microinjection compared to aCSF. The third post microinjection sample revealed NAc shell DA following 50 µM nicotine treatment was significantly higher than aCSF controls. During the fourth sample, both 10 µM and 50 µM nicotine treated animals exhibited significantly greater DA levels compared to aCSF controls. Additionally, samples five and six indicated elevated extracellular DA in adolescent drinkers that received 1 µM nicotine over aCSF microinjections. Analyses of individual nicotine concentrations within-subjects revealed a significant effect of 1 µM, 10 µM, and 50 µM nicotine ($F_{8,56}$ values > 6.98; $p < 0.001$). Post hoc comparisons demonstrated DA levels of samples 1-3 and 5 following 1 µM nicotine microinjection were significantly higher than baseline (t_8 values > 2.31, p values < 0.05). Microinjection of 10 µM nicotine resulted in significantly increased extracellular DA during the first four samples compared to baseline (t_7 values > 7.72, p values < 0.05). Lastly, adolescent drinkers that received pVTA microinjections of 50 µM nicotine had significantly greater extracellular DA during the first five samples compared to baseline levels (t_7 values > 3.80, p values < 0.01). Microinjections of 1 µM, 10 µM, and 50 µM

nicotine produced maximal DA response during the second sample reaching 197%, 223%, and 232% compared to baseline levels, respectively.

A final area under the curve (AUC) analysis of DA levels determined whether there were general effects of Drinking History or a dose-response to nicotine over the duration of the experiment. AUC is a summary analysis that provides a definite integral of a value across time to generate a quantitative value. The analysis indicated a significant Drinking History × Nicotine interaction term ($F_{6,73} = 7.11$; $p < 0.001$; Figure 16). Reducing the significant 2-way interaction by holding Nicotine constant indicated there were no significant group differences following microinjection of aCSF into the pVTA ($F_{2,18} = 0.56$; $p = 0.945$). However, there were significant group differences in animals treated with 1 μM, 10 μM, or 50 μM nicotine ($F_{2,18}$ values > 15.77; p values < 0.001; Figure 16). Post-hoc comparisons with Tukey's b revealed that at all nicotine concentrations, adolescent EtOH consuming rats had a significantly greater DA response than adult EtOH drinkers or naïve rats.

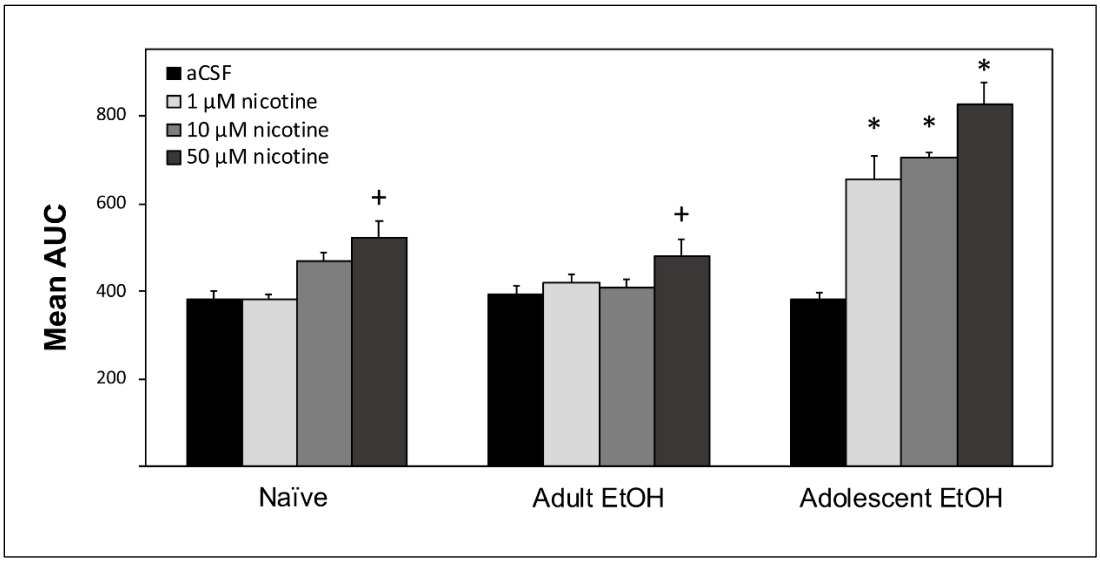

Figure 16. Average (+SEM) area under the curve (AUC) values of DA depicting general effects of drinking history and dose-response to nicotine. DA response was determined following microinjections of 0 (aCSF), 1, 10, or 50 µM nicotine administered to adult rats previously allowed to consume EtOH during adolescence (Adolescent EtOH; n = 7–9/group), during adulthood (Adult EtOH; n = 4–6/group), or water only (Naïve; n = 8–9/group). Plus sign (+) indicates significantly greater AUC in rats treated with 50 µM nicotine than respective aCSF treatment. Asterisk (*) indicates significantly greater AUC in Adolescent EtOH rats treated with 1, 10, or 50 µM nicotine compared to respective Naïve, Adult EtOH, and aCSF treatment groups.

Immunohistochemical and Western blot analyses

Immunohistochemistry was used to assess α7-IR cells in the pVTA of adolescent EtOH drinkers and naïve rats in adulthood (>PND 90). In naïve rats and adolescent drinkers, examination of α7-IR cells revealed darkly stained cell bodies and processes (Figure 17). Within the pVTA, α7-IR cells were significantly increased in adolescent drinkers compared to naïve animals ($F_{1,11} = 16.44$, $p = 0.002$). Adolescent EtOH drinking resulted in an increase of α7-IR cells/mm^3 to 193% relative to naïve rats (Figure 17).

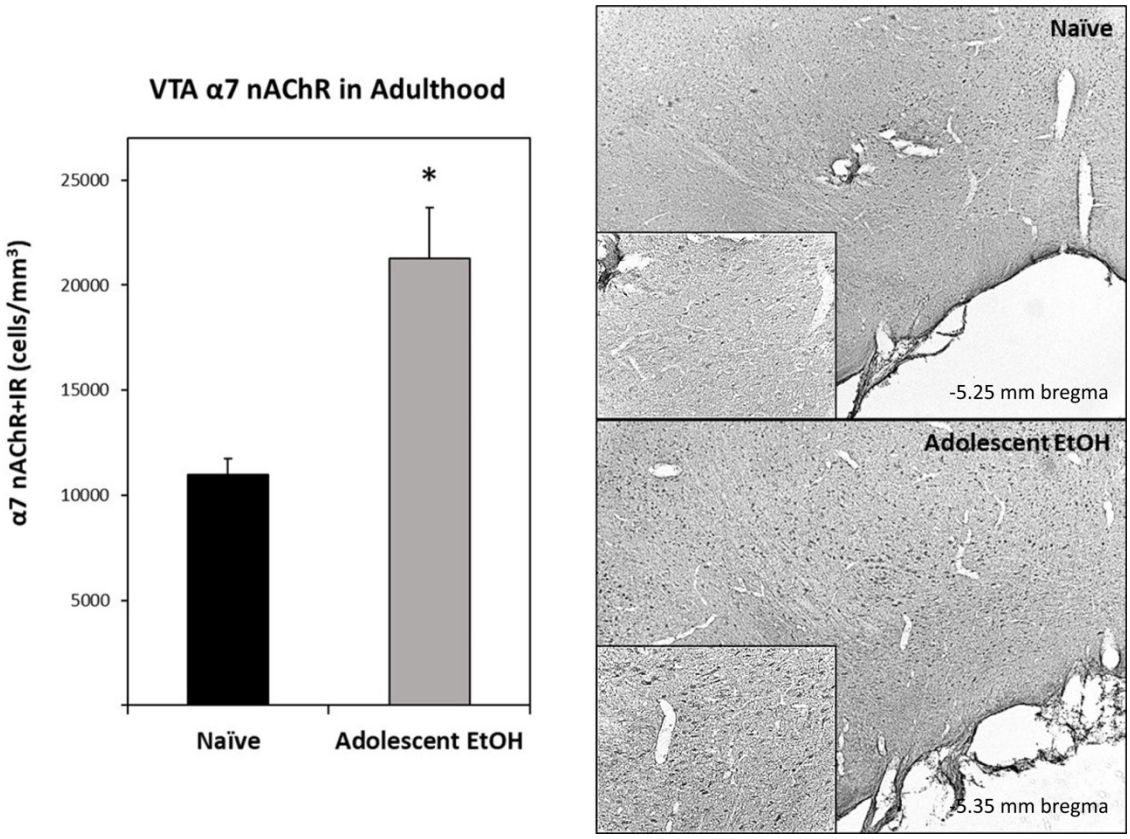

Figure 17. Average α7-IR cell counts within the pVTA and representative photomicrographs. Left: Mean (+SEM) α7-IR cell counts within the pVTA of adult rats allowed to consume water only (Naïve; n = 6) or EtOH during adolescence (Adolescent EtOH; n = 7). Asterisk (*) indicates significantly greater α7-IR cells compared to Naïve rats. Right: Representative photomicrographs (5x magnification, 20x inset) of α7-IR cells in the pVTA of Naïve and Adolescent EtOH during adulthood.

Western blot analysis was then utilized to assess α7 nAChR expression in the pVTA. Relative expression levels of α7 nAChR in the pVTA are presented in Figure 18. Adolescent EtOH consumption significantly increased α7 nAChR expression levels 33% within the pVTA compared to naïve rats ($F_{1,10} = 6.54$, $p = 0.003$).

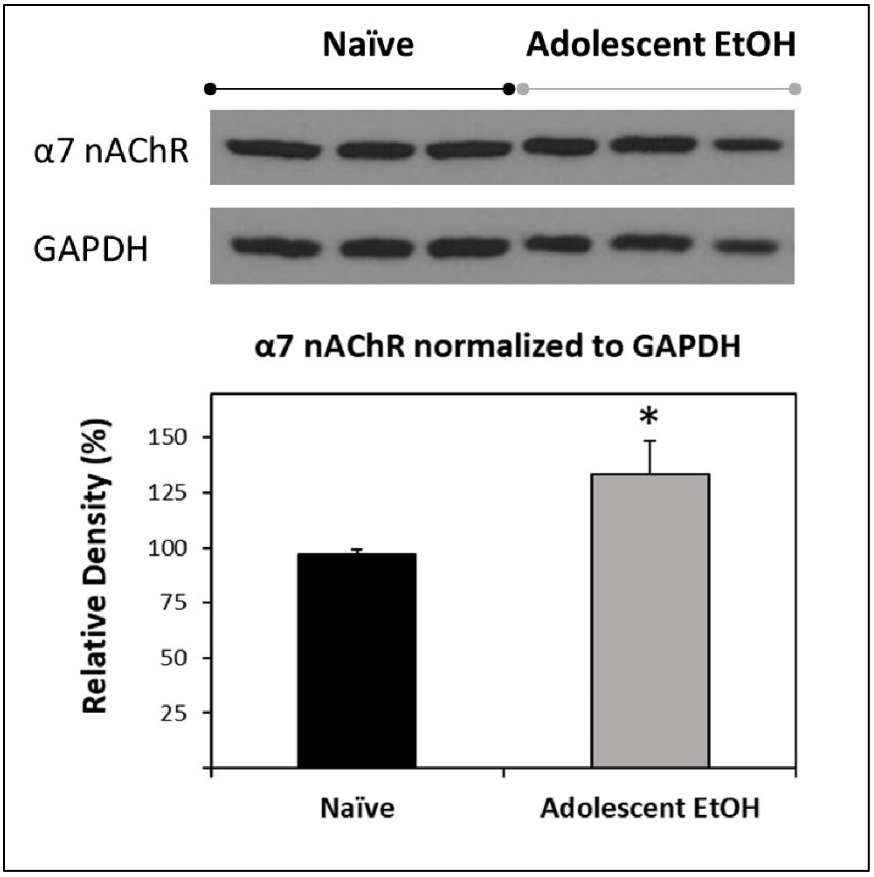

Figure 18. Representative western blots and group data illustrating increased α7 nAChR levels in the pVTA. Data were normalized to GAPDH and indicate increased α7 nAChR protein levels in the pVTA of rats allowed to consume EtOH during adolescence (Adolescent EtOH; n = 6) compared to water only controls (Naïve; n = 6) in adulthood. Asterisk (*) indicates significantly greater than controls.

Discussion

Overall, the findings of the current study indicate that voluntary adolescent, but not equivalent adult, EtOH consumption resulted in long-lasting functional and neurochemical alterations to the mesolimbic DA system. Specifically, adolescent EtOH drinkers developed a cross-sensitization to nicotine during adulthood. Adolescent EtOH consumption produced a leftward and upward shift in the ability of nicotine microinjected into the pVTA to stimulate

DA release in the NAc shell that was not present in naïve animals or adult EtOH drinkers (Figures 15 and 16). Additionally, rats allowed to consume EtOH during adolescence had significantly elevated α7 nAChR expression in the pVTA compared to naïve rats (Figures 17 and 18).

Overwhelmingly convergent data have indicated that adolescent EtOH exposure results in persistent alterations to the adult brain that mediates future drug responsivity and self-administration. The rewarding and reinforcing properties of EtOH and nicotine are well known to be associated with increased DA release within the NAc (Di Chiara and Imperato 1988; Wise and Rompre 1989). Furthermore, the associated reinforcing effects of pharmacologically relevant levels of EtOH and nicotine within the pVTA has also been established (Exley et al. 2011; Hauser et al. 2014; Rodd-Henricks et al. 2000; Truitt et al. 2014). The pVTA acts as a neural substrate where EtOH and nicotine are self-administered and interact to enhance DA release in the NAc shell (Tizabi et al. 2002, 2007; Rodd et al. 2010; Corrigall et al. 1994). It has been suggested that the hyperdopaminergic state induced by adolescent EtOH exposure could occur through both independent actions as well as through shared neurotransmitter systems of EtOH and nicotine. Repeated observations have identified neuroadaptations in the dopaminergic and cholinergic systems in adulthood following adolescent EtOH exposure (Crews et al. 2016). Alterations in DA function in adulthood is observed following voluntary consumption or experimenter administered EtOH during adolescence (Toalston et al. 2014; Sahr et al. 2004; Pascual et al. 2009).

A critical question of adolescent research is whether any observed consequence of EtOH exposure is specific to adolescence. Multiple reports now indicate that adolescent EtOH exposure produces unique adaptations in the dopaminergic system that are not observed following comparable adult consumption. Rats exposed to EtOH during adolescence expressed alterations in DRD1 and DRD2 during adulthood but adult rats treated with EtOH did not display comparable effects (Pascual et al. 2009; Coleman et al. 2011). In addition, an increased sensitivity of neuronal activity within a subset of VTA DA neurons to EtOH is observed specifically following adolescent EtOH self-administration (Avegno et al. 2016).

Importantly, it has also been demonstrated that after almost complete denervation of the NAc and the mesolimbic DA system, voluntary responding for EtOH failed to change (Rassnick et al. 1993). This undoubtedly suggests there are DA-independent neurochemical systems that contribute critically to mediating the reinforcing actions of EtOH and nicotine. The cholinergic system has also been shown to be especially susceptible to the effects of adolescent binge-like EtOH exposure. Numerous studies demonstrated long-lasting and global reductions in neurons expressing choline acetyltransferase (ChAT), a marker for cholinergic neurons and enzyme responsible for acetylcholine (ACh) biosynthesis, following an adolescent intermittent EtOH (AIE) binge protocol (Coleman et al. 2011; Ehlers et al. 2011; Vetreno et al. 2014). In addition to an overall reduction in neurons expressing ChAT, chronic EtOH reduced the endogenous function of ChAT, ACh uptake, and expression of

acetylcholinesterase (Arendt et al. 1989; Floyd et al. 1997). Alterations in the expression of nAChRs in multiple brain regions have also been reported (Figures 17 and 18; McClintick et al. 2015, 2016; Hauser et al. 2019). These data add to the current findings indicating that the persistent reduction of ChAT produced by adolescent EtOH exposure results in a compensatory upregulation of nAChRs, specifically α7, in adult brains.

Voluntary EtOH consumption in adolescence, but not adulthood, produced lasting upregulation of α7 nAChRs in the pVTA as well as alterations to the actions of EtOH and nicotine at the α7 nAChR (Doyon et al. 2013; Hauser et al. 2019). The homomeric α7 nAChRs are strongly expressed presynaptically on glutamatergic afferents to the VTA and have been found to contribute to NMDA-dependent LTP through a 20-fold greater permeability for calcium than the other nAChRs (Mansvelder and McGehee 2000; Mansvelder et al. 2002; Gao et al. 2010). The involvement of glutamate and plasticity have been implicated in several drugs of abuse including EtOH and nicotine (Gipson et al. 2013; Berglind et al. 2009; Campbell et al. 2009; Wang et al. 2008). Specifically, glutamate signaling from the mPFC has been shown to play a significant role in both EtOH and nicotine addiction. Several studies have demonstrated that experience with EtOH increases AMPA receptor mediated synaptic transmission and can induce LTP of glutamatergic synaptic afferents onto VTA DA neurons (Stuber et al. 2008; Oliva and Wanat 2016). This has also been associated with increased NMDA receptor expression as well as elevated phosphorylation of GluN2B receptor subtype (Pascual et al. 2009). Research also found elevated levels of

NR1 and GluR1 subunits, indicating higher levels of glutamate receptors, following long-term exposure to EtOH (Ortiz et al. 1995). Activation of nAChRs by nicotine induces rapid desensitization and internalization, of which α4β2* nAChRs are particularly susceptible (Fenster et al. 1997; Pidoplichko et al. 1997). Despite the rapid transition of α7 nAChRs to a desensitized state, signaling can be spatially and temporally extended through interaction with heterotrimeric GTP-binding proteins (G proteins) via a G protein-binding cluster (GPBC) located on the receptor (Kabbani and Nichols, 2018). Specifically, evidence suggests an agonist induced α7 nAChR interaction with Gαq at the GPBC that activates a signaling pathway resulting in prolonged calcium transients and increased neurotransmitter release via inositol trisphosphate (IP_3) receptor-induced calcium release (IICR; Kabbani and Nichols, 2018). This unique feature of α7 nAChRs could explain the enhanced dopaminergic response to nicotine by the associated increase in expression levels and subsequent glutamatergic signaling onto VTA DA neurons (Figures 15–18) following voluntary adolescent EtOH consumption. Collectively, enhanced DA release by nicotine during adulthood may be partly driven by increased levels of α7 nAChRs and subsequent prolonged excitatory signaling of glutamatergic inputs within the VTA. Future experiments hope to establish a causal link between α7 nAChR expression and the enhanced responsivity to nicotine within the mesolimbic system through pharmacological manipulations.

The ability of EtOH and nicotine to cross sensitize is bidirectional. Adolescent, but not adult rats, administered nicotine consumed more EtOH

during adulthood than saline treated controls as well as an alteration in $GABA_A$ signaling (Thomas et al. 2018). Similarly, exposure to cigarette smoke enhances EtOH consumption during adolescence and adulthood (Burns and Procter, 2013). The current data are first to indicate a persistent alteration in the neurochemical response to nicotine within the mesolimbic DA reward system following voluntary adolescent EtOH consumption. The research described above also suggests EtOH and nicotine actions can interact at specific sites, in addition to independent actions, to increase DA release within the NAc. Thus, for both nicotine and EtOH, adolescent exposure to drugs of abuse produce unique neuroadaptations that cross-sensitizes to others during adulthood. The upregulation of α7 nAChRs in the pVTA may be part of the biological basis for reports in humans that heavy adolescent EtOH use greatly enhances adult nicotine use (Dierker et al. 2013).

Chapter Four: Co-administration of ethanol and nicotine heightens sensitivity to ethanol reward within the nucleus accumbens (NAc) shell: increasing NAc shell BDNF is sufficient to enhance ethanol reinforcement in naïve rats

Alcohol use disorder (AUD) most commonly presents as a polydrug disorder where greater than 85% are estimated to smoke with additional patients co-administering other drugs. Published research often ignores the complex nature of drug use in humans and relies heavily on studies examining ethanol (EtOH) alone. EtOH and nicotine (NIC) co-abuse or exposure results in unique neuroadaptations that are linked to behaviors that promote drug use. The current chapter contains experiments that were conducted to identify some of the unique neuroadaptations within the mesolimbic reward system produced by concurrent EtOH and NIC exposure. Furthermore, these studies aimed to determine whether these alterations impact subsequent EtOH reinforcement. The experiments used four overall groups consisting of vehicle, EtOH or NIC alone, and EtOH+NIC. The effects of drug exposure directly into the posterior ventral tegmental area (pVTA) on stimulated release of glutamate and dopamine in addition to protein expression of brain-derived neurotrophic factor (BDNF) in the nucleus accumbens (NAc) shell were determined. A second experiment investigated the effects of drug pretreatment within the pVTA on the reinforcing properties of EtOH within the NAc shell. The concluding experiment evaluated the effect of NAc shell pretreatment with BDNF on EtOH reward within that region. The data

indicated that only EtOH+NIC administration into the pVTA simultaneously increased glutamate, dopamine, and protein expression of BDNF in the NAc shell. Moreover, only pretreatment of the pVTA with EtOH+NIC enhanced the reinforcing properties of EtOH in the NAc shell. BDNF pretreatment in the NAc shell was also sufficient to enhance the reinforcing properties of EtOH in the NAc shell. The collected data suggest that concurrent EtOH+NIC exposure results in a distinct neurochemical response and neuroadaptations within the mesolimbic reward pathway that alter EtOH reward. The failure to develop successful pharmacotherapeutics for the treatment of AUD could be linked to examining potential targets in models that fail to reflect the neuroadaptations produced by polydrug exposure.

Introduction

Alcohol is the most commonly used drug of abuse worldwide and accounts for more than 5% of global deaths annually (WHO, 2018). Nearly 14% of the US population currently meets the diagnostic criteria for alcohol use disorder (AUD) which is associated with numerous adverse health, economic, and social consequences (Hasin and Grant 2015; Grant et al. 2015, 2017). Importantly, the majority of individuals with AUD have comorbid disorders involving other drugs of abuse. This includes an estimated 65 - 90% that also use tobacco products comprising the largest group of polysubstance abusers (Falk et al. 2006; Grant et al. 2015; Saha et al. 2018). The high comorbidity of alcohol and tobacco use is associated with more severe levels of dependency and

increased tendency to consume more of each drug (John et al. 2003; McKee et al. 2007). Additionally, concurrent use decreases the likelihood of initiating abstinence (Weinberger et al. 2013; Adams 2017). The propensity to consume alcohol and nicotine together creates an obstacle where each drug hinders successful cessation of the other.

Despite the high levels of alcohol (EtOH) and nicotine (NIC) comorbidity in humans, preclinical research often focuses on investigating the effects of individual drugs of abuse. In order to develop successful therapeutics for AUD as a polydrug disorder, a better understanding of the mechanisms behind these associations is needed. It is necessary to examine the shared neurobiological mechanisms and subsequent effects on behavior by concurrent EtOH and NIC exposure, rather than individually. A growing body of research suggests the interactions between EtOH and NIC may stem from direct and indirect modulation of the mesocorticolimbic pathway as well as dysregulation of neurotransmitter regulatory systems (Van Skike et al. 2016). Previous research from our laboratory has demonstrated EtOH and NIC co-exposure consistently results in unique alterations to the mesocorticolimbic pathway that is not evident with either drug alone. For example, chronic self-administration of EtOH and NIC, but not equivalent amounts of EtOH or NIC alone, increases sensitivity to the reinforcing properties of NIC within the nucleus accumbens (NAc) shell. This chronic exposure also alters glutamate neurochemistry within the medial prefrontal cortex (Deehan et al. 2015). Tizabi and colleagues (2002, 2007) demonstrated that systemically administered EtOH and NIC generated an

additive effect when measuring stimulated dopamine release in the NAc shell. EtOH and NIC was likewise found to interact within the posterior ventral tegmental area (pVTA) and produce synergistic effects on drug reward in the pVTA (Truitt et al. 2015). Furthermore, a single administration of EtOH and NIC directly into the pVTA results in numerous gene changes within the NAc shell which was not observed following comparable acute administration of EtOH or nicotine alone (Truitt et al. 2015). Specifically, acute intra-pVTA co-exposure to EtOH and NIC produced a robust upregulation of brain-derived neurotrophic factor (*bdnf*) and a significant reduction in glial cell-derived neurotrophic factor (*gdnf*) mRNA in the NAc shell (Truitt et al. 2015).

Few studies have investigated BDNF protein expression following exposure to EtOH and NIC within the mesolimbic pathway and how these changes may influence reward sensitivity. The NAc shell is well established as a critical brain region involved in motivated behaviors and reward (Sesack and Grace 2010). Preclinical models of EtOH and/or NIC associated drug-seeking and reward indicate the behavioral changes are potentially linked to altered neurotransmitter and BDNF levels in the NAc (Deehan et al. 2015; Truitt et al. 2015).

The overall hypothesis is that limited and concurrent exposure to EtOH and NIC results in distinct neurochemical responses within the mesolimbic system that facilitate the development of neuroadaptations and promote future drug self-administration. The goal of the present study was to characterize alterations in neurochemistry and behavior that are specific to co-exposure of

naïve animals to EtOH and NIC. The first experiment determined how acute intra-pVTA microinfusions of vehicle, EtOH, NIC, or EtOH+NIC stimulate dopamine and glutamate release within the NAc shell. Building on previous work, the second experiment determined alterations in BDNF protein levels of the NAc shell produced by acute or repeated intra-pVTA exposure to vehicle, EtOH, NIC, or EtOH+NIC as well as a temporal assessment of the observed effects. The biological consequence of concurrent exposure to EtOH+NIC was assessed by determining the effects of repeated intra-pVTA pretreatment with vehicle, EtOH, NIC, or EtOH+NIC on the reinforcing properties of EtOH in the NAc shell. The significant elevation in levels of BDNF was identified as a potential mediator for the enhancement of EtOH reward within the NAc shell following exposure to EtOH+NIC in the pVTA. Thus, exogenous BDNF was directly infused into the NAc shell to determine whether elevated levels of BDNF were sufficient to enhance EtOH reward and increase self-administration.

Materials and methods

Subjects

Adult male Wistar rats (Envigo, Indianapolis, IN) were utilized in the current study. All animals were maintained in fully accredited facilities by the Association for the Assessment and Accreditation of Laboratory Animal Care. Research protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and followed the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (NRCC. 2011).

Animals were received around post-natal day (PND) 70 and double-housed in plastic shoebox cages for at least 2 weeks prior to use. All rats were kept on a 12-hour reverse light cycle with lights off at 0900. Food and water were available *ad libitum* to all animals throughout the experiment. Experiments were carried out following PND 90.

EtOH and/or NIC microinjection-microdialysis and dialysate analysis

The microinjection-microdialysis procedure was implemented as previously described (Ding et al. 2009, Toalston et al. 2014; Deehan et al. 2018). Briefly, rats were stereotaxically implanted with two ipsilateral guide cannulas in the right hemisphere. A 22-guage microinjection cannula (Plastics One, Inc., Roanoke, VA) was aimed 1.0 mm above the pVTA with an 18-guage microdialysis cannula implanted 3.0 mm above the NAc shell while under isoflurane anesthesia. Coordinates for the pVTA and NAc shell were AP -5.6 mm, ML +2.1 mm, DV -8.2 mm and +1.4 mm, ML +2.2 mm, DV -5.3 mm, respectively (Paxinos and Watson, 1998). Cannulas were implanted at a 10° angle and inserted with sterile stylets while no experiments were being carried out to prevent blockage and infection. After surgery, rats were single housed in new shoebox cages and allowed one week of recovery. Rats were also habituated to the experimental housing and handled daily during this time.

Loop-style microdialysis probes were constructed as previously described with an active length of 2.0 mm and molecular weight cut-off of 13 kDa (Kohl et al. 1998; Engleman et al. 2000; Ding et al. 2009; Toalston et al. 2014). Rats were

placed under isoflurane anesthesia and the microdialysis probes were inserted into the NAc shell by extending 3.0 mm below the guide cannula. The microinjection-microdialysis procedure was carried out the following day.
All experiments were carried out in awake freely moving animals. Subjects were placed in the experimental housing and the microdialysis probes were connected to a syringe pump to continuously perfuse the NAc shell with artificial cerebrospinal fluid (aCSF) at a rate of 1 µL/minute. Microdialysis aCSF was made up of 140.0 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 2.0 mM Na_2HPO_4 • 7H2O, and 1.0 mM $MgCl_2$ with a pH 7.2 to 7.4. Samples were collected in 20-minute intervals beginning with five baseline samples that start follow a 90-minute washout period.

Rats received challenge microinjections directly into the pVTA consisting of aCSF, EtOH only, NIC only or combinations of EtOH and NIC. Nicotine HCl (Sigma-Aldrich, St. Louis, MO) concentrations were calculated based on the salt. Ethyl alcohol (95.0%; McCormick Distilling Co., Weston, MO) was diluted to the desired concentration with aCSF. Treatment groups are as follows: aCSF, 100 mg% EtOH, 150 mg% EtOH, 10 µM NIC, 50 µM NIC, 100 mg% EtOH+10 µM NIC, or 150 mg% EtOH+50 µM NIC. Drug concentrations were selected based on previous intracranial self-administration (ICSA) studies within the pVTA (Gatto et al. 1994; Rodd et al. 2004; Hauser et al. 2014a; Truitt et al. 2015). Lower drug doses represent “subthreshold” levels that are not readily self-administered. Higher doses considered “suprathreshold” have been consistently shown to be reinforcing and are self-administered at levels significantly greater than vehicle

controls. Passive microinjections were carried out with an electrolytic microinfusion transducer (EMIT) system (Rodd-Henricks et al. 2000; Ding et al. 2009). Subjects underwent 30 pulse injections during a 10-minute period designed to match ICSA levels (Rodd-Henricks et al. 2000; Toalston et al. 2014). Pulse injections infused 100 nl over 5 seconds that was followed by a 15-second timeout for a total of 3 µl.

Following the intra-pVTA microinjection challenge, six 20-minute samples of dialysate samples were also collected from the NAc shell into tubes containing five µl of 0.1 N perchloric acid. Samples were frozen on dry ice and stored at -80 °C until analysis for dopamine and glutamate content with high performance liquid chromatography (HPLC). The current microdialysis study utilized 57 male Wistar rats. Subjects were randomly assigned to one of seven microinjection conditions with n = 7–10/group for dopamine analysis and n = 6–9/group for glutamate analysis.

Dopamine content was determined as described previously with a reversed-phase HPLC system and electrochemical detection (Engleman et al. 2000, 2006; Ding et al. 2009; Toalston et al. 2014). Dialysate samples were loaded into a 10 µl loop and injected onto an analytical column (Hypersil BDS C18, 150 mm x 2.1 mm, 3 µm, Thermo Fisher Scientific, Waltham, MA). Mobile phase was made up of 0.1 mM EDTA, 8 mM KCl, 50 mM phosphoric acid, 100 mg/L OSA, and 10% MeOH with a pH of 6.0. Detection occurred with a glassy-carbon electrode and an amperometric detector. The oxidation potential was set at 350 mV with a sensitivity of 100 pA/V (Decade II EC Detector, Antec Scientific,

Netherlands). Dopaminergic signal analysis was resolved with ChromePerfect chromatography data system (Justice Innovations, Inc., Palo Alto, CA).

Extracellular glutamate concentrations were also determined using a reversed-phase HPLC system with electrochemical detection as described previously (Donzanti and Yamamoto 1988; Ding et al. 2012, 2013; Deehan et al. 2015). Precolumn glutamate derivatization was carried out on 5 µl of dialysate with *o*-phthalaldehyde and performed using an ESA Model 542 autosampler (ESA, Inc., Chelmsford, MA). Mobile phase was composed of 35% MeOH and 100 mM $Na_2HPO_4 \bullet 7H2O$ with a pH of 6.75. Samples were injected onto a Hypersil ODS C18 column (150 mm x 2.1 mm, 3 µm, Thermo Fisher Scientific, Waltham, MA). Separation and detection of glutamate was done with an amperometric detector (BAS LC-4C, Bioanalytical Systems, Inc., West Lafayette, IN) with the oxidation potential of 550 mV and sensitivity of 0.2 µA. Glutamatergic signal analysis was again determined with ChromePerfect data system (Justice Innovations, Inc., Palo Alto, CA).

EtOH and/or NIC microinjections, tissue preparation, and BDNF ELISA

A separate cohort of male Wistar rats were stereotaxically implanted with a guide cannula aimed at the pVTA. The same passive microinjection protocol and EMIT units outlined in the microinjection-microdialysis procedure were used to administer aCSF, EtOH only, NIC only or combinations of EtOH and NIC directly to the pVTA (n = 77). Additionally, rats either underwent a single (acute) microinjection session or one microinjection session every day (repeated) for

seven consecutive days with n = 4–8/group (Figure 21, top panel). Single session treatment groups include aCSF, 100 mg% EtOH, 150 mg% EtOH, 10 μM NIC, 50 μM NIC, 100 mg% EtOH+10 μM NIC, or 150 mg% EtOH+50 μM NIC. Repeated microinjection session groups consisted of the same treatments. Following pVTA pretreatment, rats were returned to home-cage for 3 or 24 hours.

At the assigned time points, rats were deeply anesthetized with isoflurane and rapidly decapitated. Brains were removed immediately and flash frozen in isopentane on dry ice. Brains were stored at -80 °C until ready for analysis. Serial coronal sections at a thickness of 300 μm were collected with a freezing microtome. Micro-punches containing the ipsilateral NAc shell were obtained using a 1 mm diameter Harris micro-punch (Electron Microscopy Sciences, Hatfield, PA) as previously described (McBride et al. 2009; Ding et al. 2013). The tissue was immediately homogenized in 150 μl of ice-cold N-PER lysis buffer with Halt Protease inhibitor cocktail (Thermo Scientific, Waltham, MA). Samples were incubated at 4 °C for 20 minutes on a nutator followed by centrifugation at 10,000 x g for 20 minutes. Supernatant protein content was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Protein concentrations were determined with 10 μl of lysate and 200 μl of working reagent on a microplate reader (Bio-Rad, Hercules, CA). Samples were analyzed in duplicate and absorbance values averaged. The levels of BDNF in the NAc shell were determined with a rat ELISA kit for BDNF from RayBiotech, Inc. (Norcross, GA) according to the manufacturer's guidelines. ELISA samples were run in duplicate and absorbance values normalized to total protein content.

EtOH and/or NIC microinjections and ICSA

Another cohort of male Wistar rats were stereotaxically implanted with microinjection cannulas placed ipsilaterally 1.0 mm dorsal to the pVTA as well as the NAc shell. The NAc shell coordinates for this experiment were different than those of the microdialysis study with +1.4 mm, ML +2.2 mm, DV -7.3 mm relative to bregma (Paxinos and Watson, 1998). The EMIT units and passive microinjection protocol were again utilized to administer aCSF, EtOH only, NIC only, or EtOH and NIC. Subjects were randomly assigned to undergo one or seven microinjection sessions for aCSF, 100 mg% EtOH, 10 μM NIC, or 100 mg% EtOH+10 μM (n = 105). Following the final pretreatment session, rats were maintained in home-cage for 2 days prior to initiating ICSA testing for EtOH reward in the NAc shell (Figure 21, bottom panel).

Standard two-lever operant chambers contained within sound attenuating boxes were used in all ICSA experiments as described previously (Rodd et al. 2004; Engleman et al. 2009; Hauser et al. 2014a; Toalston et al. 2014; Deehan et al. 2015; Truitt et al. 2015). The EMIT system was used to control microinfusions of drug or vehicle. On testing days, subjects had stylets removed and an airtight tank fitted with an injection cannula containing the designated infusate tightened onto the NAc shell guide cannula extending 1.0 mm past the end. A single, non-contingent administration of infusate was delivered during insertion of the injector to prime the system and avoid trapping air. ICSA test sessions occurred every other day and lasted 4 hours. The position of active lever and inactive lever were counterbalanced between subjects but remained

the same for individual rats. Throughout the 4-hour session, depression of the active lever, set to a fixed ratio-1 (FR1) schedule of reinforcement, delivered a 100 nl infusion over the course of 5 seconds followed by a 5 second timeout. During this period, responses on the active lever did not generate additional infusions but were recorded. Responses on the inactive lever were always recorded but did not cause an infusion. Subjects were randomly assigned aCSF, 75 mg% EtOH, or 125 mg% EtOH to self-administer into the NAc shell with n = 6–10/pretreatment/EtOH concentration. As done with the previous experiments targeting the pVTA, the EtOH doses for ICSA were chosen based on previous work investigating EtOH reward specifically within the NAc shell of female P rats (Engleman et al. 2009). Subjects received their respective doses of aCSF or EtOH during acquisition sessions 1 through 4. All subjects then received only aCSF during extinction sessions 5 and 6. The original concentration was then made available during session 7 for reinstatement. The vehicle control group received only infusions of aCSF for all seven sessions.

BDNF microinjections and ICSA

A final cohort of male Wistar rats were stereotaxically implanted with a single microinjection cannula aimed 1.0 mm dorsal to the NAc shell (+1.4 mm, ML +2.2 mm, DV -7.3 mm, 10° angle). Rats were single housed after surgery and allowed at least one week of recovery. During this time, rats were habituated to the testing chambers and handled daily. Microinjections of BDNF (0 or 0.125 µg) into the NAc shell were delivered using a PHD ULTRA syringe pump (Harvard

Apparatus, Holliston, MA) in a volume of 1.0 µl administered over 2 minutes, with the injector left in place for an additional 2 minutes to allow sufficient diffusion. Recombinant human BDNF (Sigma-Aldrich, St. Louis, MO) was prepared in aCSF immediately prior to microinfusion. Doses of BDNF were chosen based on previously published reports (Lu et al. 2004; Vargas-Perez et al. 2014; Bobadilla et al. 2018; Haun et al. 2018).

Subjects were randomly assigned to undergo one or seven microinfusion sessions for aCSF or BDNF (n = 61). Rats were then maintained in home cage for 2 days prior to beginning the ICSA protocol for EtOH reward in the NAc shell described for the previous experiment (Figure 21, bottom panel). Rats were randomly assigned to self-administer aCSF, 75 mg% EtOH, or 125 mg% EtOH with n = 6–8/pretreatment/EtOH concentration. During the first four sessions, subjects received their assigned doses of aCSF or EtOH. Only aCSF was available to self-infuse during sessions 5 and 6. During session 7, the original concentration of aCSF or EtOH was made available. The control group only received infusions of aCSF for all seven sessions.

Histology

Upon completion of the experiments, rats were euthanized and a 1% solution of bromophenol blue was infused into each cannula. Brains were quickly removed, frozen on dry ice, and stored at -80 °C until examined. Serial sections were collected with a thickness of 40 µm on a cryostat microtome for verification of microdialysis probe, microinjection, and/or ICSA placements. Cannula

placements determined to be outside the region of interest were excluded from further analysis.

Statistical Analysis

Microdialysis data are expressed as a percentage of basal extracellular dopamine and glutamate values to correct for subject-to-subject variability (Engleman et al. 2000). Basal baseline values for dialysate were calculated as the mean of the three samples collected prior to the challenge microinjection. The effects of pVTA EtOH, NIC, or EtOH+NIC microinjections on extracellular dopamine and glutamate were analyzed using a Time × Treatment mixed analysis of variance (ANOVA). Post-hoc comparisons to identify significance were Student's two-tailed *t* for within group differences or Tukey's b for between group differences. BDNF protein levels were normalized to total protein content for each sample and presented as a percentage of control treatments. Data were analyzed with mean BDNF pg/mg protein calculated for each treatment group. BDNF protein levels were comparable to those previously reported for the NAc (Liu et al. 2018). These values were then compared using a univariate ANOVA ($p < 0.05$). The ICSA data were analyzed with a Pretreatment × EtOH Dose × Session mixed ANOVA on the mean number of self-infusions during sessions 1–4. Additionally, responses on active and inactive levers were examined for each group with a Pretreatment × EtOH Dose × Session × Lever mixed ANOVA with repeated measures on session and lever. Post hoc tests were used when significant main effects were found ($p < 0.05$). Extinction was determined through

comparison of active lever responses during sessions 4–6. Reinstatement was identified by comparing responses on the active lever during sessions 5–7.

Results

Microinjection-Microdialysis

Dopamine levels in the NAc shell dialysate had a mean of 1.31 ± 0.11 nM during baseline sample collection and were comparable to previous reports (Engleman et al. 2000; Ding et al. 2009; Deehan et al. 2018). Mean levels of glutamate during baseline were 1.42 ± 0.22 µM and are also consistent with past studies (Ding et al. 2016). Importantly, microdialysis can only provide an accurate assessment of extracellular neurotransmitter levels through a quantitative No-Net-Flux protocol that was not applied in the present study.

The overall analysis for stimulated dopamine release revealed a significant Time × Treatment interaction term ($F_{48,400} = 2.38$; $p < 0.001$; Figure 19). Individual ANOVAs indicate a significant effect of Treatment during the first three sample periods following microinjection (F values > 2.49; *p* values < 0.04). Tukey's b post-hoc comparisons found that only rats treated with combinations of EtOH and NIC (100 mg% EtOH+10 µM NIC and 150 mg% EtOH+50 µM NIC) had significantly higher dopamine levels within the NAc shell than rats treated with aCSF during the first sample period. During the second and third 20-minute samples, rats treated with 100 mg% EtOH+10 µM NIC had significantly elevated dopamine levels compared to aCSF controls.

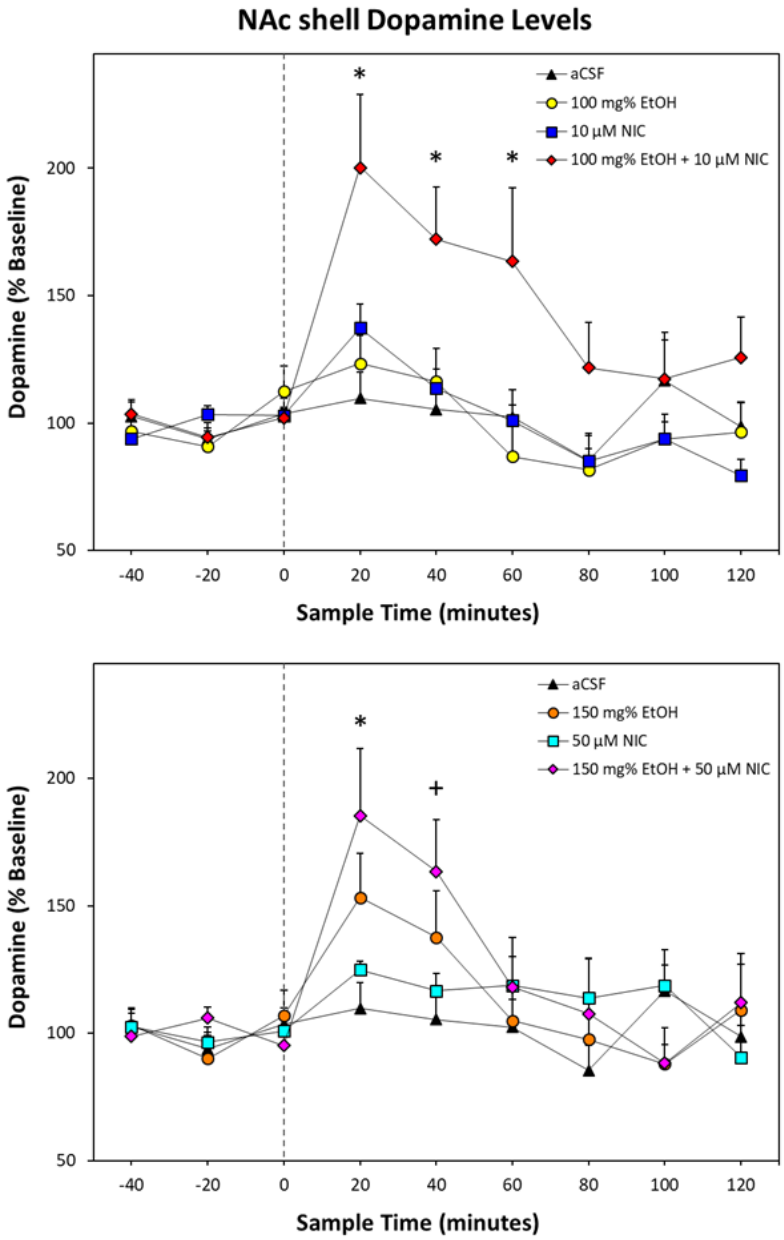

Figure 19. Average change in DA of rats microinjected with EtOH and/or NIC. Depicts the mean (+SEM) percent change in DA levels (n = 7–10/group) within the NAc shell of rats receiving intra-pVTA infusions of aCSF control, 100 mg% EtOH, 150 mg% EtOH, 10 µM NIC, 50 µM NIC, 100 mg% EtOH+10 µM NIC, or 150 mg% EtOH+50 µM NIC. Treatment groups were analyzed together but presented separately for clarity. Asterisk (*) indicates significantly elevated DA levels in both EtOH+NIC treated groups compared to aCSF controls. Plus sign (+) indicates 150 mg% EtOH+50 µM NIC and 150 mg% EtOH treatment groups were significantly higher than respective baseline levels.

Within-subject examination of individual microinjection groups revealed a significant effect of 150 mg% EtOH ($F_{8,48} = 2.95$; $p = 0.009$), 10 μM NIC ($F_{8,56} = 7.15$; $p < 0.001$), 50 μM NIC ($F_{8,56} = 2.23$; $p = 0.038$), 100 mg% EtOH+10 μM NIC ($F_{8,72} = 9.12$; $p < 0.001$), and 150 mg% EtOH+50 μM NIC ($F_{8,48} = 6.51$; $p < 0.001$). Compared to corresponding baseline levels, 10 μM NIC ($t_7 = 3.96$, $p = 0.005$) and 50 μM NIC ($t_7 = 5.26$, $p = 0.001$) significantly increased extracellular dopamine during the first sample to 137% and 125%, respectively. Additionally, 150 mg% EtOH and 150 mg% EtOH+50 μM NIC (t values > 2.61, p values < 0.04) significantly increased dopamine levels over baseline during the first and second sample periods. Finally, post hoc comparisons of rats treated with 100 mg% EtOH+10 μM NIC indicated dopamine levels of the first three post microinjection samples were significantly higher than baseline (t values > 2.38, p values < 0.05) at 201%, 172%, and 163%.

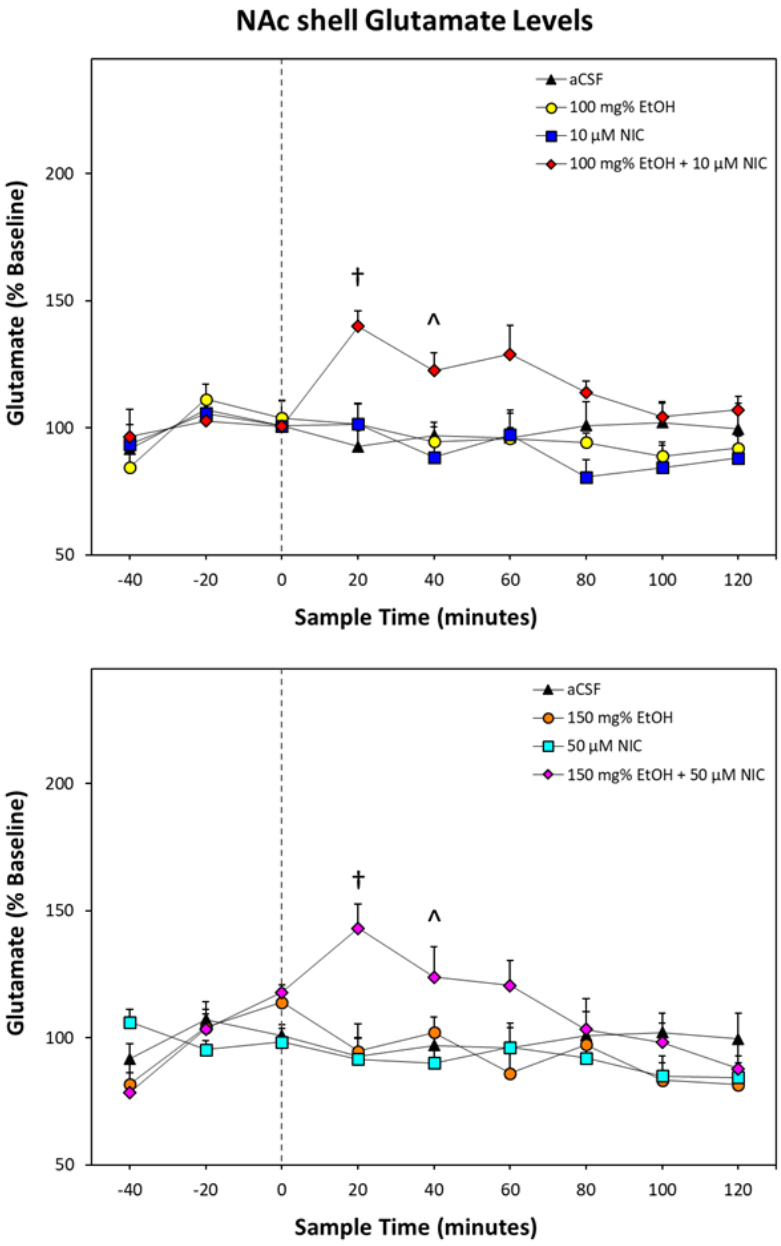

Figure 20. Average change in glutamate of rats microinjected with EtOH and/or NIC. Depicts the mean (+SEM) percent change in glutamate levels (n = 6–10/group) within the NAc shell of rats receiving intra-pVTA infusions of aCSF control, 100 mg% EtOH, 150 mg% EtOH, 10 µM NIC, 50 µM NIC, 100 mg% EtOH+10 µM NIC, or 150 mg% EtOH+50 µM NIC. Treatment groups were analyzed together but presented separately for clarity. Dagger (†) indicates significantly higher glutamate levels in both EtOH+NIC treated groups than all other treatment groups. Carrot (^) indicates significantly greater glutamate levels in both EtOH+NIC treated groups than those treated with 10 µM NIC or 50 µM NIC.

Repeated-Measure ANOVA of stimulated glutamate release indicated a significant effect of Time × Treatment interaction ($F_{48,376} = 2.28$; $p < 0.001$; Figure 20). Reducing the interaction term to individual ANOVAs performed for each sample time period revealed significant differences between the treatment groups during the first four samples collected post-microinjection (F values > 2.52; *p* values < 0.04). Tukey's b post hoc comparisons indicated during the first 20-minute sample period that rats microinjected with combinations of EtOH and NIC had significantly elevated glutamate levels compared to all other treatment groups. During the second 20-minute time period following microinjections, post hoc comparisons revealed glutamate levels in the NAc shell were higher in rats treated with combinations of EtOH and NIC compared to rats that received 10 μM NIC or 50 μM NIC. The third 20-minute time period following microinjections indicated glutamate levels in the NAc shell were elevated only in rats treated with 100 mg% EtOH+10 μM NIC compared to rats that received 150 mg% EtOH. The fourth post-microinjection sample collected indicated that glutamate levels were higher in rats that received 100 mg% EtOH+10 μM NIC compared to the 10 μM NIC treatment group. Within-subject examination of individual microinjection treatments revealed a significant effect of 100 mg% EtOH+10 μM NIC ($F_{8,64} = 4.48$; $p < 0.001$) and 150 mg% EtOH+50 μM NIC ($F_{8,40} = 7.88$; $p < 0.001$). Post hoc comparisons indicated glutamate levels within the NAc shell of the first post microinjection sample were significantly greater than baseline (*t* values > 3.97, *p* values < 0.003) at 140% and 143%.

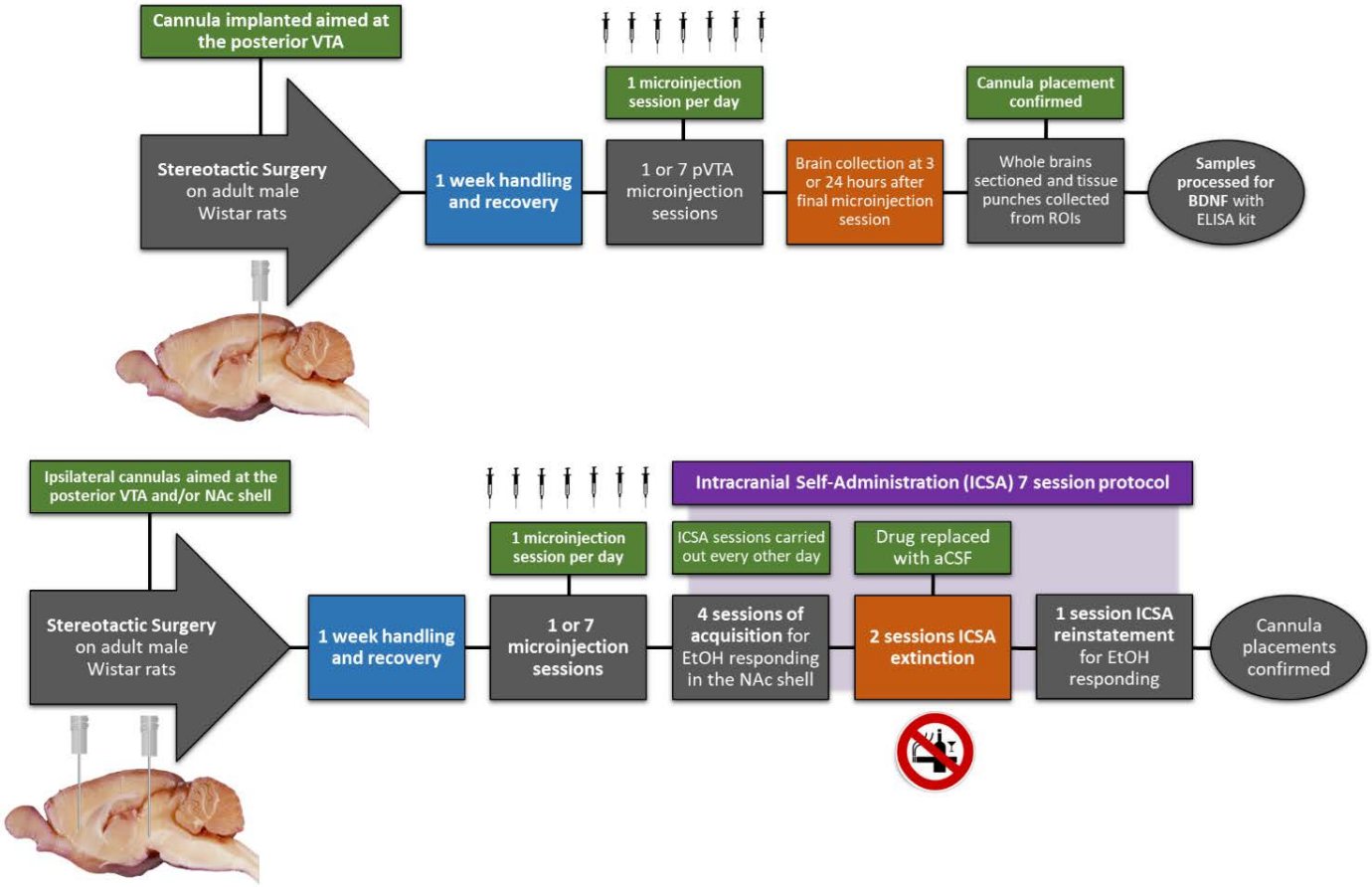

Figure 21. Illustration of the experimental timelines. Top panel: Experimental timeline that investigated the effect of 1 (acute) or 7 (repeated) intra-pVTA microinjection sessions on NAc shell BDNF protein levels. Treatment groups included doses of EtOH and/or NIC (aCSF control, 100 mg% EtOH, 150 mg% EtOH, 10 μM NIC, 50 μM NIC, 100 mg% EtOH+10 μM NIC, or 150 mg% EtOH+50 μM NIC). Bottom panel: Experimental timeline to determine the effects of repeated microinjection sessions of aCSF, EtOH, NIC, EtOH+NIC, or BDNF on EtOH reward in the NAc shell.

NAc shell BDNF following intra-pVTA drug pretreatment

Previous research has demonstrated significant changes to neurotrophic gene expression within the NAc shell following microinjections of EtOH and NIC directly into the pVTA. In order to expand on these results, an ELISA kit was used to assess BDNF protein levels in the NAc shell of rats treated with sub- and suprathreshold doses of EtOH and/or NIC within the pVTA. The results indicate that only combinations of EtOH and NIC microinjected directly into the pVTA significantly increased BDNF protein in the NAc shell compared to aCSF 3 hours following treatment ($F_{6,43} = 4.66$, $p = 0.001$; Figure 22, top panel). Microinjection of subthreshold or suprathreshold EtOH+NIC increased NAc shell BDNF to 179% and 168% of aCSF levels, respectively. Microinjections of EtOH only and NIC only showed no change to BNDF levels. The next experiment aimed to determine whether the significantly elevated BDNF is sustained over repeated exposure to EtOH+NIC or was a transient phenomenon. Repeated microinjection sessions of subthreshold EtOH+NIC into the pVTA were found to consistently increase the protein expression of BDNF in the NAc shell 3 hours after the final session over both acute and repeated aCSF treatments ($F_{4,26} = 3.11$, $p = 0.032$; Figure 22, middle panel). Next, BDNF levels in the NAc shell were determined 24 hours after both acute and repeated microinjection sessions of EtOH+NIC. The data indicated that repeated microinjections of EtOH+NIC into the pVTA resulted in a lasting elevation of BDNF in the NAc shell at 24 hours that was not present after a single administration of EtOH+NIC ($F_{2,21} = 11.37$, $p < 0.001$; Figure 22, bottom panel).

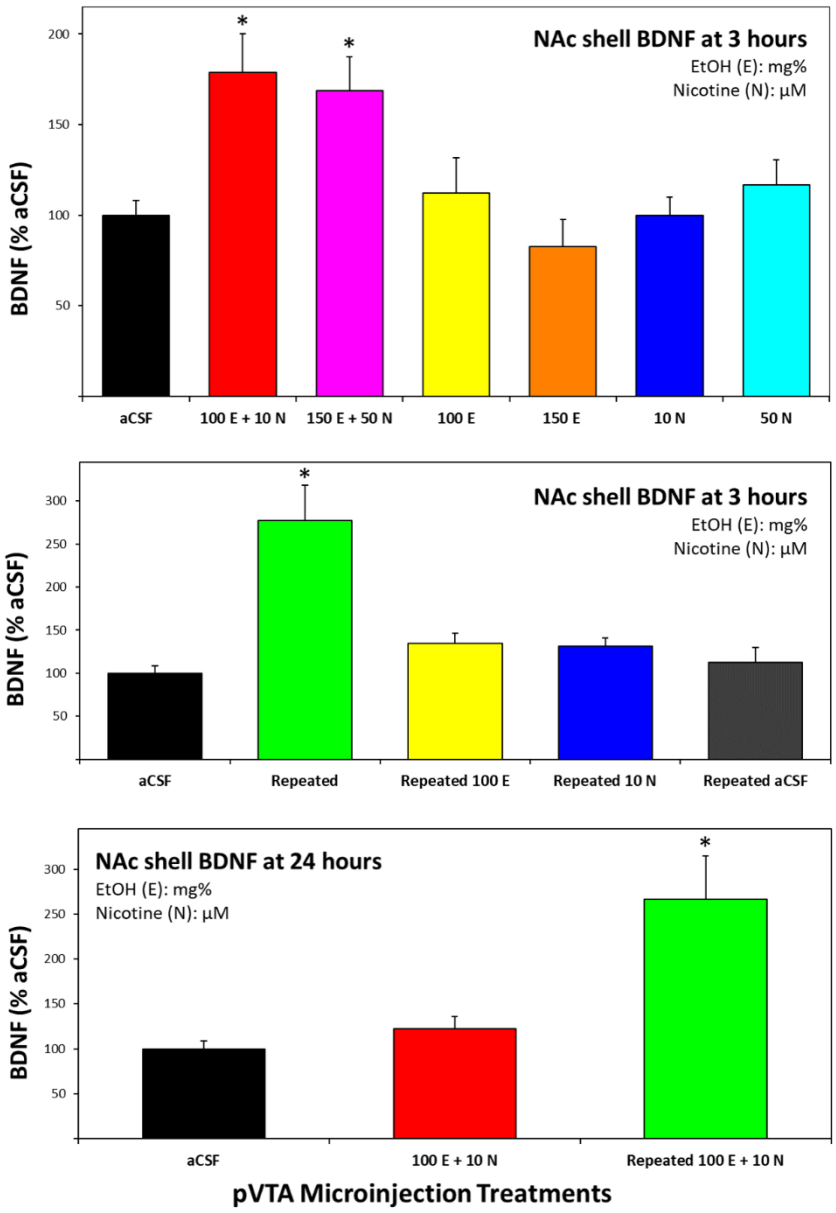

Figure 22. Average change in BDNF relative to aCSF controls of rats microinjected with EtOH and/or NIC. Top Panel: Mean (+SEM) BDNF levels in the NAc shell relative to aCSF controls 3 hours following a single microinjection session into the pVTA (n = 5–8/group). Middle Panel: Mean (+SEM) BDNF levels in the NAc shell relative to aCSF controls 3 hours following 7 microinjection sessions into the pVTA (n = 6–8/group). Bottom Panel: Mean (+SEM) BDNF levels in the NAc shell relative to aCSF controls 24 hours following 1 or 7 microinjection sessions into the pVTA (n = 4–6/group). Asterisk (*) indicates significantly greater BDNF levels compared to aCSF controls.

EtOH reward in the NAc shell following intra-pVTA drug pretreatment

A mixed ANOVA was conducted on the average number of infusions received during the first four acquisition ICSA sessions of rats pretreated with intra-pVTA EtOH and/or NIC (Figure 23, top panel). The analysis revealed a significant Pretreatment × EtOH Dose interaction ($F_{6,77} = 3.89$, $p = 0.002$). Reducing the interaction term was performed by examining the average number of infusions for each EtOH dose. Rats allowed to self-administer aCSF or 125 mg% EtOH showed no effect of pretreatment on the number of infusions received (F values < 0.59; *p* values > 0.63). Conversely, there was a significant difference in rats allowed to self-administer a subthreshold EtOH dose of 75 mg% between rats pretreated with repeated EtOH+NIC and those pretreated with repeated aCSF, EtOH only, or NIC only ($F_{3,26} = 9.15$, $p < 0.001$). Tukey's b post hoc indicated that rats pretreated with repeated EtOH+NIC received significantly more self-infusions than all other groups (Figure 23, top panel). In rats pretreated with repeated aCSF, EtOH, or NIC, there was a significant effect of EtOH dose on the number of self-infusions (F values > 6.27; *p* values < 0.01). Post hoc comparisons revealed that rats pretreated with repeated aCSF, EtOH, or NIC self-infused significantly more of the 125 mg% EtOH suprathreshold dose compared to infusions of aCSF or subthreshold 75 mg% EtOH. In rats pretreated with repeated EtOH+NIC, there was also a significant effect of EtOH dose on the number of self-infusions ($F_{2,16} = 9.56$, $p = 0.002$). Interestingly, post hoc comparisons indicated that rats given repeated EtOH+NIC pretreatment self-

infused significantly more 125 mg% EtOH as well as the subthreshold 75 mg% EtOH dose compared to aCSF self-infusions.

The number of active and inactive lever responses across the 7 ICSA sessions were examined and revealed a significant Pretreatment × EtOH Dose × Lever × Session interaction term ($F_{36,456} = 3.90$, $p < 0.001$). Overall analysis of inactive lever responses indicated no significant interaction Pretreatment × EtOH Dose × Session interaction ($F_{36,456} = 0.83$, $p = 0.743$). However, analysis of active lever responses revealed a significant Pretreatment × EtOH Dose × Session interaction ($F_{36,456} = 2.69$, $p < 0.001$). Further investigation of active lever responses by all pretreatment groups allowed to self-administer aCSF or 125 mg% EtOH into the NAc shell did not indicate significant interactions of Pretreatment × Session (F values < 0.73, $p > 0.774$). Examining active lever responses by all pretreatment groups allowed to self-administer 75 mg% EtOH revealed a significant Pretreatment × Session interaction ($F_{18,156} = 5.27$, $p < 0.001$). Performing individual ANOVAs on active lever responses for 75 mg% EtOH in each session indicated significant differences for all sessions ($F_{3,26}$ values > 5.09, $p < 0.007$) except during extinction sessions 5 and 6 ($F_{3,26}$ values < 1.09, $p > 0.371$). Tukey's b post hoc analysis revealed that rats pretreated with repeated EtOH+NIC responded significantly more on the active lever for 75 mg% EtOH than all other groups during sessions 1–4 and 7.

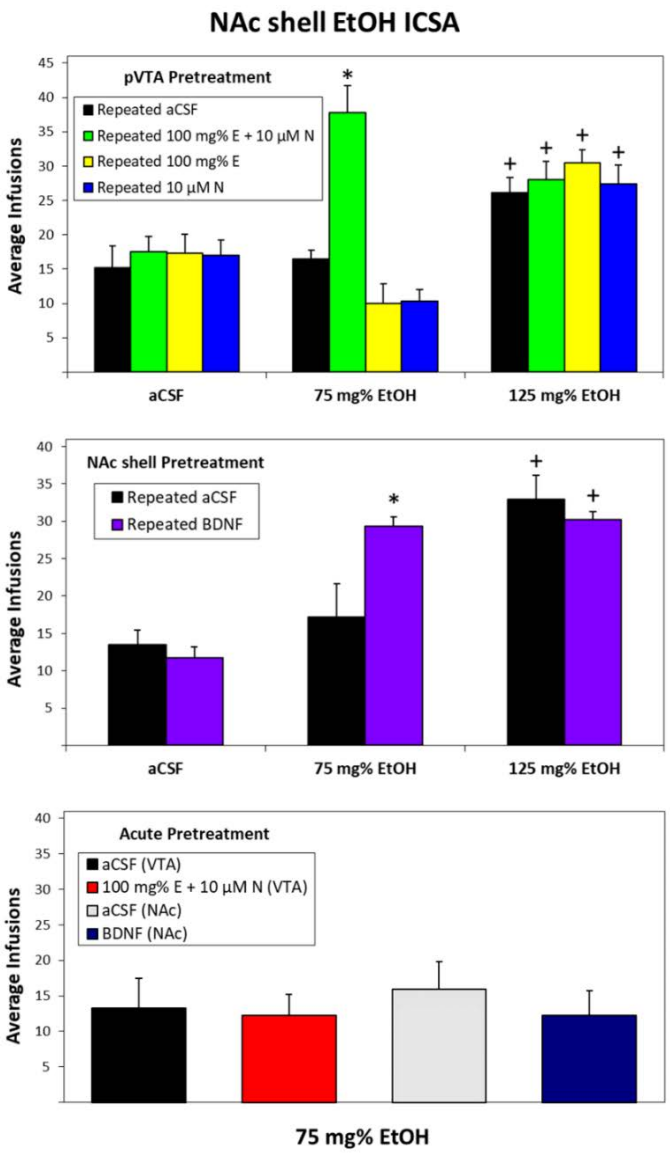

Figure 23. Average number of EtOH self-infusions into the NAc shell by rats pretreated with aCSF, EtOH and/or NIC, or BDNF. Top Panel: Mean (+SEM) number of self-infusions of EtOH during the first 4 ICSA sessions by rats pretreated with 7 microinjection sessions of aCSF, EtOH, NIC, or EtOH+NIC (n = 4–8/group). Middle Panel: Mean (+SEM) number of self-infusions of EtOH during the first 4 ICSA sessions by rats pretreated with 7 microinjection sessions of aCSF or BDNF (n = 6–8/group). Bottom Panel: Mean (+SEM) number of self-infusions of a subthreshold EtOH dose during the first 4 ICSA sessions by control rats pretreated with a single microinjection session of aCSF, EtOH+NIC, or BDNF (n = 8/group). Asterisk (*) indicates significantly more self-infusions of 75 mg% EtOH compared to all other pretreatment groups. Plus sign (+) indicates significantly more self-infusions than corresponding pretreatment groups allowed to self-administer aCSF.

EtOH reward in the NAc shell following repeated intra-NAc BDNF pretreatment

Next, a mixed ANOVA was conducted on the average number of infusions received over the first four ICSA sessions of rats pretreated with intra-NAc shell BDNF or aCSF (Figure 23, middle panel). The analysis revealed a significant interaction of Pretreatment × EtOH Dose ($F_{2,39} = 5.11$, $p = 0.011$). The interaction term was then reduced by examining the average number of infusions for each EtOH dose. There was no effect of pretreatment found on number of infusions received by rats allowed to self-infuse aCSF or 125 mg% EtOH (F values < 0.74, *p* values > 0.406). However, there was a significant difference found in rats allowed to self-administer the subthreshold dose of 75 mg% EtOH between those pretreated with repeated BDNF and rats that received repeated aCSF ($F_{1,11} = 6.05$, $p = 0.032$). Tukey's b post hoc analysis revealed rats pretreated with BDNF had significantly more self-infusions than aCSF pretreated rats. The Pretreatment × EtOH Dose interaction term was then reduced by investigating the average number of infusions for each pretreatment. In rats that received repeated intra-NAc shell infusions of aCSF, there was a significant effect of EtOH dose on the number of self-infusions ($F_{2,20} = 10.60$, $p = 0.001$). Post hoc comparisons indicated that rats pretreated with repeated aCSF within the NAc shell self-infused significantly more 125 mg% EtOH compared to infusions of aCSF or subthreshold 75 mg% EtOH. Rats that underwent repeated BDNF infusions also showed a significant effect of EtOH dose on the number of self-infusions ($F_{2,19} = 64.84$, $p < 0.001$). Tukey's b post hoc analysis revealed that unlike repeated

aCSF pretreatment, repeated BDNF pretreated rats received significantly more self-infusions for both the subthreshold 75 mg% EtOH dose as well as the suprathreshold dose of 125 mg% EtOH.

Responses across the 7 ICSA sessions on the active and inactive levers revealed a significant overall interaction term of Pretreatment × EtOH Dose × Lever × Session ($F_{12,228} = 2.26$, $p = 0.01$). Reducing this interaction with analysis of inactive lever responses indicated no significant effect of Pretreatment × EtOH Dose × Session ($F_{12,228} = 1.39$, $p = 0.173$). Next, active lever responses were examined and further analysis indicated a significant Pretreatment × EtOH Dose × Session interaction ($F_{12,228} = 2.19$, $p = 0.013$). Additional analysis of active lever responses by rats allowed to self-administer aCSF into the NAc shell did not indicate a significant interaction of Pretreatment × Session ($F_{6,84} = 0.61$, $p = 0.723$). However, examination of active lever responses by BDNF or aCSF pretreated groups allowed to self-administer 75 mg% EtOH or 125 mg% EtOH revealed significant Pretreatment × Session interactions ($F_{6,66} = 2.77$, $p = 0.021$ and $F_{6,78} = 2.64$, $p = 0.022$; respectively). Though rats pretreated with repeated BDNF had higher mean responses on the active lever than those pretreated with aCSF during all sessions, individual ANOVAs performed on active lever responses for 75 mg% EtOH revealed only statistically significant differences during sessions 2 and 7 (F values > 12.07, p values < 0.005). Analysis of active lever responses for 125 mg% EtOH indicated session 5 was significantly different between BDNF and aCSF pretreated groups.

A final analysis aimed to determine whether single microinjection sessions of intra-NAc shell BDNF, intra-pVTA 100 mg% EtOH+10 μM NIC, and corresponding aCSF controls altered subthreshold EtOH self-administration within the NAc shell (Figure 23, bottom panel). The results indicate no significant difference between pretreatment groups and self-infusion rates at similar levels to control groups from previous experiments ($F_{3,28} = 1.32$, $p = 0.287$; Figure 23).

Discussion

The results of the present study indicate that co-administration of EtOH+NIC directly into the pVTA induced a distinct neurochemical response, compared to either drug alone, within the NAc shell (Figures 19, 20, 22, and 23). A single intra-pVTA microinjection session of EtOH+NIC produced significant increases in dopamine, glutamate, and BDNF that were not present for either drug alone. Moreover, repeated exposure to EtOH+NIC resulted in a significant elevation of endogenous BDNF in the NAc shell lasting at least 24 hours that was not seen after a single pretreatment session. This repeated intra-pVTA exposure to EtOH+NIC also produced an enhancement of EtOH reward within the NAc shell (Figure 22). Repeated microinfusions of exogenous BDNF into the NAc shell recapitulated the increased sensitivity to the reinforcing properties of EtOH within the NAc shell produced by intra-pVTA pretreatment with EtOH+NIC. Collectively, these results indicate that co-administration of EtOH+NIC functions within the pVTA to generate discrete responses and subsequent

neuroadaptations of the NAc shell via BDNF that augment EtOH reward in that region.

It is well established that exposure to EtOH or NIC elevates VTA neuronal activity and is associated increased dopamine release within the NAc shell (Di Chiara and Imperato 1988; Lecca et al. 2006; Ding et al. 2009; Robinson et al. 2009). Furthermore, combinations of EtOH and NIC have been shown to interact within the VTA to enhance dopaminergic activity and increase NAc shell dopamine release beyond the levels of either drug alone (Figure 19; Clark and Little 2004; Tizabi et al. 2002, 2007). The mechanisms responsible for the exaggerated dopamine response produced by EtOH+NIC could be through interactions with shared neurotransmitter systems in addition to independent actions. A specific site of interaction between nicotine and EtOH within the pVTA occurs through α6-containing nicotinic receptors, which with sufficient activation initiates a signaling cascade resulting in enhanced AMPA receptor function (Engle et al. 2015). Other neuronal nicotinic acetylcholine receptors (nAChRs) have been investigated as sites contributing to the disproportionate co-use of EtOH and NIC (Tizabi et al. 2002, 2007; Van Skike et al. 2016; Adams 2017). Within the VTA, nAChRs are expressed on dopamine, GABA, and glutamate neurons and the distinct subunit combinations alter channel properties as well as agonist binding. Importantly, the high-affinity α4β2 is most densely expressed on dopaminergic VTA neurons while the α7-homomeric nAChRs are primarily located on excitatory glutamatergic terminals that synapse on dopamine neurons. Both of which have been demonstrated to increase dopamine signaling via

activation by EtOH and/or NIC (reviewed in Morel et al. 2018). Evidence suggests that EtOH may potentiate the actions of most nAChRs, through stabilization of the receptor open state, while inhibiting nicotine-induced receptor desensitization (Aistrup et al. 1999; Marszalec et al. 1999; Zuo et al. 2004). Furthermore, another member of the Cys-loop ion channel superfamily, the serotonin-3 (5-HT_3) receptor, has been implicated as a convergent site of action for EtOH and NIC (Lovinger and White 1991; Breitinger et al. 2001; Rodd et al. 2007, 2010; Hauser et al. 2014b). EtOH increases the excitatory action of 5-HT_3 receptor activation and promotes stimulated dopamine release within the mesolimbic system (Campbell et al. 1996; Rodd et al. 2010). Together, evidence suggests intra-pVTA co-administration of EtOH and NIC interact and promote enhanced dopamine release within the NAc shell over either drug alone through convergent actions on specific nAChRs and 5-HT_3 receptors.

It has been suggested that glutamatergic inputs of the NAc play a critical role in the initiation and learning of drug-seeking behaviors (Spencer et al. 2016). These projections into the NAc are involved in stimuli response to previously established associations and cues (Scofield et al. 2016). Several studies have demonstrated alterations in basal extracellular glutamate levels or clearance rates within the NAc following both voluntary and forced chronic exposure to EtOH and/or NIC (Figure 20; Reid et al. 2000; Liechti et al. 2007; Lallemand et al. 2011; Ding et al. 2013; Griffin et al. 2014; Carcoba et al. 2018). The significant increase seen here of stimulated glutamate release within the NAc shell of only naïve rats exposed to intra-pVTA EtOH+NIC suggests a transient alteration in

post-synaptic glutamate signaling. The prior studies examining NAc shell basal glutamate levels demonstrated changes in glutamatergic regulatory genes or proteins following chronic, repeated exposure or withdrawal conditions providing support for the reported results (Ding et al. 2013; Griffin et al. 2014; Carcoba et al. 2018). The present study reveals a response to EtOH+NIC under acute conditions by naïve rats suggesting a unique pVTA activation profile that occurs immediately and is a distinct response than either drug alone. The simultaneous increase of both dopamine and glutamate, specific to EtOH+NIC, provides some explanation for the subsequent gene changes and neurochemical alterations within the NAc shell found in previous work (Deehan et al. 2015; Truitt et al. 2015).

A single co-administration of EtOH+NIC combinations into the pVTA results in significantly increased BDNF protein in the NAc shell at three hours post-treatment (Figure 22). Repeated exposure to EtOH+NIC was found to result in BDNF increases lasting more than 24 hours, which was not observed following equivalent treatment of EtOH or NIC alone (Figure 22). These results are consistent with mounting research implicating a role for BDNF in the regulation of drug-related behaviors (Figure 23; Lu et al. 2004; Logrip et al. 2009; Vargas-Perez et al. 2014; Bobadilla et al. 2018; Haun et al. 2018).

BDNF is part of the neurotrophin family and via binding with tropomyosin receptor kinase B (TrkB) promotes neuronal development, modulates synaptic function, and regulates synaptic plasticity (Park and Poo 2013; Nakahata and Yasuda 2018). A number of studies have examined BDNF in response to acute

exposure, seeking, dependence, and relapse to EtOH or NIC (Logrip et al. 2015; Machaalani and Chen 2018). Preclinical research has demonstrated a relationship between BDNF expression and drug-related changes in the brain that is dependent upon a variety of factors including duration of exposure, drug dose, and region of interest (Lu et al. 2004; Logrip et al. 2009; Vargas-Perez et al. 2014; Bobadilla et al. 2018; Haun et al. 2018). In order to determine whether the associated neurochemical changes in the current study had any significant impact on reward-related behaviors, rats were allowed to self-administer EtOH into the NAc shell following single or repeated intra-pVTA microinjections of aCSF, EtOH, NIC, or EtOH+NIC. EtOH reward in the NAc shell was only significantly altered in rats pretreated with EtOH+NIC in the pVTA. Specifically, the average number of self-infusions for 75 mg% EtOH during the first 4 sessions by aCSF, EtOH, or NIC pretreated rats was 10-17 infusions/session. In contrast, rats pretreated with EtOH+NIC self-infused 75 mg% EtOH significantly more with an average of almost 38/session into the NAc shell. The apparent shift in sensitivity to EtOH reward within the NAc shell suggests pVTA co-exposure to EtOH+NIC results in unique alterations with the mesolimbic dopamine system.

Rats pretreated with repeated BDNF were the only group that readily self-infused 75 mg% EtOH into the NAc shell. Single infusion sessions of aCSF, BDNF, or EtOH+NIC did not alter sensitivity to EtOH reward. Additionally, there was no effect of repeated BDNF pretreatment on self-infusions for aCSF or 125 mg% EtOH, a dose known to be reinforcing within the NAc shell. Taken together, the data indicate that direct pretreatment of the NAc shell with exogenous BDNF

is sufficient to recapitulate previous results demonstrating repeated exposure to EtOH+NIC within the pVTA increases the sensitivity to the rewarding properties of EtOH in the NAc shell. One caveat of these studies is that the elevation of BDNF in the NAc shell following microinjections of EtOH+NIC into the pVTA was not blocked through direct manipulation. Future work is planned to reduce TrkB receptor expression with viral knockdown to establish the necessity of BDNF signaling via TrkB receptor and the altered EtOH sensitivity within the NAc shell. Thus, although BDNF infusions into the NAc shell are sufficient to mimic the effects of intra-pVTA EtOH+NIC and enhance EtOH reinforcement in the NAc shell, other factors could be involved in mediating these changes.

Collectively, the results of the present study support previous work demonstrating that co-administration of EtOH+NIC produces a unique initial physiological response which facilitates the development of neuroadaptations within the mesolimbic reward pathway (Figures 19, 20, 22, and 23; Tizabi et al. 2002, 2007; Truitt et al. 2015). Multiple reports have indicated that naltrexone and varenicline, the pharmacological ‘gold standards’ for treating AUD and nicotine dependence, both fail to alter the concurrent co-administration of EtOH+NIC (Van Skike et al. 2016; Waeiss et al. 2018; Maggio et al. 2018). The current results provide clear preclinical data indicating that EtOH+NIC co-administration results in a unique neurological cascade that is not observed following comparable exposure to EtOH or NIC alone. Evaluating potential pharmacotherapeutics for the treatment of AUD or nicotine dependence using a single drug of abuse fails to reflect the reality of how humans ingest EtOH and

NIC. This method of investigating individual drugs of abuse is likely a major reason that efficacious treatments for these substance disorders has not occurred. Future research should focus on co-exposure models to better elucidate the specific effects of EtOH+NIC within the CNS if better pharmacotherapeutics are to be developed.

Chapter Five: Conclusions

Summary

The series of experiments reported above examined different factors related to the disproportionate rates of alcohol and nicotine comorbidity. The regularity of concurrent use has demonstrated that each drug essentially creates a barrier to the cessation of the other. This is especially clear in research investigating naltrexone and varenicline, the preferred treatments for AUD and nicotine dependence. Previous investigations consistently demonstrate the vast majority of treated individuals will relapse within one year (Garbutt et al. 2005; Volkow and Skolnick 2012). Each chapter aimed to identify specific features associated with the interactions alcohol and nicotine with different experimental models and approaches. The overall goal was to provide some insight into the complex neurobiological mechanisms underlying the co-abuse of alcohol and nicotine to support the development of improved treatments.

The experiments in the second chapter were designed to determine the efficacy of naltrexone or varenicline in reducing drug intake with a preclinical model of voluntary concomitant consumption of alcohol and nicotine. The alcohol-preferring (P) rat provides a useful genetic model to investigate addiction-like behaviors. P rats were originally developed from a closed-colony of Wistar rats with selection for an alcohol preference using two metrics. First, rats needed to show preference for 10% alcohol over water by a ratio of 2:1 or greater. Additionally, the rats had to consume at least 5 g of alcohol/kg body weight/day.

Relative to humans, that amount would roughly equate to a 165 lb. male drinking a fifth of whiskey per day (Bell et al. 2017). Importantly, P rats will also voluntarily consume oral nicotine sweetened with saccharin or combinations of alcohol and nicotine, which produce significant concurrent blood alcohol and nicotine levels (Hauser et al. 2012b; Deehan et al. 2015). Drug intake of P rats has been demonstrated to produce binge-like blood alcohol levels and blood nicotine levels comparable to that observed in heavy smokers. This offers a powerful preclinical model to examine the neurobiological impact of alcohol and nicotine co-abuse, and as in Chapter Two, the effectiveness of standard pharmacotherapeutics on concurrent drug intake.

Varenicline was found to reduce maintenance of nicotine self-administration and inhibited the expected increase in nicotine self-administration that occurs following a period of deprivation. Notably, there was no effect present on alcohol alone or alcohol and nicotine self-administration under maintenance or relapse conditions. Naltrexone reduced alcohol maintenance and relapse intake of alcohol while demonstrating no effect on nicotine alone or alcohol and nicotine self-administration. Furthermore, relapse consumption was increased and prolonged in rats concurrently consuming alcohol and nicotine regardless of treatment with the FDA approved pharmacotherapeutics. These findings support the hypothesis that the failure to develop efficacious treatments for AUD or nicotine dependence may partly stem from investigating drugs alone, rather than with the more accurate comorbid condition.

The third chapter investigated another important aspect of alcohol and nicotine co-use by identifying potential long-term repercussions of peri-adolescent alcohol drinking on the mesolimbic pathway. Specifically, this study determined the impact of adolescent alcohol drinking on NAc shell DA response to nicotine microinjected directly into the pVTA during adulthood. P rats were again used for the ability to voluntarily consume binge-like levels of alcohol.

Peri-adolescent alcohol consumption was found to induce a shift in the dose response curve for nicotine microinjected into the pVTA to stimulate DA release in the NAc shell. Microinjections of the lowest dose of nicotine stimulated DA release in rats that consumed alcohol during adolescence, but not adult drinkers or naïve rats. Peri-adolescent alcohol drinking also increased the percentage of DA released in the NAc shell produced by all nicotine doses compared to adult drinkers or naïve rats. Additionally, alterations to a potential interaction site for alcohol and nicotine was identified. Rats allowed to consume alcohol during adolescence had significantly greater levels of α7 nAChR in the pVTA compared to naïve rats. These data indicate that exposure to alcohol during adolescence results in distinct and lasting neuroadaptations that enhance and prolong the neurochemical response to nicotine within the pVTA. Persistent alterations to the mesolimbic reward pathway provide some explanation for human studies reporting increased tobacco use during adulthood after heavy adolescent alcohol use (Dierker et al. 2013). The cross-sensitization to nicotine in adulthood and inflated adult risk for drug dependency could, in part, be

predicated upon alterations to α7 nAChR within the mesolimbic pathway produced by adolescent drinking.

The fourth chapter extended previous work by exploring the effects of acute or repeated exposure to alcohol and/or nicotine within the pVTA on the neurochemical response in the NAc shell. A second experiment aimed to determine whether repeated exposure to alcohol and nicotine within the pVTA produced neuroadaptations within the NAc shell that altered subsequent alcohol reward. Finally, the role of elevated NAc shell BDNF in the enhanced sensitivity to the reinforcing properties of alcohol following exposure to alcohol and nicotine was investigated with Wistar rats.

A single exposure to combinations of alcohol and nicotine in the pVTA resulted in a significant increase of DA and glutamate release within the NAc shell not seen with either drug alone. This acute pVTA exposure to alcohol and nicotine also increased BDNF in the NAc shell that was not observed following comparable alcohol or nicotine treatments. Increased BDNF was sustained at least 24 hours after repeated exposure to alcohol and nicotine. This effect was not present following acute alcohol and nicotine treatment. Alcohol reward in the NAc shell was found to be significantly altered in rats pretreated with repeated alcohol and nicotine in the pVTA. Interestingly, the enhancement in alcohol reward within the NAc shell could be recapitulated with exogenous BDNF infusions in the same region. These data indicate that a single exposure to pharmacologically relevant levels of alcohol and nicotine in the pVTA results in neurochemical changes within the mesolimbic pathway that, with repeated

exposure, can enhance reward sensitivity to drugs of abuse. This lasting elevation of glutamate produced specifically by combinations of alcohol and nicotine, may initially support neuroadaptations that enhance the rewarding action of alcohol within the mesolimbic pathway. The role of BDNF in plasticity, development, learning, and memory suggests the sustained increases within the NAc shell could partly mediate the enhanced propensity to self-administer lower doses of alcohol. Overall, the data clearly indicate that co-exposure within the mesolimbic reward pathway creates distinct neuroadaptations that may promote addiction.

In summary, these data demonstrate the importance of investigating AUD and nicotine dependence as a polydrug disorder rather than in isolation. Researching AUD as a comorbid condition more accurately represents the disorder as it occurs in humans. Chapter Two highlighted the utility of investigating potential therapeutics in a model of concurrent alcohol and nicotine consumption. This preclinical study reflects similar findings in humans that show some effectiveness with the targeted reinforcers while failing to reduce simultaneous intake. Chapter Three provided support for epidemiological studies indicating that adolescent alcohol drinking is associated increased risk of nicotine use as an adult. Lasting neurobiological changes within the mesolimbic system were readily apparent in response to nicotine and alterations in protein expression. Chapter Four built upon findings that consumption of either alcohol or nicotine increases the likelihood of using the other as well as the amount. Brief and repeated exposure to alcohol and nicotine within the mesolimbic pathway

increased sensitivity to alcohol reward following a specific neurochemical response. Collectively, these results demonstrate the necessity of researching alcohol and nicotine simultaneously.

It is important to develop a robust foundation of the pharmacological interactions of alcohol and nicotine to discover novel targets through valid models of polysubstance abuse. However, there is also a critical need to better understand the subpopulations within those with AUD. This integration of both components will greatly enhance the generation of novel medications and improved therapeutic approaches to treat addiction.

Limitations and future directions

Findings from the aforementioned experiments have yielded a significant amount of novel information about the mesolimbic system under conditions of, or related to, the co-use/abuse of alcohol and nicotine. Additionally, this work established a basis on which new research questions can be interrogated. Though the results described in Chapters Two and Three were compelling, a number of limitations create the opportunity for additional experiments to support and extend the conclusions. For example, the experiments in Chapters Two and Three utilized exclusively female rats. It has become increasingly apparent there is a growing need to examine drugs of abuse as well as therapeutics in both sexes. Significant differences between male and female response to drugs of abuse in preclinical models have been discovered at distinct biological levels. For example, recent work has indicated that the response to alcohol within the

amygdala is sex dependent in some cases. Outbred female rats have a blunted cell type-specific response to alcohol within the amygdala compared to male rats (Logrip et al. 2017). Additionally, unpublished data from our laboratory have indicated that Wistar females will consistently self-administer less than half the alcohol dose required for males to develop self-administering behaviors in specific brain regions. Gene expression analysis has also indicated that there are innate sex differences for cholinergic and dopaminergic related genes, which are impacted by alcohol exposure.

This research reiterates the necessity to fully develop the co-abuse model employed in Chapter Two by conducting experiments on male and female rats. The benefit becomes apparent when determining efficacy of potential therapeutics with known and unknown sex differences in drug pharmacokinetics and pharmacodynamics that can influence dependence and addiction as well as side effects of drugs. Furthermore, adding male cohorts to the experiments in Chapter Three would demonstrate whether the increased sensitivity to nicotine and elevated response during adulthood was specific to female adolescent drinkers. Chapters Two and Three would also benefit from the inclusion of a non-selected rat line. The P rat provides the tremendous advantage of voluntary self-administration and consumption. However, direct comparison between males and females as well as rat strains would generate the strongest data and the potential identification of key genetic or sex differences.

There are a number of experiments possible in Chapter Four that would substantially bolster the findings and answer key questions. Moreover, expanding

on data from Chapter Four provides the opportunity to make general hypotheses regarding co-exposure to alcohol and nicotine and subsequent mechanistic changes. First, the addition of pretreatments, as done in the ICSA experiments, to the micro-micro study would offer important temporal information on stimulated DA and glutamate. Identifying whether the increased neurotransmitter release by alcohol and nicotine was a transient phenomenon, remained at the same level, or was sensitized by repeated exposure would offer insight into the mechanisms and pathways responsible for altered alcohol reward. The addition of a complete dose-response to alcohol and nicotine combinations would also clarify whether the neurochemical response was synergistic or additive in nature. Holding one drug constant while increasing the other would indicate the individual contributions to the induced neurotransmitter release. Following these experiments with pharmacological manipulations could then identify the integral systems involved. Dependent upon the neurotransmitter temporal analysis, the concurrent administration of DA, glutamate, or BDNF antagonists during the alcohol and nicotine pretreatment would indicate which, or if, signaling through these receptor systems is necessary for the change in alcohol reward. The combination of findings described in Chapter Four with the addition of these experiments would provide a much clearer picture of the specific pathways responsible and time course of neuroadaptations following co-exposure to alcohol and nicotine.

Importantly, BDNF levels should be investigated through Western blot analysis. The use of ELISA provides a quantitative measure of BDNF content

within a given sample. However, this particular ELISA uses an antibody for an immunogen region corresponding to amino acids 128 - 247 from recombinant human BDNF (accession P23560), which is located within the mature form. BDNF is initially translated as a pre-pro-protein that is sequentially cleaved into the pro-form and then into mature BDNF. The significance of this process is that pro-BDNF binds with much higher affinity to p75 neurotrophin receptor ($p75^{NTR}$) and activates a signaling cascade that leads to cell degeneration and apoptosis (Park and Poo 2013). In order to confirm the elevated protein levels detected via ELISA are mature-BDNF, rather than pro-BDNF, a Western blot would indicate heavier bands at the predicted molecular weights of 13.2 – 15.9 kDa for the mature form rather than ~32 kDa of pro-BDNF (Nikulina et al. 2014; Foltran and Diaz 2016).

Convincing evidence for neuroadaptations within the mesolimbic pathway following co-exposure to alcohol and nicotine could be generated from a dendritic spine analysis. The significant role of BDNF in promoting neuronal development, modulating synaptic function, and regulating synaptic plasticity could be partially quantified in the NAc shell after drug treatment (Park and Poo 2013; Nakahata and Yasuda 2018). Increased spine numbers and size within the NAc shell would suggest the prolonged elevation in BDNF protein is altering the reward circuit at the synaptic level. Another important study to build on these findings should determine where the BDNF is localized within the NAc shell (Figure 2). Under physiological conditions, the NAc contains low levels of BDNF that is predominately transported to the ventral striatum from the PFC and VTA.

Previous work by Truitt and colleagues (2015) found significantly greater BDNF mRNA within the NAc shell following a similar procedure. Localizing BDNF to a specific neuronal subset within the NAc would provide important information regarding the specific circuits involved and how these relate to the behavioral output. This indicates BDNF may differentially regulate actions depending on the anatomical source (Nikulina et al. 2014; Bobadillo et al. 2018).

Results from the current report have provided important information about how co-use/abuse of alcohol and nicotine generates unique responses within the mesolimbic pathway and subsequent neuroadaptations. A consistent theme throughout the findings is that simultaneous exposure to alcohol and nicotine induces alterations distinct from either drug alone. Exposure to alcohol and nicotine is hypothesized to increase DA and glutamate release within the NAc shell through enhanced activation of the VTA (Clark and Little 2004; Tizabi et al. 2004, 2007; Figures 19 and 20). The combination of increased DA and glutamate signaling within the NAc shell then upregulates local BDNF mRNA as well as BDNF protein. Repeated exposure was found to prolong the significant elevation in BDNF allowing continued signaling via TrkB receptor. The resulting effect within the NAc shell is potentially increased spine numbers, size, and dendritic arborization of MSNs (Figure 2; Nikulina et al. 2014). This form of structural plasticity could be initiated through downstream activation of ERK and *de novo* protein synthesis following extended TrkB receptor activation in the NAc. These alterations at the synaptic level reflect a stable molecular event that may explain some of the long-term behavioral changes associated with drug addiction.

Sustained BDNF signaling in the NAc generating increased dendritic arborizations on MSNs could underlie the sensitized behavioral response to alcohol reward and potentially enhance drug-related memories fundamental to relapse and addiction. Additional work is needed on the hypothesized relationship between structural and behavioral plasticity that occurs following exposure to alcohol and nicotine.

Not only do standard pharmacological treatments in a co-abuse model fail to reduce drug intake, there are distinct changes within the mesolimbic pathway resulting from alcohol and nicotine. Undoubtedly, future research is necessary to determine the specific interactions of alcohol and nicotine responsible for the disproportionate rates of co-abuse and key for the treatment of AUD. These studies will both complete the present data and address questions that have arisen from interpretation and conclusions.

References

Abrahao KP, Salinas AG, Lovinger DM (2017) Alcohol and the Brain: Neuronal Molecular Targets, Synapses, and Circuits. Neuron 96(6):1223-1238

Adams S (2017) Psychopharmacology of Tobacco and Alcohol Comorbidity: Review of Current Evidence. Curr Addict Rep 4(1):25-34

Agaku IT, King BA, Husten CG, Bunnell R, Ambrose BK, Hu SS, Holder-Hayes E, Day HR; Centers for Disease Control and Prevention (2014) Tobacco product use among adults--United States, 2012-2013. MMWR Morb Mortal Wkly Rep 63(25):542-7

Agboola SA, Coleman T, McNeill A, Leonardi-Bee J (2015) Abstinence and relapse among smokers who use varenicline in a quit attempt-a pooled analysis of randomized controlled trials. Addiction 110(7): 1182–1193

Aistrup GL, Marszalec W, Narahashi T (1999) Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons. Mol Pharmacol 55(1):39-49

Allgaier C (2002) Ethanol sensitivity of NMDA receptors. Neurochem Int 41:377–382

Anthenelli RM, Benowitz NL, West R, St Aubin L, McRae T, Lawrence D, Ascher J, Russ C, Krishen A, Evins AE (2016) Neuropsychiatric safety and efficacy of varenicline, bupropion, and nicotine patch in smokers with and without psychiatric disorders (EAGLES): a double-blind, randomised, placebo-controlled clinical trial. Lancet 387(10037):2507-20

Anthony JC, Petronis KR (1995) Early-onset drug use and risk of later drug problems. Drug Alcohol Depend 40:9–15

Arendt T, Allen Y, Marchbanks RM, Schugens MM, Sinden J, Lantos PL, Gray JA (1989) Cholinergic system and memory in the rat: effects of chronic ethanol, embryonic basal forebrain brain transplants and excitotoxic lesions of cholinergic basal forebrain projection system. Neuroscience 33: 435–462

Avegno EM, Salling MC, Borgkvist A, Mrejeru A, Whitebirch AC, Margolis EB, Sulzer D, Harrison NL (2016) Voluntary adolescent drinking enhances excitation by low levels of alcohol in a subset of dopaminergic neurons in the ventral tegmental area. Neuropharmacology 110(Part A), 386–395

Baik JH (2013) Dopamine signaling in reward-related behaviors. Front Neural Circuits 7:152

Baker TB, Piper ME, Stein JH, Smith SS, Bolt DM, Fraser DL, Fiore MC (2016) Effects of nicotine patch vs varenicline vs combination nicotine replacement therapy on smoking cessation at 26 weeks: A randomized clinical trial. JAMA 315(4):371–379

Bates ME, Labouvie EW (1997) Adolescent risk factors and the prediction of persistent alcohol and drug use into adulthood. Alcohol Clin Exp Res 21(5):944-50

Beaulieu JM, Espinoza S, Gainetdinov RR (2015) Dopamine receptors - IUPHAR Review 13. Br J Pharmacol 172(1):1-23

Beaulieu JM, Gainetdinov RR (2011) The physiology, signaling and pharmacology of dopamine receptors. Pharmacol Rev 63:182–217

Bell RL, Hauser SR, Liang T, Sari Y, Maldonado-Devincci A, Rodd ZA (2017) Rat animal models for screening medications to treat alcohol use disorders. Neuropharmacology 122:201-243

Benowitz NL (1988) Pharmacologic aspects of cigarette smoking and nicotine addiction. N Eng J Med 319:1318–1330

Berglind WJ, Whitfield TW Jr, LaLumiere RT, Kalivas PW, McGinty JF (2009) A single intra-PFC infusion of BDNF prevents cocaine-induced alterations in extracellular glutamate within the nucleus accumbens. J Neurosci 29(12):3715-9

Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology 191(3), 391-431

Best D, Rawaf S, Rowley J, Floyd K, Manning V, Strang J (2000) Drinking and smoking as concurrent predictors of illicit drug use and positive drug attitudes in adolescents. Drug and Alcohol Dependence. 60(3) 319-321.

Biala G, Budzynska B, Kruk M (2005) Naloxone precipitates nicotine abstinence syndrome and attenuates nicotine-induced antinociception in mice. Pharmacol Rep 57(6):755–760

Blomqvist O, Engel JA, Nissbrandt H, Soderpalm B (1993) The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. Eur J Pharmacol 249:207–213

Bobadilla A, Garcia KC, Chareunsouk V, Hyde J, Camacho DM, Heinsbroek JA, Kalivas PW (2018) Accumbens brain-derived neurotrophic factor (bdnf) transmission inhibits cocaine seeking. Addict Biol [Epub ahead of print]

Bobo JK, Husten C (2000) Sociocultural influences on smoking and drinking. Alcohol Res Health 24(4) 225-232

Bolliger CT, Issa JS, Posadas-Valay R, Safwat T, Abreu P, Correia EA, Park PW, Chopra P (2011) Effects of varenicline in adult smokers: a multinational, 24-week, randomized, double-blind, placebo-controlled study. Clin Ther 33(4):465-77

Boutros N, Semenova S, Markou A (2016) Adolescent alcohol exposure decreased sensitivity to nicotine in adult Wistar rats. Addict Biol 21(4):826-34

Breitinger HG, Geetha N, Hess GP (2001) Inhibition of the serotonin 5-HT3 receptor by nicotine, cocaine, and fluoxetine investigated by rapid chemical kinetic techniques. Biochemistry 40(28):8419-29

Breese GR, Chu K, Dayas CV, Funk D, Knapp DJ, Koob GF, Lê DA, O'Dell LE, Overstreet DH, Roberts AJ, Sinha R, Valdez GR, Weiss F (2005) Stress enhancement of craving during sobriety: a risk for relapse. Alcohol Clin Exp Res 29(2):185–195

Brenhouse HC, Andersen SL (2011) Developmental trajectories during adolescence in males and females: a cross-species understanding of underlying brainchanges. Neurosci. Biobehav. Rev. 35 (8), 1687–1703

Burns BE, Proctor WR (2013) Cigarette smoke exposure greatly increases alcohol consumption in adolescent C57BL/6 mice. Alcohol Clin Exp Res 37 Suppl 1:E364-E372

Campbell AD, Kohl RR, McBride WJ (1996) Serotonin-3 receptor and ethanol-stimulated somatodendritic dopamine release. Alcohol 13(6):569-74

Campbell JC, Szumlinski KK, Kippin TE (2009) Contribution of early environmental stress to alcoholism vulnerability. Alcohol 43(7), 547-554

Carcoba LM, Flores RJ, Natividad LA, O'Dell LE (2018) Amino acid modulation of dopamine in the nucleus accumbens mediates sex differences in nicotine withdrawal. Addict Biol 23(5):1046-1054

Cardoso RA, Brozowski SJ, Chavez-Noriega LE, Harpold M, Valenzuela CF, Harris RA (1999) Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes. J Pharmacol Exp Ther 289(2):774-80

Ceylan-Isik AF, McBride SM, Ren J (2010) Sex difference in alcoholism: who is at a greater risk for development of alcoholic complication? Life Sci 87:133–138

Chi H, de Wit H (2003) Mecamylamine attenuates the subjective stimulant-like effects of alcohol in social drinkers. Alcohol Clin Exp Res 27:780–786

Clark A, Little HJ (2004) Interactions between low concentrations of ethanol and nicotine on firing rate of ventral tegmental dopamine neurones. Drug Alcohol Depend 75(2):199–206

Coleman LG Jr, He J, Lee J, Styner M, Crews FT (2011) Adolescent binge drinking alters adult brain neurotransmitter gene expression, behavior, brain regional volumes, and neurochemistry in mice. Alcohol Clin Exp Res 35(4), 671-688.

Corrigall WA, Coen KM, Adamson KL (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res 653(1–2):278–284

Covey LS, Glassman AH, Stetner F (1999) Naltrexone effects on short term and long-term smoking cessation. J Addict Dis 18(1):31–40

Crews FT, Vetreno RP, Broadwater MA, Robinson DL (2016) Adolescent Alcohol Exposure Persistently Impacts Adult Neurobiology and Behavior. Pharmacol Rev 68(4) 1074-1109.

Czachowski CL, Froehlich JC, DeLory M (2018) The effects of long term varenicline administration on ethanol and sucrose seeking and self-administration in male P rats. Alcohol Clin Exp Res 42(2):453–460

Daeppen JB, Smith TL, Danko GP, Gordon L, Landi NA, Nurnberger JI, Bucholz KK, Raimo E, Schuckit MA (2000) Clinical correlates of cigarette smoking and nicotine dependence in alcohol-dependent men and women. Alcohol Alcohol 35(2):171–175

David SP, Chu IM, Lancaster T, Stead LF, Evins AE, Prochaska JJ (2014) Systematic review and meta-analysis of opioid antagonists for smoking cessation. BMJ Open 4(3):e004393

Dawson DA, Goldstein RB, Chou SP, Ruan WJ, Grant BF (2008) Age at first drink and the first incidence of adult-onset DSM-IV alcohol use disorders. Alcohol Clin Exp Res 32:2149–2160

de Bejczy A, Löf E, Walther L, Guterstam J, Hammarberg A, Asanovska G, Franck J, Isaksson A, Söderpalm B (2015) Varenicline for treatment of alcohol dependence: a randomized, placebo-controlled trial. Alcohol Clin Exp Res 39(11):2189–2199

De Biasi M, Dani JA (2011) Reward, addiction, withdrawal to nicotine. Annu Rev Neurosci 34:105–130

Deehan GJ, Hauser SR, Waeiss RA, Knight CP, Toalston JE, Truitt WA, McBride WJ, Rodd ZA (2015) Co-administration of ethanol and nicotine: the enduring alterations in the rewarding properties of nicotine and glutamate activity within the mesocorticolimbic system of female alcohol-preferring (P) rats. Psychopharmacology 232(23): 4293–4302

Deehan GA Jr, Hauser SR, Getachew B, Waeiss RA, Engleman EA, Knight CP, McBride WJ, Truitt WA, Bell RL, Rodd ZA (2018) Selective breeding for high alcohol consumption and response to nicotine: locomotor activity, dopaminergic in the mesolimbic system, and innate genetic differences in male and female alcohol-preferring, non-preferring, and replicate lines of high-alcohol drinking and low-alcohol drinking rats. Psychopharmacology 235(9) 2755–2769.

Delnevo CD, Wackowski OA, Giovenco DP, Manderski MT, Hrywna M, Ling PM (2012) Examining market trends in the United States smokeless tobacco use: 2005–2011. Tob Control 23(2):107-12

Dhaher R, Toalston JE, Hauser SR, Bell RL, McKinzie DL, McBride WJ, Rodd ZA (2012) Effects of naltrexone and LY255582 on ethanol maintenance, seeking, and relapse responding by alcohol-preferring (P) rats. Alcohol 46(1):17–27

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85:5274–8

Dierker L, Selya A, Piasecki T, Rose J, Mermelstein R (2013) Alcohol problems as a signal for sensitivity to nicotine dependence and future smoking. Drug Alcohol Depend 132:688–693.

DiFranza JR, Guerrera MP (1990) Alcoholism and smoking. J Stud Alcohol 51(2):130–135

Digard H, Errington G, Richter A, McAdams K (2009) Patterns and behaviors of snus consumption in Sweden. Nicotine Tob Res 11: 1175–1181

Digard H, Proctor C, Kulasekaran A, Malmqvist U, Richter A (2013) Determination of nicotine absorption from multiple tobacco products and nicotine gum. Nicotine Tob Res 15(1):255–261

Ding ZM, Rodd ZA, Engleman EA, McBride WJ (2009) Sensitization of ventral tegmental area dopamine neurons to the stimulating effects of ethanol. Alcohol Clin Exp Res 33:1571-81

Ding ZM, Engleman EA, Rodd ZA, McBride WJ (2012) Ethanol increases glutamate neurotransmission in the posterior ventral tegmental area of female wistar rats. Alcohol Clin Exp Res 36(4), 633-640

Ding ZM, Rodd ZA, Engleman EA, Bailey JA, Lahiri DK, McBride WJ. (2013) Alcohol drinking and deprivation alter basal extracellular glutamate concentrations and clearance in the mesolimbic system of alcohol-preferring (P) rats. Addict Biol 18(2):297-306

Ding ZM, Ingraham CM, Rodd ZA, McBride WJ (2016) Alcohol drinking increases the dopamine-stimulating effects of ethanol and reduces D_2 auto-receptor and group II metabotropic glutamate receptor function within the posterior ventral tegmental area of alcohol preferring (P) rats. Neuropharmacology 109:41-48

Dominguez del Toro E, Juiz JM, Peng X, Lindstrom J, Criado M (1994) Immunocytochemical localization of the alpha 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. J Comp Neurol 349(3):325-42

Donoghue K, Elzerbi C, Saunders R, Whittington C, Pilling S, Drummond C (2015) The efficacy of acamprosate and naltrexone in the treatment of alcohol dependence, Europe versus the rest of the world: a meta-analysis. Addiction 110(6):920–930

Donzanti BA, Yamamoto BK (1988) An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. Life Sci 43(11), 913-922

Doyon WM, Thomas AM, Ostroumov A, Dong Y, Dani JA (2013) Potential substrates for nicotine and alcohol interactions: a focus on the mesocorticolimbic dopamine system. Biochem Pharmacol 86(8) 1181-1193.

Ehlers CL, Criado JR, Wills DN, Liu W, Crews FT (2011) Periadolescent ethanol exposure reduces adult forebrain ChAT+IR neurons: correlation with behavioral pathology. Neuroscience 199, 333-345

Engle SE, McIntosh JM, Drenan RM (2015) Nicotine and ethanol cooperate to enhance ventral tegmental area AMPA receptor function via α6-containing nicotinic receptors. Neuropharmacology 91:13-22

Engleman EA, McBride WJ, Wilber AA, Shaikh SR, Eha RD, Lumeng L, Li TK, Murphy JM (2000) Reverse microdialysis of a dopamine uptake inhibitor in the nucleus accumbens of alcohol-preferring rats: effects on dialysate dopamine levels and ethanol intake. Alcohol Clin Exp Res 24(6), 795-801

Engleman EA, Ingraham CM, McBride WJ, Lumeng L, Murphy JM (2006) Extracellular dopamine levels are lower in the medial prefrontal cortex of alcohol-preferring rats compared to Wistar rats. Alcohol 38:5–12

Engleman EA, Ding ZM, Oster SM, Toalston JE, Bell RL, Murphy JM, McBride WJ, Rodd ZA (2009) Ethanol is self-administered into the nucleus accumbens shell, but not the core: evidence of genetic sensitivity. Alcohol Clin Exp Res 33(12):2162-71

Erwin BL, Slaton RM (2014) Varenicline in the treatment of alcohol use disorders. Ann Pharmacother 48(11):1445–1455

Exley R, Maubourguet N, David V, Eddine R, Evrard A, Pons S, Marti F, Threlfell S, Cazala P, McIntosh JM, Changeux JP, Maskos U, Cragg SJ, Faure P (2011) Distinct contributions of nicotinic acetylcholine receptor subunit alpha4 and subunit alpha6 to the reinforcing effects of nicotine. Proc Natl Acad Sci U S A 108(18):7577–7582

Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K, Asplund A, Sjöstedt E, Lundberg E, Szigyarto CA, Skogs M, Takanen JO, Berling H, Tegel H, Mulder J, Nilsson P, Schwenk JM, Lindskog C, Danielsson F, Mardinoglu A, Sivertsson A, von Feilitzen K, Forsberg M, Zwahlen M, Olsson I, Navani S, Huss M, Nielsen J, Ponten F, Uhlén M (2014) Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics 13(2):397-406

Falk DE, Yi HY, Hiller-Sturmhöfel S (2006) An epidemiologic analysis of co-occurring alcohol and tobacco use and disorders: findings from the National Epidemiologic Survey on Alcohol and Related Conditions. Alcohol Res Health 29(3):162-71

Fenster CP, Rains MF, Noerager B, Quick MW, Lester RAJ. Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. J Neurosci 17(15):5747-5759

Floyd EA, Young-Seigler AC, Ford BD, Reasor JD, Moore EL, Townsel JG, Rucker HK (1997) Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol 14(1), 93–98

Foltran RB, Diaz SL (2016) BDNF isoforms: a round trip ticket between neurogenesis and serotonin? J Neurochem 138(2):204-21

Froehlich JC, Fischer SM, Dilley JE, Nicholson ER, Smith TN, Filosa NJ, Rademacher LC (2016) Combining varenicline (Chantix) with naltrexone decreases alcohol drinking more effectively than does either drug alone in a rodent model of alcoholism. Alcohol Clin Exp Res 40(9):1961–1970

Froehlich JC, Nicholson ER, Dilley JE, Filosa NJ, Rademacher LC, Smith TN (2017) Varenicline reduces alcohol intake during repeated cycles of alcohol re-access following deprivation in alcohol-preferring (P) rats. Alcohol Clin Exp Res 41(8):1510–1517

Funk D, Lo S, Coen K, Lê AD (2016) Effects of varenicline on operant self-administration of alcohol and/or nicotine in a rat model of co-abuse. Behav Brain Res 296:157–162

Gao M, Jin Y, Yang K, Zhang D, Lukas RJ, Wu J (2010) Mechanisms involved in systemic nicotine-induced glutamatergic synaptic plasticity on dopamine neurons in the ventral tegmental area. J Neurosci 30, 13814–13825

Garbutt JC, Kranzler HR, O'Malley SS, Gastfriend DR, Pettinati HM, Silverman BL, Loewy JW, Ehrich EW, Vivitrex Study G (2005) Efficacy and tolerability of long acting injectable naltrexone for alcohol dependence: a randomized controlled trial. JAMA 293:1617–1625

Garg BK, Loring RH (2017) Evaluating Commercially Available Antibodies for Rat α7 Nicotinic Acetylcholine Receptors. J Histochem Cytochem 65(9):499-512

Garris PA, Kilpatrick M, Bunin MA, Michael D, Walker QD, Wightman RM (1999) Dissociation of dopamine release in the nucleus accumbens from intracranial self-stimulation. Nature 398(6722), 67-69

Gass JT, Glen WB Jr, McGonigal JT, Trantham-Davidson H, Lopez MF, Randall PK, Yaxley R, Floresco SB, Chandler, LJ (2014) Adolescent alcohol exposure reduces behavioral flexibility, promotes disinhibition, and increases resistance to extinction of ethanol self-administration in adulthood. Neuropsychopharmacology 39 (11), 2570–2583

Gatto GJ, McBride WJ, Murphy JM, Lumeng L, Li T-K (1994) Ethanol self infusion into the ventral tegmental area by alcohol-preferring rats. Alcohol 11:557–564

George O, Lloyd A, Carroll FI, Damaj MI, Koob GF (2011) Varenicline blocks nicotine intake in rats with extended access to nicotine self-administration. Psychopharmacology 213(4):715–722

Gipson CD, Reissner KJ, Kupchik YM, Smith AC, Stankeviciute N, Hensley-Simon ME, Kalivas PW (2013) Reinstatement of nicotine seeking is mediated by glutamatergic plasticity. Proc Natl Acad Sci U S A 110(22):9124-9

Gonzales D, Rennard SI, Nides M, Oncken C, Azoulay S, Billing CB, Watsky EJ, Gong J, Williams KE, Reeves KR (2006)Varenicline, an alpha4beta2

nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking cessation: a randomized controlled trial. JAMA. 296(1):47–55

Grady SR, Drenan RM, Breining SR, Yohannes D, Wageman CR, Fedorov NB, McKinney S, Whiteaker P, Bencherif M, Lester HA, Marks MJ (2010) Structural differences determine the relative selectivity of nicotinic compounds for native α4β2*-, α6β2*-, α3β4*- and α7-nicotinic acetylcholine receptors. Neuropharmacology 58(7):1054–1066

Grant B, Hasin D, Chou S, Stinson F, Dawson D (2004) Nicotine dependence and psychiatric disorders in the United States: results from the national epidemiologic survey on alcohol and related conditions. Arch Gen Psychiatry 61(11):1107–1115

Grant BF, Goldstein RB, Saha TD, Chou SP, Jung J, Zhang H, Pickering RP, Ruan WJ, Smith SM, Huang B, Hasin DS (2015) Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry 72(8):757-66

Grant BF, Chou SP, Saha TD, Pickering RP, Kerridge BT, Ruan WJ, Huang B, Jung J, Zhang H, Fan A, Hasin DS (2017) Prevalence of 12-Month Alcohol Use, High-Risk Drinking, and DSM-IV Alcohol Use Disorder in the United States, 2001-2002 to 2012-2013: Results From the National Epidemiologic Survey on Alcohol and Related Conditions. JAMA Psychiatry 74(9):911-923

Griffin WC 3rd, Haun HL, Hazelbaker CL, Ramachandra VS, Becker HC (2014) Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. Neuropsychopharmacology 39(3):707-17

Grucza R, Bierut L (2006) Co-occurring risk factors for alcohol dependence and habitual smoking: update on findings from the Collaborative Study on the Genetics of Alcoholism. Alcohol Res Health 29(3):172–178

Grüsser SM, Wrase J, Klein S, Hermann D, Smolka MN, Ruf M, Weber-Fahr W, Flor H, Mann K, Braus DF, Heinz A (2004) Cue-induced activation of the striatum and medial prefrontal cortex is associated with subsequent relapse in abstinent alcoholics. Psychopharmacology (Berl) 175(3):296-302

Gueorguieva R, Wu R, Donovan D, Rounsaville BJ, Couper D, Krystal JH, O'Malley SS (2010) Naltrexone and combined behavioral intervention effects on trajectories of drinking in the COMBINE study. Drug Alcohol Depend 107(2–3):221–229

Gulliver S, Rohsenow DJ, Colby SM, Dey AN, Abrams DB, Niaura RS, Monti PM (1995) Interrelationship of smoking and alcohol dependence, use and urges to use. J Stud Alcohol 56(2):202–206

Gurley DA, Lanthorn TH (1998) Nicotinic agonists competitively antagonize serotonin at mouse 5-HT3 receptors expressed in Xenopus oocytes. Neurosci Lett 247(2–3):107–110

Hasin DS, Grant BF (2015) The National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) Waves 1 and 2: review and summary of findings. Soc Psychiatry Psychiatr Epidemiol 50(11):1609-40

Haun HL, Griffin WC, Lopez MF, Solomon MG, Mulholland PJ, Woodward JJ, McGinty JF, Ron D, Becker HC (2018) Increasing Brain-Derived Neurotrophic Factor (BDNF) in medial prefrontal cortex selectively reduces excessive drinking in ethanol dependent mice. Neuropharmacology 140, 35–42

Hauser SR, Getachew B, Oster SM, Dhaher R, Ding ZM, Bell RL, McBride WJ, Rodd ZA (2012a) Nicotine modulates alcohol seeking and relapse by alcohol-preferring (P) rats in a time dependent manner. Alcohol Clin Exp Res 36(1):43–54

Hauser SR, Katner SN, Deehan GA Jr, Ding ZM, Toalston JE, Scott BJ, Bell RL, McBride WJ, Rodd ZA (2012b) Development of an oral operant nicotine/ethanol co-use model in alcohol-preferring (P) rats. Alcohol Clin Exp Res 36:1963–1972

Hauser SR, Bracken AL, Deehan GA, Toalston JE, Ding ZM, Truitt WA, Bell RL, McBride WJ, Rodd ZA (2014a) Selective breeding for high alcohol preference increases the sensitivity of the posterior VTA to the reinforcing effects of nicotine. Addict Biol 19(5):800–811

Hauser SR, Deehan GA Jr, Toalston JE, Bell RL, McBride WJ, Rodd ZA (2014b) Enhanced alcohol-seeking behavior by nicotine in the posterior ventral tegmental area of female alcohol-preferring (P) rats: modulation by

serotonin-3 and nicotinic cholinergic receptors. Psychopharmacology (Berl) 231(18), 3745-55

Hauser SR, Knight CP, Truitt WA, Waeiss RA, Holt IS, Carvajal GB, Bell RL, Rodd ZA (2019) Adolescent intermittent ethanol (AIE) increases the sensitivity to the reinforcing properties of ethanol and the expression of select cholinergic and dopaminergic genes within the posterior ventral tegmental area. Alcohol Clin Exp Res doi: 10.1111/acer.14150 [Epub ahead of print]

Hendrickson LM, Guildford MJ, Tapper AR (2013) Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence. Front Psychiatry 4:29

Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S (2010) Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. Neuron 66(6), 896-907

Hodge CW, Haraguchi M, Erickson H, Samson HH (1993) Ventral tegmental microinjections of quinpirole decrease ethanol and sucrose reinforced responding. Alcohol Clin Exp Res 17(2):370–375

Holgate JY, Shariff M, Mu EH, Bartlett S (2017) A rat drinking in the dark model for studying ethanol and sucrose consumption. Front Behav Neurosci 11:29

Hoogendam JM, Kahn RS, Hillegers MHJ, van Buuren M, Vink M (2013) Different developmental trajectories for anticipation and receipt of reward during adolescence. Dev Cogn Neurosci 6:113-124

Howell LL, Kimmel HL (2008) Monoamine transporters and psychostimulant addiction. Biochem Pharmacol 75(1):196-217

Hurt RT, Ebbert JO, Croghan IT, Schroeder DR, Hurt RD, Hays JT (2018) Varenicline for tobacco-dependence treatment in alcohol dependent smokers: a randomized controlled trial. Drug Alcohol Depend 184:12–17

Ikemoto S (2010) Brain reward circuitry beyond the mesolimbic dopamine system: a neurobiological theory. Neurosci Biobehav Rev 35(2):129-50

John U, Hill A, Rumpf H, Hapke U, Meyer C (2003a) Alcohol high risk drinking, abuse and dependence among tobacco smoking medical care patients and the general population. Drug Alcohol Depend 69(2):189–195

John U, Rumpf H, Hanke M, Gerke P, Hapke U (2003b) Estimation of tobacco- or alcohol-attributable disease rates in national hospital care: an approach based on routine in-patient disease register data and systematic diagnosis of alcohol use disorders. Alcohol Alcohol 38(4):339–346

Johnson PL, Molosh A, Fitz SD, Arendt D, Deehan GA, Federici LM, Bernabe C, Engleman EA, Rodd ZA, Lowry CA, Shekhar A (2015) Pharmacological depletion of serotonin in the basolateral amygdala complex reduces anxiety and disrupts fear conditioning. Pharmacol Biochem Behav 138, 174–179.

Johnston LD, O'Malley PM, Bachman JG, and Schulenberg JE (2004) Monitoring the future national survey results on drug use, 1975-2004. Secondary School Students (NIH Publication No. 04-5507), vol. I. National Institute on Drug Abuse, Bethesda, MD.

Jones IW, Wonnacott S (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. J Neurosci 24(50) 11244-11252.

Jonas DE, Amick HR, Feltner C, Bobashev G, Thomas K, Wines R, Kim MM, Shanahan E, Gass CE, Rowe CJ, Garbutt JC (2014) Pharmacotherapy for adults with alcohol use disorders in outpatient settings: a systematic review and meta-analysis. JAMA 311(18):1889–1900

Jorenby DE, Hays JT, Rigotti NA, Azoulay S, Watsky EJ, Williams KE, Billing CB, Gong J, Reeves KR (2006) Efficacy of varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. JAMA 296(1):56–63

Kabbani N, Nichols RA (2018) Beyond the Channel: Metabotropic Signaling by Nicotinic Receptors. Trends Pharmacol Sci. 39(4):354-366

Katner SN, Toalston JE, Smoker MP, Rodd ZA, McBride WJ, Engleman EA (2015) Time-course of extracellular nicotine and cotinine levels in rat brain following administration of nicotine: effects of route and ethanol coadministration. Psychopharmacology (Berl) 232(3):551-60

Kasten CR, Frazee AM, Boehm SL 2nd (2016) Developing a model of limited-access nicotine consumption in C57Bl/6J mice. Pharmacol Biochem Behav 148L:28–37

King A, Cao D, O'Malley S, Kranzler H, Cai X, deWit H, Matthews A, Stachoviak R (2012) Effects of naltrexone on smoking cessation outcomes and weight

gain in nicotine-dependent men and women. J Clin Psychopharmacol 32(5):630–636

Kohl RR, Katner JS, Chernet E, McBride WJ (1998) Ethanol and negative feedback regulation of mesolimbic dopamine release in rats. Psychopharmacology (Berl) 139(1-2):79-85

Koob GF, Volkow ND (2010) 'Neurocircuitry of addiction': Erratum. Neuropsychopharmacology 35(4),1051

Kyzar EJ, Zhang H, Pandey SC (2019) Adolescent alcohol exposure epigenetically suppresses amygdala arc enhancer rna expression to confer adult anxiety susceptibility. Biol Psychiatry doi:10.1016/j.biopsych.2018.12.021 [Epub ahead of print]

Lajtha A, Sershen H (2010) Nicotine: alcohol reward interactions. Neurochem Res 35:1248–1258

Lallemand F, Ward RJ, De Witte P, Verbanck P (2011) Binge drinking +/- chronic nicotine administration alters extracellular glutamate and arginine levels in the nucleus accumbens of adult male and female Wistar rats. Alcohol Alcohol 46(4):373-82

Lammel S, Lim B K, Malenka RC (2014) Reward and aversion in a heterogeneous midbrain dopamine system. Neuropharmacology, 76351-359.

Lê AD, Funk D, Lo S, Coen K (2014) Operant self-administration of alcohol and nicotine in a preclinical model of co-abuse. Psychopharmacology 231(20):4019–4029

Lecca D, Cacciapaglia F, Valentini V, Gronli J, Spiga S, Di Chiara G (2006) Preferential increase of extracellular dopamine in the rat nucleus accumbens shell as compared to that in the core during acquisition and maintenance of intravenous nicotine self-administration. Psychopharmacology 184: 435–46

Liechti ME, Lhuillier L, Kaupmann K, Markou A (2007) Metabotropic glutamate 2/3 receptors in the ventral tegmental area and the nucleus accumbens shell are involved in behaviors relating to nicotine dependence. J Neurosci 27(34):9077-85

Liu D, Tang QQ, Yin C, Song Y, Liu Y, Yang JX, Liu H, Zhang YM, Wu SY, Song Y, Juarez B, Ding HL, Han MH, Zhang H, Cao JL (2019) Brain-derived neurotrophic factor-mediated projection-specific regulation of depressive-like and nociceptive behaviors in the mesolimbic reward circuitry. Pain 159(1):175

Logrip ML, Janak PH, Ron D (2009) Escalating ethanol intake is associated with altered corticostriatal BDNF expression. J Neurochem 109(5):1459-68.

Logrip ML, Barak S, Warnault V, Ron D (2015) Corticostriatal BDNF and alcohol addiction. Brain Res 1628(Pt A):60-7

Logrip ML, Oleata C, Roberto M (2017) Sex differences in responses of the basolateral-central amygdala circuit to alcohol, corticosterone and their interaction. Neuropharmacology 114:123-134

Lovinger DM, White G, Weight FF (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 243: 1721–1724

Lovinger DM, White G (1991) Ethanol potentiation of 5-hydroxytryptamine 3 receptor-mediated ion current in neuroblastoma cells and isolated adult mammalian neurons. Mol Pharmacol 40(2):263-70

Lu L, Dempsey J, Liu SY, Bossert JM, Shaham Y (2004) A Single Infusion of Brain-Derived Neurotrophic Factor into the Ventral Tegmental Area Induces Long-Lasting Potentiation of Cocaine Seeking after Withdrawal. J Neurosci 24(7) 1604-1611

Lummis SC, Thompson AJ, Bencherif M, Lester HA (2011) Varenicline is a potent agonist of the human 5-hydroxytryptamine3 receptor. J Phamacol Exp Ther 339:125–131

Machaalani R, Chen H (2018) Brain derived neurotrophic factor (BDNF), its tyrosine kinase receptor B (TrkB) and nicotine. Neurotoxicology 65:186-195

Maggio SE, Saunders MA, Baxter TA, Nixon K, Prendergast MA, Zheng G, Crooks P, Dwoskin LP, Slack RD, Newman AH, Bell RL, Bardo MT (2018a) Effects of the nicotinic agonist varenicline, nicotinic antagonist r-bPiDI, and DAT inhibitor (R)-modafinil on co-use of ethanol and nicotine in female P rats. Psychopharmacology 235(5):1439–1453

Maggio SE, Saunders MA, Nixon K, Prendergast MA, Zheng G, Crooks P, Dwoskin LP, Bell RL, Bardo MT (2018b) An improved model of ethanol and nicotine co-use in female P rats: effects of naltrexone, varenicline, and the selective nicotinic α6β2* antagonist r-bPiDI. Drug Alcohol Depend 193:154–161

Maisel NC, Blodgett JC, Wilbourne PL, Humphreys K, Finney JW (2013) Meta-analysis of naltrexone and acamprosate for treating alcohol use disorders: when are these medications most helpful? Addiction 108(2):275–293

Malin DH, Lake JR, Carter VA, Cunningham JS, Wilson OB (1993) Naloxone precipitates nicotine abstinence syndrome in the rat. Psychopharmacology 112(2–3):339–342

Malin DH, Lake JR, Payne MC, Short PE, Carter VA, Cunningham JS, Wilson OB (1996) Nicotine alleviation of nicotine abstinence syndrome is naloxone-reversible. Pharmacol Biochem Behav 53(1):81–85

Mansvelder HD, McGehee DS (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron, 27, 349–357

Mansvelder HD, Keath JR, McGehee DS (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33, 905–919

Marinelli M, McCutcheon JE (2014) Heterogeneity of dopamine neuron activity across traits and states. Neuroscience 282:176-197

Marszalec W, Aistrup GL, Narahashi T (1999) Ethanol-nicotine interactions at alpha-bungarotoxin-insensitive nicotinic acetylcholine receptors in rat cortical neurons. Alcohol Clin Exp Res 23(3):439-45

Martell N (2014) Tobacco returns to the bar, this time inside cocktails. National Public Radio. Retrieved from https://www.npr.org/

McBride WJ, Schultz JA, Kimpel MW, McClintick JN, Wang M, You J, Rodd ZA (2009) Differential effects of ethanol in the nucleus accumbens shell of

alcohol-preferring (P), alcohol-non-preferring (NP) and Wistar rats: A proteomics study. Pharmacol Biochem Behav 92(2), 304–313

McClintick JN, McBride WJ, Bell RL, Ding Z-M, Liu Y, Xuei X, Edenberg HJ (2015) Gene expression changes in serotonin, GABA-A receptors, neuropeptides and ion channels in the dorsal raphe nucleus of adolescent alcohol-preferring (P) rats following binge-like alcohol drinking. Pharmacol Biochem Beh 129:87-96

McClintick JN, McBride WJ, Bell RL, Ding Z-M, Liu Y, Xuei X, Edenberg HJ (2016) Gene Expression Changes in Glutamate and GABA-A Receptors, Neuropeptides, Ion Channels, and Cholesterol Synthesis in the Periaqueductal Gray Following Binge-Like Alcohol Drinking by Adolescent Alcohol-Preferring (P) Rats. Alcohol Clin Exp Res 40(5):955-968

McCutcheon JE, Conrad KL, Carr SB, Ford KA, McGehee DS, Marinelli M (2012) Dopamine neurons in the ventral tegmental area fire faster in adolescent rats than in adults. J Neurophysiol 108(6), 1620–1630

McKee SA, Falba T, O'Malley SS, Sindelar J, O'Connor PG (2007) Smoking status as a clinical indicator for alcohol misuse in US adults. Arch Internal Med 167:716–721

McKinzie DL, Nowak KL, Yorger L, McBride WJ, Murphy JM, Lumeng L, Li T-K (1998) The alcohol deprivation effect in the alcohol-preferring P rat under free drinking and operant access conditions. Alcohol Clin Exp Res 22:1170–1176

Melendez RI, Rodd-Henricks ZA, Engleman EA, Li TK, McBride WJ, Murphy JM (2002) Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation and operant self-administration of ethanol. Alcohol Clin Exp Res 26:318–325

Melendez RI, Rodd ZA, McBride WJ, Murphy JM (2004) Involvement of the mesopallidal dopamine system in ethanol reinforcement. Alcohol 32:137–144

Meyer JS, Quenzer LF (2005) Psychopharmacology: Drugs, the brain, and behavior. Sunderland, MA, US: Sinauer Associates

Miech RA, Johnston LD, O'Malley PM, Bachman JG, Schulenberg JE (2016) Monitoring the Future national survey results on drug use, 1975-2015: Volume I, Secondary school students (Institute for Social Research, The University of Michigan)

Mielke JG, Mealing GA (2009) Cellular distribution of the nicotinic acetylcholine receptor alpha7 subunit in rat hippocampus. Neurosci Res 65(3):296-306

Miguel-Hidalgo JJ (2009) The Role of Glial Cells in Drug Abuse. Curr Drug Abuse Rev 2(1), 76-82

Mihic SJ (1999) Acute effects of ethanol on GABAA and glycine receptor function. Neurochem Int 35: 115–123

Morel C, Montgomery S, Han MH (2018) Nicotine and alcohol: the role of midbrain dopaminergic neurons in drug reinforcement. Eur J Neurosci [Epub ahead of print]

Motschman CA, Gass JC,Wray JM, Germeroth LJ, Schlienz NJ, Munoz DA, Moore FE, Rhodes JD, Hawk LW, Tiffany ST (2016) Selection criteria limit generalizability of smoking pharmacotherapy studies differentially across clinical trials and laboratory studies: a systematic review on varenicline. Drug Alcohol Depend 169:180–189

Nakahata Y, Yasuda R (2018) Plasticity of Spine Structure: Local Signaling, Translation and Cytoskeletal Reorganization. Front Synaptic Neurosci 29;10:29

National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) The National Academies Collection: Reports funded by National Institutes of Health In Guide for the Care and Use of Laboratory Animals. Washington (DC): National Academies Press (US) National Academy of Sciences

New York Times Magazine (Marshall Selladec) (2003) The 3rd Annual Year in Ideas; Nicotini, The. Published Dec 14, 2003

Nikulina EM, Johnston CE, Wang J, Hammer RP Jr (2014) Neurotrophins in the ventral tegmental area: Role in social stress, mood disorders and drug abuse. Neuroscience 282:122-38

O'Connor EC, Parker D, Rollema H, Mead AN (2010) The α4β2 nicotinic acetylcholine-receptor partial agonist varenicline inhibits both nicotine self-administration following repeated dosing and reinstatement of nicotine seeking in rats. Psychopharmacology 208(3):365–376

Oliva I, Wanat MJ (2016) Ventral Tegmental Area Afferents and Drug-Dependent Behaviors. Front Psychiatry 7:30

O'Malley SS, Jaffe AJ, Rode S, Rounsaville BJ (1996) Experience of a "slip" among alcoholics treated with naltrexone or placebo. Am J Psychiatry 153(2):281–283

O'Malley SS, Cooney JL, Krishnan-Sarin S, Dubin JA, McKee SA, Cooney NL, Blakeslee A, Meandzija B, Romano-Dahlgard D, Wu R, Makuch R, Jatlow P (2006) A controlled trial of naltrexone augmentation of nicotine replacement therapy for smoking cessation. Arch Intern Med 166(6):667–674

Ortiz J, Fitzgerald LW, Charlton M, Lane S, Trevisan L, Guitart X, Shoemaker W, Duman RS, Nestler EJ (1995) Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system. Synapse 21(4):289-98

Ortiz NC, O'Neill HC, Marks MJ, Grady SR (2012) Varenicline blocks β2*-nAChR-mediated response and activates β4*-nAChR-mediated responses in mice in vivo. Nicotine Tob Res 14(6):711–719

Park H, Poo MM (2013) Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 14(1):7-23

Pascual M, Boix J, Felipo V, Guerri C (2009) Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. J Neurochem 108(4), 920-931

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates, 4th ed. Academic Press, New York

Peltier MR, Verplaetse TL, Mineur YS, Petrakis IL, Cosgrove KP, Picciotto MR, McKee SA (2019) Sex differences in stress-related alcohol use. Neurobiol Stress 10:100149

Petrakis I, Ralevski E, Desai N, Trevisan L, Gueorguieva R, Rounsaville B, Krystal J (2012) Noradrenergic vs serotonergic antidepressant with or without naltrexone for veterans with PTSD and comorbid alcohol dependence. Neuropsychopharmacology 37(4):996–1004

Pidoplichko VI, DeBiasi M, Wiliams JT, Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. Nature, 390, 401–404

Pierce RC, Kumaresan V (2006). The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? Neurosci Biobehav Rev 30(2), 215-238

Randall PA, Jaramillo AA, Frisbee S, Besheer J (2015) The role of varenicline on alcohol-primed self-administration and seeking behavior in rats. Psychopharmacology 232(14):2443–2454

Rassnick S, Stinus L, Koob GF (1993) The effects of 6-hydroxydopamine lesions of the nucleus accumbens and the mesolimbic dopamine system on oral self-administration of ethanol in the rat. Brain Res 623(1):16-24

Reid MS, Fox L, Ho LB, Berger SP (2000) Nicotine stimulation of extracellular glutamate levels in the nucleus accumbens: neuropharmacological characterization. Synapse 35(2):129-36

Robinson DL, Howard EC, McConnell S, Gonzales RA, Wightman RM (2009) Disparity between tonic and phasic ethanol-induced dopamine increases in the nucleus accumbens of rats. Alcohol Clin Exp Res 33(7):1–10

Rodd ZA, Bell RL, Kuc KA, Murphy JM, Lumeng L, Li TK, McBride WJ (2003) Effects of repeated alcohol deprivations on operant ethanol self-administration by alcohol-preferring (P) rats. Neuropsychopharmacology 28:1614–1621

Rodd ZA, Bell RL, Melendez RI, Kuc KA, Lumeng L, Li T-K, Murphy JM, McBride WJ. (2004) Comparison of Intracranial Self-Administration of Ethanol Within the Posterior Ventral Tegmental Area Between Alcohol-Preferring and Wistar Rats. Alcohol Clin Exp Res 28(8), 1212–1219

Rodd ZA, Bell RL, McQueen VK, Davids MR, Hsu CC, Murphy JM, Li TK, Lumeng L, McBride WJ (2005) Prolonged increase in the sensitivity of the posterior ventral tegmental area to the reinforcing effects of ethanol following repeated exposure to cycles of ethanol access and deprivation. J Pharmacol Exp Ther 315(2):648–657

Rodd ZA, Gryszowka VE, Toalston JE, Oster SM, Ji D, Bell RL, McBride WJ (2007) The reinforcing actions of a serotonin-3 receptor agonist within the ventral tegmental area: evidence for subregional and genetic differences and involvement of dopamine neurons. J Pharmacol Exp Ther 321(3):1003-12

Rodd ZA, Bell RL, Kuc KA, Murphy JM, Lumeng L, McBride WJ (2009) Effects of concurrent access to multiple ethanol concentrations and repeated

deprivations on alcohol intake of high-alcohol drinking (HAD) rats. Addict Biol 14:152–164

Rodd ZA, Bell RL, Oster SM, Toalston JE, Pommer TJ, McBride WJ, Murphy JM (2010) Serotonin-3 receptors in the posterior ventral tegmental area regulate ethanol self-administration of alcohol-preferring (P) rats. Alcohol 44(3):245–255

Rodd-Henricks ZA, McKinzie DL, Crile RS, Murphy JM, McBride WJ (2000) Regional heterogeneity for the intracranial self-administration of ethanol within the ventral tegmental area of female Wistar rats. Psychopharmacology 149:217–224

Rodd-Henricks ZA, Bell RL, Kuc KA, Murphy JM, McBride WJ, Lumeng L, Li TK (2001) Effects of concurrent access to multiple ethanol concentrations and repeated deprivations on alcohol intake of alcohol-preferring rats. Alcohol Clin Exp Res 25:1140–1150

Rodd-Henricks ZA, Bell RL, Kuc KA, Murphy JM, McBride WJ, Lumeng L, Li TK (2002a) Effects of ethanol exposure on subsequent acquisition and extinction of ethanol self-administration and expression of alcohol-seeking behavior in adult alcohol-preferring (P) rats: I. Peri-adolescent exposure. Alcohol Clin Exp Res 26:1632–1641

Rodd-Henricks ZA, Bell RL, Kuc KA, Murphy JM, McBride WJ, Lumeng L, Li TK (2002b) Effects of ethanol exposure on subsequent acquisition and extinction of ethanol self-administration and expression of alcohol-seeking

behavior in adult alcohol-preferring (P) rats: II. Adult exposure. Alcohol Clin Exp Res 26:1642–1652

Rollema H, Chambers LK, Coe JW, Glowa J, Hurst RS, Lebel LA, Lu Y, Mansbach RS, Mather RJ, Rovetti CC, Sands SB, Schaeffer E, Schulz DW, Tingley FI, Williams KE (2007) Pharmacological profile of the α4β2 nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. Neuropharmacology 52(3):985–994

Russo SJ, Nestler EJ (2013) The brain reward circuitry in mood disorders. Nat Rev Neurosci 14(9):609-25

Sable HJ, Bell RL, Rodd ZA, McBride WJ (2006) Effects of naltrexone on the acquisition of alcohol intake in male and female peri-adolescent and adult alcohol-preferring (P) rats. Int J Adolesc Med Health 18:139–149

Saha TD, Grant BF, Chou SP, Kerridge BT, Pickering RP, Ruan WJ (2018) Concurrent use of alcohol with other drugs and DSM-5 alcohol use disorder comorbid with other drug use disorders: Sociodemographic characteristics, severity, and psychopathology. Drug Alcohol Depend 187:261-269

Sahr AE, Thielen RJ, Lumeng L, Li T-K, McBride WJ (2004) Long-Lasting Alterations of the Mesolimbic Dopamine System After Periadolescent Ethanol Drinking by Alcohol-Preferring Rats. Alcohol Clin Exp Res 28(5):702-711

Schultz W (2013) Updating dopamine reward signals. Current Opin Neurobiol 23(2), 229-238

Scofield MD, Heinsbroek JA, Gipson CD, Kupchik YM, Spencer S, Smith AC, Roberts-Wolfe D, Kalivas PW (2016) The Nucleus Accumbens: Mechanisms of Addiction across Drug Classes Reflect the Importance of Glutamate Homeostasis. Pharmacol Rev 68(3):816-71

Scuppa G, Cippitelli A, Toll L, Ciccocioppo R, Ubaldi M (2015) Varenicline decreases nicotine but not alcohol self-administration in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. Drug Alcohol Depend 156:126–132

Sesack SR, Grace AA (2010) Cortico-basal ganglia reward network: microcircuitry. Neuropsychopharmacology 35, 27–47

Smith PH, Weinberger AH, Zhang J, Emme E, Mazure CM, McKee SA (2017) Sex differences in smoking cessation pharmacotherapy comparative efficacy: a network meta-analysis. Nicotine Tob Res 19(3): 273–281

Soderpalm B, Ericson M, Olausson P, Blomqvist O, Engel JA (2000) Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav Brain Res 113:85–96

Soyka M, Müller CA (2017) Pharmacotherapy of alcoholism - an update on approved and off-label medications. Expert Opin Pharmacother 18(12):1187–1199

Spanagel R, Zieglgansberger W (1997) Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. Trends Pharmacol Sci 18:54–59

Spanagel R, Hölter SM, Allingham K, Landgraf R, Zieglgänsberger W (1996) Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat. Eur J Pharmacol 305(1–3):39–44

Spanagel R (2009) Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiol Rev 89(2):649-705

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417–463

Spear LP, Swartzwelder HS (2014) Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: A mini-review. Neurosci Biobehav Rev 45:1-8

Spencer S, Scofield M, Kalivas PW (2016) The good and bad news about glutamate in drug addiction. J Psychopharmacol 30(11):1095-1098

Steensland P, Simms JA, Holgate J, Richards JK, Bartlett SE (2007) Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. Proc Natl Acad Sci U S A 104(30):12518–12523

Stuber GD, Hopf FW, Hahn J, Cho SL, Guillory A, Bonci A (2008) Voluntary ethanol intake enhances excitatory synaptic strength in the ventral tegmental area. Alcohol Clin Exp Res 32(10):1714-20

Thomas AM, Ostroumov A, Kimmey BA, Taormina MB, Holden WM, Kim K, Brown-Mangum T, Dani JA (2018) Adolescent Nicotine Exposure Alters GABAA Receptor Signaling in the Ventral Tegmental Area and Increases Adult Ethanol Self-Administration. Cell Rep 23(1) 68–77

Thompson A, Ashcroft DM, Owens L, van Staa TP, Pirmohamed M (2017) Drug therapy for alcohol dependence in primary care in the UK: a Clinical Practice Research Datalink study. PLoS One 12(3):1–14

Tizabi Y, Copeland RJ, Louis VA, Taylor RE (2002) Effects of combined systemic alcohol and central nicotine administration into ventral tegmental area on dopamine release in the nucleus accumbens. Alcohol Clin Exp Res 26(3):394–399

Tizabi Y, Bai L, Copeland RJ, Taylor RE (2007) Combined effects of systemic alcohol and nicotine on dopamine release in the nucleus accumbens shell. Alcohol Alcohol 42(5):413–416

Toalston JE, Oster SM, Kuc KA, Pommer TJ, Murphy JM, Lumeng L, Bell RL, McBride WJ, Rodd ZA (2008) Effects of alcohol and saccharin deprivations on concurrent ethanol and saccharin operant self-administration by alcohol-preferring (P) rats. Alcohol 42:277–284

Toalston JE, Deehan GA, Jr., Hauser SR, Engleman EA, Bell RL, Murphy JM, Truitt WA, McBride WJ, Rodd ZA. (2014) Reinforcing properties and neurochemical response of ethanol within the posterior ventral tegmental area are enhanced in adulthood by periadolescent ethanol consumption. J Pharmacol Exp Ther 351: 317-26

Toalston JE, Deehan GA Jr, Hauser SR, Engleman EA, Bell RL, Murphy JM, McBride WJ, Rodd ZA (2015) The reinforcing properties of ethanol are quantitatively enhanced in adulthood by peri-adolescent ethanol, but not

saccharin, consumption in female alcohol-preferring (P) rats. Alcohol 49(5) 513-518

Toll B, White M, Wu R, Meandzija B, Jatlow P, Makuch R, O'Malley S (2010) Low-dose naltrexone augmentation of nicotine replacement for smoking cessation with reduced weight gain: a randomized trial. Drug Alcohol Depend 2010(3):200–206

Truitt WA, Hauser SR, Deehan GJ, Toalston JE, Wilden JA, Bell RL, McBride WJ, Rodd ZA (2015) Ethanol and nicotine interaction within the posterior ventral tegmental area in male and female alcohol-preferring rats: evidence of synergy and differential gene activation in the nucleus accumbens shell. Psychopharmacology 232(3):639–649

Van Skike CE, Maggio SE, Reynolds AR, Casey EM, Bardo MT, Dwoskin LP, Prendergast MA, Nixon K (2016) Critical needs in drug discovery for cessation of alcohol and nicotine polysubstance abuse. Prog Neuropsychopharmacol Biol Psychiatry 65:269-87

Vargas-Perez H, Bahi A, Bufalino MR, Ting-A-Kee R, Maal-Bared G, Lam J, Fahmy A, Clarke L, Blanchard JK, Larsen BR, Steffensen S, Dreyer J-L, van der Kooy D (2014) BDNF signaling in the VTA links the drug-dependent state to drug withdrawal aversions. J Neurosci 34(23) 7899–7909

Vengeliene V, Bilbao A, Molander A, Spanagel R (2008) Neuropharmacology of alcohol addiction. Br J Pharmacol 154:299–315

Vetreno RP, Broadwater M, Liu W, Spear LP, Crews FT. (2014) Adolescent, but Not Adult, Binge Ethanol Exposure Leads to Persistent Global Reductions of Choline Acetyltransferase Expressing Neurons in Brain. PLoS ONE 9(11):1-12

Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F (2011) Addiction: beyond dopamine reward circuitry. Proc Natl Acad Sci U S A 108(37):15037-42

Volkow ND, Skolnick P (2012) New medications for substance use disorders: challenges and opportunities. Neuropsychopharmacology 37:290–292

Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP (1992) Naltrexone in the treatment of alcohol dependence. Arch Gen Psychiatry 49(11): 876–880

Volpicelli JR, Rhines KC, Rhines JS, Volpicelli LA, Alterman AI, O'Brien CP (1997) Naltrexone and alcohol dependence. Role of subject compliance. Arch Gen Psychiatry 54:737–742

Waeiss RA, Knight CP, Hauser SR, Pratt LA, McBride WJ, Rodd ZA (2019) Therapeutic challenges for concurrent ethanol and nicotine consumption: naltrexone and varenicline fail to alter simultaneous ethanol and nicotine intake by female alcohol-preferring (P) rats. Psychopharmacology (Berl) doi: 10.1007/s00213-019-5174-y. [Epub ahead of print]

Wang F, Chen H, Sharp BM (2008) Neuroadaptive changes in the mesocortical glutamatergic system during chronic nicotine self-administration and after extinction in rats. J Neurochem 106(2):943-56

Wang F, Chen H, Steketee JD, Sharp BM (2007) Upregulation of Ionotropic Glutamate Receptor Subunits within Specific Mesocorticolimbic Regions

during Chronic Nicotine Self-Administration. Neuropsychopharmacology, 32(1), 103-109

Weinberger AH, Pilver CE, Hoff RA, Mazure CM, McKee SA (2013) Changes in smoking for adults with and without alcohol and drug use disorders: longitudinal evaluation in the US population. Am J Drug Alcohol Abuse 39, 186–193

Wetter DW, Kenford SL, Smith SS, Fiore MC, Jorenby DE, Baker TB (1999) Gender differences in smoking cessation. J Consult Clin Psychol 67(4):555–562

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annu Rev Psychol 40:191–225

Wong G, Wolter T, Croghan G, Croghan I, Offord K, Hurt R (1999) A randomized trial of naltrexone for smoking cessation. Addiction 94(8):1227–1237

World Health Organization (2018) Global status report on alcohol and health http://www.who.int/substance_abuse/publications/global_alcohol_report/en/ (Accessed May 1, 2019)

Yetnikoff L, Lavezzi HN, Reichard RA, Zahm DS (2014) An update on the connections of the ventral mesencephalic dopaminergic complex. Neuroscience 282:23-48

Yu D, Zhang L, Eisele J-L, Bertrand D, Changeux J-P, Weight FF (1996) Ethanol inhibition of nicotinic acetylcholine type α7 receptors involves the amino-terminal domain of the receptor. Mol Pharmacol 50, 1010-1016.

Zhu S, Lee M, Zhuang Y, Gamst A, Wolfson T (2012) Interventions to increase smoking cessation at the population level: how much progress has been made in the last two decades? Tob Control 21(2):110–118

Zuo Y, Nagata K, Yeh JZ, Narahashi T (2004) Single-channel analyses of ethanol modulation of neuronal nicotinic acetylcholine receptors. Alcohol Clin Exp Res 28(5):688-96

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2014 – 2015	Stark Neurosciences Research Scholar Award
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Publications

Waeiss, R.A., Knight, C.P., Carvajal, G.B., Bell, R.L., Engleman, E.A., McBride, W.J., Hauser, S.R., and Rodd, Z.A. Peri-adolescent alcohol consumption increases sensitivity and neurochemical response to nicotine during adulthood in female alcohol-preferring (P) rats: alterations in α7 nicotinic acetylcholine receptor expression. *Behavioural Brain Research,* doi:10.1016/j.bbr.2019.112190

Hauser, S.R., Knight, C.P., Truitt, W.A., **Waeiss, R.A.,** Holt, I.S., Carvajal, G.B., Bell, R.L., and Rodd, Z.A. Adolescent intermittent ethanol (AIE) increases the sensitivity to the reinforcing properties of ethanol and the expression of select cholinergic and dopaminergic genes within the posterior ventral tegmental area. *Alcoholism: Clinical and Experimental Research*, doi:10.1111/acer.14150

Hauser, S.R., Deehan, G.A. Jr., Knight, C.P., **Waeiss, R.A.,** Truitt, W.A., Johnson, P.L., Bell, R.L., McBride, W.J. and Rodd, Z.A. Conditioned stimuli affect ethanol-seeking by alcohol-preferring (P) rats: The role of repeated-deprivations, cue-pretreatment, and cue-temporal intervals. *Psychopharmacology*, doi:10.1007/s00213-019-05264-6.

Waeiss, R.A., Hauser, S.R., Knight, C.P., Pratt, L.A., McBride, W.J. and Rodd, Z.A. (2019). Therapeutic challenges for concurrent ethanol and nicotine consumption: naltrexone and varenicline fail to alter simultaneous ethanol and nicotine intake by female alcohol-preferring (P) rats. *Psychopharmacology,* doi:10.1007/s00213-019-5174-y

Deehan, G.A. Jr., Hauser, S.R., Getachew, B., **Waeiss, R.A.,** Engleman, E.A., Knight, C.P., McBride, W.J., Truitt, W.A., Bell, R.L. and Rodd, Z.A. (2018). Selective breeding for high alcohol consumption and response to nicotine: locomotor activity, dopamine in the mesolimbic system, and innate genetic differences in male and female alcohol-preferring, non-preferring, and replicate lines of high-alcohol drinking and low-alcohol drinking rats. *Psychopharmacology*, 235(9):2755-2769. doi:10.1007/s00213-018-4970-0

Deehan, G.A. Jr., Knight, C.P., **Waeiss, R.A.,** Engleman, E.A., Toalston, J.E., McBride, W.J., Hauser, S.R. and Rodd, Z.A. (2016). Peripheral administration of ethanol results in a correlated increase in dopamine and serotonin within the posterior ventral tegmental area. *Alcohol and Alcoholism*, 51(5): 535-540. doi:10.1093/alcalc/agw037

Deehan, G.A. Jr., Hauser, S.R., **Waeiss, R.A.,** Knight, C.P., Toalston, J.E., Truitt, W.A., McBride, W.J. and Rodd, Z.A. (2015). Co-administration of ethanol and nicotine: the enduring alterations in the rewarding properties of nicotine and glutamate activity within the mesocorticolimbic system of female alcohol-preferring (P) rats. *Psychopharmacology*, 232(23): 4293-4302. doi:10.1007/s00213-015-4056-1

Arthur, R.A., Kohara, E., **Waeiss, R.A.,** Eckert, G., Zero, D.T. and Ando, M. (2014). Enamel carious lesion development in response to sucrose and fluoride concentrations and to time of biofilm formation: an artificial-mouth study, *Journal of Oral Diseases*, doi:10.1155/2014/348032

Ferracini, M., Sahu, R.P., Harrison, K.A., **Waeiss, R.A.,** Murphy, R.C., Jancar, S., Konger, R.L. and Travers, J.B. (2014). Topical photodynamic therapy induces systemic immunosuppression via generation of platelet-activating factor receptor ligands. *Journal of Investigative Dermatology,* 135(1): 321-323. doi:10.1038/jid.2014.313

Waeiss, R.A., Arthur, R.A., Negrini, T.C. and Bottino, M.C. (2014). Antimicrobial effects of drug-containing electrospun matrices on osteomyelitis associated biofilms. *Journal of Oral and Maxillofacial Surgery*, 72(7): 1310-1319. doi:10.1016/j.joms.2014.01.007

Bottino, M.C., Arthur, R.A., **Waeiss, R.A.,** Kamocki, K., Gregson, K.S. and Gregory, R.L. (2014). Biodegradable nanofibrous drug delivery systems: effects of metronidazole and ciprofloxacin on periodonto-pathogens and commensal oral bacteria. *Clinical Oral Investigations*, 18(9): 2151-2158. doi:10.1007/s00784-014-1201-x

Negrini, T., Arthur, R.A., **Waeiss, R.A.,** Carlos, I. and Srinivasan, M. (2014). Salivary epithelial cells: a model to study immune response against

cutaneous pathogens. *Clinical and Translational Science*, *7*(1): 48-51. doi:10.1111/cts.12113

Arthur, R.A., **Waeiss, R.A.,** Hara, A.T., Lippert, F., Eckert, G.J. and Zero, D.T. (2013). A defined-multispecies microbial model for studying enamel caries development. *Caries Research, 47*(4): 318-324. doi:10.1159/000347O5O

Manuscripts in Preparation

Waeiss, R.A., Knight, C.P., Engleman, E.A., Hauser, S.R., and Rodd, Z.A. Co-administration of ethanol and nicotine heightens sensitivity to ethanol reward within the nucleus accumbens (NAc) shell: increasing NAc shell BDNF is sufficient to enhance ethanol reward in naïve rats. [under review]

Knight, C.P., Hauser, S.R., **Waeiss, R.A.,** Molosh, A.I., Johnson, P.L., Truitt, W.A., McBride, W.J., Bell, R.L., Shekhar, A., and Rodd, Z.A. The rewarding and anxiolytic properties of EtOH within the central nucleus of the amygdala are mediated by genetic background and nociceptin but not the CRF_1 receptor. [under review]

Hauser, S.R., Deehan, G.A. Jr., Wilden, J.A., Knight, C.P., **Waeiss, R.A.,** Engleman, E.A., Ding, Z.M., Johnson, P.L., McBride, W.J., Truitt, W.A., and Rodd, Z.A. The role of the basal lateral amygdala – ventral tegmental area pathway regulating inhibitory and excitatory alcohol-seeking cues: behavior and neurochemistry. [in preparation]

Abstracts

Waeiss, R.A., Antoni, N.L., Pratt, L.A., Hauser, S.R. and Rodd, Z.A. Only co-administration of nicotine and ethanol into the posterior VTA increases BDNF in the nucleus accumbens shell (AcbSh), and parallel pretreatment of the AcbSh with BDNF results in a comparable enhancement of ethanol reward. Research Society on Alcoholism Annual Meeting. Minneapolis, MN. June 2019.

Hauser, S.R., **Waeiss, R.A.,** Antoni, N.L., Pratt, L.A. and Rodd, Z.A. Adolescent intermittent ethanol exposure increased the sensitivity of ethanol reward in the posterior VTA during adulthood in Wistar male rats. Research Society on Alcoholism Annual Meeting. Minneapolis, MN. June 2019.

Hauser, S.R., Knight, C.P., **Waeiss, R.A.,** Pratt, L.A. and Rodd, Z.A. Histone deacetylases mediate the increased sensitivity to ethanol reward in the central amygdala produced by adolescent intermittent ethanol exposure in male Wistars. Research Society on Alcoholism Annual Meeting. Minneapolis, MN. June 2019.

Waeiss, R.A., Antoni, N.L., Pratt, L.A., Hauser, S.R. and Rodd, Z.A. Ethanol and nicotine in the posterior VTA elevates nucleus accumbens shell (AcbSh) BDNF levels and AcbSh pretreatment with BDNF enhances ethanol reward. Annual Meeting of the Greater Indiana Society for Neuroscience. Indianapolis, IN. March 2019.

Waeiss, R.A., Knight, C.P., Engleman, E.A., Hauser, S.R. and Rodd, Z.A. Microinjections of ethanol and nicotine, but not ethanol or nicotine, into the posterior ventral tegmental area increases glutamate levels within the nucleus accumbens shell. American College of Neuropsychopharmacology Annual Conference. Hollywood, FL. December 2018.

Hauser, S.R., Knight, C.P., **Waeiss, R.A.,** Pratt, L.A., and Rodd, Z.A. Epigenetic factors mediate the increase in sensitivity of ethanol reward in the central nucleus of the amygdala produced by adolescent binge ethanol exposure in male, but not female Wistar rats. American College of Neuropsychopharmacology Annual Conference. Hollywood, FL. December 2018.

Waeiss, R.A., Knight, C.P., Brown E.R., Pratt, L.A., Engleman, E.A., Hauser, S.R. and Rodd, Z.A. Microinjections of ethanol and nicotine into the posterior ventral tegmental area produces a unique neurochemical response within the nucleus accumbens shell. Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Waeiss, R.A., Knight, C.P., Pratt, L.A., Hauser, S.R. and Rodd, Z.A. Despite reductions of nicotine self-administration by varenicline and ethanol self-administration by naltrexone, concurrent ethanol and nicotine self-administration is unaffected by both**.** Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Knight, C.P., **Waeiss, R.A.,** Brown E.R., Pratt, L.A., Hauser, S.R., Truitt, W.A. and Rodd, Z.A. Gene expression for nicotinic and dopaminergic factors in the central amygdala during adulthood: Sex differences and interactions with adolescent ethanol exposure. Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Knight, C.P., **Waeiss, R.A.,** Pratt, L.A., Hauser, S.R., Bell, R.L. and Rodd, Z.A. Ethanol reward in the central nucleus of the amygdala: Significant sex differences and enhancement by adolescent ethanol exposure. Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Hauser, S.R., Knight, C.P., **Waeiss, R.A.,** Pratt, L.A., Bell, R.L. and Rodd, Z.A. The ability of an excitatory conditioned cue to enhance ethanol-seeking can be transferred to another cue: Cue-Generalization. Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Pratt, L.A., Knight, C.P., **Waeiss, R.A.,** Johnson, P.L., Bell, R.L., Hauser, S.R. and Rodd, Z.A. A history of alcohol dependency alters ethanol sensitivity in the central nucleus of the amygdala: Evidence of sex differences.

Research Society on Alcoholism Annual Meeting. San Diego, CA. June 2018.

Knight, C.P., Hauser, S.R., Bell, R.L., **Waeiss, R.A.,** Pratt, L.A., and Rodd, Z.A. Alcohol in the Central Nucleus of the Amygdala: Sex Differences, Effect of Adolescent Alcohol consumption, and Modulation by Neuropeptides. Winter Conference on Brain Research. Whistler, British Columbia. January 2018.

Knight, C.P., Hauser, S.R., **Waeiss, R.A.,** Pratt, L.A., McBride, W.J., Bell, R.L. and Rodd, Z.A. Characterizing the Reinforcing Properties of Ethanol within the Central Nucleus of the amygdala: Sex Differences, Effect of Adolescent Alcohol consumption, and Modulation by Neuropeptides. American College of Neuropsychopharmacology Annual Conference. Palm Springs, CA. December 2017.

Waeiss, R.A., Knight, C.P., Brown E.R., Truitt, W.A., Hauser, S.R., McBride, W.J. and Rodd, Z.A. Microinjections of ethanol and nicotine into the posterior VTA produces unique adaptations that enhance ethanol reward in the nucleus accumbens shell. Research Society on Alcoholism Annual Meeting. Denver, CO. June 2017.

Waeiss, R.A., Knight, C.P., Brown E.R., Hauser, S.R., McBride, W.J. and Rodd, Z.A. Adolescent alcohol consumption heightens the sensitivity and neurochemical response of the mesolimbic dopamine system to nicotine during adulthood**.** Research Society on Alcoholism Annual Meeting. Denver, CO. June 2017.

Knight, C.P., **Waeiss, R.A.,** Brown E.R., McBride, W.J. Truitt, W.A., Bell, R.L., Hauser, S.R., Johnson, P.L. and Rodd, Z.A. Elucidating the reinforcing and anxiolytic properties of ethanol within the central amygdala: Involvement of nociceptin but not CRF. Research Society on Alcoholism Annual Meeting. Denver, CO. June 2017.

Knight, C.P., **Waeiss, R.A.,** Brown E.R., McBride, W.J. Truitt, W.A., Bell, R.L., Hauser, S.R. and Rodd, Z.A. Adolescent binge ethanol exposure enhances the reinforcing properties of ethanol in the central nucleus of the amygdala during adulthood. Research Society on Alcoholism Annual Meeting. Denver, CO. June 2017.

Hauser, S.R., Knight, C.P., **Waeiss, R.A.,** Brown E.R., McBride, W.J., Bell, R.L. and Rodd, Z.A. Adolescent binge ethanol exposure enhances the acquisition, but inhibits the extinction, of ethanol self-administration in alcohol-preferring (P) rats. Research Society on Alcoholism Annual Meeting. Denver, CO. June 2017.

Rodd, Z.A., **Waeiss, R.A.,** Bell, R.L., Knight, C.P. and Hauser, S.R. Exposure to ethanol or nicotine during adolescence enhances the neurochemical response to nicotine within the mesolimbic dopamine system in adulthood. Winter Conference on Brain Research. Big Sky, MT. January 2017.

Deehan Jr., G.A., Knight, C.P., **Waeiss, R.A.,** McBride, W.J., Bell, R.L., Hauser, S.R. and Rodd, Z.A. Adolescent ethanol or nicotine exposure enhances the neurochemical response to nicotine within the mesolimbic dopamine system during adulthood. American College of Neuropsychopharmacology Annual Conference. Hollywood, FL. December 2016.

Hauser, S.R., Deehan Jr., G.A., Knight, C.P., **Waeiss, R.A.,** McBride, W.J. and Rodd, Z.A. Presentation of excitatory conditioned cues result in the activation of GABA interneurons within the basolateral amygdala. American College of Neuropsychopharmacology Annual Conference. Hollywood, FL. December 2016.

Knight, C.P., **Waeiss, R.A.,** Johnson, P.L., Deehan Jr., G.A., Bell, R.L., McBride, W.J., Hauser, S.R. and Rodd, Z.A. Adolescent intermittent alcohol exposure enhances the reinforcing properties of alcohol in the central amygdala during adulthood in wistar rats. American College of Neuropsychopharmacology Annual Conference. Hollywood, FL. December 2016.

Hauser, S.R., Deehan Jr., G.A., Knight, C.P., **Waeiss, R.A.,** McBride, W.J., Truitt, W.A. and Rodd, Z.A. GABA interneurons within the basolateral amygdala are activated by presentation of an excitatory conditioned cue. ISBRA and ESBRA World Congress on Alcohol and Alcoholism. Berlin, Germany. September 2016.

Knight, C.P., Deehan Jr., G.A., **Waeiss, R.A.,** Hauser, S.R., McBride, W.J. and Rodd, Z.A. Determining the neurochemical basis of ethanol reward and anxiolysis in the central amygdala. ISBRA and ESBRA World Congress on Alcohol and Alcoholism. Berlin, Germany. September 2016.

Deehan Jr., G.A., Bracken, A.L., Knight, C.P., **Waeiss, R.A.,** McBride, W.J., Bell, R.L. and Rodd, Z.A. Voluntary ethanol consumption during adolescence primes the brain reward system to be more responsive to nicotine during adulthood. ISBRA and ESBRA World Congress on Alcohol and Alcoholism. Berlin, Germany. September 2016.

Hauser, S.R., Bracken, A.L., Knight, C.P., Deehan Jr., G.A., **Waeiss, R.A.,** McBride, W.J., Bell, R.L. and Rodd, Z.A. Exposure to nicotine during adolescence increases the neurochemical response to nicotine within the mesolimbic dopamine system during adulthood. Society for Neuroscience Annual Meeting. San Diego, CA. November 2016.

Rodd, Z.A., Bracken, A.L., Deehan Jr., G.A., Knight, C.P., **Waeiss, R.A.,** McBride, W.J., Bell, R.L. and Hauser, S.R. Voluntary ethanol consumption during adolescence enhances the sensitivity and response to nicotine within the mesolimbic dopamine system during adulthood. Society for Neuroscience Annual Meeting. San Diego, CA. November 2016.

Waeiss, R.A., Knight, C.P., Deehan Jr., G.A., Hauser, S.R., Truitt, W.A., Shekhar, A. and Rodd, Z.A. Unique effects of ethanol and nicotine in the mesocorticolimbic dopamine system: Alterations of BDNF within the

nucleus accumbens shell. Research Society on Alcoholism Annual Meeting. New Orleans, LA. June 2016.

Knight, C.P., Deehan Jr., G.A., Hauser, S.R., **Waeiss, R.A.,** McBride, W.J. and Rodd, Z.A. The CRF1 receptor and nociception do not modulate the reinforcing effects of ethanol within the central nucleus of the amygdala. Research Society on Alcoholism Annual Meeting. New Orleans, LA. June 2016.

Deehan Jr., G.A., **Waeiss, R.A.,** Hauser, S.R., Engleman, E.A., McBride, W.J. and Rodd, Z.A. The co-abuse of alcohol and nicotine: Examining the underlying behavioral and neurobiological mechanisms. 15th European Society for Biomedical Research on Alcoholism (ESBRA) Congress. Valencia, Spain. September 2015.

Rodd, Z.A., Deehan Jr., G.A., Knight, C.P., **Waeiss, R.A.** and Hauser, S.R. Alcohol and cocaine interact with discrete brain structures to promote reinforcement. 15th ESBRA Congress. Valencia, Spain. September 2015.

Waeiss, R.A., Deehan Jr., G.A., Hauser, S.R., Knight, C.P., Engleman, E.A., McBride, W.J. and Rodd, Z.A. Co-abuse of ethanol and nicotine increases basal glutamate levels within the nucleus accumbens shell. Research Society on Alcoholism Annual Meeting. San Antonio, TX. June 2015.

Knight, C.P., Toalston, J.E., Lungwitz, E.A., Deehan Jr., G.A., Hauser, S.R., **Waeiss, R.A.,** McBride, W.J. and Rodd, Z.A. Is anxiolysis reinforcing? Ethanol has both rewarding and anxiolytic properties within the central nucleus of the amygdala. Research Society on Alcoholism Annual Meeting. San Antonio, TX. June 2015.

Deehan Jr., G.A., **Waeiss, R.A.,** Hauser, S.R., Engleman, E.A., McBride, W.J. and Rodd, Z.A. Local repeated exposure to ethanol sensitizes the posterior ventral tegmental area to the dopamine stimulatory properties of acetaldehyde. Research Society on Alcoholism Annual Meeting. San Antonio, TX. June 2015.

Rodd, Z.A., Knight, C.P., Toalston, J.E., **Waeiss, R.A.,** Deehan Jr., G.A., Truitt, W.A., McBride, W.J. and Hauser, S.R. Ethanol is Self-Administered Directly into the Central Nucleus of the Amygdala in Wistar Rats. American College of Neuropsychopharmacology Annual Conference. Phoenix, AZ. December 2014.

Waeiss, R.A., Arthur, R.A., Negrini, T.C. and Bottino, M.C. Inhibitory effect of local drug delivery via electrospun mats on Staphylococci biofilm formation. Indiana University School of Dentistry Research Symposium. IN-AADR Indianapolis, IN. April 2013.

Bottino, M.C., Kamp, T., **Waeiss, R.A.,** Yassen, G., Platt, J.A. and Arthur, R.A. Drug-containing periodontal membranes – Effects on non-periodontitis related bacteria. International Association of Dental Research General Session. Iguaçu Falls, Brazil. June 2012.

Arthur, R.A., Kohara, E., **Waeiss, R.A.,** Eckert, G., Zero, D.T. and Ando, M. Sucrose / Fructose effects on enamel caries initiation produced by a microbial model. Indiana University School of Dentistry Research Symposium. IN-AADR Indianapolis, IN. April 2012.

Arthur, R.A., **Waeiss, R.A.,** Hara, A.T., Lippert, F. and Zero, D.T. *In vitro* biofilm-associated demineralization under distinct sugar exposure. American Association of Dental Research Annual Conference. Tampa, FL. March 2012.