NEUROBIOLOGICAL MECHANISMS LINKING STRESS AND NICOTINE TO INCREASED ALCOHOL CONSUMPTION

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"No one can whistle a symphony. It takes an orchestra to play it."

- H.E. Luccock

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ABSTRACT

NEUROBIOLOGICAL MECHANISMS LINKING STRESS AND NICOTINE TO INCREASED ALCOHOL CONSUMPTION

Alyse M. Thomas

John Dani, Ph.D.

Alcohol use is a leading cause of preventable disease, disability and death worldwide. Among other factors, exposure to stress or nicotine promotes drinking in humans, yet the neurobiological mechanisms mediating these interactions are unknown. Decades of research indicate that alcohol, stress hormones and nicotine act within the mesolimbic dopamine system to promote behavioral reinforcement. Based off this literature, the central hypothesis guiding my dissertation was that exposure to stress or nicotine promoted drinking via adaptations within ventral tegmental area (VTA).

The findings presented in the second chapter of my thesis describe a novel adaptation within the mesolimbic dopamine system contributing to increased alcohol self-administration. Exposure to stress blunted subsequent dopamine responses to alcohol and increased alcohol consumption via VTA stress hormone receptors. These adaptations arose from excitatory GABA transmission onto VTA GABA neurons. Further investigation revealed that excitatory shifts in GABA transmission were associated with the downregulation of the chloride transporter KCC2. Pharmacological enhancement of KCC2 function within the VTA prevented stress-induced drinking, identifying a novel mechanism of stress-induced alcohol consumption.

The results in the third and fourth chapters reveal that similar adaptations within the VTA may also contribute to the co-use of nicotine and alcohol. As was observed after stress,

exposure to acute nicotine blunted dopamine responses to alcohol and increased alcohol self-administration. Blocking glucocorticoid receptors during nicotine normalized the dopamine signaling and drinking to control levels, indicating that nicotine recruits stress hormone receptors to influence subsequent responses to alcohol.

The fourth chapter examines the effects of adolescent nicotine exposure on adult responses to alcohol, since early-life tobacco use confers a major risk for subsequent alcohol abuse. Animals treated with nicotine during adolescence show attenuated dopamine signaling and increased self-administration throughout adulthood. Pharmacological enhancement of KCC2 in adulthood prevented the elevated alcohol intake, highlighting a potential therapeutic role of this drug to reduce alcohol consumption long after the initial nicotine exposure. Taken together, this body of work suggests that exposure to stress or nicotine boosts drinking via anionic plasticity mechanisms within the VTA and implicates KCC2 activation as a potential therapeutic target in the treatment of excessive alcohol consumption.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1 – Stress and Alcohol Consumption: A General Introduction

Chapter 2 – Stress Increases Ethanol Self-Administration via a Shift toward Excitatory GABA Signaling in the Ventral Tegmental Area

CHAPTER 1

STRESS, NICOTINE AND ALCOHOL USE: A GENERAL INTRODUCTION

Alyse M. Thomas

> "Not all addictions are rooted in abuse or trauma, but I do believe they can all be traced to painful experience. A hurt is at the center of all addictive behaviors. It is present in the gambler, the Internet addict, the compulsive shopper and the workaholic. The wound may not be as deep and the ache not as excruciating, and it may even be entirely hidden—but it is there. As we'll see, the effects of early stress or adverse experiences directly shape both the psychology and the neurobiology of addiction in the brain."
>
> —Dr. Gabor Maté, *In the Realm of Hungry Ghosts (2010)*

The Prevalence and Burden of Alcohol Consumption

Alcohol consumption is common across many cultures worldwide, with 38 percent of the global adult population consuming an alcoholic beverage in the past year (WHO, 2014). Drinking is nearly twice as prevalent in the United States, with 71 percent of adults consuming alcohol in the past year (SAMHSA, 2015). Several societal factors contribute to the prevalence of alcohol use in the United States, including economic prosperity, widespread drug availability and general social acceptance (WHO, 2014). From annual celebrations to weekly sporting events, drinking defines many aspects of American culture. For example, 93 percent of popular American movies and 71 percent of regular TV programming portrayed alcohol consumption (Roberts, 1999; Christensen, 2000).

Recent studies examining social media reveal that 85 percent of Facebook profiles make reference to alcohol (Egan & Moreno, 2011). Of 11 million drinking-related tweets recorded during a single month, 79 percent portray alcohol use in a positive light (Cavazos-Rehg *et al.*, 2015).

Despite its popularity, many people misuse alcohol and experience negative consequences. Among individuals aged 15-49, alcohol misuse is the leading cause of premature disability and death, taking the lives of 3.3 million people around the globe (WHO, 2014). Drinking excessively is a common form of alcohol misuse. Nearly a quarter of American adults report binge drinking within the last month, defined as having 4-5 drinks within a two-hour period (SAMHSA, 2015). This level of intoxication is associated with compromised decision-making and impaired motor function, posing risk self and others. It follows that binge-drinking accounts for 75 percent of costs (186 billion dollars) related to alcohol misuse, including loss of productivity, motor vehicle accidents, criminal justice, and health care (Sacks *et al.*, 2015).

In addition to binge consumption, alcohol misuse can refer to excessive intake over longer timescales. Approximately 7 percent of the adult population reported heavy drinking over the past month, defined as 8-15 drinks per week (SAMHSA, 2015). The combination of binge and heavy drinking (i.e. risky drinking) predisposes an individual to alcohol-related harm and disease (NIAAA, 2010). While alcohol is entirely attributable for conditions like fetal alcohol syndrome, it is also a known risk factor for hundreds of other conditions such as liver cirrhosis, cardiovascular disease, sleep disorders, depression, cancer, and epilepsy (NIAAA, 2010; WHO, 2014). Worldwide, approximately 139 million disability-adjusted life years are lost in the form premature death or living with an alcohol-associated disease (WHO 2014).

Another major risk associated with excessive alcohol consumption is the development of alcohol use disorders (AUDs), which are characterized by the compulsive desire to consume alcohol despite negative consequences (NIAAA, 2010). Addicted individuals enter a persistent cycle of drug use, leading to the progressive development of drug tolerance, withdrawal, and dependence (Koob & Le Moal, 1997). Alcohol addiction ultimately imparts a diminished quality of life, including interpersonal strain, poor health, and a loss of productivity in the workplace (Donovan *et al.*, 2005). Reflecting the persistency of this struggle, AUDs encompass 25 percent of the alcohol-attributable disease burden compared to only 4 percent of alcohol-attributable deaths (WHO, 2014; Grant *et al.*, 2015). Importantly, one-third of Americans develop an AUD during their lifetime, revealing a major national public health concern (Grant *et al.*, 2015). The widespread prevalence and burden of excessive alcohol consumption has prompted broad research efforts towards identifying effective prevention and treatment strategies.

Stress and Alcohol Consumption

In an effort to mitigate the burden of alcohol on society, it is necessary to first identify the risk factors associated with alcohol misuse. Decades of research reveal complex interactions between environmental conditions and individual predisposition, with no single known cause of alcoholism. However, many prominent theories of addiction argue that exposure to stress can trigger pathological alcohol use (Conger, 1956; Cappell & Herman, 1972; Critchlow, 1986; Koob & Le Moal, 1997; Sinha, 2008).

The corresponding epidemiological literature suggests a positive correlation exists between exposure to stress and alcohol use, but differences have been reported (Keyes *et al.*, 2011). Differences in the effect of prior stress on subsequent alcohol consumption

likely arise from the complexity of stress-alcohol interactions, as well as aspects of study design. Variables to consider across studies include the type of stressor measured, type of questionnaire use, history of alcohol intake, size/diversity of the population under investigation, and the temporal structure of the study (cross-sectional vs. longitudinal). In general terms, stress refers to any external stimuli perceived as threatening or harmful and often elicits a negative impact or outcome (Sinha, 2001; 2008). Stressful stimuli vary across dimensions of severity, chronicity, expectedness, and consequence to mental health (Keyes *et al.*, 2011). Examples of more commonly experienced, less-severe life stressors that negatively influence adult mental health include financial/legal crisis, workplace tension, and interpersonal conflict. Relevant to the experiments presented in this thesis, even common, acute stressors experienced in adulthood have been shown promote alcohol misuse (San Jose *et al.*, 2000; Dawson *et al.*, 2005).

Comparison across populations, communities, and individuals provides further insight into the relationship between general life stressors and alcohol consumption. Larger-scale studies are critical to detect the prevalence of this interaction across the general population. Though few in number, results from these representative cohorts confirm a positive correlation between increasing number of stressors and heavy drinking over the past 6 months (San Jose *et al.*, 2000) or 12 months (Dawson *et al.*, 2005). The National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) found that individuals who experienced six or more stressful events tripled their average daily alcohol intake when compared to a non-stressed cohort with similar drinking patterns (Dawson *et al.*, 2005).

Community samples of adults also report positive correlations between stressful life events and elevated alcohol consumption (Cole *et al.*, 1990; King *et al.*, 2003). Follow-up

diary studies with the same individuals reveal more specifically that negative work and non-work events predict same day increases in alcohol consumption (Carney *et al.*, 2000). Not all communities are equally vulnerable, however. Longitudinal studies examining older adults did not correlate acute stressful life events with increased alcohol consumption (Skaff *et al.*, 1999). This result could be explained by overall reductions in alcohol consumption amongst elderly populations (Skaff *et al.*, 1999). Thus, variation in this relationship exists across different subpopulations.

At the individual level, the vulnerability to stress-induced drinking can be influenced by age, sex, race, and personal coping strategies. For example, males report drinking more after stressful experiences than females (San Jose *et al.*, 2000; Dawson *et al.*, 2005) and demonstrate higher rates of AUDs (WHO, 2014). However, females still report alcohol misuse after stressful experiences (Kilpatrick *et al.*, 1997). In addition to sex differences, stress associated with perceived discrimination correlates with more severe alcohol outcomes (McCabe *et al.*, 2010; McLaughlin *et al.*, 2010; Yoo *et al.*, 2010). Across all social subgroups, individual coping strategies can promote pathological drug use. People who report drinking to cope with distress often develop solitary drinking practices (Smith *et al.*, 1993) and engage in abusive drinking over social drinking (Abbey *et al.*, 1993; Moos *et al.*, 2010). Integration of this literature sheds light on the complex personal and environmental conditions that may render an individual vulnerable to stress-induced alcohol consumption and confirms the pervasive nature of this interaction.

Tobacco and Alcohol Consumption

Like stress, tobacco use is associated with increased alcohol consumption (Carmody *et al.*, 1985; Batel *et al.*, 1995; Barrett *et al.*, 2006). Smoking cigarettes is highly addictive,

claiming more users that show clinical symptoms for drug dependence than any other drug (Anthony & Echeagaray-Wagner, 2000). Factors such as genetic vulnerability, drug availability, social demographics, and age at first exposure influence the propensity to use tobacco and alcohol (Schorling *et al.*, 1994; Bobo & Husten, 2000; Weitzman & Chen, 2005; McKee *et al.*, 2007). Despite this complexity, epidemiological studies consistently show a positive correlation between tobacco and alcohol use (Schorling *et al.*, 1994; Bobo & Husten, 2000; Weitzman & Chen, 2005; McKee *et al.*, 2007). Amongst adults that consume alcohol, 37 percent also smoke cigarettes (Bobo & Husten, 2000). Smokers consume nearly twice as much alcohol as nonsmokers, highlighting a significant dose-response relationship (Carmody *et al.*, 1985).

Tobacco users are also more likely to misuse alcohol and experience alcohol-related harm (DiFranza & Guerrera, 1990; Kozlowski & Ferrence, 1990; Grant *et al.*, 2004; Larsson & Engel, 2004). The combined use of these drugs generates overlapping risk for the development of diseases like cancer and AUDs (Miller & Gold, 1998). Over 83% of alcoholics smoke cigarettes and alcoholism is approximately 10 times more prevalent in smokers than in non-smokers (DiFranza & Guerrera, 1990). In clinical terms, smoking is sufficiently predictive of alcoholism to warrant a screen for assessment of drinking patterns (Kozlowski & Ferrence, 1990). Thus, there are major health risks associated with the co-use of tobacco and alcohol.

Accumulating evidence suggests that the comorbidity of tobacco and alcohol use may originate in adolescence (Cross *et al.*, 2017). Tobacco is often the first drug used by young people (Fleming *et al.*, 1989; Grant, 1998) and 84 percent of smokers have their first cigarette before the age of 18 (Yuan *et al.*, 2015; Richter *et al.*, 2017). Thus, adolescence represents a unique period of vulnerability for smoking habit initiation. Young smokers are

more likely to be heavier drinkers throughout adolescence and adulthood (Harrison & McKee, 2008). Further, individuals that experiment with tobacco at any point are twice as likely to develop an alcohol use disorder compared to never smokers (Grucza & Bierut, 2006). For this reason, adolescent tobacco use is hypothesized to serve as a gateway drug, acting to promote excessive alcohol use later in life (Torabi *et al.*, 1993; Parra-Medina *et al.*, 1995; Lai *et al.*, 2000; Chen *et al.*, 2002; Degenhardt *et al.*, 2010; Kandel & Kandel, 2015; Cross *et al.*, 2017). Gateway theories primarily function to identify life exposures preceding pathological drug use; however, the compelling epidemiology linking alcohol abuse with prior exposure to stress or tobacco has motivated laboratory research into the causal mechanisms underlying these interactions.

Modeling Alcohol Consumption in Rodents

Due to the correlative nature of epidemiological surveys, it is necessary to carry out causal experiments under controlled laboratory conditions. The most rigorous animal model used to study the effect of stress or nicotine on alcohol intake is operant self-administration, where animals are required to press a lever in order to gain limited access to an alcohol drinking solution. This model of drug use possesses high face validity to the human condition and draws upon associative learning mechanisms inherent to operant conditioning (Panlilio & Goldberg, 2007). That is, drugs of abuse reinforce the behavior associated with their delivery by strengthening associative learning in the brain (Everitt & Robbins, 2005; Sanchis-Segura & Spanagel, 2006). Recording lever presses and the corresponding alcohol consumption thus provides a quantitative approach for studying the mechanisms of behavioral reinforcement.

The development of operant alcohol self-administration in rodents generally involves two phases of behavior: the acquisition phase and the maintenance phase (46 Lynch et al 2010). The acquisition phase refers to the initial alcohol exposure period when the patterns and preference of alcohol intake are first established. In an effort to minimize aversive taste and stimulus properties, alcohol is gradually introduced (or faded) into a sweetened, non-caloric drinking solution like saccharin (Samson, 1986; Boyle *et al.*, 1994). Animals quickly adjust their intake to the taste and sensory cues of the drinking solution. After this fading procedure is complete and intake levels stabilize, animals transition to the maintenance phase of self-administration. In addition to acquisition and maintenance, operant self-administration enables investigation into other drug-related behaviors, such as extinction and relapse (Le *et al.*, 2000b; Funk *et al.*, 2014).

Effects of Stress on Alcohol Self-Administration

For the purposes of *in vivo* research, stress refers to any stimulus that perturbs normal homeostasis (Selye, 1950) and promotes the secretion of stress hormones (Herman & Cullinan, 1997). Rodent models of stress-induced drinking show mixed behavioral results and likely reflect differences in experimental design, heterogeneous responses to stress, and limited mechanistic insight (Becker *et al.*, 2011). There are an array of acute and sub-chronic stressors used in experimental research, including foot shock, restraint stress, forced swim test, social defeat, and social isolation. In addition, experience with alcohol consumption prior to stress exposure varies greatly across studies. Differences are also noted between rodent strains, with Long-Evans rats more consistently showing stress-induced drinking than Sprague-Dawley rats for a given stressor (Casey, 1960; Myers & Cicero, 1969; Mills *et al.*, 1977; Mills & Bean, 1978; Brunell & Spear, 2005).

Within-subject variability at baseline and in response to stress could also contribute to equivocal behavioral results. For example, Long-Evans rats show wide variability in their basal saccharin preference during operant self-administration (Ostroumov *et al.*, 2016). Since alcohol is often faded into a sweetened solution for operant self-administration studies, differences in basal saccharin preference could confound the readout of alcohol intake. In addition, efforts to minimize environmental stress in controls presents a constant challenge for animal research, yet it is critical to ensure homogenous, non-stressed rodent populations at the onset of these studies. Finally, rodents can differ in their response to the same stressor (Pfau & Russo, 2015). For example, only a subset of rodents subjected to social defeat show susceptibility to subsequent social aversion and anxiety (Golden *et al.*, 2011). Reflecting divergent stress responses in the context of alcohol consumption, recent studies confirm that isolating stress-sensitive populations more reliably leads to increases in subsequent alcohol consumption (Edwards *et al.*, 2013).

Explicitly addressing these experimental considerations and taking efforts to minimize within-subject variability for a given stressor paradigm is thus critical to the consistency and reproducibility of stress-alcohol studies. In addition to these experimental considerations, it remains unknown how stressful experiences alter neurobiological alcohol responses in drug-naïve rodents. Given the prominent contribution of stress to the development of dependence, withdrawal, and relapse (Le *et al.*, 2000b; Funk *et al.*, 2014), these stress-related neuroadaptations are of high interest to the alcohol field.

Effects on Nicotine on Alcohol Self-administration

In order to examine effects of tobacco on alcohol consumption, animals are either passively exposed or actively self-administer nicotine, the main psychoactive component in tobacco smoke. Passive administration offers enhanced precision with drug dosing

whereas active administration offers greater insight into individual preference (Matta *et al.*, 2007). In rodent behavioral experiments, the reinforcing effects of nicotine exhibit an inverted U-shaped dose–response curve upon peripheral injection (Iyaniwura *et al.*, 2001; Picciotto, 2003), reaching maximal brain concentrations approximately 15 min post-injection (Turner, 1975). Due to rapid drug metabolism in rats ($t_{1/2}$ = 45 min) compared to humans ($t_{1/2}$ = 2 h), larger doses are often used in rodent models (Matta *et al.*, 2007).

Rodent models show that exposure to nicotine can increase subsequent alcohol self-administration. Differences between studies include nicotine administration route, chronicity of exposure, age upon exposure, and the phase of drinking under consideration. Several studies have examined alcohol intake in response to proximal nicotine exposure (Dyr *et al.*, 1999; Nadal & Samson, 1999; Le *et al.*, 2000a; Sharpe & Samson, 2002; Le *et al.*, 2003; Bito-Onon *et al.*, 2011). In these studies, the nicotine was injected prior to daily alcohol self-administration sessions. Overall, the evidence indicates that nicotine treatment can increase alcohol self-administration. For example, three different groups have shown that moderate to high doses of nicotine (0.2–0.8 mg/kg) increase operant responding and alcohol intake in three different rat strains (Le *et al.*, 2000a; Le *et al.*, 2003; Bito-Onon *et al.*, 2011; Leao *et al.*, 2015).

However, a few studies have also reported that repeated nicotine exposure decreases alcohol intake or has no effect on it (Nadal & Samson, 1999; Sharpe & Samson, 2002). If there were methodological differences between studies, it is not clear which one specifically contributed to these results. The studies employed comparable rat strains and nicotine doses, and the nicotine pretreatment was administered at a similar time (15–30 min) prior to the alcohol self-administration session. One possibility is that increases in alcohol intake after nicotine pretreatment might require longer self-administration sessions

to observe the increased consumption (Ahmed & Koob, 1999; Le, 2002). Most studies that report increased alcohol intake used 60-min self-administration sessions, whereas the studies by Samson and colleagues used 30-min sessions. In addition, differences in the schedule of reinforcement (fixed-ratio vs. response requirement) could also factor into these results. In summary, under certain conditions, repeated daily nicotine pretreatment can increase alcohol self-administration, but more work is needed to understand the basis for some discrepancies in the literature.

In contrast to adulthood, adolescent nicotine exposure has longer-lasting influences on subsequent alcohol self-administration (Kemppainen *et al.*, 2009; Larraga *et al.*, 2017). Adolescent rats exposed to nicotine or nicotine plus alcohol (but not alcohol alone) showed significantly higher alcohol intake as adults (Larraga *et al.*, 2017). Continuous peri-adolescent exposure, on the other hand, did not show similar elevations in consumption (Smith *et al.*, 2002). In a separate study, animals treated with nicotine during adolescence showed sensitization to nicotine-induced drinking upon nicotine re-exposure as adults (Kemppainen *et al.*, 2009). Behavioral studies examining nicotine-alcohol interactions thus provide evidence in favor of nicotine exposure, particularly during adolescence, influencing subsequent responses to alcohol. Though underlying mechanisms mediating this interaction have yet to be determined, altered alcohol self-administration likely arises from neural adaptations induced by nicotine or stress hormones.

Neurobiological Effects of Stress, Nicotine, and Alcohol

Influence of Alcohol on Brain Function

The behavioral responses to alcohol arise from its cellular and molecular actions in the brain. In pharmacological terms, alcohol (ethanol) is considered a “dirty drug” because it modulates a wide range of membrane receptors and other molecular targets in neurons, limiting our ability to link specific molecular targets to their corresponding behavioral effects (Harris, 1999). For example, mild to moderate levels of intoxication potentiate inhibitory (GABA) and inhibit excitatory (NMDA) synaptic transmission (Dopico & Lovinger, 2009). Specifically, a concentration of 3-30 mM ethanol enhances $GABA_A$ receptor function while 5-50 mM is sufficient to inhibit NMDA receptor function. Importantly, this dose-response relationship varies further across different $GABA_A$ and NMDA receptor subunits expressed throughout the brain (Alfonso-Loeches & Guerri, 2011). Ethanol also enhances serotonin and nicotinic ligand-gated ion channels as well as non-ligand ion channels like L-type Ca2+, HCN, and GIRK (Luscher & Ungless, 2006).

Despite many targets within the brain, behavioral pharmacological experiments reveal that $GABA_A$ and NMDA receptors contribute to the discriminative stimulus properties (i.e. the ability to report subjective effects of the drug) of ethanol (Grant, 1999). Furthermore, nicotinic and serotonergic signaling can amplify ethanol’s stimulating effects (Kostowski & Bienkowski, 1999). Taken together, ethanol influences brain function and produces a state of behavioral intoxication that arises through the functional modulation of multiple ion channels and intracellular targets. Despite targets across the brain, evidence suggests that alcohol’s action within the mesolimbic dopamine system elicits its addictive properties.

Influence of Stress Hormones on Brain Function

Stressful or arousing events activate the hypothalamic-pituitary-adrenal (HPA) axis to induce long-term adaptive changes in the brain and in behavior (Selye, 1950). Exposure to drugs of abuse activates these same brain stress pathways (Armario, 2010), hindering the ability to tease apart stress-mediated from drug-mediated adaptations under conditions of repeated drug exposure. The stress response is governed by neurons in the paraventricular nucleus (PVN) of the hypothalamus that release corticotropin-releasing factor (CRF) into the hypophyseal portal system. CRF then activates corticotrophs in the pituitary that release adrenocorticotropic hormone (ACTH) into the bloodstream. Peripheral ACTH stimulates the secretion of stress hormones, such as glucocorticoids, from the adrenal cortex (Tsigos & Chrousos, 2002), which modulate the function of nearly every cell in the brain and body.

Glucocorticoid stress hormones influence neuronal activity via numerous genomic and non-genomic cellular cascades (Stahn & Buttgereit, 2008). Upon activation, genomic glucocorticoid receptors translocate to the nucleus, bind glucocorticoid response elements, and broadly modify gene transcription through transactivation or transrepression (Beato & Sanchez-Pacheco, 1996). Though genomic effects initiate within minutes to hours, they can persist over timescales of hours to days. In contrast, non-genomic glucocorticoid effects initiate more quickly, within seconds to minutes, and modulate the function of numerous ionotropic receptors in the cell membrane as well as intracellular signaling cascades (Evanson *et al.*, 2010).

In addition to glucocorticoid signaling, stress hormones can serve as precursors for the production of neuroactive steroids, which exert rapid effects on neural transmission and

regulate neuroendocrine responses (Morrow *et al.*, 2009). More specifically, progesterone and other structurally related molecules can act as potent allosteric modulators of nicotinic, $GABA_A$, and NMDA receptors (Majewska *et al.*, 1988; Wu *et al.*, 1991; Ke & Lukas, 1996; Bullock *et al.*, 1997). Therefore, stress hormones and neuroactive steroids may act in concert through multiple mechanisms to influence the responses to drugs of abuse.

Adding further complexity to the issue, glucocorticoids can produce opposing effects in different brain regions (Koob & Le Moal, 2001). Within the PVN of the hypothalamus, for example, glucocorticoids act as a negative feedback signal to inhibit CRF release and HPA reactivity (Albeck *et al.*, 1994). However, in extra-hypothalamic regions, such as the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST), glucocorticoids deliver a positive feedback signal to potentiate CRF activity (Shepard *et al.*, 2000). CRF activity in extra-hypothalamic regions, including the DA system, contributes to different aspects of drug abuse (Koob & Le Moal, 2001). These complex effects have challenged our ability to link specific stress-mediated adaptations with subsequent alcohol use.

Influence of Nicotine on Brain Function

Nicotine acts upon nicotinic acetylcholine receptors (nAChRs) within the brain. The nAChR is a pentameric ligand-gated ion channel formed by different combinations of subunits (α2-α10 and β2-β4) (Dani & Bertrand, 2007). Heteromeric αβ subunit combinations include α2–α6 and β2–β4. Homomeric subunit combinations of nAChRs include α7–α9 subunits, with α7 nAChRs widely distributed throughout the mammalian brain. Ligand binding to different receptor subtypes produces a diverse range of neurophysiological effects due differing ligand affinity and desensitization dynamics. For

example, α7 nAChRs exhibit low ligand affinity but rapid desensitization whereas α4β2 nAChRs exhibit high ligand affinity and slow desensitization (Wooltorton *et al.*, 2003). Nicotinic receptors can also vary across cellular subtype and subcellular compartmentalization offering multiple opportunities to influence cellular and circuit activity (Laviolette & van der Kooy, 2004b). Like alcohol and stress hormones, nicotine targets receptors within the mesolimbic system to modulate reward-related behaviors.

The Mesolimbic Dopamine System: A Locus for Interactions

Overview of mesolimbic dopamine system

The development of behavioral reinforcement and acquisition of operant self-administration involves the dopamine (DA) system, suggesting a potential locus for interactions between alcohol, stress, and nicotine. (Piazza *et al.*, 1993; Gonzales *et al.*, 2004). This circuitry is known to regulate mood, emotional responses, and incentive-based behavior (Grace *et al.*, 2007; Schultz, 2007). In addition, dysregulation of this system is a hallmark of the drug-addicted state (Volkow *et al.*, 1996; Volkow *et al.*, 2007; Luscher & Malenka, 2011). Neurons in the ventral tegmental area (VTA) are the primary source of DA within the mesolimbic system. Those neurons project to many cortical and forebrain limbic structures, including the nucleus accumbens (NAc), ventral pallidum, amygdala, and the medial prefrontal cortex (mPFC) (See Figure 1.1). DA neurons represent an abundant cell type in the VTA, but the exact percentage of DA neurons varies between sub-regions (Swanson, 1982; Nair-Roberts *et al.*, 2008; Yamaguchi *et al.*, 2011).

The VTA also contains glutamate neurons and GABA neurons that project to the forebrain and can form local synapses with other VTA neurons (Van Bockstaele & Pickel, 1995; Dobi *et al.*, 2010; Yamaguchi *et al.*, 2011). A fourth cell type synthesizes both DA and

glutamate, and there are potentially other mixed transmitter neurons in this area (Hnasko *et al.*, 2010; Stuber *et al.*, 2010). Numerous afferent inputs regulate the activity of DA and non-DA neurons within the VTA (see Figure 1.1). Major excitatory inputs arise from the laterodorsal and pendunculopontine tegmentum, the lateral hypothalamus, the bed nucleus of the stria terminalis, and the prefrontal cortex (Carr & Sesack, 2000; Omelchenko & Sesack, 2005; Massi *et al.*, 2008; Watabe-Uchida *et al.*, 2012). The major GABAergic inhibitory inputs to the VTA arise from the rostromedial tegmental nucleus, the ventral pallidum, the laterodorsal tegmentum, and the nucleus accumbens (NAc) (Geisler & Zahm, 2005; Jhou *et al.*, 2009; Xia *et al.*, 2011). The serotonergic (5-HT) neurons of the dorsal raphe nucleus constitute another important afferent projection, which transmits mainly excitatory signals to the VTA (Herve *et al.*, 1987). Some inputs target specific subsets of VTA neurons. The excitatory inputs from the laterodorsal tegmentum, for example, mainly target VTA DA neurons that project to the nucleus accumbens (Omelchenko & Sesack, 2005), whereas the inhibitory inputs from the nucleus accumbens target primarily non-dopaminergic VTA neurons (Xia *et al.*, 2011).

VTA neuron properties

DA neurons are the most well studied class of cells in the VTA. The classic DA neurons (found mainly in the lateral VTA and in the substantia nigra compacta) display various modes of firing activity *in vivo*, including tonic and phasic activation (Grace & Bunney, 1984; Hyland *et al.*, 2002). Tonic activity involves slow irregular patterns of single action potentials, whereas phasic activity involves short-latency bursts of action potentials (typically 2–4 action potentials, each separated by less than 80ms).

DA neurons in brain slices do not display normal burst activity (Grace & Onn, 1989), indicating that burst firing depends on afferent synaptic input, which is altered and disrupted in brain slices. The tonic firing frequency of DA neurons in freely moving animals is approximately 2–10 Hz. However, within a single burst of action potentials the average firing frequency increases to approximately 15–28 Hz (Hyland *et al.*, 2002; Li *et al.*, 2011). The transition from tonic to phasic firing activity is hypothesized to be a key mechanism for transmitting behaviorally relevant information related to addictive drug reinforcement (Grace *et al.*, 2007). Other molecular substrates, such as DA transporters and DA auto-receptors, further filter DA neuron activity and are modulated by drugs of abuse. Due to circuitry differences in these molecular substrates, phasic DA signals lead to greater extracellular DA levels in the ventral striatum compared to the dorsal striatum (Gonon, 1988; Chergui *et al.*, 1994; Zhang *et al.*, 2009).

In addition to DA neurons, VTA GABAergic and glutamatergic neurons provide input to the mesocorticolimbic target areas. A high firing frequency (typically above 20 Hz) and relatively short action potential duration characterize a well-studied subset of GABAergic neurons (Steffensen *et al.*, 1998; Luo *et al.*, 2008; Li *et al.*, 2011). Recent studies indicate that VTA GABA neurons make selective synaptic connections with cholinergic interneurons in the NAc that influence local DA transmission and associative learning (Brown *et al.*, 2012; van Zessen *et al.*, 2012). Glutamatergic VTA neurons also innervate the NAc and other structures, and their properties and their regulation continue to be studied (Hnasko *et al.*, 2010; Stuber *et al.*, 2010; Yamaguchi *et al.*, 2011).

Alcohol's Action in the Dopamine System

Alcohol self-administration coincides with an increase in DA levels in the NAc (Weiss *et al.*, 1993; Melendez *et al.*, 2002; Boileau *et al.*, 2003) and this DA learning signal transfers to drug-associated cues over time (Doyon *et al.*, 2005). Local infusion of DA receptor antagonists into the VTA and the NAc reduces subsequent operant responses for alcohol (Rassnick *et al.*, 1992; Samson *et al.*, 1993; Hodge *et al.*, 1997), and neurotoxic lesions of the DA system decrease alcohol intake (Rassnick *et al.*, 1993; Ikemoto *et al.*, 1997). The lesions only appear to alter alcohol intake when applied prior to the acquisition of drinking behavior (i.e., prior to training), and not after self-administration behavior becomes established, suggesting that DA signaling is particularly critical during the acquisition and development of alcohol reinforcement. Many other addictive drugs share these dopamine-dependent features of alcohol self-administration.

At concentrations achieved during intoxication (20–100 mM), alcohol moderately excites VTA DA neurons in brain slices, as well as in acutely dissociated DA neurons lacking synaptic inputs. This excitation reflects direct action of alcohol on DA neurons (Brodie & Appel, 1998; Okamoto *et al.*, 2006). For example, alcohol reduces the amplitude of the after-hyperpolarization phase of the action potential and modulates ionic conductances as well as metabotropic signals to increase DA neuron excitability (Brodie & Appel, 1998; Lewohl *et al.*, 1999; Okamoto *et al.*, 2006; Mulholland *et al.*, 2011). In addition to these direct actions, the *in vivo* effects of alcohol on DA neurons arise from a complex interplay between alcohol and many neurotransmitter systems.

Substantial evidence indicates a role of GABA signaling in alcohol self-administration (Boyle *et al.*, 1993; Chester & Cunningham, 2002). Local blockade of $GABA_A$ receptors in

the VTA and the ventral pallidum and knockout of $GABA_A$ receptors in the NAc decreases alcohol self-administration (Nowak *et al.*, 1998; June *et al.*, 2003; Nie *et al.*, 2011). At the molecular level, alcohol binds to specific amino acid residues on the $GABA_A$ receptor, which enhances ionic conductance and positively modulates $GABA_A$ receptor function (Harris, 1999; Glykys *et al.*, 2007).

Application of alcohol to VTA brain slices increases presynaptic GABA release onto DA neurons (Theile *et al.*, 2008) and evidence suggests that *in vivo* alcohol exposure induces a long lasting (at least 24 h to 1 week) potentiation of GABA inhibition in the VTA (Melis *et al.*, 2002). The precise mechanism by which alcohol enhances presynaptic GABA release is not understood, but could involve 5-HT transmission or stress signaling pathways (Nie *et al.*, 2004; Theile *et al.*, 2008). *In vivo* recordings from putative VTA GABA neurons indicate that GABAergic responses to alcohol can vary substantially between cells. Importantly, GABA cells can show transient inhibition and excitation during a single recording (Gallegos *et al.*, 1999). The dose of alcohol and the timing between injections may thus contribute to differing GABAergic responses (Gallegos *et al.*, 1999; Steffensen *et al.*, 2009).

Stress hormones and Alcohol in the Dopamine System

Stressful or arousing experiences promote DA signaling within the mesolimbic system via direct and indirect mechanisms. Exposure to restraint stress, for example, produces a bi-phasic increase in accumbal DA release, with levels peaking at the onset and termination of restraint (Imperato *et al.*, 1991; Imperato *et al.*, 1992; Enrico *et al.*, 2013). This signal is thought to be mediated, in part, by glucocorticoid-dependent facilitation of glutamatergic input onto DA (Yuen *et al.*, 2009; Krugers *et al.*, 2010; Satoh & Shimeki, 2010). Stress hormones can also potentiate DA transmission at target regions by impairing its synaptic

clearance via non-genomic effectors (Graf *et al.*, 2013). Further, neurosteroid metabolites indirectly modulate dopamine transmission and alcohol consumption via $GABA_A$ receptors within the VTA (Follesa *et al.*, 2006; Tanchuck *et al.*, 2013; Cook *et al.*, 2014). These results indicate that the mesolimbic DA system is responsive to stressful experiences and is a primary target of stress hormone signaling.

Extending these findings, several studies support the hypothesis that stress hormones contribute to the motivational properties of alcohol. Alcohol directly activates stress pathways to elicit glucocorticoid release (Ellis, 1966). Adrenalectomized rodents show reduced alcohol self-administration (Fahlke *et al.*, 1994; Shoaib & Shippenberg, 1996), and local activation of glucocorticoid receptors in the ventral striatum has been shown to increase alcohol intake (Fahlke & Hansen, 1999). Further, the development of compulsive drinking and alcohol dependence involves the activation of glucocorticoid receptors within the DA system (Vendruscolo *et al.*, 2012). Taken together, these findings suggest that exposure to stress could influence subsequent responses to alcohol via biological adaptations within the mesolimbic dopamine system.

Nicotine and Alcohol within the Dopamine System

Nicotine acts upon nAChRs within the ventral tegmental area to promote drug use (Corrigall *et al.*, 1994). The most common nAChRs in the rodent VTA are the high affinity β2-containing subtype (often in combination with α4 and/or α6) and the lower affinity α7-containing subtype (Jones & Yakel, 1997; Klink *et al.*, 2001; Wooltorton *et al.*, 2003). DA and GABA neurons, express the high affinity β2-containing nAChR (Klink *et al.*, 2001; Mansvelder *et al.*, 2002). In comparison, the α7-containing nAChR is located predominately on presynaptic glutamatergic inputs and to a lesser extent on VTA GABA neurons (Jones & Yakel, 1997; Klink *et al.*, 2001). The low brain concentrations of nicotine

obtained from tobacco (~20–100 nM) activate the β2-containing nAChRs (Calabresi *et al.*, 1989; Pidoplichko *et al.*, 1997; Picciotto *et al.*, 1998). Within minutes of exposure, nicotine desensitizes the high affinity nAChRs (Pidoplichko *et al.*, 1997; Wooltorton *et al.*, 2003). This process leads to a decrease particularly in GABA inhibition of VTA DA neurons (Mansvelder *et al.*, 2002; Pidoplichko *et al.*, 2004). Due to differences in agonist affinity, these low concentrations of nicotine do not readily desensitize α7- containing nAChRs (Wooltorton *et al.*, 2003; Pidoplichko *et al.*, 2004). This distinction may allow α7-containing nAChRs to exert a prolonged excitatory effect over glutamatergic afferents onto DA neurons, favoring the induction of long-term synaptic potentiation (Mansvelder *et al.*, 2002; Pidoplichko *et al.*, 2004).

It is important to note, however, that the β2-containing, and not the α7-containing, nAChRs are of greater importance during the initiation of nicotine self-administration (Picciotto *et al.*, 1998; Brunzell *et al.*, 2006). A single exposure is sufficient to induce long-term synaptic potentiation of glutamatergic afferents onto DA neurons, as indicated by an increase in the AMPA/NMDA receptor ratio and an increase in the probability of glutamate release (Saal *et al.*, 2003; Gao *et al.*, 2010; Mao *et al.*, 2011). These synaptic changes contribute to learning processes associated with the reinforcement of drug-seeking behaviors.

There is a direct interaction between alcohol and the nicotinic cholinergic system via the nAChRs. Low concentrations of alcohol increase the affinity of acetylcholine for neuronal nAChRs and potentiate the nicotinic currents produced by acetylcholine (Aistrup *et al.*, 1999; Narahashi *et al.*, 1999). Heterologously expressed recombinant nAChRs that contain the α2 or α4 subunit are particularly sensitive to activation by alcohol (Cardoso *et al.*, 1999; Borghese *et al.*, 2003). Evidence indicates that alcohol potentiates receptor function by stabilizing the open state of the α4-containing nAChR (Zuo *et al.*, 2004), but

prolonged exposure to alcohol may increase nAChR desensitization (Nagata *et al.*, 1996). In contrast, alcohol seems to inhibit the nicotinic responses of some α7-containing nAChRs, but the results can vary depending on the expression system or the duration of the bath alcohol application (Covernton & Connolly, 1997; Cardoso *et al.*, 1999). Interestingly, chronic exposure to alcohol was shown to upregulate high affinity nAChRs in the thalamus and hypothalamus (Yoshida *et al.*, 1982), similar to chronic nicotine exposure. Whether alcohol induces similar nAChR upregulation within the DA system remains to be tested.

In addition to any direct actions on nAChR function, alcohol may influence the DA system via activation of afferent cholinergic inputs. Alcohol self-administration elevates extracellular acetylcholine levels in the VTA (Larsson *et al.*, 2005), and a single dose of alcohol activates cholinergic interneurons in the nucleus accumbens, as measured by Fos expression (Herring *et al.*, 2004). Substantial evidence indicates an involvement of distinct nAChRs in alcohol-induced DA release and self-administration. Intra-VTA or systemic blockade of nAChRs antagonists with mecamylamine (a general nAChR antagonist) reduces alcohol consumption and alcohol-induced DA release in the NAc (Ericson *et al.*, 1998; Smith *et al.*, 1999; Le *et al.*, 2000a). Blocking α3β2-containing nAChRs (and/or β3* nAChRs) with α-conotoxin MII prevents alcohol-induced DA release and alcohol self-administration ((Larsson *et al.*, 2004; Jerlhag *et al.*, 2006; Kuzmin *et al.*, 2009). Further, using a novel nAChR ligand, one study has suggested that α4β2-containing nAChRs participate in self-administration of both nicotine and alcohol (Rezvani *et al.*, 2010). Likewise, recent work using α4 nAChR knockout models demonstrates that alcohol-induced DA responses and alcohol conditioned place preference require α4-containing nAChRs (Liu *et al.*, 2013). Knockout studies also suggest a role for the α7- containing

nAChRs in alcohol consumption (Kamens *et al.*, 2010). Interestingly, varenicline, an anti-smoking agent and a partial agonist at several nAChR subtypes, effectively reduces alcohol consumption in rodents and in humans (Steensland *et al.*, 2007; McKee *et al.*, 2009). Varenicline administration also appears to decrease DA release induced by alcohol (Ericson *et al.*, 2009). Although the role of specific nAChR subtypes remains unclear, these findings suggest that nAChRs mediate many actions of alcohol.

It is important to emphasize that these variables do not necessarily occur in isolation. Stress hormone signaling is likely to participate in nicotine-alcohol interactions since both drugs activate stress pathways to elicit glucocorticoid release (Ellis, 1966; Fu *et al.*, 1997; Porcu *et al.*, 2003). Stress hormones can also inhibit nAChRs and influence reward-related responses to nicotine (Shoaib & Shippenberg, 1996; Caggiula *et al.*, 1998). This is supported by the fact that stress causes a down regulation of the nAChR in the rat cerebral cortex and midbrain (Takita & Muramatsu, 1995). Therefore, there are multiple, overlapping pathways within the dopamine system through which stress and nicotine could modulate reward-related responses to alcohol.

Summary

Excessive alcohol use is a persistent phenomenon despite well-documented risks to individual health and general well-being. Epidemiological surveys indicate that exposure to stress or nicotine can predispose certain populations to develop risky drinking habits. Human and animal studies reveal a complex relationship between stress, nicotine, and alcohol consumption and their actions in the brain, highlighting the need for mechanistic investigation. A growing body of literature points to the mesolimbic dopamine system as

a locus for their interaction. The work presented in this thesis identifies a novel adaption within the dopamine system that promotes subsequent alcohol consumption after exposure to stress or nicotine.

Figures

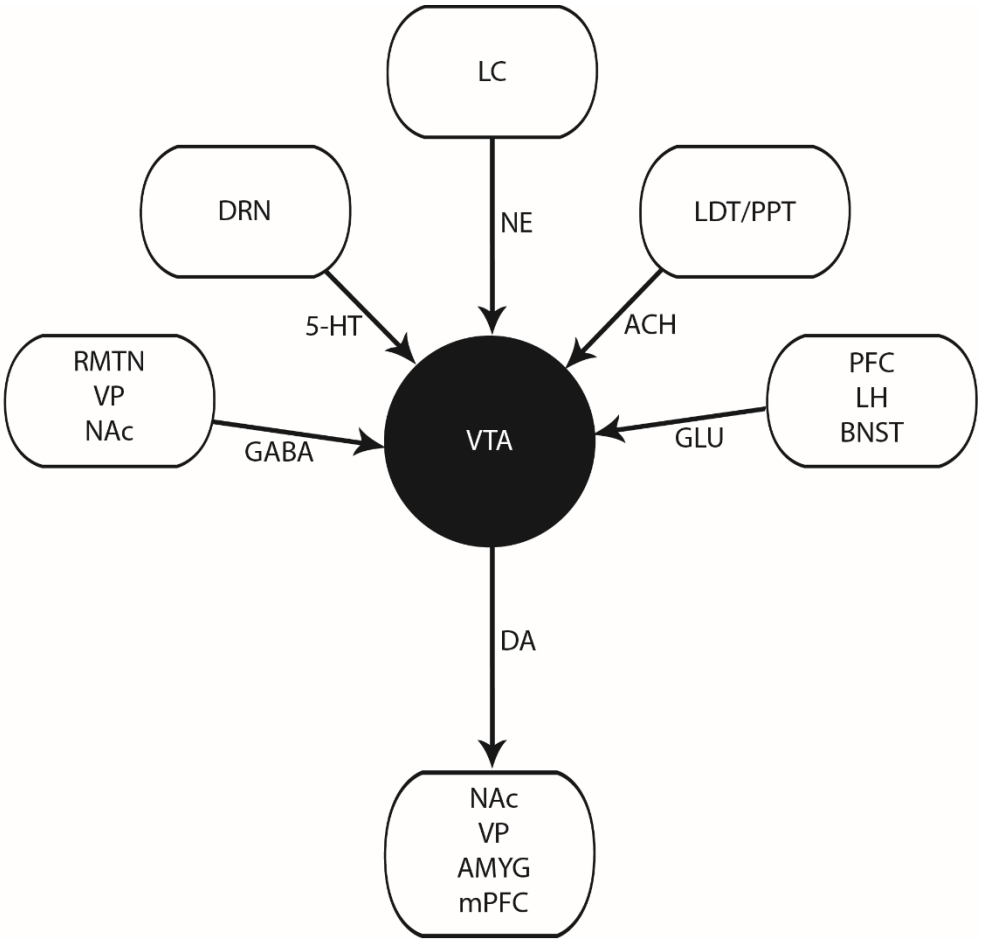

Figure 1.1 Simplified schematic of the mesocorticolimbic reward circuitry. Primary dopaminergic (DA) targets of the ventral tegmental area (VTA) include the nucleus accumbens (NAc), ventral pallidum (VP), amygdala (AMYG), and medial prefrontal cortex (mPFC). Inhibitory (GABA) inputs to the VTA include the rostral medial tegmental nucleus (RMTG), as well as feedback from the VP and NAc. Excitatory (GLU) inputs include the prefrontal cortex (PFC), lateral hypothalamus (LH), and basal nucleus of stria terminalis (BNST). Serotonergic (5-HT) projections from the dorsal raphe nuclei (DRN) as well as noradrenergic (NE) projections from the locus coereleus (LC) provide neuromodulatory input. Cholinergic (ACH) projections arise from the laterodorsal tegmentum and pendunculopontine tegmentum (LDT/PPT).

CHAPTER 2

Stress Increases Ethanol Self-Administration via a Shift toward Excitatory GABA Signaling in the Ventral Tegmental Area

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Author Contributions

A.O. designed and performed ex vivo and in vivo electrophysiological experiments assisted by B.A.K. A.M.T. and W.M.D. designed and performed in vivo experiments. J.S.K. performed western blot and histology experiments. J.A.D. originated, planned, and oversaw the experiments with A.O.'s, A.M.T.'s, and W.M.D.'s assistance. Led by J.A.D., all the authors contributed to write the manuscript.

Abstract

Stress is a well-known risk factor for subsequent alcohol abuse, but the neural mechanisms underlying interactions between stress and alcohol remain largely unknown. We demonstrate in rodents that pre-exposure to stress attenuates alcohol-induced dopamine responses and increases alcohol self-administration. The blunted dopamine signaling resulted from ethanol-induced excitation of GABA neurons in the ventral tegmental area. Excitation of GABA neurons was mediated by $GABA_A$ receptor activation and involved stress-induced functional downregulation of the K+, Cl− cotransporter, KCC2. Blocking stress hormone receptors, enhancing KCC2 function, or preventing excitatory GABA signaling by alternative methods all prevented the attenuated alcohol-induced dopamine response and prevented the increased alcohol self-administration. These results demonstrate that stress alters the neural and behavioral responses to alcohol through a neuroendocrine signal that shifts inhibitory GABA transmission toward excitation.

Introduction

Excessive alcohol use is among the leading causes of preventable death worldwide (WHO, 2014). While many variables contribute to the development of alcohol use disorder (AUD), exposure to stressful life events represents a significant risk factor (Keyes *et al.*, 2012). Stress increases alcohol consumption in alcohol-dependent and non-dependent populations (Ayer *et al.*, 2011; Thomas *et al.*, 2011; Tamers *et al.*, 2014), and stress is thought to underlie a transition to pathological drug (Koob & Le Moal, 2005).

Stress-induced changes in alcohol use likely arise from an interaction between the stress and reward systems of the brain (Uhart & Wand, 2009; Spanagel *et al.*, 2014). At the cellular level, both stress hormones and ethanol influence the DA system by direct actions on DA neurons or indirectly via changes in excitatory and inhibitory synaptic inputs (Saal *et al.*, 2003; Niehaus *et al.*, 2010). Stress hormone signaling also may alter midbrain $GABA_A$ receptor signaling, but the molecular mechanism underlying this adaptation has not been identified and may arise from changes in GABA synthesis, in release, or in expression of specific $GABA_A$ receptor subunits (Maguire, 2014). Alternatively, acute stress exposure has been shown to induce a paradoxical shift toward excitatory $GABA_A$ receptor signaling within the HPA axis by altering the intracellular anion homeostasis (Hewitt *et al.*, 2009; Sarkar *et al.*, 2011). Given that the $GABA_A$ receptor is a target of ethanol, we postulated that alterations in $GABA_A$ receptor transmission could contribute to an interaction between stress and ethanol self-administration.

To examine the interaction between stress and ethanol, we exposed drug-naive rats to stress and then measured their subsequent ethanol intake. Concomitant with increases in ethanol self-administration, we show that acute stress attenuates ethanol-induced DA

release at target regions. These glucocorticoid receptor-dependent effects on DA signaling were mediated by an increase in VTA GABAergic inhibition onto DA neurons. Stress induced the functional downregulation of KCC2 in VTA GABA neurons, shifting GABAA receptor signaling from inhibition towards excitation. Pharmacological activation of KCC2 restored the GABAergic circuitry and DA neuron signaling and prevented the escalation in ethanol self-administration induced by stress. These results indicate that a shift toward excitatory GABA signaling within the mesolimbic system is associated with increased drinking after exposure to stress.

Experimental Procedures

Subjects

Male Long-Evans rats (Harlan-Sprague) weighing 300-500 g were used for in vivo studies. All animals were handled at least 5 days prior to the onset of surgery/behavioral testing and were singly housed in a quiet, temperature- and humidity-controlled satellite facility under at 12-hour light/dark cycle. Rats had food and water available ad libitum in their home cage and were rewarded with sweetened cereal when handled. All procedures were carried out in compliance with guidelines specified by the Institutional Animal Care and Use Committee at University of Pennsylvania.

Drugs and Experimental Design

Unless otherwise noted, all drugs were obtained from Sigma Aldrich and were dissolved in sterile saline. CLP 290 was a generous gift from Drs. Y. De Koninck and A. Castonguay (Laval University, Quebec, Canada). CLP 290 is a carbamate prodrug of CLP257 (Gagnon *et al.*, 2013). Driven by the findings of carboxylesterase expression in the brain (Yamada *et al.*, 1994; Yamada *et al.*, 1995; Holmes *et al.*, 2009; Jones *et al.*, 2013) and by the knowledge that carbamate bonds can be broken even in brain extracts (Fernandez *et al.*, 2000; Fernandez *et al.*, 2003), we hypothesized that CLP290 would be effective even when applied locally in the VTA. Before injecting CLP290 into the VTA, we showed that incubation of VTA brain slices with CLP290 recovered the chloride homeostasis after stress.

RU486 (40 mg/kg) was dissolved in DMSO and administered intraperitoneally (i.p.) 15 min before stress (Saal *et al.*, 2003). All VTA microinfusions were administered at a flow rate of 0.5 µL/min for a duration of 2 min, except for microdialysis (0.5 µL/min for a duration of

1 min). The injector was removed after an additional 1-2 minutes to permit diffusion away from the injection site. For VTA microinfusion experiments, RU486 was first dissolved in DMSO (2 mg/ml) then diluted in ACSF to a final concentration of 2 µg/ml in 10% DMSO. Similarly, acetazolamide and CLP290 were first dissolved in DMSO (1 mM) then dissolved in ACSF to a final concentration of 40-50 uM in 5% DMSO. Intra-VTA microinfusions of acetazolamide or CLP290 occurred 15-30 min before microdialysis or ethanol self-administration. Upon experiment completion, infusions sites were identified post mortem by the location of the injectors in the tissue. Infusions outside the VTA were used for comparison as a negative control. Chicago Sky Blue was infused into the VTA to estimate spatial diffusion of the drugs.

Stressed animals were subjected to a 1 hour of immobilization in a clear cylindrical Broome-style restrainer. Immobilization potently activates the stress hormone systems (Pitman *et al.*, 1988). Behavioral responses to prolonged immobilization include increased vocalization, dander release, defecation, and urination (DeTurck & Vogel, 1982). Animals that did not show defecation were excluded from the study. Restraint stress typically occurred between 5-8 P.M., towards to the beginning of the animals' dark cycle and 15-20 hours prior to ethanol exposure or testing. The 15-hour timeframe between the stress and the ethanol exposure was chosen to examine the long-term effects of acute stress on alcohol responses and to allow the animals to recover from any physical discomfort experienced during the restraint.

Operant ethanol self-administration

Standard operant chambers (Med Associates Inc., St. Albans, VT, USA) were used for the self-administration experiments. Illumination of interior chamber light and presentation of

lever accompanied the start of each session. Depression of the lever triggered 15 sec of access to a retractable drinking spout (a fixed ratio-1 reinforcement schedule). Three rats in the stress treatment group responded for ethanol on a single fixed ratio-4 schedule. Ethanol intake in these rats was not significantly different from other rats in the same group. Each drinking session lasted 60 min/day and occurred daily in most cases, with an occasional day off. Spout licks were recorded by a lickometer.

Animals were initially water-restricted overnight and trained to lever press for a saccharin solution (0.125%, w/v). Once trained, the animals were no longer water restricted and their baseline saccharin intake was monitored for at least 3 days until intake was stable (less than 20% variation in the 2 days preceding ethanol exposure). Importantly, to reduce variability within and between groups, animals were excluded if their saccharin intake was below 5 ml or above 15 ml on average per session. If the animals underwent surgery, saccharin intake was re-established. The effects of stress on acquisition of ethanol self-administration were then measured. Ethanol was introduced into the saccharin drinking solution in the following way: 2% ethanol on day 1 and 4% ethanol for all subsequent days (Fig 1). Consumption was monitored by measuring the volume of liquid in the drinking bottle before and after the session. Lever presses, lickometer responses, and body weight were also measured daily. To achieve consistent behavioral results with this protocol, it is imperative to maintain a quiet, stress-free environment for the animals. Importantly, noise in the animal facility should be kept to a minimum and the animals should be calm and at ease with their handlers.

To assess whether the effect of stress was specific to ethanol and not related to the saccharin in the drinking solution, we included a separate control group that responded for saccharin alone (no ethanol) (n = 8). This saccharin control group did not consume

significantly more fluid after stress exposure compared to their pre-stress baseline: 10.7 ± 1.3 ml/session before stress, 11.6 ± 1.1 ml/session after stress ($p > 0.05$). Moreover, saccharin intake after stress was not statistically different from intake in the non-stressed ethanol control group over a comparable number of days. As an additional saccharin control experiment, we faded saccharin out of the ethanol solution over a period of 5 days. After fading out the saccharin completely, a difference in ethanol intake between control and stress groups was still present. Lastly, stress did not alter intake of a novel high-palatable food (6.0 ± 0.5 g in control vs. 6.8 ± 0.8 g after stress, n = 4 rats/group, $p > 0.05$), suggesting that stress did not enhance novelty-related learning of a reward. For the analysis of blood-ethanol levels, blood samples were taken immediately following the completion of the 60-min self-administration session (within 10 min) and stored in a sealed vial. Blood samples were analyzed on the same day by gas chromatography-mass spectrometry (Atlantic Diagnostic Laboratories, Bensalem, PA).

In vivo Microdialysis

The active dialysis membrane (2.0 mm), was made of hollow cellulose fiber (inner diameter = 200 μm; molecular weight cutoff = 18,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The inlet and outlet to the membrane was composed of fused-silica tubing (inner diameter = 40 μm; Polymicro Technologies, Phoenix, AZ, USA). The microdialysis probes were perfused with artificial cerebral spinal fluid (ACSF in mM): 149.0 NaCl, 2.8 KCl, 1.2-3.2 $CaCl_2$, 1.2-2.4 $MgCl_2$, and 0.25 ascorbic acid, 5.4 D-glucose. The perfusion flow rate was set to 2.0 μl/min. Each sample vial was manually changed and immediately stored at −80° C until analyzed.

Animals were habituated to tethering and microdialysis chambers one day prior to testing. Microdialysis probes were implanted into the NAc after habituation. Dialysis samples were collected every 5 min on the day of testing. Baseline DA samples were collected (15-30 min), followed by a timed intravenous (i.v.) infusion of ethanol (1.5 g/kg, 20% in sterile saline, v/v) over the course of 5 minutes. The i.v. route was chosen to circumvent handling-related disturbances in DA levels associated with i.p. injections (Dong *et al.*, 2010). The dose of ethanol (1.5 g/kg) falls within the typical range tested in rodents (Gonzales *et al.*, 2004) and produces brain ethanol concentrations in rodents that humans can achieve (Howard *et al.*, 2008). Following i.v. infusion of 1.5 g/kg ethanol, the estimated brain ethanol concentration peaks near 50 mM approximately 10 min after the infusion (Doyon *et al.*, 2013a).

Dopamine Analysis

Dialysis samples were analyzed for DA content using high-performance liquid chromatography (HPLC) coupled to an electrochemical detector. The HPLC system included a pump (Model 582; Thermo Scientific, West Palm Beach, FL, USA), an autosampler (Model 542; Thermo Scientific), and an Acclaim 210-2.1 x 50 mm column (3-µm particle size; Thermo Scientific). A coulometric cell (5014B; Thermo Scientific) was connected to a Coulochem II detector. The mobile phase comprised of citric acid (4.0 mM), sodium dodecyl sulfate (3.3 mM), sodium dihydrogen phosphate dehydrate (100.0 mM), and ethylenediaminetetraacetic acid (0.3 mM), acetonitrile (15%), and methanol (5%). The autosampler mixed 9.5 µl of the dialysate with ascorbate oxidase (EC 1.10.3.3; 162 units/mg; Sigma-Aldrich Inc.) prior to injection. DA signals were acquired with 501 chromatography software and Chromeleon Software (Thermo Scientific). Quantification of

dialysate DA concentration was carried out by comparing the peak area to external standards (0–5 nM).

Surgical procedures

Cannulation surgeries were performed for microdialysis and self-administration experiments as described previously (Doyon *et al.*, 2013a). For microdialysis experiments, animals were implanted with a catheter in the jugular vein and a stainless steel guide cannula (21G, Plastics One) above the NAc (mm from Bregma: AP +2.1, ML -1.1, DV -4.0). For microdialysis experiments involving intra-cranial infusions, animals were implanted with an additional 24 G guide cannula above the VTA (mm from Bregma: AP -5.85, ML -0.8, DV -6.5) ipsilateral to the NAc cannula. For behavioral studies, animals were implanted with bilateral cannulae (30G, Plastics One) targeting the VTA (mm from Bregma: AP -5.8, ML ±0.8, DV -6.5). Recovery from surgery took approximately 3-5 days and was accompanied by daily handling and stable increases in body weight.

Histology

The rats were deeply anesthetized with a combination of Ketamine and Xylazine (0.1 mL/100 g bodyweight). Saline was then perfused through the heart, followed by 10% formalin (v/v). The brains were removed and immersed in 10% formalin for at least 2 days. The brains were cut into 75-µm coronal sections on a vibratome (Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with cresyl violet to determine the anatomical placements of the microdialysis probes and microinfusion injectors.

Midbrain slices and electrophysiology

Horizontal slices (230 μm) containing the VTA were cut on a vibratome (Leica Microsystems) from Long-Evans rats (21–30 days old) in ice-cold, oxygenated (95% O2/5% CO2) high-sucrose ACSF (in mM): 205.0 sucrose, 2.5 KCl, 21.4 NaHCO3, 1.2 NaH2PO4, 0.5 CaCl2, 7.5 MgCl2, 11.1 dextrose. Immediately after cutting, slices were transferred to normal ACSF buffer (in mM): 120.0 NaCl, 3.3 KCl, 25.0 NaHCO3, 1.2 NaH2PO4, 2.0 CaCl2, 1.0 MgCl2, and 10.0 dextrose, 20.0 sucrose. The slices were constantly oxygenated (95% O2/5% CO2) and maintained at 32°C in ACSF for 40 min, then at room temperature for at least 60 min.

For incubation experiments, slices were bathed in corticosterone (1 μM), RU486 (10 μM) or CLP290 (10 μM) for an additional hour (Gagnon et al., 2013; Pitman et al., 1988; Yoshiya et al., 2013). To perform electrophysiological recordings, slices were transferred to a holding chamber and perfused with normal ACSF at a constant rate of 2-3 ml/min at 32°C. Patch electrodes made of thin-walled borosilicate glass (1.12mm ID, 1.5 mm OD; WPI) had resistances of 1.0–2.0 MΩ when filled with the internal solution (in mM): 135.0 KCl, 12.0 NaCl, 2.0 Mg-ATP, 0.5 EGTA, 10.0 HEPES, and 0.3 Tris-GTP, pH 7.2–7.3.

DA neurons were identified in the lateral VTA by their morphology (> 20 μm soma size), low firing frequency (1–5 Hz), and the presence of a large Ih current, which together correlate (> 94%, 34 of 36) with tyrosine hydroxylase (TH)-positive cells (Chen et al., 2008; Zhang et al., 2010). In contrast, VTA GABA neurons were identified by the combination of factors including small somata size, high firing rate (> 7 Hz), and the lack of Ih current. Cells with these properties were consistently TH-negative (> 95%, 47 of 49) (Klink *et al.*, 2001; Korotkova *et al.*, 2006; Margolis *et al.*, 2006).

In whole-cell configuration, spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded in voltage clamp mode while holding VTA DA neurons at −60 mV. To isolate sIPSCs, ionotropic glutamatergic synaptic transmission was inhibited by AP5 and DNQX in the perfused ACSF. Ethanol (50 mM) was added to the perfused ACSF to assess ethanol-induced alterations in sIPSCs. sIPSCs were blocked by picrotoxin. Synaptic GABAA input was isolated using DNQX, AP5 and CGP55845. The liquid junction potential between the bath and the pipette solutions was corrected before either recordings or data analysis.

The firing rates VTA GABA neurons were recorded in cell-attached configuration in passive voltage-follower mode. For repetitive synaptic stimulation recordings in cell-attached configuration, a bipolar tungsten stimulating electrode (World Precision Instruments, Inc) was placed 100–150 μm away from the recording electrode. Trains of constant-current pulses (20 Hz frequency, 1 s duration, 200 μA amplitude) were applied with an inter-stimulus interval of not less than 1 minute. At least 3 stimulation trains were conducted for each cell and the responses were averaged. Synaptic GABAA receptor stimulation was isolated using AMPA, NMDA and GABAB receptor antagonists (DNQX, 20 μM; AP5, 50 μM, and CGP55845 1 μM respectively). Contributions of GABAergic synaptic inputs to ethanol (50 mM) induced alterations in firing rate were assessed with the GABAA-receptor antagonist, picrotoxin (50-100 μM). Some cells were also backfilled with neurobiotin for immuno-identification.

Immunohistochemistry

To validate the identification of VTA DA and VTA GABA neurons, neurobiotin backfills and TH double labeling were used. The recording pipette contained 0.3% neurobiotin (Vector

Laboratories). Slices were fixed with 10% neutral formalin phosphate buffer for 12–24 hours, incubated in a blocking solution containing 3% normal goat serum solution and 0.3% triton X-100 for 2 hours, and then incubated overnight with primary anti-TH (1:100; Millipore, #AB152) at 4 °C. The slices were then rinsed with PBS and treated with the secondary antibody Cy3- conjugated anti-rabbit IgG (1:200) and AMCA-conjugated streptavidin (1:1000; both from Jackson ImmunoResearch).

To analyze KCC2 immunolabeling in the VTA, rats were perfused with PBS (Chemicon), followed by 4% paraformaldehyde (Boston BioProducts). Brains were post-fixed for additional 2 hours in 4% paraformaldehyde and then kept in 30% sucrose for 24-48 hours. VTA sections were cut at 25-30 µm and processed with antibodies against KCC2 (1:500, Millipore, #07-432) and TH (1:1000, Millipore, #MAB318) overnight at 4ºC. After washing in PBS, immunofluorescence reactions were visualized using secondary antibodies labeled with AlexaFluor 488 or AlexaFluor 594 (1:1000; Invitrogen) and confocal microscopy.

Western Blots

The VTA was harvested in horizontal brain slices from adult animals (VTA slices were prepared as described in Midbrain slices and electrophysiology section). Membrane fractions were prepared [Mem-PER Plus Membrane Protein Extraction Kit (Model 89842; Thermo Scientific, Rockford, IL). Samples (30 µg of protein) in 2.5% 2- mercaptoethanol were run through a 4-15% Precast Protein Gel (4561083; Bio-Rad). The sample was transferred to nitrocellulose membrane (Bio-Rad). Primary antibodies used were rabbit anti-KCC2 antibody at 1:400 (07-432; Millipore, Temecula, CA), rabbit anti-Phospho-Ser940 KCC2 antibody (p1551-940; PhosphoSolutions, Aurora, CO) at 1:1000, and

mouse anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (MAB374; Millipore) at 1:400. Secondary anti-bodies used were goat anti-rabbit IgG secondary antibody (T2191; Applied Biosystems, AB Foster City, CA) or goat anti-mouse IgG/IgM (T2192, Applied Biosystems). All antibobodies were diluted in SignalBoost solution (407207; EMD Millipore Corp., Billerica, MA).

Membranes were developed using Tropix CDP-Star solution (T2218; Applied Biosystems) for 5 minutes, then scanned using the Protein Simple FluorChem R chemiluminescence detector, and analyzed using AlphaView SA software. The optical densities of KCC2- and Phospho-Ser940 KCC2-specific bands were measured and normalized to the GAPDH values.

Statistical analysis

Analysis of variance (ANOVA) with repeated measures (in SPSS for Windows) was used to analyze the dialysate and the % of basal DA concentrations, and the DA and GABA neuron firing rates. For analysis of action potential firing, the raw data (in Hz) were converted into a percentage of basal, and the last three bins (2-min each) before bath application of ethanol were used as the baseline. For analysis of repetitive synaptic stimulation, the last ten bins (0.5-s each) before the stimulus were used as the baseline. A two-tail t-test assuming equal variance was used to assess differences between the mean sIPSC frequency, the mean firing rates, as well as the mean ethanol intake levels. For western blot analysis, a paired t-test was used to compare protein levels from control and stress littermates that were run on the same gel. Significance for all analyses was determined by $p < 0.05$.

Results

Stress increases ethanol self-administration via glucocorticoid receptors

We first examined how a single episode of restraint stress alters subsequent ethanol intake measured during daily operant self-administration sessions (Figure 2.1A). Stable lever pressing for saccharin (0.125%, w/v) was first established followed by the introduction of ethanol (2%–4%) into the drinking solution (Doyon *et al.*, 2013a). Animals were subjected to restraint stress (1 hr) approximately 15 hr prior to the first ethanol self-administration session. The 15 hr separation between the stress and ethanol self-administration was chosen to examine the lasting impact on neural circuits, not the immediate proximal influence of the stressor itself (Noori *et al.*, 2014).

Pre-exposure to stress caused a significant and long-lasting increase in ethanol self-administration compared to the non-stressed control group (Figure 2.1A): group: $F(1,21) = 19.32$, $p < 0.01$. Blood-ethanol levels were measured in a subset of animals and were correlated with ethanol intake (Figure 2.1B). Stressed rats showed significantly higher blood-ethanol levels (120.8 ± 13.6 mg/dL, n = 5) than non-stressed rats (61.3 ± 4.3 mg/dL, n = 10, $p < 0.01$). Mean intake of ethanol over the first 7 days was 0.74 ± 0.03 g/kg for the control group (n = 19) and 0.95 ± 0.03 g/kg for the stressed group (n = 16, $p < 0.01$) (Figure 2.1C, black and red bars). Elevated drinking after stress was also observed at higher ethanol concentrations (7%–10%) over a 3-week period Figure 2.2). Therefore, acute restraint stress induced robust changes in the acquisition and maintenance of ethanol-drinking behavior.

Restraint stress is known to increase circulating glucocorticoid levels (Pitman *et al.*, 1988). To determine whether the effect of stress on drinking was mediated by stress hormone signaling, a separate group of rats was pretreated with RU486 (a glucocorticoid receptor antagonist) prior to stress. Pretreatment with systemic RU486 prevented the stress from increasing subsequent ethanol self-administration (Figure 2.1C, dark blue bar; 0.67 ± 0.07 g/kg). To determine whether stress hormone signaling acted locally within the DA system (Uhart & Wand, 2009; Spanagel *et al.*, 2014), RU486 or vehicle was microinfused bilaterally into the VTA prior to stress exposure (Figure 2.3). Intra-VTA administration of RU486 prevented stress from increasing subsequent ethanol self-administration (Figure 2.1C, light blue bar; 0.55 ± 0.05 g/kg), revealing that the effect of stress requires glucocorticoid receptor activation within the VTA. In non-stressed control rats, RU486 did not significantly influence ethanol intake when administered systemically or by local microinfusion (Figure 2.1C, gray bar on the right; 0.67 ± 0.04 g/kg), demonstrating a selective effect of RU486 on stress-induced drinking. Additional control experiments with saccharin and palatable food suggest that the effect of stress was specific to ethanol self-administration.

Stress Attenuates Ethanol-Induced DA Activity In Vivo via Glucocorticoid Receptors

Because ethanol self-administration involves DA signaling in the nucleus accumbens (NAc) (Gonzales *et al.*, 2004), we hypothesized that stress might also alter ethanol-induced DA release in the NAc. To test this hypothesis, we first subjected rats to 1 hr restraint stress 15 hr prior to ethanol exposure. Then we collected microdialysis samples to measure ethanol-induced changes in the extracellular DA concentration (Figure 2.4A). We observed a sustained increase in DA levels in the control group (Figure 2.4B, black trace), but stressed animals showed a blunted DA response to ethanol (Figure 2.4B, red

trace): group × time: $F(10,160) = 2.79$, $p < 0.01$. No significant differences in baseline DA levels were detected between control and stress groups: 2.0 ± 0.2 nM in control versus 2.1 ± 0.2 nM after stress.

Next, we tested whether glucocorticoid receptor activation mediated the stress-induced decrease in ethanol-evoked DA release. Systemic pretreatment with RU486 prevented the inhibitory effect of stress on ethanol-induced DA release (Figure 2.4B, gray data). The distribution of the microdialysis probe placements within the NAc is shown in Figure 2.5.

Stress Enhances GABA Release onto VTA DA neurons Ex Vivo

Ethanol stimulates DA release in the NAc by increasing the firing rate of VTA DA neurons (Foddai *et al.*, 2004). No adaptations in basal DA cell excitability or glutamatergic inputs were detected after stress (data not shown). However, blunted DA responses to ethanol in stressed animals were associated with increased GABAergic inhibition onto VTA DA cells (Figure 2.6). Whole-cell patch-clamp recordings of VTA DA neurons measured spontaneous inhibitory postsynaptic currents (sIPSC) in the presence of ethanol (Figure 2.6A,B). In control animals, bath-applied ethanol produced a small increase in sIPSC frequency (116.1% ± 5.0%). In contrast, DA neurons from stressed animals showed significantly greater ethanol-induced potentiation of sIPSC frequency compared to the control response (171.5% ± 7.2%) (Figures 2.6C, black and red data; $n = 8–10$, $p < 0.01$). Systemic injection of RU486 prior to stress prevented the stress-mediated increase in sIPSC frequency observed after ethanol application (Figure 2.6C, gray bar): 110.6% ± 4.5%, $n = 8$.

To further demonstrate glucocorticoid actions within the VTA were mediating the effects of stress, brain slices from control rats were incubated in corticosterone (1 μM) for 1 hr

(Pitman *et al.*, 1988). DA neurons from control animals treated with corticosterone showed a potentiation of ethanol-induced sIPSC frequency that was indistinguishable from stressed animals (Figure 2.6C, dark blue bar): 182.7% ± 10.7%, n = 8. Importantly, incubation with RU486 prevented this corticosterone-mediated increase in sIPSC frequency (Figure 2.6C, light blue bar): 114.5% ± 5.5%, n = 6. Increased frequency, but not amplitude, of sIPSCs suggests that the change caused by stress and corticosterone resides with the presynaptic neuron (i.e., the GABA neuron) not with the postsynaptic neuron (i.e., the DA neuron).

Stress Promotes Excitatory GABA Input onto VTA GABA Neurons Ex Vivo

Our results revealed that stress increased VTA GABA cell firing upon ethanol exposure via a GABAA-dependent mechanism (data not shown). To determine how stress influenced VTA GABA activity, we measured VTA GABA neuron firing rates in response to repetitive stimulation of synaptic GABAA receptor inputs with ionotropic glutamate receptors inhibited (Figure 2.7). Upon electrical stimulation (20 Hz for 1 s), GABA neurons from control animals showed decreased firing (Figure 2.7A-B, black data), indicative of GABAergic inhibition of the recorded GABA neuron. In marked contrast, slices from stressed animals showed increased GABA neuron firing after GABAA receptor stimulation (Figure 2.7A-B, red data): group × time: $F(29,464) = 10.03$, $p < 0.01$. This finding directly demonstrates excitation mediated by high-frequency stimulation of GABAA receptors. Importantly, this effect was blocked by picrotoxin (Figures 2.7C, top and 2.7D, Stress + picrotoxin), providing further confirmation that the observed excitation of VTA GABA neurons following stress was mediated by GABAA receptors. In control animals, similar GABAA receptor-mediated excitation was also observed following 1 hr incubation of brain slices in corticosterone (Figure 2.7D, blue bar, Cort), suggesting that prolonged exposure

to corticosteroids is sufficient to promote excitatory GABAA transmission onto VTA GABA neurons.

It has been reported (Staley *et al.*, 1995) that GABAA receptor-mediated excitation can be prevented by application of acetazolamide, an inhibitor of carbonic anhydrase. Based on these findings, we postulated that acetazolamide would prevent the transition from GABAA receptor-mediated inhibition to excitation of GABA neurons observed after stress. Bath application of acetazolamide (10 μM) did not change basal GABA neuron firing rate between control and stress groups, nor did it change control responses to repetitive stimulation. However, repetitive stimulation in the presence of acetazolamide blocked the increase in GABA neuron firing after stress (Figures 2.7C, bottom, and 2.7D, Stress + ACTZ).

Acetazolamide Prevents Stress and Ethanol Interactions In Vivo

Given that stress promoted excitatory GABA input onto VTA GABA neurons, we tested whether this phenomenon mediated the stress-induced alterations in alcohol responses. To determine if excitatory GABA transmission contributed to blunted ethanol-induced DA release in the NAc observed after stress, we infused acetazolamide into the VTA prior to the microdialysis experiments (Figure 2.8A). In contrast to intra-VTA infusion of vehicle, acetazolamide prevented the inhibitory effect of stress on ethanol-induced [DA] (Figure 2.8B): group × time: $F(10,120) = 2.96$, $p < 0.01$. The effect of acetazolamide was indistinguishable from the non-stressed control group (Figure 2.8B, black dotted trace). Microinfusion of acetazolamide outside the VTA did not reverse the inhibitory effect of stress exposure (Figure 2.9A). In unstressed control animals, the microinfusion of acetazolamide in the VTA did not alter the DA response to ethanol (data not shown, $n = 6$, $p > 0.05$).

To prevent stress from escalating ethanol intake, we bilaterally infused acetazolamide into the VTA prior to each ethanol self-administration session (Figure 2.8C). Intra-VTA infusion of acetazolamide significantly decreased the average daily ethanol intake in the stressed group (0.67 ± 0.06 g/kg) compared to the stressed group that received intra-VTA infusions of vehicle (0.92 ± 0.08 g/kg) (Figure 2.8D; n = 10–14, $p < 0.05$). These data were indistinguishable from the non-stressed control group (Figure 2.8D, dotted horizontal line). VTA infusions of acetazolamide did not alter ethanol consumption in control animals (Figure 2.9B). All microinfusion sites of acetazolamide in the VTA are shown in Figures 2.9C-D.

Stress and Glucocorticoids Dephosphorylate KCC2 at Serine 940

Excitatory GABA transmission can arise from high intracellular chloride concentrations, which are mediated by decreases in Cl− extrusion capacity. Stress-induced reductions in Cl− extrusion capacity have been associated with dephosphorylation of the K+, Cl− cotransporter, KCC2, at serine 940 (S940) (Sarkar *et al.*, 2011; Kahle *et al.*, 2013). To examine stress-induced alterations in KCC2 protein expression and its phosphorylation in the VTA, we performed western blot analysis using an antibody against total KCC2 protein, as well as a phospho-specific antibody against the KCC2 phosphorylation site S940 (Sarkar *et al.*, 2011). Immunoblots revealed two prominent bands (~140 and ~270 kDa) for both total and S940 KCC2 antibodies, indicating the presence of monomeric and dimeric structures of KCC2 protein (Figure 2.10A) (Hewitt *et al.*, 2009). No significant differences in the expression of total KCC2 protein between control and stressed groups were observed (Figure 2.10B, red data) or in controls tissue incubated with corticosterone ex vivo (Figure 2.10C, blue data). In contrast, the ratio of phosphorylated-S940 KCC2 to total KCC2 protein after stress was significantly lower compared to control (Figures

2.10D): 78.3% ± 5.5% for monomer, 79.6% ± 4.7% for dimer. Incubating control tissue in corticosterone recapitulated this effect (Fig2.10E): 75.5% ± 7.6% for monomer, 78.6% ± 9.4% for dimer. This finding confirms that glucocorticoid exposure influences KCC2 phosphorylation state. Importantly, as also reported previously (Taylor *et al.*, 2015), immunolabeling analysis in the VTA suggested that KCC2 protein was expressed exclusively on non-DA neurons (Figure 2.10F), which is consistent with the presence of another chloride extrusion mechanism in DA neurons (Gulacsi *et al.*, 2003). Taken together, these results suggest that stress or corticosterone leads to dephosphorylation of KCC2 protein at S940, which decreases KCC2 function and alters anion homeostasis.

Enhanced Cl− Extrusion Restores Anion Homeostasis in VTA GABA Neurons

Based on our findings in Figure 2.10, we hypothesized that enhancement of Cl− extrusion would restore normal alcohol self-administration in stressed animals. To enhance Cl− extrusion specifically, we used CLP290, a recently developed pro-drug that activates KCC2 (Gagnon *et al.*, 2013). CLP290 or vehicle was bilaterally infused into the VTA prior to the first ethanol self-administration session and measured ethanol intake over 7 days (Figure 2.11). Intra-VTA infusion of CLP290 (Figure 2.12) significantly decreased the average daily ethanol intake in the stressed group back to control levels (0.79 ± 0.05 g/kg) compared to the stressed group that received intra-VTA infusions of vehicle (1.00 ± 0.08 g/kg) (Figure 2.11; $n = 12–13$, $p < 0.05$). These data were indistinguishable from the non-stressed control group (Figure 2.11, dotted horizontal line). VTA infusions of CLP290 did not alter ethanol consumption in control animals (0.84 ± 0.05 g/kg, data not shown).

Discussion

While epidemiological studies consistently report associations between stress and ethanol consumption (Keyes *et al.*, 2012), the underlying neuronal effects have not been well delineated. We found that alterations in GABAA receptor responses on GABAergic neurons of the VTA correlate with an increase in ethanol self-administration induced by temporally distant, acute stress. After stress, we detected enhanced VTA GABAergic inhibition of DA neurons and reduced mesolimbic DA release in response to ethanol. Blunted DA signaling was mediated by a transition toward excitatory GABAA receptor signaling in the VTA and was associated with decreased functional expression of KCC2. Stress-induced adaptations were prevented by acetazolamide (Staley *et al.*, 1995) or by CLP290 (Gagnon *et al.*, 2013). The effect of stress on GABA transmission was recapitulated in vitro by corticosterone exposure and was prevented by pharmacological blockade of glucocorticoid receptors (Cadepond *et al.*, 1997). Most importantly, when acetazolamide, CLP290, or RU486 were locally infused in the VTA *in vivo*, stress no longer increased ethanol self-administration.

The decreased DA response to ethanol was correlated to the stress event and to the excitatory GABA signaling. Although the DA response was not directly examined as a cause of the increased self-administration, others have reported that enhancing DA signaling exogenously attenuates voluntary drinking in rats (Bass *et al.*, 2013). Furthermore, the correlation between decreased ethanol-induced DA release and increased self-administration has been previously reported in rodent studies (Brodie & Appel, 2000; Ramachandra *et al.*, 2007; Doyon *et al.*, 2013a).

The shift toward excitatory GABAA receptor signaling was correlated to the stress event and was required for the stress to cause increased ethanol self-administration (Figure 2.8). Although GABAA signaling normally mediates inhibitory synaptic transmission in the adult mammalian nervous system, it can shift toward excitation under certain pathological conditions, including epilepsy, neuropathic pain, and neuronal trauma (De Koninck, 2007). The shift arises from the decreased function of the chloride extrusion pump, KCC2 (Kaila *et al.*, 2014). Upon strong GABAA receptor stimulation, diminished function of KCC2 leads to the accumulation of chloride ions inside the cell and subsequent loss of the chloride gradient. Activity-dependent loss of the hyperpolarizing chloride gradient unmasks an outward flux of bicarbonate ions through GABAA receptors, resulting in neuronal depolarization/excitation (Staley *et al.*, 1995). Consistent with this model, we found that the GABAergic circuitry responds as expected in the basal condition. However, when GABAA receptors are highly engaged by strong stimulation (Figure 2.7) or by ethanol (Figures 2.8A), a compromised extrusion capacity leads to a collapse in the Cl^- gradient, excitation of VTA GABAergic neurons, and blunted dopamine responses are detected. This shift toward excitatory GABA may occur elsewhere in the brain after stress, but the increased ethanol self-administration was prevented if this shift was blocked in the VTA. Although an excitatory shift in GABA transmission in the adult brain is usually associated with pathological conditions, similar transitions were found in the HPA axis following stress and in the VTA following chronic exposure to opiates (Laviolette *et al.*, 2004; Hewitt *et al.*, 2009; Sarkar *et al.*, 2011). When our results are taken with the accumulation of evidence in the literature, it suggests that the shift toward excitatory GABA signaling may be a more common phenomenon than is presently appreciated (Chung, 2012; Astorga *et al.*, 2015).

Here we demonstrated that glucocorticoid receptor signaling within the VTA was necessary to increase ethanol self-administration after stress. Moreover, prolonged exposure to corticosterone in vitro (in midbrain slices) was sufficient to induce neuroadaptations associated with in vivo stress. These findings highlight the importance of glucocorticoid signaling within the VTA, but we do not rule out the participation of other stress signaling molecules or hormones, such as CRF, in mediating stress-induced adaptations (Ungless *et al.*, 2003; Hwa *et al.*, 2016). Furthermore, the effect of glucocorticoids on VTA GABA neurons may involve the activity of noradrenaline, glutamate, and glial cells (Coull *et al.*, 2003; Hewitt *et al.*, 2009; Lee *et al.*, 2011; Taylor *et al.*, 2015; 2016).

Although animal studies generally support the hypothesis that stress increases ethanol consumption, some results have shown that stress decreases intake or has no effect (Becker *et al.*, 2011). These differences arise from a combination of factors, including the type of stressor used, the duration or timing of the stressor, as well as the type of drinking paradigm employed (Noori *et al.*, 2014). An important parameter in our experimental design is that the stress exposure was well-separated (15–20 hr) from the ethanol self-administration, which allowed us to examine the lasting neural circuit consequences of the treatment not the proximal effect of stress itself. In our study, we kept the ethanol content of the drinking solutions relatively low during the acquisition phase, which likely resulted in less variability in self-administration. The rats experienced less of the aversive stimulus cues of ethanol while still achieving significant blood-ethanol levels. In addition, some animals show resilience to the effects of stress (Pfau & Russo, 2015), so it is essential to verify that there is a physiological response to the stressor and to exclude animals that do not show that response.

In summary, we showed that acute stress exposure decreases the sensitivity of the DA system to ethanol and increases subsequent ethanol self-administration. These effects required a shift toward excitatory GABAA signaling in the VTA and were associated with decreased chloride extrusion capacity in VTA GABA neurons. The temporally distant, acute, intense stress experience produced long-lasting neuroadaptations within the mesolimbic systems of the brain that were expressed upon exposure to ethanol. This overall process represents one mechanistic pathway linking life stress experiences to increased alcohol use. However, future work should determine whether similar mechanisms also contribute to the effects of stress during chronic alcohol use and relapse.

Figures

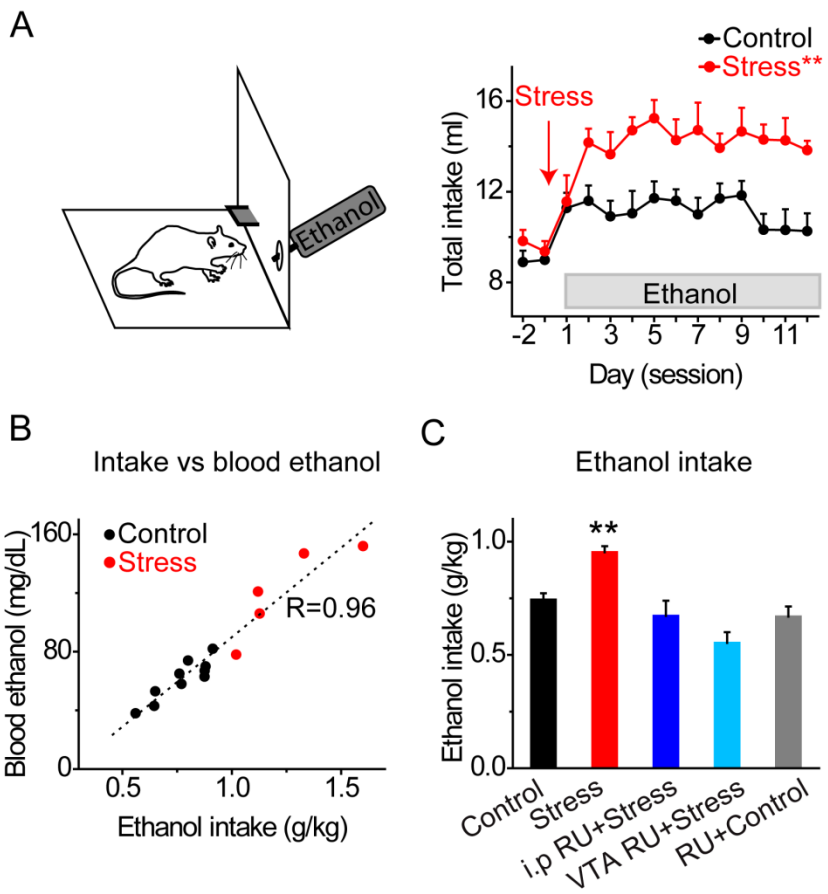

Figure 2.1 Stress increases ethanol self-administration. (A) Rats self-administered saccharin prior to fading ethanol into the drinking solution. Rats were subjected to a single restraint stress 15–20 hr before the first ethanol exposure (red arrow). Daily fluid intake was measured in control and stressed rats. Stressed rats showed greater ethanol intake compared to unstressed control rats. **Significantly different from the control group by ANOVA with repeated measures, $p < 0.01$, n = 16–19 rats/group**. (B)** Ethanol intake (g/kg) versus blood ethanol levels (mg/dL). Blood ethanol was measured immediately after the self-administration session in control (black) and stressed (red) animals. A regression analysis showed a significant and positive correlation between ethanol intake and blood ethanol levels, $F(1,13) = 162.7$, $p < 0.01$. **(C)** Mean daily ethanol intake over the first seven self-administration sessions. Stressed rats (red bar) consumed significantly more ethanol (g/kg) compared to control rats (black bar). Blockade of glucocorticoid receptors with RU486 systemically (dark blue, 40 mg/kg, i.p.) or locally in the VTA (light blue, 40 ng/1 mL) prior to stress prevented increases in ethanol intake, n = 10, 14. RU486 administered systemically or intra-VTA to control animals did not alter ethanol intake, n = 9, gray bar. **Significantly different from all groups by t test, $p < 0.01$.

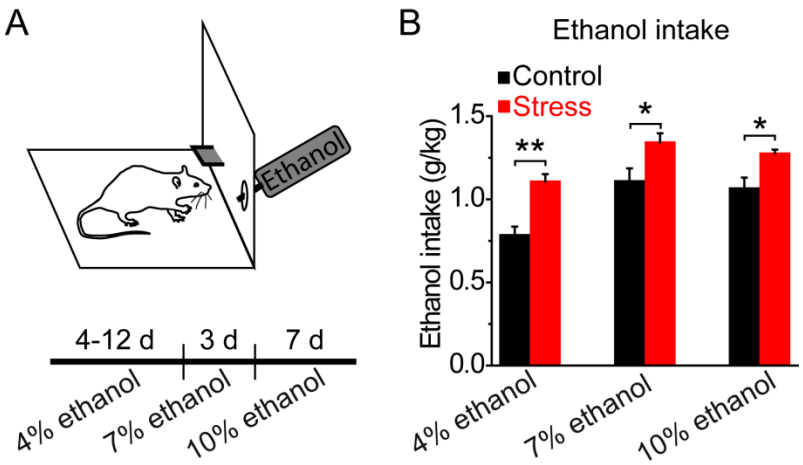

Figure 2.2 Stress increases ethanol self-administration via glucocorticoid receptor activation. (A) Control and stressed animals self-administered 4% ethanol for 4-12 days, then concentrations were increased to 7% ethanol for 3 days, followed by 10% ethanol for 7 days. **(B)** Mean daily intake for 4%, 7% and 10% ethanol are plotted. Significance of difference between control and stressed groups for each ethanol concentration was determined by t-test (*$p < 0.05$; **$p < 0.05$), n = 5-7 rats/group.

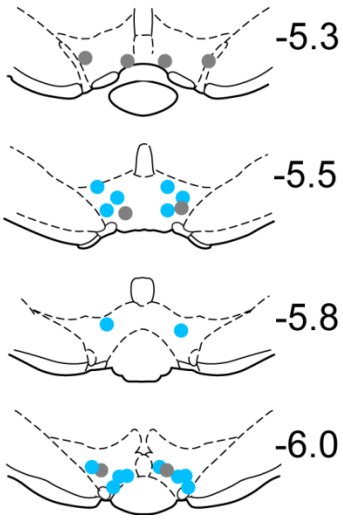

Figure 2.3 VTA microinfusion sites. Following self-administration experiments, the microinfusion sites of RU486 were determined. The distribution of injection sites in the VTA was similar between control (gray circle) and stressed animals (light blue circle) and distributed across coronal sections 5.3-6.0 mm posterior from bregma.

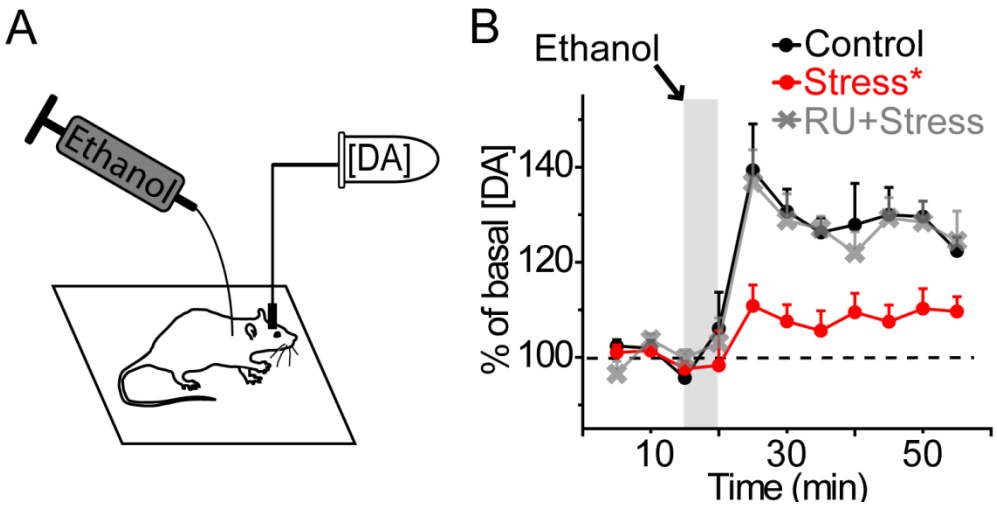

Figure 2.4 Stress attenuates ethanol-induced accumbal DA release in vivo.
(A) Animals were exposed to a 1 hr restraint stress and microdialysis experiments were conducted 15 hr later. **(B)** Time course of DA release in the NAc following in vivo ethanol administration in control rats (black), in stressed rats (red), and in rats injected with RU486 (i.p.) prior to stress exposure (gray). Ethanol (1.5 g/kg) was injected i.v. during the 5 min period (shaded vertical gray bar). *Significantly different from the control group and from the RU486+Stress group by ANOVA with repeated measures, $p < 0.05$, $n = 7–9$ rats/group.

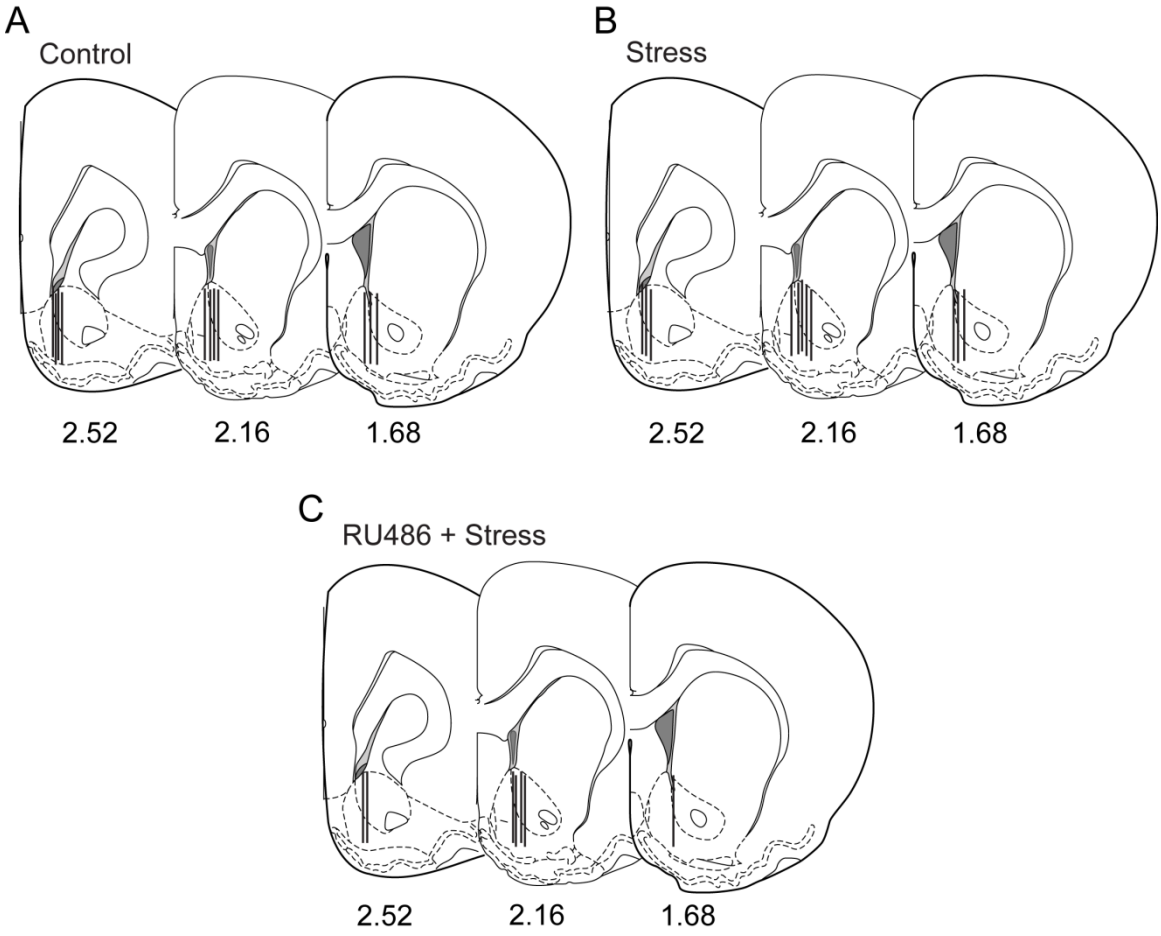

Figure 2.5 Microdialysis probe locations in the nucleus accumbens. Following microdialysis experiments the anatomical placements of the microdialysis probes were determined as described in Supplemental Experimental Procedures section. The distribution of microdialysis probe locations in the NAc were similar between the cohort of **(A)** unstressed, **(B)** stressed and **(C)** RU486-pretreated stressed animals, indicating that regional differences in DA release do not account for observed differences in dopamine release.

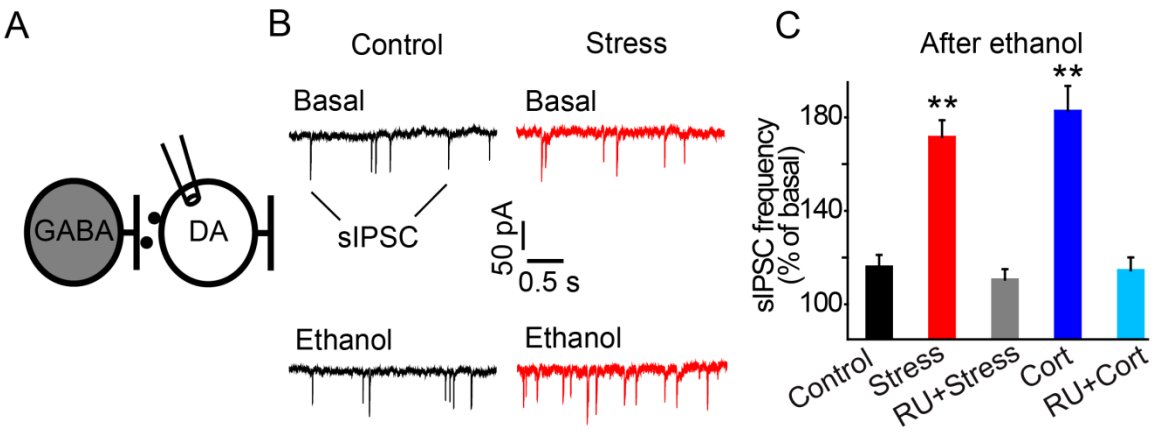

Figure 2.6 Stress and corticosterone increase GABA release onto DA neurons ex vivo. (A) Spontaneous inhibitory postsynaptic currents (sIPSCs) onto VTA DA neurons were recorded using the whole-cell patch-clamp configuration. No significant differences were detected in the mean basal sIPSC frequency or amplitude between stressed and control groups before ethanol: frequency, 2.4 ± 0.6 Hz in control versus 2.3 ± 0.3 Hz after stress; amplitude, 26.2 ± 4.0 pA in control versus 30.8 ± 3.6 pA after stress, $p > 0.05$, n = 8–10. **(B)** Representative recordings of sIPSCs before and after ethanol administration in the control (black) and stressed (red) groups. **(C)** Mean changes in the sIPSC frequency after ethanol application in VTA DA neurons. DA neurons from stressed animals (red) demonstrated a significantly increased ethanol-mediated sIPSC frequency compared to neurons from unstressed controls (black). Systemic inhibition of glucocorticoid receptors with RU486 (40 mg/kg) prior to stress prevented elevated sIPSC frequency (gray). Incubation of VTA slices from control animals with corticosterone increased ethanol-mediated sIPSC frequency in DA neurons up to stress levels (dark blue). Co-incubation with RU486 prevented this increase (light blue). Incubation of brain slices with RU486 and/or corticosterone did not alter basal parameters of sIPSCs (data not shown). Across all groups, ethanol application did not produce significant changes in the sIPSC amplitudes (data not shown, n = 6–10, $p > 0.05$). **Significantly different from control and RU486-treated groups by t test,
$p < 0.01$, n = 6–10 cells/group.

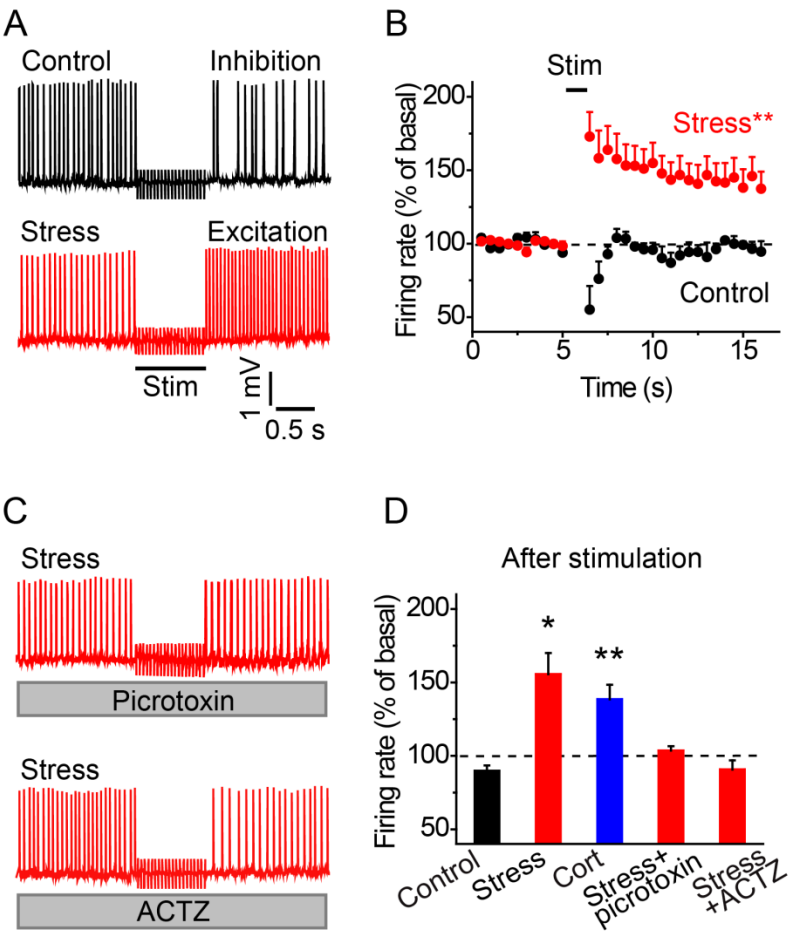

Figure 2.7 Stress promotes GABA-mediated excitation of GABA neurons ex vivo.

(A) VTA GABA neurons were recorded in a cell-attached configuration before and after electrical stimulation of GABAA receptor inputs. Representative GABA neuron recording from a control animal demonstrated decreased firing rate in response to stimulation of GABAergic input (black). Similar stimulation enhanced the firing rate of VTA GABA neurons from stressed animals (red). For display, the traces were filtered and stimulus artifacts were removed. **(B)** Mean changes in VTA GABA neuron firing rate from control (black) and stressed (red) rat slices following repetitive stimulation of synaptic GABA inputs. **Significantly different from the control by ANOVA with repeated measures, $p < 0.01$, $n = 8–10$ cells/group. **(C)** Representative VTA GABA neuron recording from a stressed rat demonstrated that in the presence of picrotoxin (top) or acetazolamide (ACTZ, bottom), repetitive stimulation of GABA inputs failed to increase the firing rate. **(D)** Normalized mean changes in the firing rates of VTA GABA neurons in response to stimulation for each treatment group. Values were averaged over 5 s immediately following termination of the stimulation. In controls, GABAA receptor-mediated increase in the firing rate was observed after slice incubation with corticosterone (blue). Significantly different from the control by t test (*$p < 0.05$), $n = 8–10$ cells/group or (**$p < 0.01$), $n = 6–8$ cells/group.

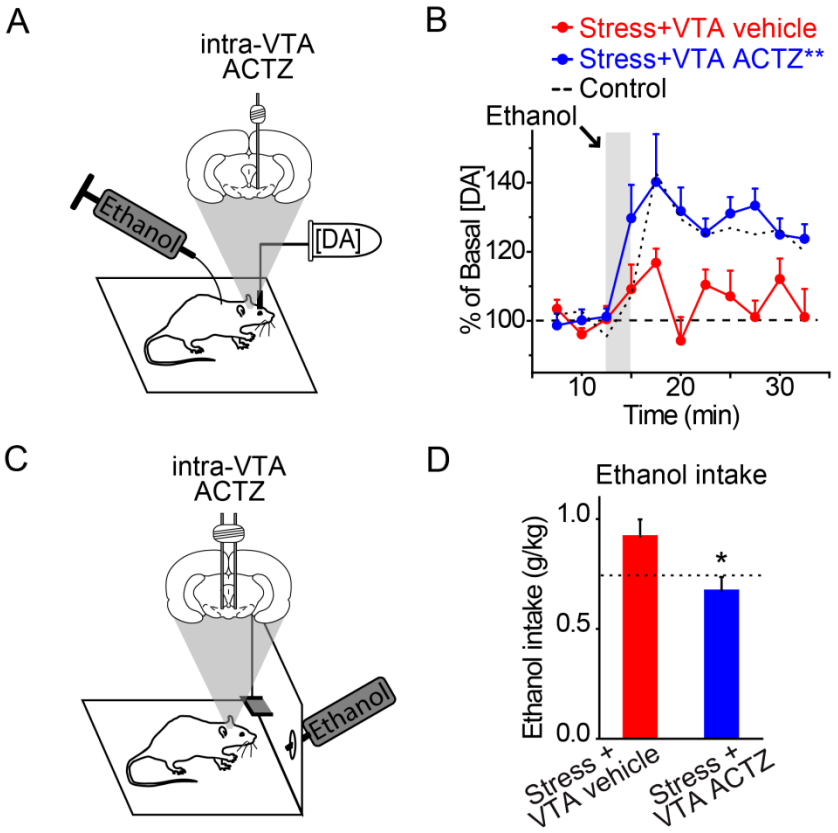

Figure 2.8 Stress requires excitatory GABA shifts to attenuate ethanol-induced DA release and increase ethanol self-administration in vivo. (A) Stressed animals received intra-VTA infusion of ACTZ (1 mL at 50 mM) or vehicle prior to the onset of baseline sample collection. Subsequent ethanol-induced DA release in the NAc was measured. **(B)** In contrast to vehicle injection (red), ethanol-induced DA levels in the NAc following ACTZ infusion were not blunted (blue) and were similar to the control response from Figure 2B (dotted black line). **Significantly different from the VTA vehicle group by ANOVA with repeated measure, $p < 0.01$, $n = 7$ rats/ group. **(C)** Stressed animals received bilateral intra-VTA infusions of ACTZ (1 mL at 50 mM) or vehicle prior to the onset of each self-administration session. **(D)** ACTZ-infused stressed animals consumed significantly less ethanol (blue) compared to vehicle-infused stressed animals (red). Ethanol consumption in unstressed control rats from Figure 1C is shown for comparison (dotted horizontal line). *Significantly different from the VTA vehicle group by t test, $p < 0.05$, $n =$ 10–14 rats/group.

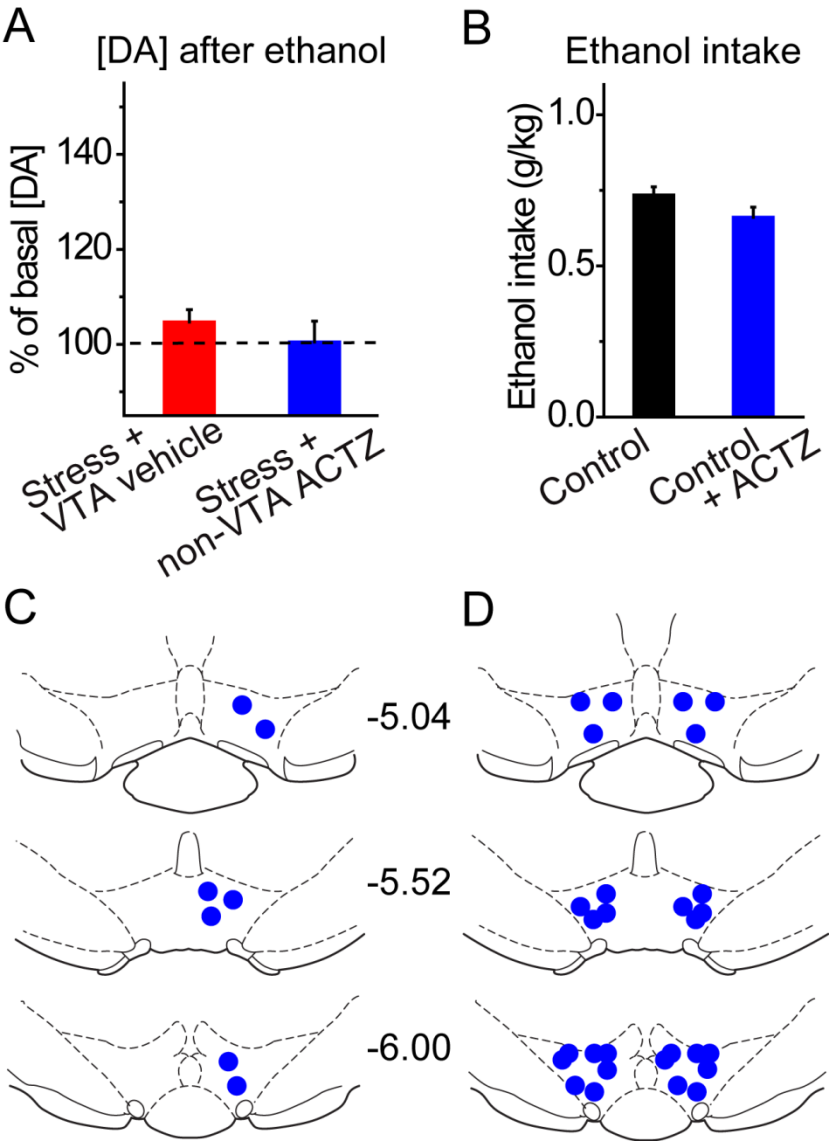

Figure 2.9 Microinfusions of acetazolamide into the VTA. (A) In stressed animals, mean changes in DA levels after exposure to ethanol were not significantly altered if acetazolamide (ACTZ) was infused outside and adjacent to the VTA (104.7% ± 2.6% stress + VTA vehicle vs. 100.5% ± 4.4% stress + non-VTA ACTZ, $p > 0.05$, $n = 5$-7). Changes in DA levels were averaged over 8 post ethanol samples for display. **(B)** In unstressed control animals, mean intake of ethanol over the first 7 days was not significantly altered by intra-VTA infusions of acetazolamide (ACTZ) (0.74 ± 0.03 g/kg control vs. 0.66 ± 0.08 g/kg control + ACTZ, $p > 0.05$, $n = 6$-19). **(C)** Unilateral VTA microinfusions of acetazolamide were administered ipsilateral to the microdialysis probe and were equally distributed along the anterior/posterior axis. **(D)** Bilateral VTA microinfusions of acetazolamide were similarly distributed along the anterior/posterior axis.

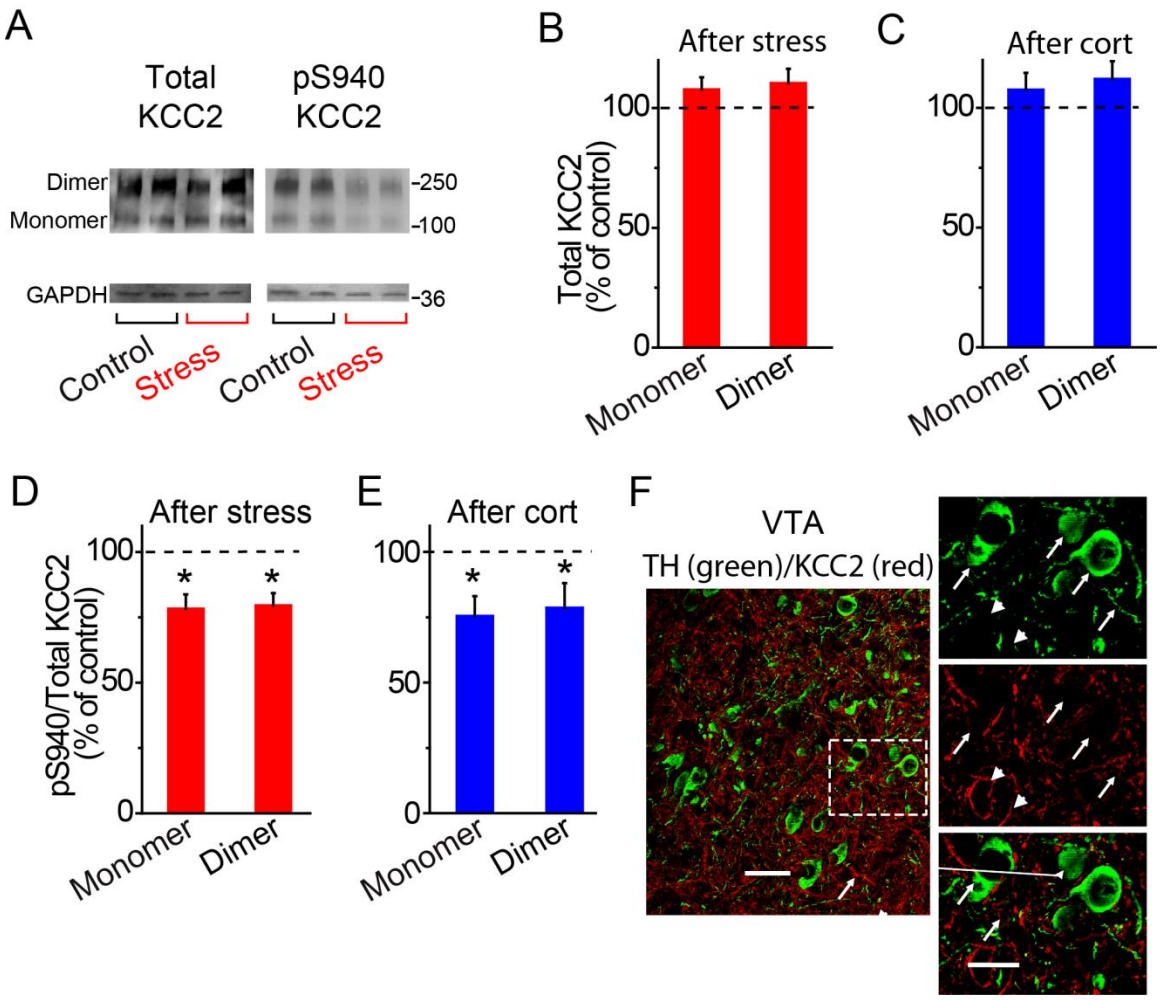

Figure 2.10 KCC2 expression is downregulated after stress and restricted to non-DA neurons. (A) Western blot analysis was conducted for total KCC2 and phosphorylated- S940 KCC2 with GAPDH as a loading control. A representative western blot indicates no differences in total KCC2 expression after stress. However, stressed animals showed reduced expression of pS940 KCC2 relative to total KCC2 when compared to non-stressed controls. **(B)** Densiometric analysis revealed no significant differences in total KCC2 expression levels after stress (red bar graphs) or after corticosterone incubation **(C)** of control slices (blue bar graphs) ($p > 0.05$, $n = 10$-16 rats/group). **(D)** Densiometric analysis revealed a significant reduction in the ratio of pS940 KCC2 to total KCC2 protein in stressed animals compared to non-stress controls (horizontal dashed line). *Significantly different from the control by t test, $p < 0.05$, $n = 10$ animals/group. **(E)** Densiometric analysis revealed a significant reduction in the ratio of pS940 KCC2 to total KCC2 protein in corticosterone-incubated slices from controls. *Significantly different from the control by t test, $p < 0.05$, $n = 16$ animals/group. **(F)** Immunolabeling analysis in the VTA revealed that tyrosine hydroxylase (TH) labeling of dopamine neuron (green) did not overlap with KCC2 labeling (red), suggesting an alternative chloride homeostasis mechanism exists in VTA DA neurons.

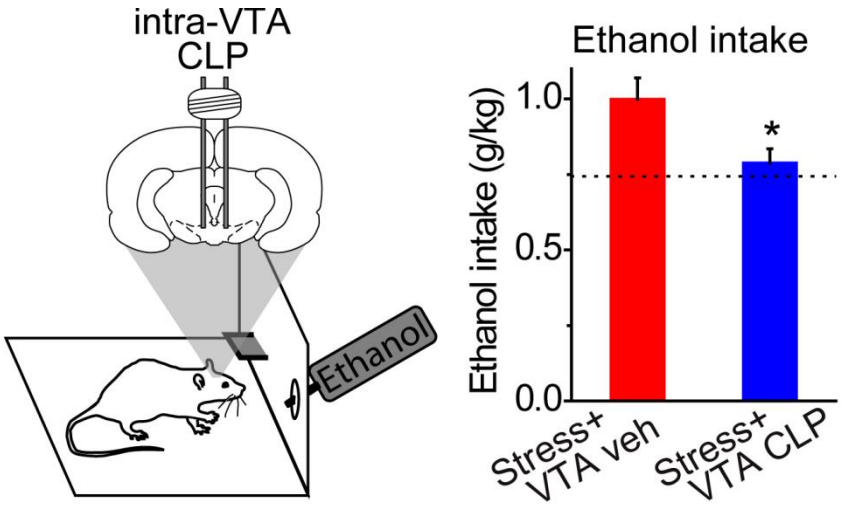

Figure 2.11 KCC2 Activation in the VTA prevents stress-induced alcohol consumption. CLP290 was infused bilaterally intra-VTA (40 mM at 0.5 mL/min). After CLP290 administration, stressed animals consumed significantly less ethanol (blue) compared to vehicle-injected stressed animals (red). Ethanol consumption in unstressed control rats is shown for comparison (dotted horizontal line). *Significantly different from the VTA vehicle group by t test, $p < 0.05$, n = 12–13 rats/group.

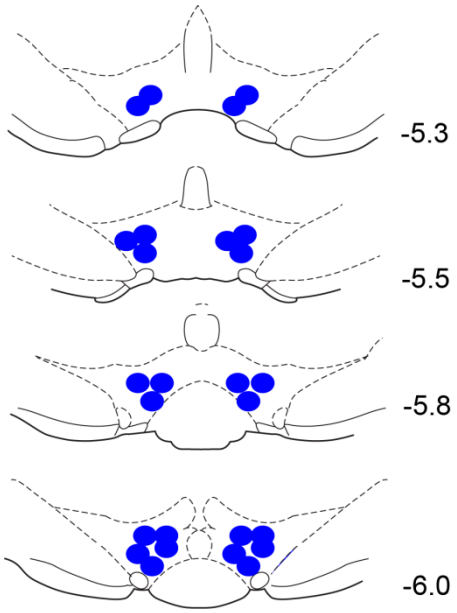

Figure 2.12 CLP290 Microinfusion Sites. Bilateral VTA microinfusions of acetazolamide were similarly distributed along the anterior/posterior axis.

CHAPTER 3

Nicotine Decreases Ethanol-induced Dopamine Signaling and Increases Self-administration via Stress Hormones

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Author Contributions

W.M.D., Y.D., and A.M.T. designed and performed in vivo experiments. A.O. designed and performed ex vivo electrophysiological experiments assisted by T.A.Z. J.A.D. originated, planned, and oversaw the experiments with W.M.D.'s, A.O.'s, and A.M.T.'s, assistance. Led by J.A.D., all the authors contributed to write the manuscript.

Abstract

Tobacco smoking is a well-known risk factor for subsequent alcohol abuse, but the neural events underlying this risk remain largely unknown. Alcohol and nicotine reinforcement involve common neural circuitry, including the mesolimbic dopamine system. We demonstrate in rodents that pre-exposure to nicotine increases alcohol self-administration and decreases alcohol-induced dopamine responses. Blocking stress hormone receptors prior to nicotine exposure prevented the decreased dopamine responses, and the increased alcohol self-administration. These results indicate that nicotine recruits neuroendocrine systems to influence neurotransmission and behavior associated with alcohol reinforcement.

Introduction

Tobacco (nicotine) and alcohol are the two most abused and costly drugs to society. Epidemiological studies consistently find a positive correlation between nicotine and alcohol use, with alcoholism approximately 10 times more prevalent in smokers than in non-smokers (Weitzman *et al.*, 2005; Barrett *et al.*, 2006; Harrison & McKee, 2008). Several studies also show that nicotine exposure increases alcohol self-administration (Smith *et al.*, 1999; Barrett *et al.*, 2006); and smoking, particularly at an early age, is a significant risk factor for subsequent alcohol abuse (Sher *et al.*, 1996; Grant, 1998).

In addition to psychosocial and genetic factors (Bobo & Husten, 2000; Schlaepfer *et al.*, 2008), evidence suggests that the interactions between nicotine and alcohol arise from shared pharmacological actions (Larsson & Engel, 2004; Funk *et al.*, 2006). These drugs activate common neural substrates, including the mesolimbic dopamine (DA) system (Di Chiara, 2000; Gonzales *et al.*, 2004; De Biasi & Dani, 2011) and the hypothalamic-pituitary-adrenal (HPA) axis associated with stress hormone signaling (Richardson *et al.*, 2008; Armario, 2010; Lutfy *et al.*, 2012).

To simplify this complex and multifaceted interaction between nicotine and alcohol, we studied how acute nicotine exposure in naïve animals alters subsequent responses to alcohol, including alcohol-induced DA signals and alcohol self-administration. We found that pretreatment with nicotine increased subsequent alcohol self-administration and decreased alcohol-induced dopamine signals in the ventral tegmental area (VTA) and the nucleus accumbens (NAc). The decreased dopamine responses to alcohol arose via an initial activation of stress hormone receptors in the ventral tegmental area. These results

identify the mesolimbic dopamine system as a locus for multiple neurophysiological interactions between nicotine, stress hormones, and alcohol.

Experimental Procedures

Subjects

Long-Evans rats (Harlan Sprague Inc., Indianapolis IN, USA) weighing between 300–500 g were used. The rats were handled and weighed for at least 3 days and commonly more than a week prior to surgery and testing, and the rats were housed in a humidity and temperature-controlled (22°C) environment under a 12-hr light/dark cycle. The rats had food and water available ad libitum in the home cage. All procedures complied with guidelines specified by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Surgical procedures

For the microdialysis experiments, each animal was implanted with an intravenous catheter through the jugular vein and a stainless steel guide cannula (21 gauge) (Plastics One, Inc., Roanoke, VA, USA). The surgery occurred under isoflurane anesthesia (1.5–2.5% in 100% O2, 1 L/min). The catheters were constructed with Silastic tubing (0.30 mm ID, 0.64 mm OD; Dow Corning, Midland, MI, USA) with one end modified with a 22-gauge cannula (Plastics One, Inc.). The microdialysis guide cannulae were positioned as follows (in mm relative to bregma): +2.1 anterior-posterior, +1.1 medial-lateral, −4.0 ventral to the skull surface (Paxinos, 2007). The experiments were conducted after a minimum recovery period of 3 days.

Drugs and experimental design

All drugs (Sigma-Aldrich Inc., St. Louis, MO, USA) were dissolved in sterile saline, except Mifepristone (RU486), which was dissolved in dimethyl sulfoxide (DMSO). Pretreatment

with nicotine tartrate (0.4 mg/kg, freebase, i.p.), or an equivalent volume of saline, occurred 3–40 hrs prior to the experiments. RU486 was administered 15 min prior to nicotine pretreatment at a dose of 40 mg/kg (Saal *et al.*, 2003). We opted for this dose because of the limited capacity of RU486 to cross the blood brain barrier (Heikinheimo & Kekkonen, 1993). The intra-VTA concentration of RU486 was (10 ng/0.5 µl) and 0.5 µl of the solution was delivered by pump over 1 min (Segev *et al.*, 2012). The microinfusion injector was left in place for 2 additional min and then removed. The infusion cannula was aimed at the following VTA coordinates (in mm relative to bregma): +5.7 anterior-posterior, +1.0 medial-lateral, −7.1 ventral to the skull surface (Paxinos, 2007). Following the experiments, Chicago Sky blue was injected into the VTA to determine the location of the microinfusion.

Baseline samples were collected (15–30 min), followed by a timed intravenous (i.v.) drug infusion (i.e., ethanol or nicotine). The i.v. administration route circumvents handling-related stress associated with a needle injection (Dong *et al.*, 2010). For the i.v. ethanol experiments, the rats received 1.5 g/kg ethanol (20% in sterile saline, v/v, i.v.) over 5 min. Two hrs prior to the experiment, rats were administered a similar volume of vehicle (sterile saline) to habituate them to the stimulus effects of the infusion.

In vivo microdialysis

The active dialysis membrane (2.0 mm), was made of hollow cellulose fiber (inner diameter = 200 µm; molecular weight cutoff = 18,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The inlet and outlet to the membrane was composed of fused-silica tubing (inner diameter = 40 µm; Polymicro Technologies, Phoenix, AZ, USA). The microdialysis probes were perfused with artificial cerebral spinal fluid (ACSF): 149 mM

NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, and 0.25 mM ascorbic acid, 5.4 mM D-glucose. At least 14 hrs before the experiment, we lowered the probes into the brain through the guide cannula. The perfusion flow rate was set to 2.0 µl/min. Each sample vial was manually changed and immediately stored at −80° C until analyzed.

Dopamine analysis

The HPLC system included a pump (Model 582; Thermo Scientific, West Palm Beach, FL, USA), an autosampler (Model 542; Thermo Scientific), and a HR-3.2 × 80 mm column (3-µm particle size; Thermo Scientific). A coulometric cell (5014B; Thermo Scientific) was connected to a Coulochem II detector. The mobile phase comprised of citric acid (4.0 mM), sodium dodecyl sulfate (3.3 mM), sodium dihydrogen phosphate dehydrate (100.0 mM), and ethylenediaminetetraacetic acid (0.3 mM), acetonitrile (15%), and methanol (5%). The autosampler mixed 9.5 µl of the dialysate with ascorbate oxidase (EC 1.10.3.3; 162 units/mg; Sigma-Aldrich Inc.) prior to injection. DA signals were acquired with 501 chromatography software and Chromeleon Software (Thermo Scientific). Quantification of dialysate DA concentration was carried out by comparing the peak area to external standards (0–2.5 nM).

Operant ethanol self-administration

Standard operant chambers (Med Associates Inc., St. Albans, VT, USA) were used for the self-administration experiments. Activation of an interior chamber light and presentation of a retractable lever accompanied the start of each session. Depression of the lever triggered the entry of a retractable drinking spout on the opposite side of the wall. Each lever press resulted in 15 sec of access to the drinking spout (a fixed ratio-1 reinforcement schedule). Each session lasted 45 min. The rats lived in the same quiet room in which

daily training sessions occurred, and the rats were typically trained one at a time to avoid any auditory distractions from activity in neighboring chambers.

The rats were trained to lever press for saccharin reinforcement (0.125%, w/v). Consistent responses for saccharin occurred in ~4–8 days. Three hrs prior to their first ethanol exposure, the animals were injected with nicotine (0.4 mg/kg) or saline. The rats were exposed to ethanol by gradually adding ethanol (2–4 %, v/v) into their saccharin solution over a 4-day period (Doyon *et al.*, 2005). Consumption was monitored by measuring the volume of liquid in the drinking bottle before and after the session. Body weights were measured each day.

Histology

The rats were overdosed with pentobarbital (120 mg/kg, i.v.). Saline was perfused through the heart, followed by 10% formalin (v/v). The brains were removed and immersed in 10% formalin for at least 2 days. The brains were cut into 75-µm coronal sections (Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with cresyl violet as indicated by the figures defining anatomical placements.

Statistical analysis

Analysis of variance (ANOVA) with repeated measures (in SPSS for Windows) was used to analyze the dialysate DA concentrations and the daily ethanol intake. A two-tail t-test assuming equal variance was used to assess differences between mean behavioral responses. Significance for all analyses was determined by $p < 0.05$.

Results

Nicotine attenuates ethanol-induced DA release

The initial administration of addictive drugs, such as nicotine and ethanol, increases basal DA levels in the nucleus accumbens (NAc) as measured by microdialysis (Di Chiara & Imperato, 1988). We found that simultaneous co-administration of nicotine and ethanol produces an additive increase in NAc DA release relative to the response of each drug alone (data not shown). To determine whether prior exposure to nicotine influences ethanol-induced DA release in the NAc, we injected rats with nicotine or saline 3 hrs prior to administering ethanol. Guided by nicotine's metabolic half-life in rats of 45 min (Matta *et al.*, 2007), we chose a 3-hr pretreatment period to decrease any carryover in the pharmacological effects of nicotine.

Microdialysis samples were collected to follow the change in extracellular DA levels induced by ethanol administration (Fig. 3.1A-D). After pretreatment with saline or nicotine, there was no difference in the basal DA concentrations prior to ethanol exposure: 1.0 ± 0.2 nM after nicotine pretreatment and 1.0 ± 0.1 nM after saline pretreatment. To avoid handling-related stress, ethanol was administered intravenously over a 5-min period (Fig. 3.1A; shaded columns). Ethanol induced a sustained increase in DA release in the saline control group (Fig. 1A–C, black circles). Nicotine pretreatment (0.4 mg/kg, i.p., 3-40 hrs prior) significantly attenuated the ethanol-induced increase in DA release (Fig. 3.1A-C, red circles) (group x time: $F(10,100) = 2.37$, $p < 0.05$). The administered ethanol dose falls within the typical range tested in rodents (Gonzales *et al.*, 2004) and produces brain ethanol concentrations in rodents that humans commonly achieve (Howard *et al.*, 2008).

The distribution of the microdialysis probe placements within the NAc were similar between the cohort of animals pretreated with nicotine and those pretreated with saline (Fig. 3.1D), indicating that regional differences in DA release do not account for these results.

Nicotine increases ethanol self-administration

Given that the single nicotine pretreatment decreased ethanol-induced DA release, we determined whether that same nicotine pretreatment influenced ethanol self-administration (Smith *et al.*, 1999). To parallel the time course of our microdialysis experiments (see Fig. 3.1), we examined ethanol intake during the early acquisition of drinking behavior. Early acquisition was defined as the first four sessions of ethanol self-administration (1 session/day for 45 min/session). Operant responses to saccharin (0.125%, w/v) were first established, followed by an introduction of ethanol (2–4%) into the drinking solutions over four days (Doyon *et al.*, 2005).

We pretreated the rats with either nicotine (0.4 mg/kg, i.p.) or saline 3 hrs prior to an initial ethanol exposure, as in the microdialysis experiments (see Fig. 3.1A). Ethanol intake across the first four self-administration sessions was significantly higher after nicotine pretreatment (0.97 g/kg, n = 20) compared to the saline pretreatment control (0.75 g/kg, n = 17) ($p < 0.01$) (Fig. 3.1E). Rats pretreated with nicotine also initiated significantly more operant responses (44 ± 2) than the saline pretreatment control (36 ± 2).

To confirm that these effects were specific to ethanol and not related to the saccharin in the drinking solution, we included a separate control group that responded for saccharin alone (no ethanol) (n = 10). This group did not drink significantly more fluid following

nicotine pretreatment (15.6 ± 1.8 ml/session) across four drinking sessions than the ethanol control rats pretreated with saline (13.9 ± 0.8 ml/session) ($p > 0.05$).

Interactions between nicotine and ethanol require stress hormones

We hypothesized that nicotine administration altered the DA and GABA responses to alcohol through a neuroendocrine signal (Armario, 2010). Stress-related hormones, such as glucocorticoids, cause long-term homeostatic changes in neural function and influence DA and GABA transmission (Barrot *et al.*, 2000; Joels & Baram, 2009; Butts *et al.*, 2011).

Nicotine activates the HPA axis to increase plasma levels of corticosterone (Lutfy *et al.*, 2012), the principle glucocorticoid in rodents, which we confirmed (Fig. 3.2). To determine whether glucocorticoid receptor activation during nicotine pretreatment contributes to subsequent alterations in ethanol-induced DA release, we systemically blocked glucocorticoid receptors with RU486 (Cadepond *et al.*, 1997) prior to nicotine pretreatment. Pretreatment with RU486 (Fig. 3.3A, blue circles) prevented the inhibitory effect of nicotine on ethanol-induced DA release (group x time: $F(10,240) = 4.75$, $p < 0.01$). This increased DA response to ethanol following RU486 and nicotine pretreatment was not distinguishable from the control rats pretreated with saline alone or RU486 alone (Fig. 3.3A, dashed trace).

These results suggested that stress receptor activation within the VTA, following nicotine pretreatment, attenuated the subsequent DA response to ethanol. To test this hypothesis, we blocked glucocorticoid receptors locally in the VTA with RU486 prior to nicotine pretreatment. The control group that received a local intra-VTA microinfusion of vehicle followed by nicotine pretreatment showed a decreased DA response to ethanol 15 hrs later (Fig. 3.3B, red circles), consistent with our previous data (see Fig. 1). This inhibitory

effect of nicotine pretreatment was prevented by intra-VTA microinfusion of RU486 prior to nicotine pretreatment (Fig. 3.3B, blue circles) (group x time: $F(10,140) = 2.43$, $p < 0.05$). We should note that the intra-VTA RU486 did not completely reverse the effect of nicotine pretreatment. A post hoc comparison indicated a significant difference between the saline control (Fig. 3.3B, dashed line) and the group pretreated with intra-VTA RU486 + Nic ($F(10,220) = 2.01$, $p < 0.05$). The microinfusion sites were dispersed mainly in the more ventral VTA, including the anterior and posterior regions (Fig. 3.3C). There was no consistent relationship between the microinfusion site and the individual DA responses to ethanol in either group. As a negative control, microinfusion of RU486 outside and adjacent to the VTA did not reverse the inhibitory effect of nicotine pretreatment (n =3).

To determine whether stress-related signals also contributed to increased ethanol consumption after nicotine pretreatment (as in Fig 3.1E), we pretreated rats with RU486 prior to nicotine administration and then monitored early acquisition of ethanol self-administration over the first 4 sessions. RU486 pretreatment prevented the increased ethanol self-administration induced by nicotine pretreatment (Fig. 3.3D). The mean ethanol intake for the group pretreated with RU486 and nicotine (0.74 ± 0.06 g/kg/session, n = 12) was significantly lower than the nicotine pretreatment alone (0.97 g/kg; n = 17) ($p < 0.01$) and nearly identical to the saline pretreatment control (Fig. 3.3D, dashed line). Thus, nicotine required the activation of stress hormone receptors to enhance subsequent ethanol self-administration.

Discussion

Acute pretreatment with nicotine induced a long-lasting attenuation of ethanol-induced DA signals within the mesoaccumbens pathway. These nicotine-induced neuroadaptations required a stress hormone signal that acted significantly within the VTA. Concomitant with these physiological changes, we also show that increases in ethanol self-administration induced by nicotine were prevented by RU486, a glucocorticoid/progesterone receptor antagonist (Cadepond *et al.*, 1997).

In addition to other interactions with ethanol (Al-Rejaie & Dar, 2006; Lopez-Moreno *et al.*, 2008), nicotine exposure influences subsequent ethanol consumption and abuse (Grant, 1998; Smith *et al.*, 1999; Barrett *et al.*, 2006). Although the development of drug abuse involves the mesolimbic DA system, there is little mechanistic data indicating how nicotine influences DA responses to ethanol. Our results suggest that nicotine acts through stress hormone signaling pathways in the VTA to decrease ethanol-induced DA signals. A blunted DA system has been associated with increased impulsivity (Reuter *et al.*, 2005) and increased susceptibility to drug and alcohol (Volkow *et al.*, 1996; Martinez *et al.*, 2005). Previous studies have shown that higher ethanol preference in mice corresponds to lower DA neuron responses to ethanol (Brodie & Appel, 2000).

We should note that RU486 is an antagonist for both the glucocorticoid and the progesterone receptor (Cadepond *et al.*, 1997). Progesterone and its metabolites are produced in the brain and participate in stress responses (Wirth). Thus, progesterone and glucocorticoid receptors could contribute to interactions between nicotine and ethanol. These results complement previous studies showing a critical role for

glucocorticoids in alcohol reward and in the transition to compulsive alcohol drinking (Rotter *et al.*; Vendruscolo *et al.*, 2012).

Activation of nAChRs in the brain stem are known to contribute to the initial response of the HPA axis to nicotine (Armario, 2010). Blocking stress hormone receptors locally in the VTA prevented the long-term alterations in ethanol-induced DA release (Fig. 3.3B), and thus identified the VTA as a locus for mechanistic interactions between nicotine and ethanol. Interestingly, local VTA infusion of RU486 to antagonize stress receptors did not completely reverse the effects of nicotine pretreatment on ethanol-induced DA release compared to the saline control. This incomplete effect could arise from a partial diffusion of RU486 throughout the VTA, but it is also feasible that adaptations outside of the VTA to induce neuroadaptations that regulate DA signals.

In summary, we provide evidence that nicotine pretreatment decreases ethanol-induced DA transmission and increased ethanol self-administration. These responses to nicotine pretreatment, required an initial stress hormone signal. These results support the hypothesis that drugs of abuse recruit neuroendocrine pathways to promote addictive behaviors (Koob & Kreek, 2007; Koob *et al.*, 2014). Our data suggest a neurophysiological basis for the observation that nicotine use can increase the reinforcing properties of alcohol.

Figures

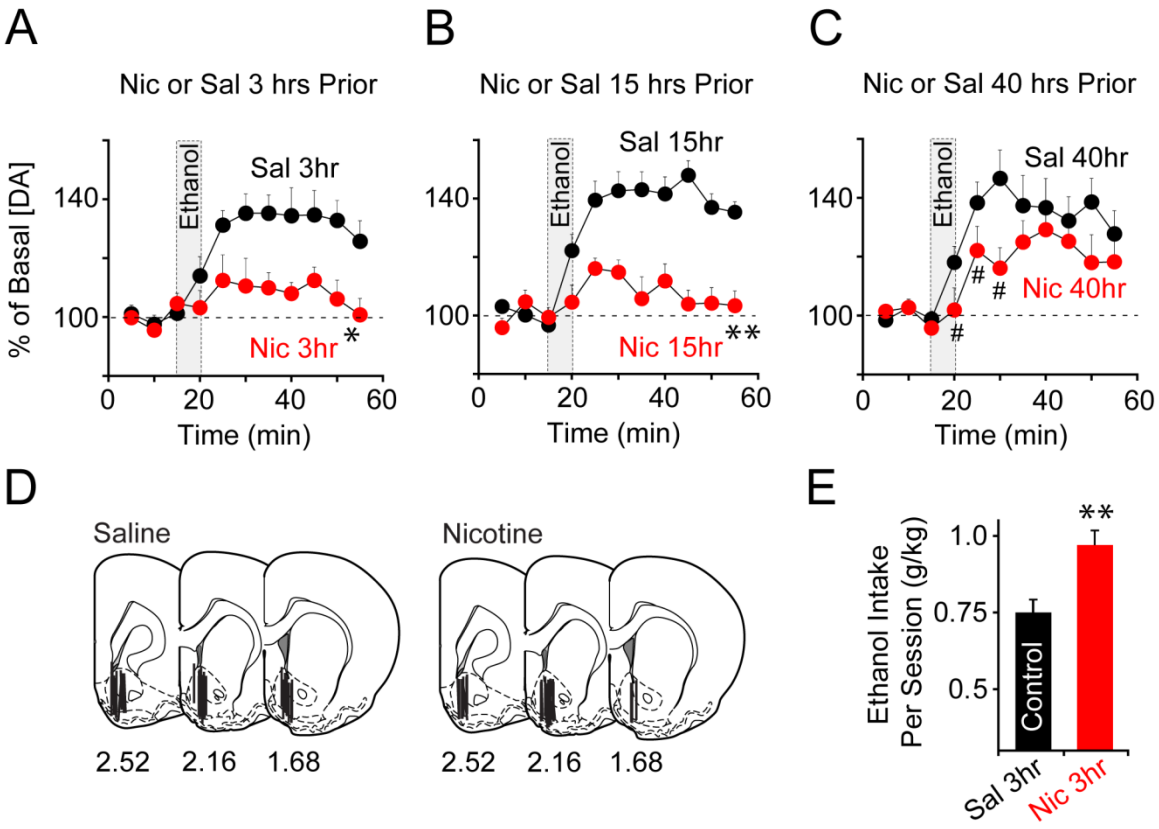

Figure 3.1 Nicotine pretreatment attenuates ethanol-induced DA release and increases ethanol self-administration. Rats were injected once with nicotine (0.4 mg/kg) or saline, which occurred either **(A)** 3 hrs prior to in vivo ethanol administration, **(B)** 15 hrs prior to in vivo ethanol administration, or **(C)** 40 hrs prior to in vivo ethanol administration. Changes in [DA] were measured in 5-min intervals using microdialysis with HPLC. Ethanol (1.5 g/kg) was infused i.v. over a 5-min period (shaded vertical bars). * Significantly different from the control by ANOVA with repeated measures ($p < 0.05$); ** $p < 0.01$; n = 6–16 rats/group. # Significantly different from the control by posthoc ANOVA with repeated measures. **(D)** Microdialysis probe placements in the NAc for all rats pretreated with saline (left) or nicotine (right) (Paxinos and Watson, 2007). **(E)** Nicotine increases acquisition of ethanol self-administration. Rats were pretreated once with either saline (black bar) or nicotine (0.4 mg/kg, i.p.; red bar) 3 hrs prior to an initial ethanol exposure. The mean ethanol intake was then measured over the first four self-administration sessions (45 min/session). ** Significantly different from the control by t-test ($p < 0.01$); n = 20, 17. Data presented as the mean ± SEM.

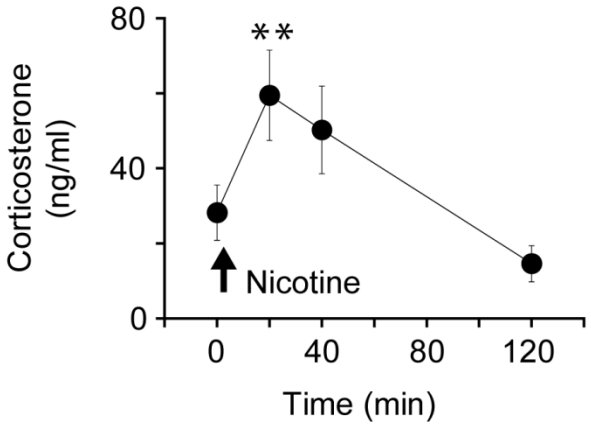

Figure 3.2. Administration of nicotine increases plasma corticosterone levels. Effect of nicotine pretreatment on plasma corticosterone levels with basal defined at time = 0. ** Significantly different from basal ($p < 0.01$; $n = 8$ rats).

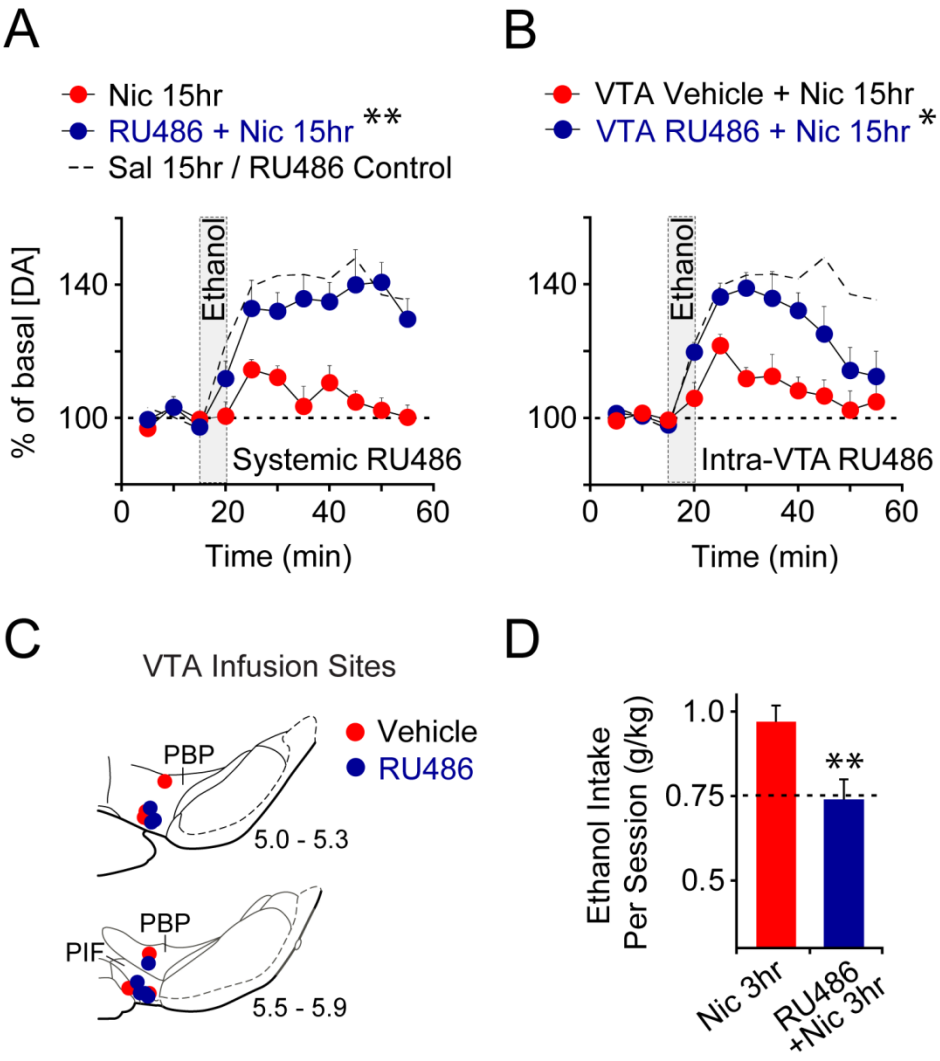

Figure 3.3 Nicotine requires glucocorticoid signaling to alter ethanol-induced DA release and ethanol self-administration. (A) Rats were injected with RU486 (40 mg/kg) 15 min prior to nicotine (0.4 mg/kg) or saline pretreatment. 15 hrs later, changes in extracellular [DA] in response to ethanol (shaded vertical bar) were measured. Control subjects that received RU486 alone were combined with the saline controls from Fig. 1B as these groups were not statistically different. n = 9–16 rats/group. **(B)** The effect of microinfusion of RU486 or vehicle into the VTA prior to nicotine pretreatment on the DA response to ethanol 15 hrs later. ** Significantly different from the nicotine pretreatment by ANOVA with repeated measures ($p < 0.01$), * $p < 0.05$, n = 8/group. **(C)** VTA microinfusion sites for all rats pretreated with RU486 (blue circle) or vehicle (red circle. PIF: parainterfascicular nucleus, PBP: parabrachial pigmented nucleus. **(D)** Systemic RU486 blocked the increase in ethanol self-administration induced by nicotine pretreatment, as shown in Fig. 1E. Ethanol intake in the saline pretreatment control is indicated by the dashed line. ** Significantly different from the nicotine pretreatment by t-test ($p < 0.01$), n = 20, 12. Data presented as the mean ± SEM.

CHAPTER 4

Adolescent Nicotine Exposure Increases Adult Ethanol Self-administration via Altered Chloride Homeostasis in the Ventral Tegmental Area

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A.M.T. designed and performed in vivo microdialysis and self-administration experiments with the help of K.K. A.O. designed and performed electrophysiological experiments assisted by T.A.Z. J.A.D. originated, planned, and oversaw the experiments with A.M.T.'s and A.O.'s assistance. Led by J.A.D., all the authors contributed to write the manuscript.

Abstract

Adolescent smoking is a well-known risk factor for subsequent alcohol abuse, but the neurobiological mechanisms mediating this interaction remain largely unknown. The mesolimbic dopamine system is implicated in drug reinforcement and addiction, suggesting a potential locus for nicotine-alcohol interactions. We demonstrate in rodents that exposure to nicotine during adolescence increases alcohol self-administration in adulthood and attenuates alcohol-induced dopamine responses. Blunted dopamine responses corresponded with increased inhibition onto DA neurons and altered anion homeostasis in VTA GABA neurons. Enhancing KCC2 function in adult animals prevented the increased alcohol self-administration, suggesting that long-lasting alterations in anion homeostasis persist within the VTA after adolescent nicotine exposure and promote ethanol self-administration. These results reveal a novel mechanism linking adolescent nicotine exposure to elevated alcohol consumption in adulthood.

Introduction

Epidemiological studies consistently highlight positive correlations between smoking and excessive alcohol consumption (DiFranza & Guerrera, 1990; Schorling *et al.*, 1994; Andersen, 2003; Weitzman *et al.*, 2005; Harrison & McKee, 2008). Compared to non-smokers, smokers drink nearly twice as much alcohol, are more likely to experience alcohol-related harm, and have significantly higher risk for developing alcohol use disorders (DiFranza & Guerrera, 1990; Kozlowski & Ferrence, 1990; Grant *et al.*, 2004; Larsson & Engel, 2004). Accumulating evidence suggests that the comorbidity of tobacco and alcohol use in adulthood may originate in adolescence at the time of initial tobacco exposure (Cross *et al.*, 2017). Adolescent smokers are more likely to be heavier drinkers and individuals that experiment with tobacco are twice as likely to develop an alcohol use disorder compared to never smokers (Grucza & Bierut, 2006).

Adolescence is considered to be a critical period of brain development (Chambers *et al.*, 2003), and exposure to nicotine during this timeframe produces long-lasting neurobehavioral effects (Spear, 2016). Rodent studies reveal that adolescent nicotine exposure increases subsequent self-administration of nicotine (Adriani *et al.*, 2003; Adriani *et al.*, 2006), alcohol (Larraga *et al.*, 2017), and other drugs of abuse (McQuown *et al.*, 2007; Dao *et al.*, 2011), suggesting that early nicotine exposure may indeed serve as a gateway for subsequent drug use (Torabi *et al.*, 1993; Baler & Volkow, 2011). Despite this compelling literature, the specific neuroadaptations induced by adolescent nicotine exposure to promote subsequent alcohol consumption remain unknown.

Evidence suggests that the interactions between nicotine and alcohol arise from shared pharmacological actions (Larsson & Engel, 2004; Funk *et al.*, 2006). These drugs activate common neural substrates, including the mesolimbic dopamine (DA) system ((Di Chiara,

2000; Gonzales *et al.*, 2004; Dani & Harris, 2005; Doyon *et al.*, 2013b). Since the dopamine system is known to regulate ethanol reinforcement (Gatto *et al.*, 1994; Rodd-Henricks *et al.*, 2000; Grace *et al.*, 2007) and sensitive to adolescent nicotine-induced neuroadaptations (Counotte *et al.*, 2009; Doura *et al.*, 2010; Ehlinger *et al.*, 2016), this circuitry could mediate nicotine-ethanol interactions from adolescence to adulthood. Prior work from our group also suggests that acute nicotine may promote drinking via adaptations within the mesolimbic circuitry. Exposure to acute nicotine 3-15 hrs prior increased subsequent alcohol consumption, blunted dopamine responses to ethanol, and increased inhibition onto DA neurons within the ventral tegmental area (VTA) (Doyon *et al.*, 2013a). Separately, we showed that elevated alcohol intake and increased inhibition of dopamine neurons arose via altered chloride homeostasis within the VTA (Ostroumov *et al.*, 2016). Based off this work, we hypothesized that adolescent nicotine increased alcohol consumption in adulthood through similar neuroadaptations in the mesolimbic circuitry.

This study sought to examine if adolescent nicotine altered mesolimbic responses to ethanol and to test the contribution of altered chloride homeostasis to this interaction. Animals treated with nicotine as adolescents, but not adults, showed persistent elevated alcohol consumption throughout adulthood. Further investigation revealed adolescent nicotine pretreatment reduced dopaminergic responses to ethanol in vivo, increased inhibition of dopamine neurons, and altered chloride homeostasis in VTA GABA neurons. Pharmacological enhancement of chloride transporter function within the VTA prevented elevated alcohol consumption in adolescent nicotine-treated animals. These results implicate anionic plasticity mechanisms within the VTA in long-term elevations in alcohol

consumption and reveal that intervention during adulthood can counter the effects of adolescent drug exposure.

Experimental Procedures

Male Long-Evans rats (Harlan-Envigo) were singly housed in a quiet, temperature and humidity- controlled satellite facility under at 12 hr light/dark cycle. Rats had food and water available ad libitum in their home cage. All procedures were carried out in compliance with guidelines specified by the Institutional Animal Care and Use Committee at University of Pennsylvania.

Drugs and Experimental Design

All drugs (Sigma-Aldrich Inc., St. Louis, MO, USA) were dissolved in sterile saline unless otherwise specified. Animals were administered daily injections of saline or nicotine tartrate (0.4 mg/kg, freebase, i.p.) during adolescence (p28-p42) and subsequent responses to ethanol were assessed in adulthood (p70-p90) (Figure 1A). Comparable injections were carried out in adult animals (~p60-p74) and subsequent responses to ethanol were assessed 4 weeks post-nicotine exposure (Figure 2A). Intra-VTA microinfusions of CLP290 occurred approximately 30 min prior to ethanol self-administration. The intra-VTA concentration of CLP290 was (45 μM) and 1.0 μl of the solution was delivered by pump at a rate of 0.5 μL/min (Ostroumov *et al.*, 2016). Animals were treated with CLP290 (45 μM) before the first day of ethanol self-administration. The microinfusion injector was left in place for 2 additional min and then removed. The infusion cannula was aimed at the following VTA coordinates (in mm relative to bregma): +5.7 anterior-posterior, +1.0 medial-lateral, −7.1 ventral to the skull surface (Paxinos and Watson, 2007). A separate group of animals treated with 50 μM CLP290 prior to self-administration over 3 non-consecutive days showed no significant differences in effect, so these animals were combined with the 45 μM CLP290 treatment group.

Operant Ethanol Self-Administration

Standard operant chambers (Med Associates) were used for the self-administration experiments. Animals were initially water restricted overnight and trained to lever press for a saccharin solution (0.125%, w/v). Once trained, the animals were no longer water restricted and their baseline saccharin intake was monitored until intake appeared stable for 3 consecutive days. If the animals underwent surgery, saccharin intake levels were re-established. The effects of nicotine or saline pretreatment on subsequent ethanol self-administration were then measured. Ethanol was introduced into the saccharin drinking solution in the following way: 2% ethanol on day 1 and 4% ethanol for all subsequent days (Ostroumov *et al.*, 2016), unless otherwise noted. We previously confirmed that this self-administration protocol produces pharmacologically relevant blood alcohol levels (Ostroumov *et al.*, 2016). For 8% ethanol studies, initial ethanol fading (2-8%) occurred over the first eight days (Doyon *et al.*, 2005). Intake of 8% ethanol consumption was then monitored for seven days. A separate cohort of animals followed the same ethanol fading procedure (2-8%) and saccharin was faded out of the drinking solution over 6 additional days. Intake of 8% ethanol consumption was then monitored for seven days.

In Vivo Microdialysis

Microdialysis studies were carried out as previously reported (Doyon *et al.*, 2013a; Ostroumov *et al.*, 2016). Briefly, animals were habituated to tethering and the microdialysis chambers 1 day prior to testing. Baseline DA samples were collected (15–30 min), followed by a timed intravenous (i.v.) infusion of ethanol (1.5 g/kg, 20% in sterile saline, v/v) over 5 min. The i.v. route (using a cannula) was chosen to circumvent handling-related disturbances in DA levels associated with i.p. injections (Dong *et al.*, 2010).

Dialysis samples were analyzed for DA content using high-pressure liquid chromatography (HPLC) coupled to an electrochemical detector.

In Vivo Electrophysiology

Rats were anesthetized with isoflurane and implanted with a catheter in the jugular vein. Animals were positioned on a stereotaxic apparatus and burr holes were drilled to accommodate recording and ground electrodes. Rat body temperature was maintained throughout the experiment at 37°C using an isothermal pad. Glass electrodes backfilled with 0.5 M Na+-acetate and 2% Chicago Sky Blue (5–15 MU) were positioned in the lateral VTA (coordinates: 5.3–6.0 mm posterior from bregma, 0.8–1.4 lateral to midline and 7.5–8.5 ventral to brain surface). Electrical signals were filtered at 0.3–5 kHz. DA neurons were identified in vivo using established electrophysiological and pharmacological criteria. After 6–20 min of stable baseline recording, we infused 0.3 g/kg of ethanol i.v. every 3 min (for a final dose of 1.5 g/kg) and recorded single-unit activity. Drug-induced changes were calculated as a percent of baseline for each 3 min period. Following ethanol administration, quinpirole and eticlopride were infused (i.v., 0.25 mg/kg) to aid in the identification of VTA neurons. Chicago Sky Blue injections were used to identify the recording sites.

Ex Vivo Electrophysiology

Horizontal slices (230 mm) containing the VTA were cut (Leica Microsystems) from adult Long-Evans rats. Most cells were also backfilled with neurobiotin for immune identification. Spontaneous inhibitory postsynaptic currents were recorded in voltage-clamp mode in the whole-cell configuration. Synaptic $GABA_A$ inputs were isolated pharmacologically. The liquid junction potential between the bath and the pipette solutions was corrected.

Gramicidin perforated-patch recordings were applied to maintain the anionic gradient during reversal potential measurements.

Statistical Analysis

ANOVA with repeated-measures (in SPSS for Windows) was used to analyze the daily ethanol intake, dialysate DA concentrations, and DA firing rate. A two-tailed t-test assuming equal variance was used to assess differences between the mean sIPSC frequency and mean ethanol intake levels. Significance for all analyses was determined by $p < 0.05$.

Results

Adolescent Nicotine Increases Ethanol Self-Administration

We first examined how exposure to nicotine during adolescence influenced subsequent ethanol self-administration in adulthood. Animals were administered daily nicotine or saline injections throughout adolescence (p28-p42), and ethanol self-administration was measured in adulthood p70-p90) (Figure 1A). Operant responses to saccharin (0.125%, w/v) were first established, followed by the introduction of ethanol (2–4%) into the drinking solutions over the first seven days (Ostroumov *et al.*, 2016). Adolescent nicotine pretreatment significantly increased ethanol self-administration in adulthood compared to adolescent saline-treated controls (Figure 1B): group $F(1,12) = 22.43$, $p<0.01$). Mean ethanol intake across the first 7 days was 1.10 ± 0.07 g/kg for the nicotine-pretreated group and 0.71 ± 0.05 g/kg for the saline pretreated group (Figure 1C; $p < 0.01$ n = 7 animals/group). The average intake in the adolescent nicotine group (1.83 ± 0.11 g/kg) was also significantly elevated at higher ethanol concentrations compared to saline controls (1.30 ± 0.16 g/kg) (Figure 1D; $p < 0.05$; n = 9, 5 animals/group). Adolescent nicotine animals also drank significantly more ethanol compared to saline controls after complete removal of saccharin from the drinking solution (Figure 1E; $p < 0.01$, n=11, 9 animals/group). Therefore, adolescent nicotine induced robust changes in acquisition and maintenance of ethanol-drinking behavior throughout adulthood.

To examine the effect of adult nicotine exposure on subsequent ethanol consumption, adult animals were administered the same daily nicotine or saline injections over a two week period. Allowing a similar delay between nicotine and ethanol, self-administration was assessed in these animals approximately 30 days later (Figure 2A). Adult nicotine pretreatment failed to increase subsequent ethanol self-administration compared to adult

saline controls (Figure 2B): group $F(1,9) = 0.01$, $p > 0.05$. The average intake in the adult nicotine group (0.69 ± 0.07 g/kg, red bar) was not statistically different from adult saline controls (0.68 ± 0.06 g/kg, black bar) or adolescent saline controls (0.71 ± 0.05 g/kg, dotted line) (Figure 2C; $p > 0.05$, n= 7, 4 animals/group). These results suggest adolescent, but not adult, nicotine exposure produces long-lasting elevations in subsequent ethanol self-administration.

Adolescent Nicotine Attenuates Ethanol-induced DA Activity In Vivo

Given that ethanol self-administration involves DA signaling in the nucleus accumbens (NAc) (Gonzales et al., 2004), we hypothesized that adolescent nicotine might also alter ethanol-induced DA signaling. To test this hypothesis, we conducted accumbal microdialysis to measure DA responses in adult animals that were treated with either nicotine or saline during adolescence (Figure 3A). A sustained increase in DA levels were observed in saline-treated controls upon ethanol administration (Figure 3B, black trace). Adolescent nicotine-treated animals, in comparison, showed a blunted DA response to ethanol (Figure 3B, red trace): group x time: $F(7,98) = 2.23$, $p < 0.05$. No significant differences in baseline DA levels were detected between saline and nicotine groups: 1.01 ± 0.24 nM in saline-treated versus 1.13 ± 0.24 nM in nicotine-treated animals.

Ethanol stimulates DA release in the NAc by increasing the firing rate of VTA DA neurons (Foddai et al., 2004). To determine whether adolescent nicotine exposure altered DA neuron firing rate in vivo, we conducted single-unit recordings of VTA DA neurons in anesthetized adult rats. DA neurons were recorded in the lateral VTA and were identified based on their electrophysiological and pharmacological properties. The spontaneous firing rate of VTA DA neurons was measured before and after intravenous infusion of

ethanol (0.6–1.5 g/kg) (Figure 3C). Ethanol administration induced an increase in the spontaneous firing rate of VTA DA neurons in saline-treated controls (123.7 ± 4.9 % of basal). In contrast, adolescent nicotine treated animals failed to demonstrate a significant firing-rate increase upon ethanol administration (97.4 ± 2.2 % of basal) (Figures 3D; n = 7-17 rats/group, $p < 0.01$). Together with the microdialysis experiments, these data indicate that ethanol-induced DA signaling in adulthood was blunted in animals treated with nicotine during adolescence.

Adolescent Nicotine Alters GABA transmission and Anion Homeostasis Ex Vivo

Our previous results showed that nicotine pretreatment attenuated dopamine responses to ethanol via enhanced GABA release onto DA neurons (Doyon *et al.*, 2013a). To examine if adolescent exposure similarly altered GABAergic neurotransmission, slices were prepared from adult animals treated with nicotine or saline during adolescence (Figure 4A). We performed whole-cell patch-clamp recordings of VTA DA neurons and measured spontaneous inhibitory postsynaptic currents (sIPSC) in the presence of ethanol (Figure 4B). In control animals, bath-applied ethanol produced a small increase in sIPSC frequency (114.3 ± 4.1 % of basal). In contrast, DA neurons from nicotine animals showed significantly greater ethanol-induced potentiation of sIPSC frequency compared to the control response (177.4 ± 5.6 % of basal) (Figures 4C-D, black and red data; n = 8-13 cells/group, $p < 0.01$). These results suggest adolescent nicotine exposure leads to greater inhibition of DA neurons upon ethanol exposure in adulthood.

Increased GABA release onto DA neurons upon ethanol exposure was previously shown to arise from depolarizing shifts in the $GABA_A$ reversal potential (E_{GABA}) in VTA GABA neurons (Ostroumov *et al.*, 2016). E_{GABA} is the membrane potential at which evoked IPSCs

change their direction from inward to outward. We hypothesized that adolescent nicotine produced depolarizing shifts in E_{GABA}. To test this, we performed gramicidin perforated patch-clamp recordings in VTA GABA neurons to preserve the intracellular anion concentrations (Figure 4E), and we measured $GABA_A$ IPSCs at different membrane potentials (Figure 4F). VTA GABA neurons from adolescent nicotine-treated animals showed a significantly more depolarized E_{GABA} value compared to saline-treated controls (Figures 4G): -66.1 ± 1.5 mV after nicotine (red data) versus -87.0 ± 3.9 mV in controls (black data), n = 6-7 cells/group, $p < 0.01$. This result suggests that depolarizing shifts in E_{GABA} give rise to altered responses to ethanol.

Enhancing chloride extrusion in the VTA prevents elevated intake after adolescent nicotine exposure

A depolarizing shift in E_{GABA} reflects a higher intracellular chloride concentration, which in adult neurons is often mediated by a decrease in chloride extrusion capacity. Given that adolescent nicotine altered anion homeostasis in VTA GABA neurons, we next determined whether this adaptation mediated increased self-administration after adolescent exposure to nicotine. Depolarizing shifts in E_{GABA} can be restored by enhancement of chloride extrusion with the KCC2 agonist CLP290. Adolescent-treated animals received bilateral, intra-VTA infusions of CLP290 (45 µM) or vehicle prior to the first ethanol self-administration session (Figure 5A). However, compared to adolescent nicotine animals that received intra-VTA infusion of vehicle, intra-VTA infusion of CLP290 significantly decreased ethanol consumption (Figure 5B): group $F(2,31) = 19.66$, $p<0.01$). Mean ethanol intake over seven days was also significantly lower than those animals that received infusions of CLP290 (0.79 ± 0.04 g/kg) compared to animals that received infusions of vehicle (1.06 ± 0.04 g/kg) (Figure 5C n= 9-11 animals/group). VTA infusions

of CLP290 did not significantly alter ethanol consumption in adolescent saline animals (0.66 ± 0.04 g/kg, n=4, data not shown).

Discussion

Though epidemiological studies consistently report associations between adolescent tobacco exposure and pathological drinking (DiFranza & Guerrera, 1990; McKee *et al.*, 2007), the neuronal adaptions promoting ethanol consumption after early nicotine exposure have not been well-delineated. In adult animals exposed to nicotine during adolescence, we found altered chloride homeostasis in GABAergic neurons of the VTA correlated with an increase in ethanol self-administration. Adolescent nicotine pretreatment enhanced inhibition of DA neurons and attenuated mesolimbic DA responses to ethanol, both of which are known consequences of depolarizing shifts in $GABA_AR$ (Ostroumov *et al.*, 2016). Most importantly, enhancing chloride transporter function within the VTA of adolescent nicotine-treated animals blocked the observed elevated ethanol intake during adulthood.

Although $GABA_A$ signaling normally mediates inhibitory synaptic transmission in the adult mammalian nervous system, it can shift toward excitation under certain pathological conditions (De Koninck, 2007) and may be an important form of plasticity in adult animals (Chung, 2012; Astorga *et al.*, 2015; Doyon *et al.*, 2016). Depolarizing shifts in $GABA_AR$ signaling correlated with prior adolescent nicotine exposure (Figure4G) and were required subsequent increased ethanol consumption (Figure 5). This form adaptation arises from compromised chloride extrusion and can be rescued using CLP290, an activator of the chloride transporter KCC2 (Gagnon *et al.*, 2013; Kaila *et al.*, 2014; Ostroumov *et al.*, 2016). Shifts toward excitatory GABA may occur elsewhere in the brain, but the increased ethanol self-administration was prevented if this shift was blocked in the VTA of nicotine-treated animals (Figure 5). Thus, adolescent nicotine induces long-lasting, adaptions in anion

homeostasis within the VTA that may serve as a gateway for subsequent pathological drug use.

Decreased DA responses to ethanol in adulthood correlated with prior adolescent nicotine exposure and altered chloride homeostasis (Figure 3). Although the DA response was not directly examined as a cause of the increased self-administration, decreases in drug reward sensitivity are often associated with compensatory increases in drug self-administration under relatively low levels of response effort (Koob & Le Moal, 2001). Others have reported that enhancing DA signaling exogenously attenuates voluntary drinking in rats (Bass *et al.*, 2013). Furthermore, the correlation between decreased ethanol-induced DA release and increased self-administration has been previously reported in rodent studies (Brodie & Appel, 2000; Ramachandra *et al.*, 2007; Doyon *et al.*, 2013a).

Comparable nicotine injections administered to adult animals failed to increase subsequent ethanol self-administration (Figure 2), suggesting that the long-lasting effects of nicotine on mesolimbic responses to alcohol are unique to the adolescent window of exposure. Other studies support the notion that nicotine exposure, particularly during adolescence, induces robust and long-lasting changes in VTA gene expression. (Doura *et al.*, 2010). Future work should determine if expression of transporters known to regulate anion homeostasis are directly affected by adolescent exposure to nicotine.

Adolescence is thought to be critical period of neural development and drug exposure during this time may induce persisting neural and behavioral alterations (Chambers *et al.*, 2003; Spear, 2016). Our study describes the adaptations that persist into adulthood following adolescent nicotine exposure. Further, we identified altered chloride

homeostasis as an important alteration that influences subsequent drinking in adulthood and highlights novel potential therapeutic intervention strategies. Future work should consider the involvement of chloride homeostasis in other phases of drug addiction and disorders of motivated behavior.

Figures

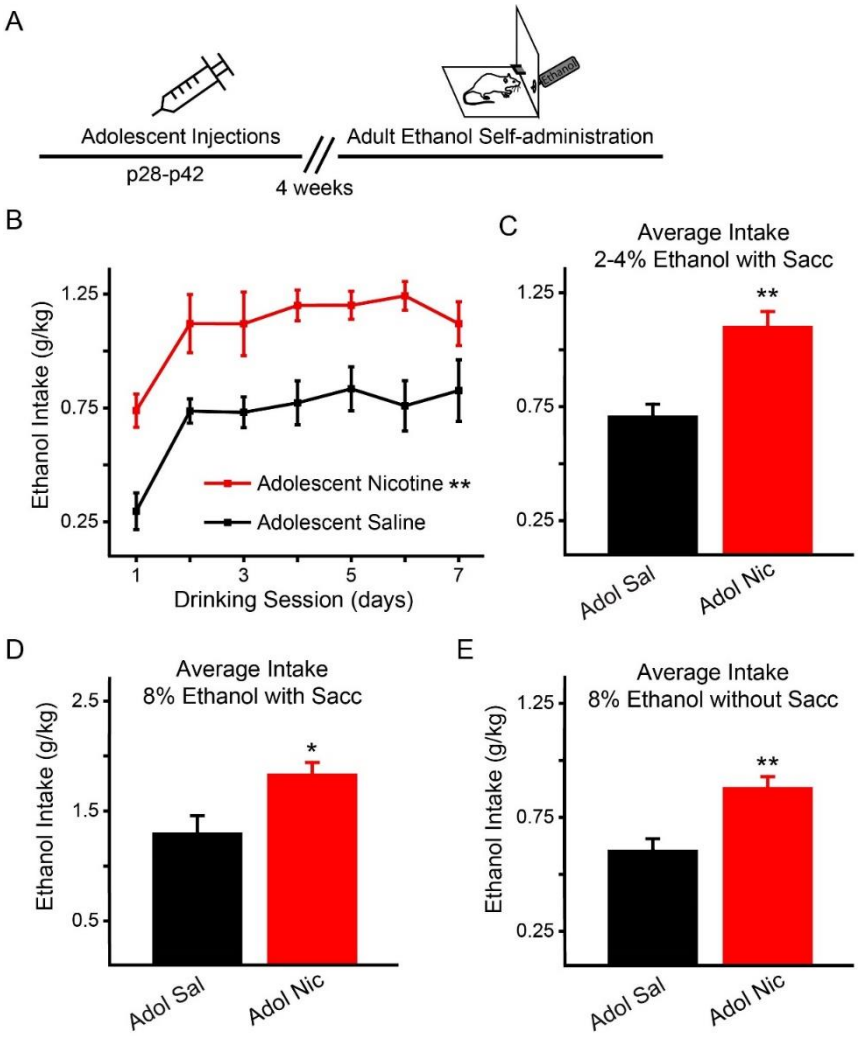

Figure 4.1 Adolescent nicotine increases ethanol self-administration. (A) Adolescent animals were administered daily injections of saline or nicotine from postnatal day (p) 28-42. Adult ethanol self-administration was assessed four weeks later. **(B)** Adolescent nicotine-treated rats showed greater ethanol intake compared to adolescent saline-treated controls. **Significantly different from the control group by ANOVA with repeated measures, $p < 0.01$, n = 7-9 per group **(C)** Mean daily ethanol intake over the first seven self-administration sessions. **Significantly different by t test, $p < 0.01$. **(D)** After six days of ethanol fading, intake of 8% ethanol with saccharin was significantly elevated in adolescent nicotine-treated animals compared to saline-treated controls. The mean intake was measured over seven self-administration sessions. *Significantly different by t test, $p< 0.05$, n = 5-8 animals per group. **(E)** After saccharin removal, intake of 8% ethanol was significantly elevated in adolescent nicotine treated animals compared to saline treated controls. The mean intake was measured over seven self-administration sessions. **Significantly different by t test, $p< 0.05$, n = 9-11 animals per group.

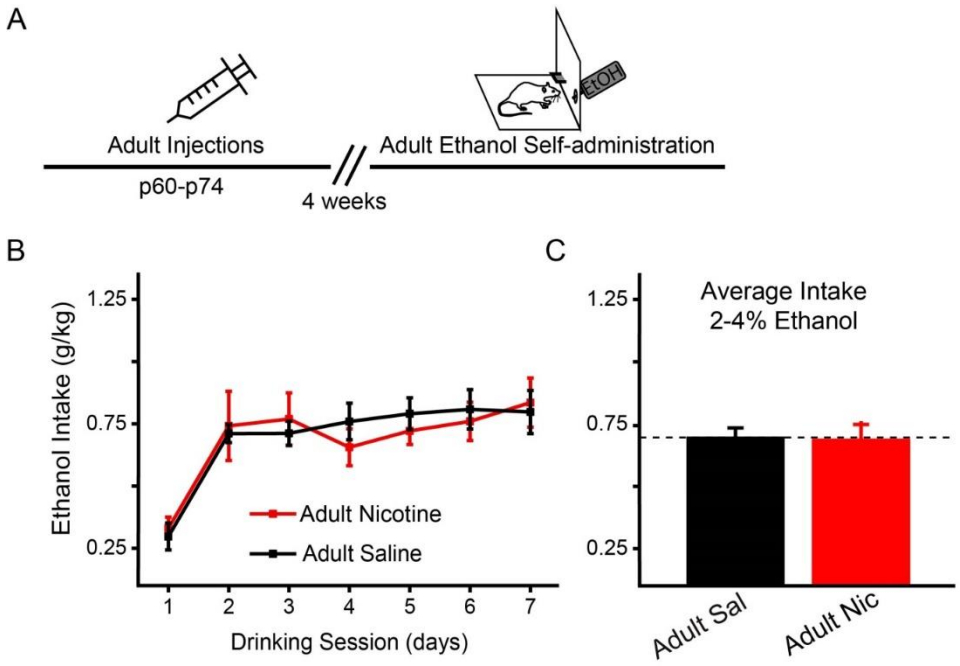

Figure 4.2 Adult nicotine does not influence subsequent ethanol self-administration. (A) Adult animals were administered daily injections of saline or nicotine from postnatal day (p) 60-74. Adult ethanol self-administration was assessed four weeks later. **(B)** Average daily intake between animals treated with saline vs. nicotine during adulthood. Adult nicotine-treated animals did not drink significantly greater ethanol compared adolescent-saline treated controls, $p > 0.05$, n= 5, 7 per group **(C)** Mean daily ethanol intake over the first seven self-administration sessions. Ethanol consumption in adolescent saline control rats is shown for comparison (dotted horizontal line). Adult nicotine-treated animals (red bar) did not consume significantly more ethanol than saline-treated controls (black bar), $p > 0.05$, n = 5, 7 per group.

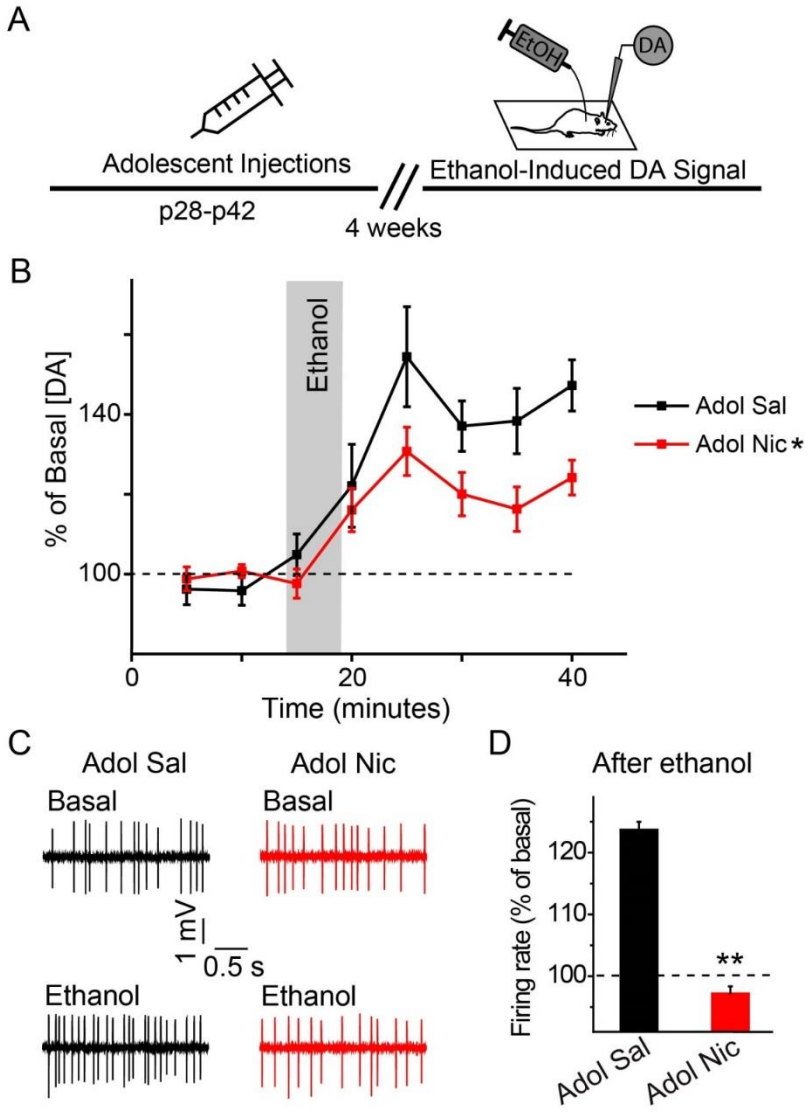

Figure 4.3 Adolescent nicotine attenuates dopamine responses to ethanol in vivo. **(A)** Animals were exposed to saline or nicotine throughout adolescence (p28-42) and in vivo dopamine responses to ethanol experiments were measured in adulthood. **(B)** Time course of DA release in the NAc measured via microdialysis following ethanol administration in saline-treated controls (black) and nicotine-treated rats (red). Ethanol (1.5 g/kg) was injected i.v. during the 5 min period (shaded vertical gray bar). *Significantly different by ANOVA with repeated measures, $p < 0.05$, $n = 7, 9$ per group. **(C)** Representative recordings from putative DA neurons before and after ethanol administration (0.6–1.5 g/kg) in the saline-treated (black) and nicotine-treated (red) groups. No significant differences in the mean basal firing rate were detected. (**C)** In saline-treated controls (black), ethanol increased the firing rate of putative DA neurons. In the nicotine-treated group (red), ethanol failed to increase the firing rate of putative DA neurons. **Significantly different from the control group by t test, $p < 0.01$, $n = 7, 17$ rats per group.

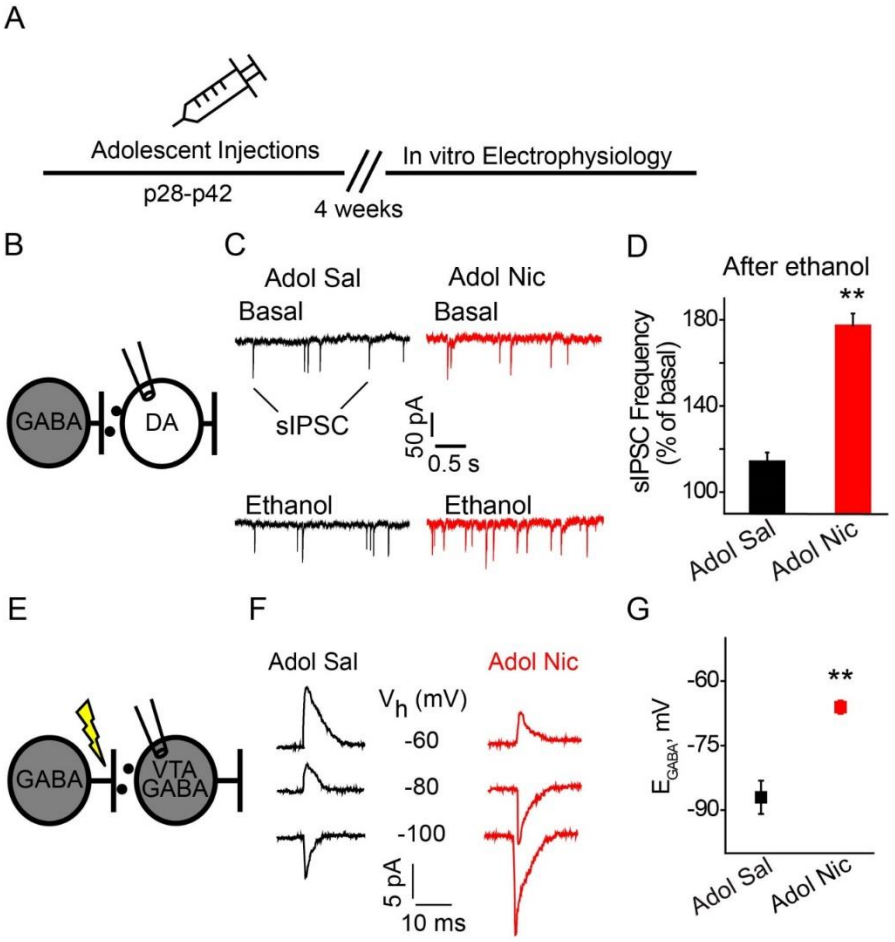

Figure 4.4 Adolescent nicotine alters GABA transmission and anion homeostasis ex vivo. (A) Animals were exposed to saline or nicotine throughout adolescence (p28-42) and in vitro electrophysiology experiments were conducted in adulthood. **(B)** Spontaneous inhibitory postsynaptic currents (sIPSCs) onto VTA DA neurons were recorded using the whole-cell patch-clamp configuration. **(C)** Representative recordings of sIPSCs before and after ethanol administration in the saline-treated (black) and nicotine-treated (red) groups. **(D)** Mean changes in the sIPSC frequency after ethanol application in VTA DA neurons. DA neurons from nicotine-treated animals (red) demonstrated a significantly increased ethanol-mediated sIPSC frequency compared to neurons from saline-treated controls (black). **Significantly different from control and RU486-treated groups by t test, $p < 0.01$, n = 8,13 cells per group. **(E)** GABAergic input onto VTA GABA neurons was recorded using gramicidin perforated patches at different holding potentials to measure nicotine-induced alterations in anion homeostasis. $GABA_A$ IPSCs were evoked by electrical stimulation in the presence of DNQX, AP5, and CGP55845. **(F)** Representative IPSCs recordings from saline (black) and nicotine-treated (red) animals at the given holding potentials. The IPSCs reverse direction at the EGABA. For display, the traces were filtered and stimulus artifacts were removed. **(G)** VTA GABA neurons from nicotine-treated animals (red, **$p < 0.01$ by t test) demonstrated a significantly more positive E_{GABA} value compared to neurons from saline-treated control animals (black square), n = 6, 7 cells/group.

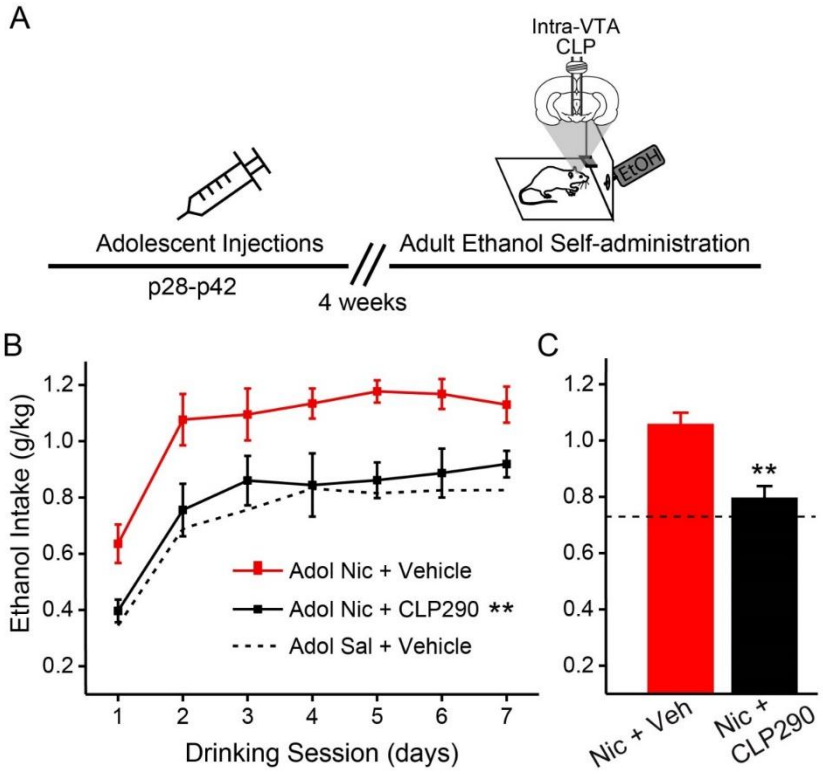

Figure 4.5 Enhancing chloride extrusion in the VTA prevents elevated intake after adolescent nicotine exposure. (A) Vehicle or CLP290 was infused bilaterally intra-VTA (45 µM at 0.5 mL/min over 2 min) prior to the first ethanol self-administration session. **(B)** Average daily intake between adolescent nicotine animals treated with CLP290 or vehicle prior to self-administration. Adolescent nicotine animals that receive CLP290 rats showed reduced ethanol intake compared to adolescent nicotine animals that receive vehicle infusions and were indistinguishable from adolescent saline controls. **Significantly different by ANOVA with repeated measures, $p < 0.01$. **(C)** Mean daily ethanol intake over the first seven self-administration sessions. After CLP290 administration, adolescent nicotine animals consumed significantly less ethanol compared to the vehicle injected group (red). Ethanol consumption in adolescent saline vehicle-treated control rats is shown for comparison (dotted horizontal line). **Significantly different from the VTA vehicle group by t test, $p < 0.01$, $n = 9–13$ rats/group.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Alyse M. Thomas

Overview

Alcohol is the oldest and most commonly used psychoactive substance in the world (McGovern, 2009; WHO, 2014). Like other drugs of abuse, alcohol's reinforcing properties can promote pathological drinking patterns (NIAAA, 2010). The risks of excessive alcohol use include alcohol-related injury, disease, and the development of alcohol use disorders (NIAAA, 2010; WHO, 2014).

Epidemiological studies reveal that stressful conditions and tobacco use are associated with elevated alcohol consumption (Anthony & Echeagaray-Wagner, 2000; Keyes *et al.*, 2011), leading to the hypothesis that stress or nicotine promote pathological drug use via adaptions in the brain (Koob & Kreek, 2007; Spear, 2016). It is well established that alcohol, stress hormones, and nicotine act within the mesolimbic dopamine circuitry to promote behavioral reinforcement (Piazza *et al.*, 1993; Corrigall *et al.*, 1994; Gatto *et al.*, 1994; Rodd-Henricks *et al.*, 2000), implicating this region as a locus for their interaction. Based on previous work implicating glucocorticoid and nicotinic receptors in ethanol reinforcement (Ericson *et al.*, 1998; Fahlke & Hansen, 1999; Le *et al.*, 2000a; Vendruscolo *et al.*, 2012), we hypothesized that prior exposure to stress or nicotine promoted drinking via adaptations in the mesolimbic circuitry.

The findings presented in the second chapter of this thesis describe a novel mechanism by which stress hormones influence subsequent behavioral and biological responses to alcohol. Drug-naïve animals subjected to acute restraint stress subsequently showed elevated alcohol intake compared to non-stressed controls. *In vivo* microdialysis experiments revealed that stress blunted subsequent accumbal dopamine responses to alcohol via excitatory GABA transmission in the ventral tegmental area. Importantly, this study identified compromised function of the potassium chloride co-transporter KCC2 as the molecular adaptation underlying excitatory GABA transmission. Moreover, we demonstrated that excitatory GABA transmission and functional KCC2 downregulation within the mesolimbic circuitry were causally related to the observed increases in alcohol self-administration.

The third chapter demonstrated how stress hormone signaling within the ventral tegmental area may contribute to the co-use of addictive drugs like nicotine and alcohol. Exposure to acute nicotine blunted dopamine responses to alcohol and increased alcohol self-administration. Importantly, my work revealed that stress hormones acting locally within the VTA mediated this nicotine-alcohol interaction. Blocking the activation of stress hormone receptors during nicotine exposure prevented blunted dopamine responses to alcohol and normalized drinking to control. These findings reveal that nicotine recruits stress hormone pathways to promote subsequent alcohol use.

The fourth chapter described that adolescent exposure to nicotine produces long-lasting alterations in reward-related responses to alcohol, suggesting a biological mechanism through which nicotine could act as a gateway drug. Adolescent nicotine treated animals showed attenuated dopamine signaling and elevated alcohol self-administration throughout adulthood. These adaptations corresponded with depolarizing shifts in GABA

transmission and increased inhibition of dopamine neurons. Pharmacological enhancement of KCC2 restored drinking to control levels in adolescent nicotine-treated animals, suggesting that compromised chloride extrusion contributes to elevated alcohol self-administration after adolescent exposure to nicotine. Taken together, this body of work describes a previously unknown mechanism within the mesolimbic circuitry that promotes alcohol consumption after exposure to nicotine or stress.

KCC2 as a Novel Regulator of Mesolimbic Activity and Alcohol Consumption

The results presented here identify KCC2 as a regulator of mesolimbic responses to alcohol at a synaptic, cellular, circuit, and behavioral level. KCC2 is a neuron-specific chloride (Cl^-) extruder (Payne *et al.*, 1996; Payne, 1997; Williams *et al.*, 1999; Karadsheh & Delpire, 2001) found predominantly in non-dopamine neurons of the midbrain (Gulacsi *et al.*, 2003; Taylor *et al.*, 2016) and is a critical determinant of $GABA_AR$ synaptic responses (Chamma *et al.*, 2012; Kahle *et al.*, 2013; Kaila *et al.*, 2014). In mature neurons, internal [Cl^-] is normally relatively low (~5 mM), which yields a hyperpolarized $GABA_A$ reversal potential (E_{GABA}) relative to the resting membrane potential. Under these conditions, $GABA_A$ receptor activation results in an inward Cl^- gradient, decreasing the probability of action potential generation. However, in the context of reduced KCC2 function, even small elevations of internal [Cl^-] can produce depolarizing shifts in GABAergic responses that increase the probability of action potential generation (Raimondo, 2017). In cases of severe KCC2 downregulation, or as seen during development, excitatory GABA transmission dominates (Ben-Ari, 2002).

After exposure to stress or adolescent nicotine, we observed a depolarizing shift in GABAergic responses within VTA GABA neurons. Illustrating the dominant influence of

compromised chloride extrusion on cell firing properties, computational models of CA1 pyramidal neurons indicate that increasing internal [Cl^-] by 2.5 mM generates a 40 percent increase in cellular firing rate (Saraga *et al.*, 2008). Accordingly, reduced KCC2 function and excitatory GABA transmission promoted VTA GABA neuron firing upon ethanol exposure to influence broader circuit function and behavior, as measured by *in vivo* microdialysis and operant self-administration. My results indicated that excitatory GABA transmission in the VTA blunts subsequent accumbal dopamine responses to ethanol and increases alcohol self-administration, given that blocking excitatory GABA responses with acetazolamide normalized dopamine signaling and intake levels.

Using the KCC2 agonist, CLP290, we demonstrated that increasing Cl^- extrusion selectively reduced alcohol intake in stress and adolescent nicotine-treated animals. Importantly, this is the first study demonstrate the efficacy of VTA infusions of CLP290 in regulating drug self-administration. This compound restores KCC2 function, thereby producing hyperpolarizing shifts in E_{GABA} (Gagnon *et al.*, 2013). Given that KCC2 is found strictly in the CNS and the extended bioavailability of the prodrug CLP290 (Gagnon *et al.*, 2013), it is reasonable to propose that this compound holds therapeutic potential. Future experiments should explore if compromised KCC2 function and altered GABA transmission contribute to other addiction phenotypes such as withdrawal and relapse.

Vulnerability to Nicotine during Adolescence

Adolescence is recognized to be a critical period of cognitive development that confers risk for drug use and addiction (Chambers *et al.*, 2003; Crews *et al.*, 2007). During this time, there is a developmental dissociation involving heightened reward processing in subcortical brain regions and diminished inhibitory control in frontal cortical areas

(Doremus-Fitzwater *et al.*, 2010; Casey *et al.*, 2011). Adolescents are more likely to initiate drug use than any other age group, with most smokers having their first cigarette before the age of 18 (Richter *et al.*, 2017). This early exposure to tobacco is associated with a significant risk for subsequent drug use disorders throughout adulthood (Miller & Gold, 1998; Chen *et al.*, 2002; Riala *et al.*, 2004).

Our study examined how exposure to nicotine during adolescence influenced responses to alcohol in adulthood. We identified long-lasting adaptions in VTA GABA transmission that gave rise to elevated ethanol consumption in adulthood. These effects were unique to adolescent nicotine exposure, since similar injections of nicotine in adults failed to increase subsequent ethanol self-administration. Future work should consider how such long-lasting adaptations are unique to nicotine exposure during adolescence. A related study suggests that chronic nicotine primes responses to cocaine via epigenetic mechanisms. Nicotine enhanced subsequent cocaine-induced transcriptional responses of the FosB gene through inhibition of histone deacetylase (Levine *et al.*, 2011). Interestingly, recent models of chronic pain have shown epigenetic suppression of KCC2 expression through histone modification (Lin *et al.*, 2017). It would be of great interest to determine if adolescent exposure to nicotine acts through acetylation mechanisms to influence KCC2 expression and mesolimbic responses to alcohol.

Ethanol Reinforcement and the Mesolimbic Dopamine System

We showed that prior exposure to stress or nicotine promoted subsequent ethanol self-administration and blunted dopamine responses to ethanol. Accumbal dopamine signaling is thought to mediate the positive reinforcing effects of ethanol (Berke & Hyman, 2000; Grace, 2000; Weiss & Porrino, 2002; Gonzales *et al.*, 2004; Stuber *et al.*, 2012).

While it is generally accepted that inhibiting dopamine neurons interrupts learning and activating dopamine neurons promotes learning (Chambers *et al.*, 2003; Steinberg *et al.*, 2013), the consequences of intermediate dopamine signaling on behavioral reinforcement have not directly been tested. Our data suggests that blunted dopamine responses elicits compensatory increases in reward-seeking behaviors. Other groups have reported similar correlations between blunted dopamine responses and elevated drinking in rodents (Brodie & Appel, 2000; Ramachandra *et al.*, 2007). Human neuroimaging studies also report that attenuated mesolimbic activity predicts problematic drug use and addiction (Volkow *et al.*, 1997; Melis *et al.*, 2005; Buchel *et al.*, 2017). Thus, dampened dopamine responses may promote drug use. To test this hypothesis, endogenous dopamine responses should first be characterized during operant self-administration, followed by direct manipulation of dopamine signaling using pharmacological or optogenetic approaches. Based off our results and others, we predict that reductions in dopamine signaling would increase ethanol intake. Alternatively, in animals previously exposed to stress or nicotine, exogenously enhancing dopamine should reduce overall consumption.

Since dopamine signaling was not causally linked to drinking in our studies, it is possible that VTA GABA transmission promotes drinking via dopaminergic as well as non-dopaminergic reward signaling pathways. Blocking $GABA_A$ receptors in the VTA was previously shown to reduce ethanol intake in rats (Nowak *et al.*, 1998), suggesting their involvement in ethanol reinforcement. Further, non-dopaminergic pathways including the brainstem tegmental pedunculopontine nucleus have been previously reported in the context of opiate and ethanol reward (Bechara & van der Kooy, 1989; Laviolette & van der Kooy, 2001; Laviolette *et al.*, 2004; Laviolette & van der Kooy, 2004a; Ting *et al.*, 2013a; Ting *et al.*, 2013b), and are associated with depolarizing shifts in VTA $GABA_A$ receptors

(Laviolette *et al.*, 2004). Selective manipulation of VTA GABA projections in the TPP (and other VTA GABA projection sites) during ethanol self-administration would help demonstrate an independent role for VTA GABA transmission in ethanol reward. The overarching goal of such experiments is to develop a circuit map of reward pathways underlying ethanol reinforcement.

Our studies measured ethanol-induced accumbal dopamine release using microdialysis, a technique that permits detection of dopamine levels over long timescales (i.e. minutes) (Di Chiara & Imperato, 1988; Floresco *et al.*, 2003). Though this approach provides a useful readout of ethanol-induced dopamine signaling, conducting cyclic voltammetry experiments would permit examination of sub-second changes in dopamine firing patterns (Floresco *et al.*, 2003; Robinson *et al.*, 2009; Howard *et al.*, 2011). Dopamine neurons can exhibit either single-spike or burst-firing patterns (Grace & Bunney, 1984; Samson *et al.*, 1993; Weiss *et al.*, 1993; Myers & Robinson, 1999; Kaczmarek & Kiefer, 2000; Czachowski *et al.*, 2001; Hyland *et al.*, 2002; Samson & Chappell, 2003). Salient or rewarding stimuli trigger burst firing (Hyland *et al.*, 2002) and this signal thought to mediate reward-prediction learning (Schultz, 1998; 2007). Acting through multiple brain targets, alcohol influences dopamine firing and release over a broad timescale (Mereu *et al.*, 1984; Imperato & Di Chiara, 1986; Yoshimoto *et al.*, 1992; Weiss *et al.*, 1993; Yim *et al.*, 2000; Foddai *et al.*, 2004; Howard *et al.*, 2009; Robinson *et al.*, 2009; Carrillo & Gonzales, 2011). The data we obtained with microdialysis provide a clear demonstration of differences in dopamine signaling in stressed or nicotine-treated animals compared to controls, yet these results can mask adaptations phasic dopamine release within the accumbens (Robinson *et al.*, 2009). Thus, the application of voltammetric techniques would greatly aid the

interpretation of our results and provide a clearer picture of how dopamine responses to ethanol are altered after exposure to stress or nicotine.

Glucocorticoid receptor influence over KCC2

The observed downregulation of KCC2 was dependent on glucocorticoid receptor (GR) activation and persisted 15 hours after acute restraint stress. Similar effects were observed after 1-hour corticosterone incubation *ex vivo*. GR activation is typically associated with transcription-dependent signaling mechanisms, and GR activity could directly influence ion transport function via genomic action (Pondugula *et al.*, 2013). However, the specific intracellular signaling cascades linking VTA GR activation to altered chloride homeostasis remain unknown. It is increasingly appreciated that glucocorticoids act via both genomic and non-genomic signaling cascades (Joels & Baram, 2009; McEwen, 2012). It is hypothesized that these cascades work in concert across time: fast non-genomic modulation occurring over a timescale of seconds to minutes, followed by slower genomic modulation occurring over subsequent minutes to hours (Haller *et al.*, 2008; Teng *et al.*, 2013). Thus, adaptations in KCC2 expression following GR activation could occur through a number of signaling pathways.

The literature surrounding activity-dependent changes in KCC2 also suggests that downregulation could occur via a number of mechanisms. Activity-dependent regulation of KCC2 expression has been observed in response to long-term potentiation (Wang *et al.*, 2006), repetitive pairing of pre- and post-synaptic activities (Woodin *et al.*, 2003), repetitive post-synaptic spiking (Fiumelli *et al.*, 2005), and NMDAR activation (Kitamura *et al.*, 2008). In one study, increased cellular activity was shown to reduce KCC2 expression via endogenous BDNF–TrkB signaling (Rivera *et al.*, 2004) implicating BDNF as one possible mediator of interactions between stress hormones and KCC2.

Although initially characterized by its neurotrophic role in neuronal survival and differentiation, it is now widely accepted that BDNF acts as a neuromodulator to influence activity-dependent synaptic plasticity (Kuczewski *et al.*, 2009) and is implicated the development of drug addiction (McGough *et al.*, 2004; Nestler, 2005). Given that (i) stressful experiences are known to recruit BDNF signaling within the VTA (Berton *et al.*, 2006; Krishnan *et al.*, 2007; Fanous *et al.*, 2010; Walsh *et al.*, 2014) and that (ii) BDNF is a known modulator of KCC2 function (Rivera *et al.*, 2002; Rivera *et al.*, 2004; Wake *et al.*, 2007), it follows that stress hormones could regulate chloride homeostasis through BDNF signaling in the VTA. Future studies should therefore examine the recruitment of BDNF signaling cascades in the functional regulation of KCC2.

Multiple transcription factors, kinases, and phosphatases are known to regulate KCC2 expression (Medina *et al.*, 2014). We observed functional KCC2 downregulation via reduced phosphorylation at serine 940 (S940). Phosphorylation at S940 by protein kinase C (PKC) contributes to membrane stabilization whereas dephosphorylation by protein phosphatase 1 (PP1) contributes to membrane destabilization and endocytosis (Lee *et al.*, 2011). Recent studies have described the PKC-dependent regulation of KCC2 during physiologically relevant processes: (i) activity-dependent attenuation of KCC2 function (Fiumelli *et al.*, 2005), (ii) tonic activation of the KCC2 by group I metabotropic glutamate receptors (mGluR1s) (Banke & Gegelashvili, 2008), and (iii) activation of the KCC2 via serotonin 2A receptors (Bos *et al.*, 2013). However, these studies failed to demonstrate the direct involvement of PKC over other intermediate signaling molecules. In contrast, NMDA receptor activation produced rapid, direct PP1-dependent dephosphorylation of KCC2 at S940 (Lee *et al.*, 2011). In order to confirm involvement of PKC in our study, VTA slices could be incubated in corticosterone and OKA (PP1 inhibitor) prior to western

blot analysis of KCC2 S940 phosphorylation levels. This approach represents an important next step towards a more comprehensive understanding of KCC2 regulation within the VTA after exposure to stress hormones.

Concluding Remarks

Stress and drugs of abuse create a permissive environment for cellular plasticity and behavioral adaptation. The work presented here sheds light on a novel mechanism by which stress hormones and nicotine can influence mesolimbic responses to promote alcohol consumption. Though we temporally separated stress and nicotine exposures from subsequent ethanol self-administration to permit mechanistic investigation, substantial evidence indicates that alcohol and nicotine are used together, and can independently recruit stress hormone signaling within the mesolimbic dopamine system. Upon each drug exposure, the distinct phases of intoxication and withdrawal serve as separate activators of the HPA axis and downstream corticosterone release (Ellis, 1966; Tabakoff *et al.*, 1978; Rivier *et al.*, 1984).

Given this recruitment, it should be no surprise that glucocorticoids play a role in the development of addictive phenotypes, including compulsive drinking (Vendruscolo *et al.*, 2012), cognitive deficits (Jacquot *et al.*, 2008), drug-craving (Fox *et al.*, 2007; Sinha *et al.*, 2009), and the reinstatement of drug-seeking behaviors (Simms *et al.*, 2012). Based off this literature and others, the GR antagonist mifepristone is currently being tested in humans for the treatment of alcohol dependence and withdrawal (Vendruscolo *et al.*, 2015; Donoghue *et al.*, 2016). In addition to improved addiction treatment strategies, it is imperative to bolster research efforts to determine the cause of pathological behaviors in the first place. With that effort in mind, we characterized changes in mesolimbic adaptations after exposure to stress or nicotine and identified KCC2 as a novel molecular

adaptation contributing to elevated alcohol consumption. Looking forward, the overarching goal of our work is to illuminate how environmental exposure influences brain function to promote certain behaviors, and then apply this knowledge in the treatment of mental health disorders.

APPENDIX

Accumbal Cannulation and Jugular Catheterization Surgery Protocol

Surgery setup

1. Turn on germinator one hour before and autoclave tools
2. Check anesthesia/O2 levels
3. Xylocaine
4. Ketoprofen
5. Sodium chloride
6. Iodine with non-sterile qtips dipped
7. Sterile qtips
8. Diapers
9. Drape
10. Paper towels with dry qtip
11. Thermometer with heating pad
12. Ear bars in EtOH then allow to air dry
13. Nair
14. Cauterizer
15. EtOH spray bottle
16. Surgical tools
17. Drill bit
18. 5-0 suture thread
19. Catheter tube (**run with ethanol then fill with saline/heparin in 1mL syringe and spray entire thing with ethanol)
20. Giant screw, catheter cap, cannula, 3 small screws in Ethanol

Animal Prep

1. Weigh animal
2. Isoflurane Anesthesia to 4 to put animal down then turn to 2-2.5
3. Once down move to heating blanket (Do not turn on yet)
4. Place tube around mouth
5. Put gel on eyes
6. Apply nair
    a. Remove hair using qtips and wipes
    b. Apply iodine

Surgery: Part 1 Vertical cut and catheter implantation

1. Cut vertical line down to skull with the scalpel
2. Place qtip over cut and then lay down a wipe as you turn animal over

3. Turn heating blanket on but be sure it is only warming upper portion of animals body
4. Apply nair to pulse
    a. Remove hair with qtips and wipes
    b. Add iodine
5. Pull skin in center of pulse
    a. Pull skin up and cut a small bit of skin off
    b. Using two teethed forceps, tear away outer tough tissue and then inner fatty tissue until you reach a blood vessel that is closely associated to muscle.
    c. Using the forceps to grab a piece of muscle, pull away the tissue from the vein. Do this until you are able to pinch the forceps under the vessel. Do not hesitate to use force or pull away more tissue. (The main concern is that we do not damage the vein)
    d. Once the vein is out on the forceps, put saline on the scoopula and place it under the vein
6. Cut partially with scissors
    a. Insert end of catheter tube into the hole
    b. Once this is done, suck up blood to ensure you are inside the vein and then inject a small amount of saline (yellow tipped catheter)
7. With suture thread do 2 horizontal knots (3-4 per knot), leaving tails. Then do 2 diagonal knots (3-4/knot)
    a. Inject saline each time
8. Dry off and add cement
    a. Wait until it takes form and then push off the scoopula and back into skin
    b. Inject saline
9. Carefully turn animal over and use clamp scissors to enter back of vertical cut and stay close to skin as you directly move toward catheter line
    a. Place cannula cap up into the clamp and pull back through the scalp just until the tube disappears from the chest.
10. Turn animal back over and suture
    a. Inject .1 mL antibiotics and finish several knots until skin is taught
    b. Add iodine
11. Set up stereotaxic apparatus
    a. Place front teeth in slot and secure nose bar
    b. Set up ear bars so that each side is approximately 7-8 and is flat in line with edges
    c. Feel head to be sure it is stable and secure
    d. Tape down drape
    e. Apply iodine
12. Scrape skill until lambda and bregma are visible
    a. Scrape to the outer lip

    b. Scratch skull surface
    c. Cauterize bleeding spots
    d. Clamp down inner layer
    e. Inject epinephrine superficially
13. Check anesthesia
14. Drill 3 holes in 3 zones of brain (UL,LL,LR) leaving room for cannula wire to wrap around small screw and the big screw
15. Use tweezers to hold circular shaft of screw as you turn it and add pressure
    a. Screw in until it no longer wobbles
16. Define bregma coordinates
    a. Make sure anterior to posterior is flat
    b. Draw line to connect posterior lambda with anterior suture
    c. Once you have bregma location write down coordinates
        i. Front/back (A/P)= 19.0 + 1.4 = 20.4
        ii. Left/right (M/L) = 18.0 + 1.1 = 19.1
        iii. Up/down (D/C) = 24.5 - 4.0 = 20.5
17. Draw circle around correct nucleus accumbens location and then a middle point and drill using larger drill bit
    a. once hole is drilled, make sure cannula can enter without touching bone because it will bend
        i. Slowly go down to coordinate (2 units at a time)
        ii. Use qtip torn off to prevent bleeding and leave it until bleeding stops
18. Add ointment to skin where cement will go over near catheter cannula
19. Tape the catheter cannula up in preferred orientation and glue down the big screw
    a. Again ensure skull is dry and clean
20. Add cement to secure the screw and the probes and remove the qtip
    a. Minimize sutures by filling all gaps with cement
    b. Pull skin out as glue takes form
    c. Once dry undo clamps and pull skin up
21. Inject gentamicin antibiotic: 2 drops front and back
22. Suture
    a. Add iodine
    b. Flush more saline then wait and take off tube and place cap on
23. Inject timentin intravenously
24. Turn down isoflurane to 1.5 and allow animal to wake up, monitor until awake
25. Inject ketoprofen
26. Return animal to home
27. Clean tools

Microdialysis Probe Protocol

1) Start with a clean surface and replace desk covering if necessary
2) Cut fused-silica:
    a) Two 35 cm pieces for the inlet and outlet (Polymicro technologies: ID~40, OD~100)
    b) Use scoring or glass surface to ensure precision cuts
        i) Use a new razor blade to make cross-sectional cuts in the silica. Around a glass bottle, hold silica down firmly with your thumb and index finger so that there is no slack in the section you are interested in cutting. Score silica by gently moving across it in a left to right motion.
    c) Verify that silica has been cut flush at the ends using a microscope. Jagged or crushed ends may lead to fluid blockage within the probe.
    d) Place any shards of broken silica in the sharps container for disposal

3) Prepare connector:
    a) Supplies -
        i) Two-pronged solid state connectors (use recycled ones first)
            (1) Inspect connectors for overall quality (no loose pieces).
            (2) Clearance through one prong must be open for silica passage
        ii) 8 mm guide cannula
        iii) Glass beaker
        iv) Mounting putty on clear box
        v) 6 mm pieces of medium tygon tubing (Norton performance plastics ID~0.020 OD~0.060)
4) Assemble connectors:
    a) Slip a 6 mm piece of medium tygon tubing over the OPEN silver prong of the connector (before inserting fused-silica).
    b) Screw guide cannula into connector so that it fits snug. Be mindful of an under-torqued guide cannula as this will result in inaccurate probe placement *in vivo*.
    c) Mount the connector-guide cannula unit onto clear box using mounting putty to secure it from moving.
    d) Thread the two 35 mm pieces of fused silica through the connector and guide cannula unit.
    e) Check quality of silica through microscope and cut again if necessary.
5) Calibrate microscope using a 2 mm calibrating slide
    a) 42 r.u. = 2 mm and 84 r.u. = 4 mm; reticular units are fixed within the eyepiece of the scope.
6) Align silica
    a) Mount threaded connectors under the scope so that the end of the guide cannula is at the zero point of the reticular scale.
    b) Adjust the inlet so that it extends 4 mm (or 84 r.u.) past the end of the cannula.
    c) Adjust the outlet so that it extends 2 mm (or 42 r.u.) past the end of the cannula. Advice: adjusting one piece of silica tends to move the other piece simultaneously. To avoid this twist the silica as you are adjusting it.
7) Once silica are positioned precisely, pipette a small amount of superglue into the medium tygon tubing in which the silica are surrounded in order to permanently fixate the silica at

their respective positions. Caution: do not pipette excess superglue into the tygon tubing. This may result in glue reaching the prong of the connector and clogging it, thereby making future reuse impossible.

8) Sheath the exposed silica (this includes two junctions and Tygon covering)
    a) Supplies -
        i) Two sample vials
        ii) Two ≈12 cm of medium Tygon tubing
        iii) One ≈15 cm piece of large Tygon tubing
        iv) Blue-handled wire cutting pliers
    b) Assembly of first juncture -
        i) Take two sample vials and cut 3 mm off the tip and 1 cm off the rim of each with pliers. Advice: after cutting the tip make sure that a piece of large Tygon tubing is able to fit snug through it. This will make the gluing process easier.
        ii) Cut one 15 cm piece of large Tygon tubing.
        iii) Thread both silica (inlet and outlet) through a shortened sample vial (rim end first) until it reaches the silver prongs of the connector. This will serve as the first junction.
        iv) Thread both silica (inlet and outlet) through the 15 cm piece of large Tygon tubing, pushing the tubing 3-4 mm through the cut end of the already inserted sample vial.
9) Mount probes vertically for gluing.
    a) Fix juncture to silica and tubing using clear DEVCON 5-min epoxy. A needle or toothpick is useful for entombing the sample vial with epoxy.
    b) Caution: (1) connector must be able to turn in order to function, so it cannot be covered with epoxy at its base. Do not fix connector too close to the juncture. (2) 5-min epoxy solidifies fairly quickly so only mix small amounts at one time. (3) Ideally the entire juncture should be filled with glue.
    c) Allow juncture to dry for 15 min.
10) Assembly of second juncture –
    a) Cut two pieces of medium Tygon tubing, the lengths of which should be cut so that they are shorter than the exposed silica (inlet beginning and outlet end).
    b) Thread both silica (inlet and outlet) through a shortened sample vial (tip end first) until it reaches the large Tygon tubing. Push the tubing 3-4 mm through the sample vial as mentioned before. This will serve as the second junction.
    c) Thread each silica through its pre-measured piece of Tygon tubing until the tubing reaches the second junction.
    d) Mount probes vertically but upside down from before.
    e) Fix juncture to silica and tubing using clear DEVCON 5-min epoxy. Again, a needle or toothpick is useful for entombing the sample vial with epoxy.
    f) Once you have entombed the juncture about half way, push down the two pieces of Tygon tubing into the juncture and continue filling with glue until the entire juncture has been filled with glue

11) Construct inlets:
    a) Gather metal pieces from small parts drawer (an 8 mm 30G and a 5 mm 22G).
    b) Verify that silica fits through the thin long metal tube and that this tube fits through the shorter fatter metal tube.

c) Score the outside surface of the thin long metal tube with a metal file; this facilitates its adhesion to the short tube.
d) Mount the two metal tubes on putty as they would appear in final form.
e) Load a P20 (pipette tip) with superglue, and viewing through a microscope, glue the two metal pieces together. Superglue should be sucked up between the pieces by capillary action.
f) Repeat 2-3 times on each end to completely fill, making sure that glue is not bulging outward
g) Allow to dry in oven for 24 hr

12) Attach inlet to probe
a) Thread previously constructed inlets (see step 4) through exposed silica inlet. Advice: be sure to leave at least a few cm of exposed silica beyond the metal inlet. Silica must be cut to fit later.
b) Apply DEVCON 5-min epoxy around the end of the metal inlet to be connected.
c) With one set of hemostats grip the metal inlet and with your hands grip the medium Tygon tubing in which the metal inlet will enter.
d) Push together so that metal inlet is inserted partially into tubing. Caution: do not force the metal inlet into the tubing. This could bend the silica beyond its limits, causing it to break. This is an easy way to ruin a probe and make your life miserable.
e) Allow 15 min to dry.
f) Under a microscope, pipette a small amount of superglue to the very end of the metal inlet. Glue should only be applied between the exposed silica and the thin metal tube of the inlet. Caution: do not apply superglue beyond the tip of the thin metal tube. Superglue-covered silica does not cut in a flush manner.
g) Allow to dry for 15 min
h) Cut the extending silica piece very carefully so that it extends approximately 2 mm past the thin metal inlet. Use the same technique for cutting silica as in step 2. Advice: it is important that these cuts are clean. So give yourself some room for error if you happen to be forced to re-do the cut.
i) Slide a 1-3 cm piece of medium tygon tubing over the trimmed silica and metal inlet.

13) Cover outlet for later use
a) Cut the very tip off of a pipette tip with wire cutting pliers and fit it into a sample tube.
b) Thread the exposed outlet silica through the pipette tip so that it touches, but is not mashed against, the bottom of the sample tube.
c) Wrap the bottom of the pipette tip with tape to secure it from moving. The attached sample tube will serve as a collection point during dialysis.

14) Fix membrane
a) Remove guide cannula from connector.
b) Use tweezers with plastic tips to pick up sealed membranes (see step 3). Advice: pick up membranes close to their middle. If you pick up a membrane close to its end you may risk permanently closing it or compromising its ability to house the silica.
c) Carefully slide each piece of silica (inlet end first and outlet second) into the membrane.
i) Advice: use a second set of tweezers to hold the silica on the opposite side of the membrane to prevent it from moving or bowing while you are working.
ii) Advice: use a microscope to do this.
iii) Advice: moisten membrane and tweezers with water to expand membrane

15) Under the scope, adjust the membrane so that it is 4-5 r.u. from the glue that seals the tip of the membrane.
16) Under the scope, use a needle or toothpick to coat the silica and part of the membrane with DEVCON 2-ton epoxy. Coat of glue should be applied to the silica approximately 1 cm from the connector and up to, but NO FURTHER than, the outlet silica (the shorter piece of silica) within the membrane.
    a) Caution: coat of epoxy should completely surround the silica and membrane, but should only be thinly applied. Any bulging epoxy that is not removed may interfere with probe entry into the guide cannula *in vivo*.
17) Seal membranes: Spectra/Por (#132 28)
    a) Place a roll of scotch tape on a glass slide as a base for the membranes to rest upon.
    b) Use special scissors and tweezers with plastic tips when handling membranes so as not to damage them.
    c) Membrane should extend 3-4 mm past tape end.
    d) Use DEVCON 2-ton white epoxy to seal ends.
    e) Viewing through a scope, use a needle to plug membrane opening with epoxy.
    f) Dab the opening of membrane with a small amount of epoxy and pull back. Caution: epoxy is easily taken up by the membrane via capillary action. Plug should only be 4-5 r.u. in thickness from the tip of the membrane.
    g) Allow epoxy to dry 24 hrs in sealed container to prevent contamination.
18) Store finished probes in a sealed container and if possible away from people

Microdialysis Protocol

Day 1

1. Print microdialysis sheets
2. Mark down ID of all instruments
3. Put springe around ejection valve and tubing from swivel
4. Spray 70% EtOH on frame and connect sprayed probe to swivel
5. Get pump syringe and wash 2x with HPLC water test after making acsf
6. Fill with HPLC water, no bubbles
    a. Set flow to 2 ul/min and diameter to 4.61
7. Make acsf (add glucose and aa each day!) and filter
8. Check flow rate after 30 minutes
    a. 9.7 ul and up ok
9. Use 7ml acsf (wash 2x then fill tube with acsf)
10. Get bedding
11. Inject animals with saline to habituate
12. Implant probe
    a. Bring pump/frame, Diaper, anesthesia tube, iv tube filled with saline, kim wipe
    b. Cut tape so that you have 3 narrow 3 thicker pieces
    c. Weigh animal
    d. Put down using 4 percent anesthesia
    e. Infuse .4ml saline, use rest to clean out the canulla
    f. First put the spring on and screw down being sure that nothing touches the probe
    g. Then holding top of the probe attachment to the spring, do ½ turn every 30 seconds
    h. When it feels tight release connection and do one final turn
    i. Tape at all joints so that animal cannot split tube/spring
    j. Take note of when anesthesia turned off and when animal turns up
13. Turn flow rate down
14. TURN OFF OXYGEN
15. Injection 15 hours prior to start of experiment
    a. .4 mg/kg so for a 400 kg animal inject .4 mL

Day 2

1. Refill tube with ascf being careful to leave no bubbles in the line
2. Turn flow rate up and allow 2 hrs to equilibrate in brain
3. Check flow rate after 30 minutes
4. Habituation injection of saline asap (us EtOH mg/kg sheet to determine injection amount and weight then refill and reconnect line with ethanol solution)
5. Prepare dry ice and baggie and tubes to collect samples
6. Upon completion of ethanol infusion, remove tubing and recap cannula

Mobile Phase Recipe

Materials:
2L graduated cyclinder
500 mL graduated cylinder
Citric acid
Monosodium phosphate
EDTA
Sodium dodecyl phosphate
Acetonitrile
Methanol

1. Wash graduated cylinder 3x tap water 3x deionized water and 1x hplc water
2. In 1600 mL HPLC water measure the following:
   1.68 g citric acid (mw 210.15)
   27.6 g NaH2PO4 (mw 137.99)
   0.1461 g EDTA (mw 292.2)
   1.903 g CH3(CH2)11OSO3Na (mw 288.18)
3. Adjust buffer pH to 5.8 using NaOH (4g NaOH in 10 mL H20) adding ~ 4 mL to bring pH from 3.6-5.8
4. Add 350 mL acetonitrile to the final bottle using marked graduated cylinder
5. Add 120 mL methanol
6. Set up vacuum. Put stir bar in final bottle. Pour liquid. Turn gas on. Stir and degass for 10-15 minutes by placing wet paper towels between lid and plastic filter cup.

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