A Subset of VTA DA Neurons Demonstrates High Sensitivity to Acute Ethanol and Enhanced Sensitivity after Adolescent Drinking

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2016

ABSTRACT

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Ethanol (EtOH) is a commonly used drug which exerts many of its effects by altering neurotransmission in the mesolimbic dopamine (DA) system. Although there is little debate that EtOH acts to increase the activity of DA neurons in the ventral tegmental area (VTA), and that this action is necessary for some of the reinforcing effects of EtOH, research *in vitro* has only been able to demonstrate an excitatory effect on VTA DA neurons in response to very high concentrations of EtOH. These concentrations, typically in the range of 50-100 mM, correspond to sedative or lethal levels for typical humans. Therefore, the significance of findings from *in vitro* experiments can be difficult to interpret. We sought to determine why high concentrations of EtOH are needed *in vitro* and whether this could be explained by simple experimental factors, including cytosolic washout from whole cell electrophysiological recordings; heterogeneity among VTA DA neurons, where previous studies may have inadvertently focused on an EtOH-insensitive population; or selection of animal population, where perhaps low EtOH response is characteristic in naïve, rather than EtOH-experienced, animals. To achieve this, we performed cell-attached recordings on a large number of midbrain DA neurons of EtOH-naïve and experienced mice.

We report evidence for a highly EtOH-responsive, medially located population of VTA DA neurons. These neurons, found within the rostral linear and interfascicular nuclei and considered "atypical" in terms of physiological criteria ascribed to DA neurons, exhibited a concentration-dependent increase of firing activity in response to EtOH, with some neurons responsive to as little as 20 mM EtOH. In contrast, DA neurons in the lateral VTA and substantia nigra were either unresponsive or responded only to 100 mM EtOH.

We then examined neuronal activity following adolescent binge-like alcohol drinking in mice, to determine whether EtOH experience drives increased EtOH sensitivity of DA neurons. We find that in medial VTA DA neurons, drinking experience greatly increased firing activity driven by subsequent exposure to EtOH itself, without altering other measures of intrinsic excitability. This enhanced sensitivity was no longer significant in the presence of glutamate receptor blockade. We attempted to further characterize the EtOH-sensitive, medially located VTA DA neurons by utilizing retrograde tracing to identify a population of nucleus accumbens medial shell-projecting neurons. We find that this population exhibits an increased sensitivity to 50 mM EtOH after adolescent drinking.

As a result of these experiments, we have identified a previously uncharacterized, highly EtOH-responsive population of DA neurons in the medial VTA. This population demonstrates an excitatory response to 10 and 20 mM EtOH, concentrations which are more pharmacologically relevant than those typically tested *in vitro*. We further demonstrate evidence for experience-induced neural adaptations which result in enhanced sensitivity to EtOH *in vitro*. These adaptations are only apparent in medial VTA DA neurons, and this phenomenon only occurs in response to adolescent drinking. These data provide evidence for a novel form of plasticity in which neurons respond to a primary reinforcer, in this case EtOH, after drinking

experience. These findings provide an anatomical and pharmacological distinction between DA neuron subpopulations that will facilitate future mechanistic studies on the actions of EtOH in the VTA.

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of variance
AP5	(2*R*)-Amino-5-phosphonovaleric acid
AUD	Alcohol use disorder
BAC	Blood alcohol concentration
BLA	Basolateral amygdala
D_2R	Dopamine D_2 receptor
DA	Dopamine
DAPI	40, 6-diamidino-2-phenylindole
DAT	Dopamine transporter
DLS	Dorsolateral striatum
DSM	Diagnostic and Statistical Manual of Mental Disorders
EPSC	Excitatory postsynaptic current
EtOH	Ethanol
FF	Firing frequency

GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GIRK	G-protein gated, inwardly rectifying potassium channels
HCN	Hyperpolarization-activated, cyclic nucleotide-gated cation channels
HIC	Handling-induced convulsion
IA	Intermittent access
I_h	Hyperpolarization-activated inwardly rectifying non-specific cation current
IF	Interfasicular nucleus
i.p.	Intraperitoneal
ISI CV	Interspike interval coefficient of variation
LDTg	Laterodorsal tegmental nucleus
LTP	Long-term potentiation
MK801	(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine maleate
NAc	Nucleus accumbens
NBQX	2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NIH	National Institutes of Health
NK3R	Tachykinin receptor 3
NMDA	N-methyl-D-aspartate

mPFC	Medial prefrontal cortex
p	Postnatal day
PBP	Parabrachial pigmented nucleus
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PN	Paranigral nucleus
PPTg	Pedunculopontine tegmental nucleus
QP	Quinpirole
RLi	Rostral linear nucleus
RMP	Resting membrane potential
RMTg	Rostromedial tegmentum
SEM	Standard error of the mean
SK	Small-conductance Ca^{2+}-sensitive K^{+} channel
SN	Substantia nigra
SNc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
vGluT2	Vesicular glutamate transporter 2
VMAT2	Vesicular monoamine transporter 2

VTA Ventral tegmental area

VTAR Rostral part of the ventral tegmental area

ACKNOWLEDGEMENTS

There are many individuals whom I would like to acknowledge for their assistance during my years in graduate school. Firstly, I would like to thank my mentor, Neil Harrison, as well as my co-mentor, David Sulzer, for their encouragement and support. Under their guidance, I was able to learn several new techniques, establish an independent project, share my findings with national and international audiences, and obtain a fellowship award from the National Institute of Alcoholism and Alcohol Abuse. They have also fostered my enthusiasm for research in the alcohol field, which I will continue in my postdoctoral studies.

Members of the Harrison and Sulzer labs have helped me tremendously over the years. Ana Mrejeru, a former postdoc in the Sulzer lab, taught me the basics of electrophysiology. Anders Borgkvist of the Sulzer lab taught me how to do stereotactic injections and has helped me to troubleshoot various issues with the rig. Michael Salling, a postdoc in the Harrison lab, taught me the intermittent access procedure. In addition to their assistance on various techniques, Ana, Anders, and Mike have given me invaluable advice in countless discussions on life in science and have assisted me as I made decisions navigating a future in the alcohol research field.

I would also like to thank Candace Castagna and Vanessa Morales for their assistance in genotyping and maintaining the TH-GFP mouse colony. Several people have helped me to run drinking mice over the years, including Anastasia Barnett, Dan Lowes, and Mary Jane Skelly. Sejoon Choi and Eugene Mosharov have helped me with various troubleshooting problems on several occasions. I must also thank Ellen Kanter, the laboratory manager of the Sulzer lab, and Dawn LaSalle, the administrator of the Harrison lab, for keeping the labs functioning smoothly.

In the pharmacology department, I thank Karen Allis for her assistance and organization over the years. I would also like to acknowledge several faculty members at Columbia, including Rich Robinson, for meeting with me to discuss graduate school requirements. My thesis committee, consisting of Neil, Dave, Christoph Kellendonk, Joachim Scholz, and Steve Siegelbaum, provided advice on my project as it progressed. I thank my dissertation committee, including Neil, Dave, Christoph, Henry Colecraft, and Diana Martinez, for agreeing to critically review and discuss the work that I've performed over the years.

Outside of Columbia, I would like to thank my friends and family for sharing my journey to New York and seeing it through to the end. To my best friends in New Orleans, Ella, Izzy, and Callie, thanks for helping me to maintain a life outside of the lab. To my New Orleans-turned New York pal and confidant Elizabeth 2, thank you for being a friend! To my parents, Donice and Bowdre, thanks for your love and encouragement. To my siblings, Donice, Bowdre, and Chesley, thanks for looking out for your weird little sister.

Family is not limited to your blood relatives; your family is composed of the individuals you choose to share your life with. Finally, I would like to thank the newest member of my family, Martin Avegno. Over the last decade, I have shared some of the best moments of my life and traveled the world with Martin. We have also endured several hardships together, including Hurricanes Katrina and Gustav, the 2007, 2012, 2014 and 2015 Saints seasons, and various aspects of living in New York. Thank you for your companionship, love, and support. And thank you for choosing me.

To my best friend, Martin,

and our son, Tycho

CHAPTER 1

Introduction[1]

Alcohol is certainly the most ancient and arguably the most widely abused substance in human society. The costs associated with alcoholism and alcohol use disorders (AUDs) are enormous, particularly in terms of both economic performance from lost productivity and human suffering caused by alcohol-associated disease, death and family breakdown (Rehm et al., 2009). Ethanol (EtOH) is readily self-administered by most mammalian species and is easily studied in rodent models (Carnicella et al., 2014; Crabbe et al., 2011). Rodent models of self-administration can model some of the aspects of human AUDs, including escalated intake over time in a two bottle choice paradigm. Most, if not all, drugs of abuse have been shown to activate the mesolimbic reward system to enhance dopamine (DA) transmission in the striatum (Sulzer, 2011), albeit via distinctly different mechanisms. Drugs such as cocaine and amphetamine act in part within the striatum via their effects on the DA transporter of axon terminals. Opiates and benzodiazepines target the inhibitory interneurons in the ventral tegmental area (VTA) to disinhibit DA cell firing and indirectly promote DA release (Lüscher & Malenka, 2011; Tan et al., 2010). In contrast to these well-characterized drugs of abuse, the manner in which alcohol alters DA physiology remains unclear and controversial (Morikawa & Morrisett, 2010).

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[1] Part of this introduction was adapted from the published article below. I am grateful to Ana Mrejeru, Michael Salling, David Sulzer, and Neil Harrison for their contributions to the ideas, text, and figures of this chapter.
Mrejeru A, Martí-Prats L, Avegno EM, Harrison NL, Sulzer D (2015). A subset of ventral tegmental area dopamine neurons respond to acute ethanol. Neuroscience 290: 649-658.

Ethanol Use during Adolescence

AUDs represent a significant public health and economic burden (Bouchery et al., 2011; Rehm et al., 2009). The consequences of EtOH abuse on public health are profound for individual well-being and impact on family structure, and in terms of lost productivity and associated health care expenses (Harwood, 2000). The neuroadaptations that occur during social drinking, which ultimately lead to development of AUDs, are not entirely understood.

Late adolescence and early adulthood comprise a particularly important period of consideration, as epidemiological research points to adolescence as the critical period in the development of AUDs (Grant et al., 2001; Schuckit, 1998). Exposure of the nervous system of adolescents and young adults to alcohol initiates a process of neuroadaptation that can exacerbate heavy drinking even in the face of learned negative consequences, and increases the probability of AUDs during adulthood (Dawson et al., 2006). The prevalence of current drinking tends to increase during this time, reaching a peak around ages 25 and 26, while episodic heavy drinking tends to peak between ages 21 and 24 (Chen et al., 2005, Johnston et al, 2014).

In some individuals, drinking during this time is associated with risky behavior and detrimental effects (Hingson et al., 2006), as well as an increased potential to develop AUDs (Figure 1.1; Grant et al., 2001). Those who develop AUDs during this time frame are also less likely to seek treatment and more likely to display a greater severity of dependence-related problems, including multiple episodes of alcohol dependence and episodes of longer duration (Li et al., 2004). Research aimed towards understanding the effects of alcohol on the brain during this time is therefore important. In mice, postnatal day 28 (p28) – p60 is considered to correspond to human adolescence (Laviola et al., 2003; Spear, 2000), with early adulthood comprising ~p60-p76 (Moore et al., 2010). Focusing on the neuroadaptations that occur during

this critical period has the potential to elucidate the neural mechanisms that underlie the transition from moderate drinking to alcohol dependence.

Ethanol's Actions on Mesocorticolimbic Circuitry

EtOH exerts many of its effects by altering neurotransmission in mesocorticolimbic circuitry (Figure 1.2). Like many drugs of abuse, EtOH causes an increase in DA transmission in the nucleus accumbens (NAc), an area strongly implicated with reward and reinforcement (for review, see Di Chiara et al., 2004). Whether this increase in local DA concentration is caused by increasing the activity of DA neurons, as with opiates; preventing DA reuptake, as with cocaine; or via another mechanism, is unknown (Sulzer, 2011). This DA release is considered to be an integral part of EtOH's actions; studies have demonstrated that lesioning DA terminals in the NAc or blocking DA receptors suppresses voluntary EtOH consumption in rodents (Dyr et al., 1993; Ikemoto et al., 1997).

The VTA is a source of dopaminergic neurons that send projections to the NAc and elsewhere throughout the brain. These neurons are implicated in EtOH reinforcement; studies have demonstrated that animals will self-administer EtOH directly into the VTA and that silencing of DA neurons by application of quinpirole, a D_2R agonist, blocks EtOH self-administration (Gatto et al., 1994; Rodd et al., 2004). Previous research has shown that EtOH increases the activity of DA neurons in the VTA, which is associated with reward and addiction (Brodie et al., 1990; Gessa et al., 1985).

Regulation of Spontaneous Dopamine Neuron Activity in the VTA

DA neurons display pacemaking activity, with intrinsic excitability influenced by various ion channels. Regulators of DA neuron activity that have been proposed as molecular targets of

EtOH include L-type voltage-gated Ca^{2+} channels, small-conductance Ca^{2+}-sensitive K^+ (SK) channels, hyperpolarization-activated, cyclic nucleotide-gated cation (HCN) channels, and A-type K^+ channels (Morikawa & Morrisett, 2010). In addition, these neurons are modulated by both inhibitory and excitatory synaptic inputs.

GABAergic neurons exert an inhibitory influence on spontaneous DA neuron activity. GABAergic inputs originate locally within the VTA (Omelchenko & Sesack, 2009) and in separate brain regions, including the NAc (Walaas & Fonnum, 1980), ventral pallidum (Kalivas et al., 1993), substantia nigra (SN; Saitoh et al., 2004), and rostromedial tegmental nucleus (RMTg; Jhou et al., 2009). EtOH has been proposed to decrease inhibitory transmission onto VTA DA neurons via actions on $GABA_A$ receptors (Xiao & Ye, 2008), although other groups have demonstrated that EtOH can enhance $GABA_B$-mediated transmission onto DA neurons (Federici et al., 2009).

DA neurons receive extensive excitatory glutamatergic input from diverse brain regions, including the prefrontal cortex (PFC; Carr & Sesack, 2000; Geisler et al., 2007), pedunculopontine nucleus (Charara et al., 1996), laterodorsal tegmentum (Clements et al., 1991), and bed nucleus of the stria terminalis (Georges & Aston-Jones, 2002). Excitatory input also stems from local glutamatergic neurons (Dobi et al., 2010), which can influence DA neuron excitability. Acute EtOH has been shown to increase local glutamatergic levels in the midbrain (Ding et al., 2012) and increase AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) onto VTA DA neurons (Xiao et al., 2009).

Properties of Dopamine Neurons

"Conventional" DA neurons, such as those found in the SN, have been classified by several properties *in vitro*, including spontaneous firing rate (1-5 Hz), action potential duration (2-3 ms), hyperpolarization in response to quinpirole (a D_2 receptor agonist), and I_h (hyperpolarization-activated inwardly rectifying non-specific cation current) magnitude. The expression of tyrosine hydroxylase (TH), the enzyme which catalyzes the rate-limiting step in dopamine synthesis, is suggested to be the gold-standard in identifying DA neurons in the VTA (Margolis et al., 2010). Studies have demonstrated that VTA DA neurons cannot all be classified based on canonical DA neuron properties. For example, not all TH+ cells are sensitive to quinpirole, and even some TH- cells are sensitive to the drug. Additionally, not all TH+ cells display a prominent I_h, and some TH- cells are I_h+ (Margolis et al., 2008b). VTA DA neurons have also been shown to have variable firing ranges, from 3-15 Hz (Lammel et al., 2008). Researchers have therefore sought additional ways to classify VTA DA neurons.

Use of Retrograde Tracers to Identify Subpopulations of VTA Neurons

There is considerable heterogeneity among DA neurons in the VTA (Lammel et al., 2008; Margolis et al., 2010; Margolis et al., 2008b; Mrejeru et al., 2015). Not surprisingly, these neurons show varied responses to EtOH. VTA neurons have been subdivided based on their projection target, and these have shown differential responses to other drugs of abuse, including cocaine (Lammel et al., 2011).

Identification of neurons based on their projection target has been achieved by the use of fluorescently labeled retrobeads, which are taken up by nerve terminals and retrogradedly transported back to the cell body. Researchers have used this method to classify VTA neurons

based on their projection targets, which include the medial prefrontal cortex (mPFC), amygdala, NAc core, and NAc shell (Lammel et al., 2008). In the study performed by Lammel and colleagues, it was shown that NAc lateral shell-projecting VTA neurons closely resembled dorsolateral striatum (DLS)-projecting SN neurons, with all other VTA cells studied (projecting to the mPFC, NAc core, NAc medial shell, and basolateral amygdala [BLA]) displaying varied molecular and functional properties. These include firing properties, size, and expression of DAT, the dopamine transporter; VMAT2, the vesicular monoamine transporter; and calbindin, a calcium binding protein.

Other studies have demonstrated a differential response to rewarding or aversive stimuli based on projection target (Lammel et al., 2011; Lammel et al., 2012). In the former study, Lammel and colleagues demonstrated that cocaine administration in mice resulted in modification of excitatory synapses on VTA neurons projecting to the NAc medial shell (expressed as an increased AMPA/NMDA ratio), while a formalin injection (modeling an aversive experience) strengthened the AMPA/NMDA ratio of VTA neurons projecting to the mPFC. Neurons projecting to the NAc lateral shell displayed a slight increase in AMPA/NMDA ratio in response to both rewarding and aversive stimuli. The group later showed that selective activation of VTA neurons projecting to the NAc shell elicits conditioned place preference in mice, while activation of VTA neurons projecting to the mPFC can cause conditioned place aversion (Lammel et al., 2012). The data presented in Chapters 2 and 3 provide evidence for regional heterogeneity within the VTA in response to acute and repeated EtOH exposure. It is likely, then, that a subset of neurons with a shared projection target display a similarly high sensitivity to EtOH, as has been demonstrated with cocaine and other stimuli.

Self-Administration of Drugs of Abuse

There is a consensus that drug self-administration is primarily driven by drug induced synaptic plasticity in excitatory pathways that impinge on the reward circuitry of the mesolimbic system (Gipson et al., 2014; Lüscher & Malenka, 2011). For EtOH, this has been suggested to occur via enhanced excitatory drive to VTA DA neurons and the expression of long-term potentiation (LTP; Stuber et al., 2008a; Wanat et al., 2009). Many studies in this field, however, have used passive rather than self-administration of EtOH to examine synaptic plasticity. Few studies have examined the relationship between voluntary drinking history or chronic alcohol exposure and DA neuron sensitivity. Studies on the effects of passive chronic i.p. administration of EtOH have generated conflicting results (Brodie, 2002; Didone et al., 2014; Okamoto et al., 2006).

Chronic administration of drugs of abuse has been shown to cause stable changes in neurons (for review, see Lüscher & Malenka, 2011). For example, repeated administration of cocaine, morphine, or EtOH has been shown to result in increased expression of TH in VTA DA neurons, as well as GluR1 and NR1, subunits of AMPA and NMDA receptors, respectively; all of which result in increased activity in these neurons (Fitzgerald et al., 1996; Ortiz et al., 1995). A comparison of the firing rate of cells from alcohol naïve mice to cells from mice chronically exposed to EtOH may reveal the neuronal adaptations that occur after repeated alcohol use.

The Mouse as a Model of Human Behavior

Studies of complex cellular behavior and neural circuits can be difficult in the human, given the limitations of current experimental techniques, such as computerized tomography,

magnetic resonance imaging, positron emission tomography, or electroencephalography (Carter & Shieh, 2010). Human studies can provide valuable information about the brain's architecture, including regions of the brain activated during a certain task and connectivity between regions. Research in non-humans, particularly rodents, affords the use of techniques such as single-cell recordings, retrograde and anterograde tracing, and genetic manipulations, which are invaluable in understanding neural components underlying behavior. While limitations certainly exist, and data from rodent studies do not always extend to human findings (discussed further in the Discussion section of this thesis), rodent studies have proven valuable in understanding the basis of neurological conditions and developing therapeutic treatments to human disorders (e.g., Baselga et al., 1998; Kawashima et al., 1994; Suh et al., 2014).

The mouse as a model for understanding physiology has been accepted as early as the turn of the twentieth century; by 1929, recognition of this model's potential led to the establishment of Jackson Laboratory to aid scientific research (Cryan & Holmes, 2005). Using the mouse offers practical and economic advantages to the researcher, including a relatively low cost of housing and ease of breeding, as mice have a short gestational period (21 days or fewer) and a litter size of 5-6 mice on average (Jackson Laboratory, 2009). More recent advances in genetic manipulation have made the mouse an especially attractive model, as it is particularly amenable to modifications such as gene knock-in or knock-out (O'Sullivan et al., 2006).

The mouse shares the key components of the mesolimbic system with the human (Figure 1.2), and structures involved in EtOH response are common between the two species. For example, EtOH has been shown to cause an increase in DA concentration in the ventral striatum of humans using positron emission tomography imaging (Boileau et al., 2003); similar increases have been observed in rodents, including mice, using microdialysis (Doyon et al., 2004;

Gonzales et al., 2004). Evidence suggests that EtOH exposure can be reinforcing to rodents, as rodents will voluntarily self-administer EtOH (Stuber et al., 2008a), and EtOH experience can induce strong conditioned place preference (Melis et al., 2007; Shimizu et al., 2015). Rodents can also develop characteristics of dependence and tolerance, similar to humans (Tabakoff & Hoffman, 2000). Thus, despite limitations of the mouse as a model of human physiology, this species can prove useful in investigating the effects of EtOH on mesolimbic circuitry.

Hypotheses of this Study

The purpose of the studies detailed in this text was to test the hypotheses that (1) an understudied, highly EtOH-sensitive population of DA neurons exists within the VTA, and (2) voluntary EtOH intake during adolescence results in an increased sensitivity of a subset of VTA DA neurons to EtOH. To test these, we performed a large survey of midbrain DA neuron response to EtOH using cell-attached patch clamp recordings.

Chapter 2 details the results obtained in EtOH-naïve animals, where we find evidence for regional heterogeneity within the VTA in response to acute EtOH. Chapter 3 follows up by repeating these experiments after EtOH self-administration, demonstrating an enhanced sensitivity to EtOH in a subset of VTA DA neurons after adolescent drinking. Chapter 4 attempts to further characterize this EtOH-responsive population in the VTA by determining whether medial VTA DA neurons projecting to the NAc medial shell demonstrate a uniformly high response to EtOH *in vitro*. Together, these data provide novel findings of significant cellular response to pharmacologically relevant concentrations of EtOH *in vitro* by focusing on an understudied population of VTA DA neurons in transgenic mice.

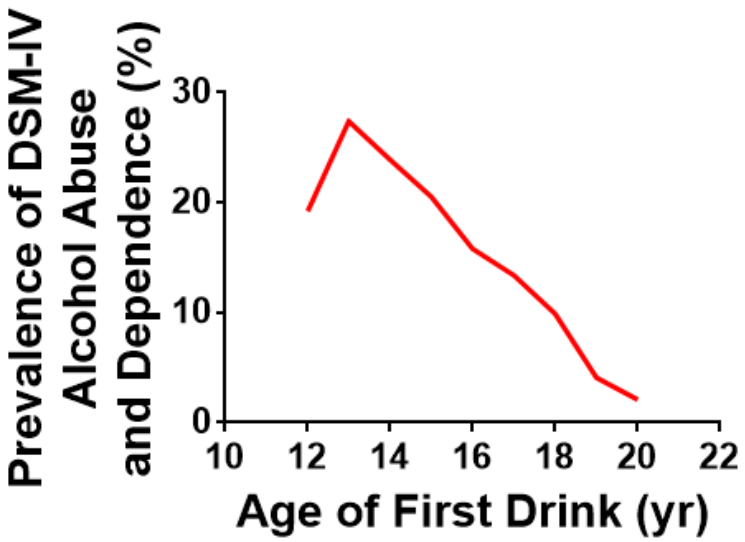


Figure 1.1: **Age of First Drink is Associated with Prevalence of Alcohol Dependence**. Data taken from 1994 study that assessed risk factors associated with prevalence of alcohol abuse and dependence in individuals. "Age of first drink" corresponds to self-reported age at which respondents drank two or more drinks in a week. Prevalence of alcohol use and dependence decreased with increased age of first drink. Adapted from Grant et al., 2001.

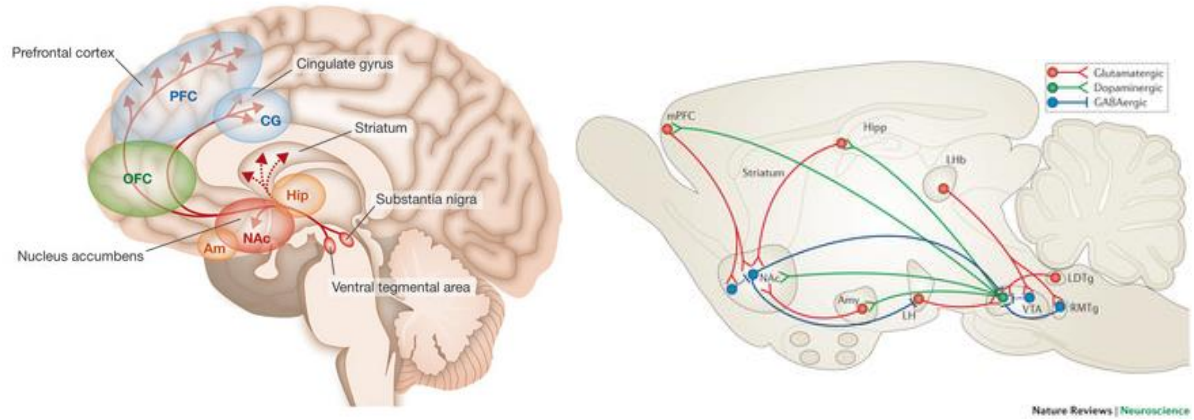


Figure 1.2: **Overview of the Mesolimbic System**. Left, diagram of human brain (reproduced from Lee et al., 2012). This system comprises regions of the brain involved in reward prediction (red), learning and memory (orange), motivation, drive, and salience evaluation (green), and cognitive control (blue). Right, major connections of rodent brain (reproduced from Russo & Nestler, 2013). Inhibitory GABAergic projections are shown in blue; excitatory glutamatergic projections shown in red; dopaminergic projections shown in green. Amy, amygdala; CG, cingulate gyrus; Hip/hipp, hippocampus; LHb, lateral habenula, LH, lateral hypothalamus; LDTg, lateral dorsal tegmentum; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex; NAc, nucleus accumbens, RMTg, rostromedial tegmentum; VTA, ventral tegmental area.

CHAPTER 2

A Subset of Ventral Tegmental Area Dopamine Neurons Responds to Acute Ethanol[2]

Abstract

The mechanisms by which alcohol drinking promotes addiction in humans and self-administration in rodents remain obscure, but it is well known that alcohol can enhance DA neurotransmission from neurons of the VTA and increase DA levels within the nucleus accumbens and prefrontal cortex. We recorded from identified DA neuronal cell bodies within ventral midbrain slices prepared from a transgenic mouse line (TH-GFP) using long-term stable extracellular recordings in a variety of locations and carefully mapped the responses to applied EtOH. We identified a subset of DA neurons in the medial VTA located within the rostral linear and interfascicular nuclei that fired spontaneously and exhibited a concentration-dependent increase of firing frequency in response to EtOH, with some neurons responsive to as little as 20 mM EtOH. Many of these medial VTA DA neurons were also insensitive to the D_2 receptor agonist quinpirole. In contrast, DA neurons in the lateral VTA (located within the parabrachial pigmented and paranigral nuclei) were either unresponsive or responded only to 100 mM EtOH. Typically, these lateral VTA DA cells had very slow firing rates, and all exhibited inhibition by quinpirole via D_2 "autoreceptors". VTA non-DA neurons did not show any significant response

[2] This chapter is adapted from a manuscript published in 2015. I am grateful to Ana Mrejeru, Lucia Martí-Prats, David Sulzer, and Neil Harrison for their contributions to the ideas, text, and figures of this chapter. The data presented in this chapter was collected by Ana Mrejeru, Lucia Martí-Prats, and Elizabeth Avegno; Figures were prepared by Ana Mrejeru.
Mrejeru A, Martí-Prats L, Avegno EM, Harrison NL, Sulzer D (2015). A subset of ventral tegmental area dopamine neurons respond to acute ethanol. *Neuroscience* **290**: 649-658.

to low levels of EtOH. These findings are consistent with evidence for heterogeneity among midbrain DA neurons and provide an anatomical and pharmacological distinction between DA neuron subpopulations that will facilitate future mechanistic studies on the actions of EtOH in the VTA.

Introduction

There have been many previous studies of alcohol action in VTA *in vitro*, many of which were quite elegant in their methodology. As a result, numerous synaptic and intrinsic targets for EtOH have been suggested in recent years (Brodie et al., 1990; Nimitvilai et al., 2013; Okamoto et al., 2006). The significance of some of these findings has been difficult to interpret because of the universal observation that very high concentrations of EtOH application have been required to observe responses. Typically, 50-100 mM EtOH has been used, a level of the drug that would result in unconsciousness or death in naïve animals, although tolerated in some alcoholics. The question therefore remains as to how the VTA responds to modest levels of EtOH that are typically associated with social intoxication. Indeed, low and moderate concentrations of EtOH (equivalent to several glasses of wine in humans) have been little studied since the early days of the field (Gessa et al., 1985), yet there is no question that these provide more accurate models of early-stage drinking.

It has been suggested by some authors that these difficulties have been amplified by other problems, such as ambiguous identification of DA neurons in VTA (Margolis et al., 2006; Ungless & Grace, 2012). Microdialysis studies in freely-moving rats have demonstrated that low doses of EtOH injection (0.5 g/kg i.p.) preferentially stimulated DA release in the nucleus accumbens (Di Chiara & Imperato, 1985). Furthermore, precise mapping of DA release sites using cyclic voltammetry *in vivo* after acute EtOH injection (0.5 g/kg injected intravenously) revealed the existence of "hot spots" of EtOH-responsive regions in NAc core and shell, as well as clearly unresponsive regions nearby (Robinson et al., 2009). The anatomical basis for the heterogeneity of these EtOH responses is unknown, but a reasonable supposition is that this may originate in the cell bodies in the VTA.

Considerable attention has recently focused on the concept of regional heterogeneity of VTA cells, which had formerly been lumped together as a homogenous population of DA neurons bearing considerable similarity to the neighboring population in the SN pars compacta (SNc; Bjorklund & Dunnett, 2007; Borgkvist et al., 2011; Lammel et al., 2008; Lammel et al., 2014; Marinelli & McCutcheon, 2014; Neuhoff et al., 2002; Ungless et al., 2004). Unlike the nigral DA neurons, however, the identification of DA neurons in VTA by physiological criteria or pharmacology alone appears to be insufficient (Margolis et al., 2006). It is now generally accepted that verification of TH expression is necessary to confirm DA identity (Fields et al., 2007; Ungless & Grace, 2012).

VTA neurons appear to exhibit regional differences in responses to other drugs of abuse, including opioids (Ford et al., 2006; Margolis et al., 2008b), nicotine (Ericson et al., 2008; Zhao-Shea et al., 2011), and cocaine (Lammel et al., 2011). Retrograde labeling studies have demonstrated that midline VTA DA neurons are most sensitive to cocaine, and that the axons of these cells project to the medial shell of NAc and prefrontal cortex (Lammel et al., 2011). It has been suggested that responsiveness to EtOH may also exhibit regional differences (Robinson et al., 2009). Here we undertook a simple but careful study of the alcohol responses of a large sample of 81 DA neurons in the mouse VTA, in an attempt to locate the cells that might be most sensitive to EtOH, in order to facilitate future characterization of target molecules. We selected a technologically simple, yet highly stable recording technique ("loose-patch" recording of action potentials) that obviates any problems associated with cytoplasmic dialysis during long recordings (Carta et al., 2004). Midbrain slices were prepared from a transgenic mouse line (TH-GFP) expressing green fluorescent protein under the TH promoter (Sawamoto et al., 2001)

in order to facilitate the identification of the DA neuron phenotype. Our results show that EtOH can accelerate DA neuron firing of a subpopulation of medial VTA DA neurons.

Materials and Methods

All animal procedures were performed following NIH guidelines, and were approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center. Wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bay Harbor, MA, USA). Wild-type and TH-GFP mice, in which neuronal GFP expression showed >87% co-localization with TH immunoreactivity (Sawamoto et al., 2001), were sacrificed at 3-12 weeks of age, and their brains removed for acute slice recordings.

Electrophysiological Recordings

Coronal midbrain slices (250 µm-thick) were prepared using a vibratome (Leica VT1200; Nussloch, Germany) with VTA between bregma -3.0 to 3.8 mm (primarily near bregma -3.5 mm). Brains were submerged in ice-cold cutting solution containing (in mM): 100 glucose, 75 NaCl, 26 $NaHCO_3$, 2.5 KCl, 2 $MgCl_2$-6H20, 1.25 NaH_2PO_4-$6H_2$0, and 0.7 $CaCl_2$. Slices were allowed to recover in the solution for 30 min at 34°C and then transferred to a recording solution (artificial cerebrospinal fluid, ACSF) containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 2.4 $CaCl_2$, 1.8 KCl, 1.2 $MgCl_2$-$6H_2$0, 1.0 NaH_2PO_4-$6H_2$0, and 10 glucose. The recording chamber temperature was maintained at 32°C (± 2°C) with an in-line heater and temperature controller (Warner Instruments, Hamden, CT, USA). Extracellular "on-cell" recordings were obtained with pipettes (tip resistance 2-4 MΩ) pulled from borosilicate glass (G150F-4, Warner Instruments) on a P-97 Flaming-Brown micropipette puller (Sutter Instruments) and filled with ACSF solution. Seal resistances ranged from 10 MΩ to 1 GΩ, but most recordings were between 10 and 30 MΩ and were monitored in voltage-clamp mode at a command potential of 0 mV throughout the recordings.

In a subset of experiments, whole-cell patch clamp recordings were performed with pipettes (tip resistance 3–4 MΩ) filled with internal solution containing (in mM):
115 K-gluconate, 20 KCl, 10 HEPES, 2 $MgCl_2$, 2 ATP-Mg, 2 ATP-Na_2, 0.3 GTP-Na, (pH = 7.3, 290 mOsm). DA neurons expressing GFP were visualized under a 40X water immersion objective by fluorescence and DIC optics (Olympus, Waltham, MA, USA). Voltage-clamp and whole cell current-clamp recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitized at 10 kHz with a Digidata 1332 (Molecular Devices). Data were acquired using Clampex 8 software (Molecular Devices). For whole-cell patch-clamp recordings, the resting membrane potential (RMP), spontaneous firing frequency (FF), and input resistance (measured by 100 pA, 100 ms duration hyperpolarizing pulses) were monitored throughout the recording. Only cells in which a baseline FF was stable for at least 5 min were retained and analyzed for tonic firing. The coefficient of variation (CV) of interspike intervals (ISIs) was calculated as CV = (SD of ISIs)/mean ISI. The resting RMP in these neurons was arbitrarily measured at the trough of the after-hyperpolarization. All drugs were purchased from Sigma Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Minneapolis, MN, USA) unless otherwise specified.

Imaging of TH-GFP Expression

Acute coronal slices (prepared as above) were placed in the bath chamber of a multiphoton microscope and continuously perfused with ACSF bubbled with carbogen. VTA cells were visualized at >30 µm depth in the slice under a 10X objective using a Prairie Ultima Multiphoton Microscopy System (Prairie Technologies, Middleton, WI, USA). TH-GFP was excited at 900 nm with a Mai Tai laser and visualized using an emission range of 440-500 nm. Images were acquired with Prairie v 4.0 software scanning 10 µm steps through a z-stack of

200 µm, and image stacks were flattened using Image J software and tiled in Photoshop CS3 (Adobe).

For Fig. 2.1B, acute brain slices (150 µm-thick) were cut on a vibratome as described above and stained with 40, 6-diamidino-2-phenylindole (DAPI) (Calbiochem, Billerica, MA, USA) diluted at 1:5000 in phosphate buffered saline (PBS) (Sigma) for 5 minutes. Slices were mounted on glass slides for imaging with a confocal microscope (Nikon Eclipse Ti, Tokyo, Japan) under 10X and 20X objective lenses. Images were acquired with NIS Elements software.

Statistical Analysis

Statistical analysis was performed in Prism 6 (GraphPad Software, La Jolla, CA, USA). The distribution of firing frequencies did not always pass the Kolmogorov-Smirnov normality test, and a non-parametric Mann-Whitney test was used to compare spontaneous FF distributions (in Fig. 2.2). A non-parametric Mann-Whitney test was used to compare control recordings with EtOH-treated recordings as percentage change in FF above baseline (mean ± SEM). Correlation coefficients were calculated by a Pearson analysis. Significance level was $p < 0.05$.

Results

Spontaneous Firing Pattern Properties of VTA DA and Non-DA Neurons

Electrical extracellular recordings were made from 81 VTA DA neurons identified by TH-GFP fluorescence. To obtain an unbiased representation of all VTA neurons, the entire VTA region was sampled, as shown in Fig. 2.1. We recorded from "medial VTA" neurons within 250 μm of the midline, including the rostral linear nucleus (RLi) and interfascicular (IF) nucleus (red outline in Fig. 2.1C; Supplementary Figure 1), and from the "lateral VTA" consisting of the parabrachial pigmented (PBP) nucleus, paranigral nucleus (PN), and rostral part of VTA (VTAR) (blue outline in Fig. 2.1C; Supplementary Figure 1).

DA neurons in the VTA were spontaneously active, with a mean FF of 2.3 ± 0.2 Hz (n = 81), and the measure of firing regularity (expressed as the CV in inter-spike intervals) was 0.28. DA neurons of medial VTA showed spontaneous firing rates similar to lateral VTA and SN DA cells. As shown in Fig. 2.2A, medial VTA DA neurons fired at mean FF of 2.8 ± 0.3 Hz (n = 33) versus lateral VTA DA neurons that fired at 2.2 ± 0.3 Hz (n = 21). SN DA neurons fired at a mean frequency of 2.0 ± 0.2 Hz (n = 19). The FF of these three populations of DA neurons was not statistically different from each other, as FF was somewhat heterogeneous even within these subgroups ($p > 0.05$, Mann-Whitney test). In contrast, VTA non-DA neurons fired at a mean frequency of 6.9 ± 0.4 Hz (n = 15) with a CV of ISI = 0.23 ± 0.07. The FF of non-DA neurons was significantly faster than all DA neuron groups ($p < 0.0001$). Consistent with previous *in vitro* studies (Margolis et al., 2006; Margolis et al., 2012; Neuhoff et al., 2002), the midline VTA DA neurons (CV of ISI = 0.30 ± 0.04, n = 32) and lateral VTA DA neurons (CV of ISI = 0.28 ± 0.11, n = 21) exhibited significantly more variability in FF than the SNc DA neurons (CV of ISI = 0.12 ± 0.02, n = 18; $p < 0.0001$, Mann-Whitney test), as shown in Fig.

2.2B. Thus, VTA neurons appear to have unique spontaneous firing properties distinct from nigral DA neurons.

To determine whether DA neuron firing frequencies were stable over long duration extracellular recording conditions, we recorded from a subset of VTA DA neurons for 30 min during saline (ACSF) application. The firing rates of these TH-GFP-positive neurons were measured at the time points 13, 22, and 30 min after baseline (Fig. 2.3A). A representative recording of stable firing rate is shown in Fig. 2.3B, with a baseline FF of 4.5 Hz that showed a maximal 12% increase after 22 min. For the entire VTA DA population, we observed a mean increase in FF of 13 ± 9%, 18 ± 10%, and 23 ± 18%, respectively, after 13, 22, and 30 min (n = 27), as shown in Fig. 2.3C, D. These firing rates were remarkably stable, and any changes observed were not statistically significant ($p > 0.05$).

A Subset of Identified Midbrain DA Neurons Responds to EtOH

Responses to acute EtOH (bath-applied) were measured in VTA DA neurons, and some of these neurons exhibited a concentration-dependent increase in FF. Fig. 2.4A shows a representative recording from a medial VTA neuron in the IF nucleus (TH-GFP+, quinpirole-sensitive cell) that displayed a concentration-dependent increase of firing in response to EtOH. Spontaneous FF of 3.3 Hz increased by 21%, 41%, and 62% with increasing [EtOH] (20, 50, and 100 mM), respectively (Fig. 2.4B, C). These changes were reversible upon washout of EtOH. Other DA neurons showed even more robust changes with EtOH, showing an increase of FF by 200%, 329%, and 299% at 20, 50, and 100 mM concentrations, respectively (Fig. 2.4D). For the entire population of VTA DA neurons, the mean increase in FF was 31 ± 9% at 20 mM (n = 38), 55 ± 13% at 50 mM (n = 35), and 63 ± 14% at 100 mM EtOH (n = 46; Fig. 2.5A, B). All three concentrations of EtOH were significant compared to control recordings in Fig. 2.3 (at 20 mM

EtOH, $p < 0.05$; at 50 and 100 mM EtOH $p < 0.01$; Mann-Whitney test). There was considerable variability in the responses of VTA DA cells, and half of the cells showed less than 40% change in FF at 100 mM EtOH. Several DA neurons showed a robust increase of firing rate in EtOH (maximum: 200% increase at 20 mM, and 415% increase at 100 mM) as illustrated by Fig. 2.4D, while others showed a reduced firing rate (minimum: 35% decrease at 100 mM). It thus appeared that some DA neurons in naïve mice are uniquely sensitive to acute EtOH at low concentrations, while many DA neurons in VTA do not react at all to EtOH.

Reversal of EtOH effects on DA neurons occurred after 15-30 min in 18 out of 25 washout recordings, with FF returning to within 30% of baseline. Complete washout of EtOH was not possible for all long-duration recordings (60 min) due to instability of seal resistance. EtOH is a highly membrane-permeable substance and is known to equilibrate fully and rapidly across biological cell membranes, and so does not create an osmotic gradient. We nonetheless tested whether an increase in extracellular osmolarity might alter firing rates of DA neurons. The osmolarity of our ACSF was measured as 295 mOsm (0 mM EtOH), 316 mOsm with 20 mM EtOH added, 346 mOsm with 50 mM EtOH added, and 394 mOsm with 100 mM EtOH added (Fiske osmometer, Norwood, MA, USA). In a series of control experiments, we added sucrose to ACSF at concentrations of 12, 48, and 94 mM, respectively, corresponding to the same osmotic equivalent as 20, 50, and 100 mM EtOH. DA neuron firing rates showed a mean change in FF of 0.7 ± 7.0% (n = 8) with 48 mM sucrose (346 mOsm), and a 25 ± 19% decrease in FF at 94 mM sucrose (n = 2). Firing rates were thus unchanged or slower when sucrose was added. We conclude that the FF increases observed in VTA in response to EtOH were not due to changes in osmolarity during the experiment.

We measured the FF of DA neurons by loose-patch extracellular recordings, using both the voltage-clamp (command voltage = 0 mV) and current-clamp (holding current = 0 pA) configurations to compare baseline FF and EtOH responses, and determined that recording mode did not alter these parameters ($p > 0.05$, n = 66 voltage-clamp; n = 25 current-clamp). In addition, to further determine whether recording configuration has any impact on FF of DA neurons, we performed whole-cell current-clamp recordings from midbrain DA neurons. The RMP of VTA DA neurons was -54 ± 3 mV (n = 7) and RMP for SNc DA cells was -56 ± 2 mV (n = 11). VTA neurons showed spontaneous firing with a mean FF of 2.6 ± 0.5 Hz (n = 6), and SNc neurons had mean FF of 2.0 ± 0.3 Hz (n = 10). These values were similar to our extracellular recordings (e.g., voltage-clamped at a holding potential of 0 mV) from Fig. 2.2, and suggest that resting membrane properties were unaltered by the recording configuration. We measured the amplitude of the "sag" in the electrotonic potential elicited by a hyperpolarizing current injection. VTA neurons recorded in lateral and medial regions had considerable variability in sag amplitudes during a 200 pA injection, with a mean of 15 ± 5 mV, as compared to SNc neurons with mean of 20 ± 2 mV. As noted by others, the VTA shows greater heterogeneity in resting and active membrane properties than the SNc, and this consideration may be fundamental in understanding how EtOH affects these DA neuron regions.

Based on studies suggesting that VTA DA neurons with elevated basal excitability have stronger responses to cocaine (Lammel et al., 2011), we tested whether the baseline FF of DA neurons were correlated with EtOH responses. Baseline FF of VTA DA cells was plotted versus changes in FF with EtOH at 20 mM (Pearson r = 0.25), 50 mM (Pearson r = 0.26), and 100 mM (Pearson r = 0.20). There was no significant correlation between FF and EtOH response. This

suggests that the mechanisms that regulate spontaneous firing rates may be distinct from those mediating EtOH responsiveness.

VTA Non-DA Neurons Were Unresponsive to Low Doses of EtOH

We then tested the response of VTA non-DA neurons to acute EtOH *in vitro*. These non-DA neurons (putative GABA) were identified by lack of TH-GFP under fluorescence in TH-GFP mice, in which GFP expression had >87% co-localization with TH immunoreactivity (Sawamoto et al., 2001). Since some GFP-negative neurons may be dopaminergic, additional criteria for identification included: (a) lack of response to quinpirole (1 µM, bath), and (b) spontaneous FF > 4 Hz. Extracellular recordings were obtained from non-DA neurons, and we tested EtOH responses. Baseline spontaneous FF of non-DA neurons in VTA was 6.9 ± 0.4 Hz (n = 15). Non-DA neurons exhibited small changes in FF, with a mean increase in FF of 12 ± 13% at 20 mM (n = 11), 12 ± 10% at 50 mM (n = 11), and 32 ± 13% at 100 mM EtOH (n = 15), as shown in Fig. 2.5C, D. These changes were not significantly different from controls ($p > 0.05$). Thus, unlike the DA neurons in VTA, VTA non-DA neurons were not sensitive to low amounts of EtOH and did not exhibit a concentration-dependent response.

Medial and Lateral VTA DA Neurons Differ in Sensitivity to EtOH

We postulated that the VTA DA neurons most responsive to EtOH may be located at the midline, analogous to the observations made with cocaine (Lammel et al., 2011). To test this hypothesis, we recorded cells near the midline and tested response to three concentrations of EtOH. Medial VTA neurons were located in the RLi and IF nuclei, within 250 µM of the midline, as outlined in Fig. 2.1B. The mean increase in FF was 34 ± 13% at 20 mM ($p < 0.05$), 73 ± 22% at 50 mM ($p < 0.01$), and 78 ± 22% at 100 mM EtOH ($p < 0.05$, n = 19), as

summarized in Fig. 2.6A. All concentrations of EtOH significantly increased firing rates compared to medial VTA control neurons with no EtOH added. Of the midline DA neurons recorded, 37% (n = 7 of 19) showed >40% change in FF at 20 mM EtOH, 53% (n = 10 of 19) responded at 50 mM, and 58% (n = 11 of 19) responded at 100 mM. Thus, over a third to half of all DA neurons located at the midline VTA nuclei respond to EtOH with greater than a 40% increase in FF. In the medial VTA, one third of neurons tested with quinpirole (1 μM) were unresponsive (n = 9 of 27). The medial DA neurons most strongly excited by EtOH (> 40% increase of FF) included a mix of quinpirole-sensitive (n = 6 of 10), and insensitive cells (n = 4 of 10).

In comparison, DA neurons in the lateral VTA (PBP nucleus) exhibited a mean increase in FF of 4 ± 4% at 20 mM, 8 ± 6% at 50 mM, and 23 ± 7% at 100 mM EtOH (n = 13), as shown in Fig. 2.6B. These changes were not significant ($p > 0.05$). In the lateral VTA, all DA neurons tested with quinpirole showed sensitivity to this agonist (n = 13 of 13). SNc DA neurons were the least responsive to EtOH, and nearly half of these cells were in fact slightly inhibited by high levels of EtOH (100 mM; n = 4 of 10). SNc cells exhibited a mean change in FF of 14 ± 9% at 20 mM, 29 ± 22% at 50 mM, and 21 ± 29% at 100 mM EtOH (n = 10). These changes were not significant ($p > 0.05$) and did not appear to be concentration-dependent.

Discussion

We report that pharmacologically relevant levels of EtOH (20 mM, corresponding to a blood alcohol level of 0.09, or 3-4 glasses of wine for humans) increased neuronal firing in a subset of medial VTA DA neurons. VTA neurons *in vivo* receive strong glutamatergic and cholinergic drive from brainstem laterodorsal (LDTg) and pedunculopontine (PPTg) tegmental nuclei, and inhibitory drive from rostromedial tegmentum (RMTg), which has been suggested to regulate DA neurons responses to drugs of abuse (Geisler & Wise, 2008; Lecca et al., 2012; Lodge & Grace, 2006; Sesack & Grace, 2010; Watabe-Uchida et al., 2012). The strong response to EtOH we find in a subset of medial VTA neurons is, however, present in coronal ventral midbrain slices that are largely deafferented from excitatory and inhibitory inputs. In contrast to *in vivo* recordings in which SN GABA neurons were potently inhibited by EtOH (Mereu & Gessa, 1985), we did not observe significant inhibitory or excitatory effects of EtOH on non-DA neuron firing in our brain slices. A possible contribution of synaptic inputs to the EtOH effect *in vitro* remains to be determined, including the possibility of transmitter release from excitatory and inhibitory terminals.

The identification of a specific subset of EtOH-responsive medial VTA neurons contrasts with some previous studies. In particular, I_h-containing DA neurons of the VTA have been reported to exhibit minimal responses to lower concentrations of EtOH *in vitro*. Using extracellular recordings similar to ours, Brodie and colleagues reported that 20 mM EtOH increased FF by less than 20% above baseline (Brodie et al., 1990). Okamoto and colleagues later showed that 25 mM EtOH only caused a 5% increase in one of five DA neurons tested, while 50 and 100 mM EtOH increased FF by 19% and 18% above baseline, respectively (Okamoto et al., 2006). Note, however, that this study included only VTA DA neurons that

resemble nigral DA cells (calbindin-negative, I_h-positive) and that they reported no difference between SNc and VTA responses to EtOH. In contrast, our study revealed a 33% increase at 20 mM EtOH in VTA DA neurons. If I_h were indeed the primary target underlying the mechanism of EtOH excitation, SNc neurons would be expected to show the largest response, but we find that SNc DA cells are the least EtOH-responsive subset of ventral midbrain DA neurons. Two groups have reported that I_h regulates FF in VTA DA cells, as the I_h inhibitor ZD7288 (30 μM) decreased spontaneous firing by 33% (McDaid et al., 2008), and 28% (Okamoto et al., 2006). Other studies, however, present different findings. Neuhoff and colleagues found in VTA DA neurons that express I_h, ZD7288 (30 μM) had no effect on pacemaking (Neuhoff et al., 2002). Khaliq and Bean, moreover, demonstrated that 3 M CsCl did not stop or slow pacemaking in medial VTA neurons that express I_h from TH-GFP mice (Khaliq & Bean, 2010). These contrasting results may reflect the heterogeneity of DA neurons, and the significance of I_h for pacemaking in VTA DA neurons remains unresolved at this time. The robust responses to EtOH reported here were recorded from identified TH-GFP-positive DA neurons. These recordings included a large number of medial VTA DA neurons reported to be I_h-negative (Lammel et al., 2008; Neuhoff et al., 2002) and highly sensitive to acute cocaine (Lammel et al., 2011), and are thought to project to the medial shell of NAc and PFC. Of the medial VTA DA neurons that we identified as high responders to EtOH, one third were quinpirole-insensitive and may therefore be PFC-projecting cells that are likely to have been missed in previous studies of EtOH response. Our data thus suggest that EtOH sensitivity may be mediated, at least in part, by a subset of midbrain DA neurons that lack D_2 receptors (Ford, 2014).

The DA neurons of the medial VTA appear to be unique in their high level of expression of the synaptic vesicle glutamate transporter vGluT2 (Gorelova et al., 2012; Hnasko et al., 2010; Li et al., 2013; Trudeau et al., 2014; Yamaguchi et al., 2011). Our recordings were primarily in the posterior VTA at the level of the prominent fibers of the oculomotor nerve (bregma -3.52 mm; Paxinos and Franklin, 2001). Distinct subnuclei within the VTA have been described by immunostaining and are readily identifiable (Del-Fava et al., 2007; Ferreira et al., 2008; Fu et al., 2012). Differential responsiveness of anterior and posterior VTA has also been reported to impact alcohol drinking behavior (Melon & Boehm, 2011; Rodd et al., 2005), and the idea that these represent distinct populations of DA neurons warrants further study *in vitro*. In summary, our results identify a subset of DA neurons that are activated by levels of alcohol attained during social intoxication. These observations may have future impact for the treatment of alcoholic patients by addressing the relevant neural targets for the initial rewarding effects prior to addiction onset. There are currently no widely effective therapies for treating alcoholism, and improved clinical approaches may be possible in future with the advent of novel therapies using drugs or gene therapy approaches targeted to the sub-populations within the medial VTA to interact with the (as yet unknown) molecular targets of EtOH within the midbrain.

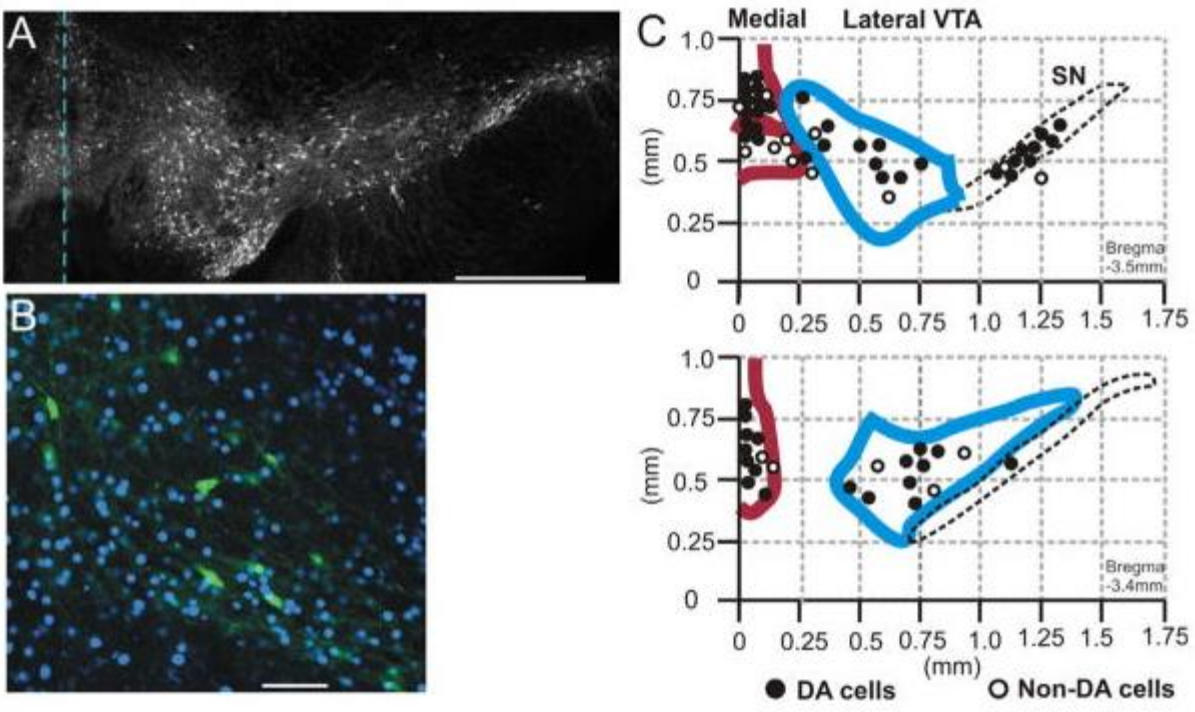

A
B
C
Medial
Lateral VTA
SN
(mm)
1.0
0.75
0.5
0.25
0
0 0.25 0.5 0.75 1.0 1.25 1.5 1.75
Bregma
-3.5mm
Bregma
-3.4mm
(mm)
DA cells
Non-DA cells

Figure 2.1: Recording Loci within the Ventral Midbrain. (**A**) Acute coronal brain slice (-3.4 mm from bregma) of TH-GFP mouse as prepared for electrophysiological recording under fluorescence. The dashed line indicates the position of the midline. GFP expression in living DA neurons can be observed in neurons of the medial VTA, lateral VTA, and SN. Scale bar, 500 μm. (**B**) Lateral VTA from TH-GFP brain slice with TH-GFP+ neurons (green) and nuclei stained with DAPI (blue). Scale bar, 100 μm. (**C**) Map of recording locations in coronal midbrain slices. Top: A caudal slice (posterior to bregma -3.5 mm). Bottom: A more rostral slice (at bregma -3.4 mm). Recordings were in the medial VTA (red), lateral VTA (blue), and the substantia nigra pars compacta (black dotted line). Filled circles represent TH+ (putative DA) neurons, and open circles indicate TH- (putative non-DA) neurons. Reproduced from Mrejeru et al., 2015.

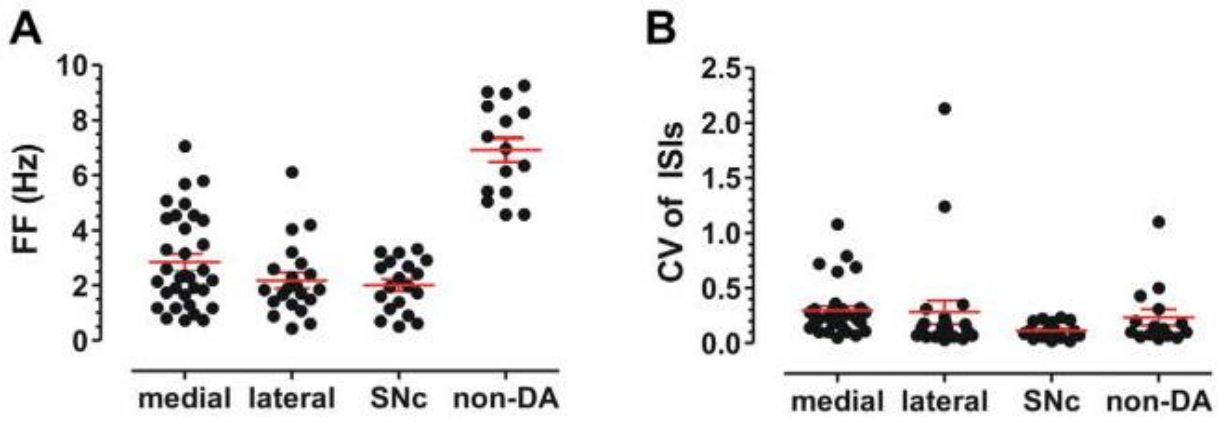

A
FF (Hz)
10
8
6
4
2
0
medial
lateral
SNc
non-DA
B
CV of ISIs
2.5
2.0
1.5
1.0
0.5
0.0
medial
lateral
SNc
non-DA

Figure 2.2: Spontaneous Firing Rates of VTA DA and non-DA Neurons. (**A**) Midline VTA DA neurons fired at a mean frequency of 2.8 ± 0.3 Hz (n = 33), while lateral VTA DA neurons fired at 2.2 ± 0.3 Hz (n = 21), and substantia nigra DA neurons fired at 2.0 ± 0.2 Hz (n = 19). This is in contrast to a population identified as non-DA neurons which fired at a mean frequency of 6.9 ± 0.4 Hz (n = 15). (**B**) CV of ISIs was more variable in medial VTA (mean CV = 0.3) and lateral VTA (mean CV = 0.3) than in SNc (mean CV = 0.1). Error bars show SEM. Reproduced from Mrejeru et al., 2015.

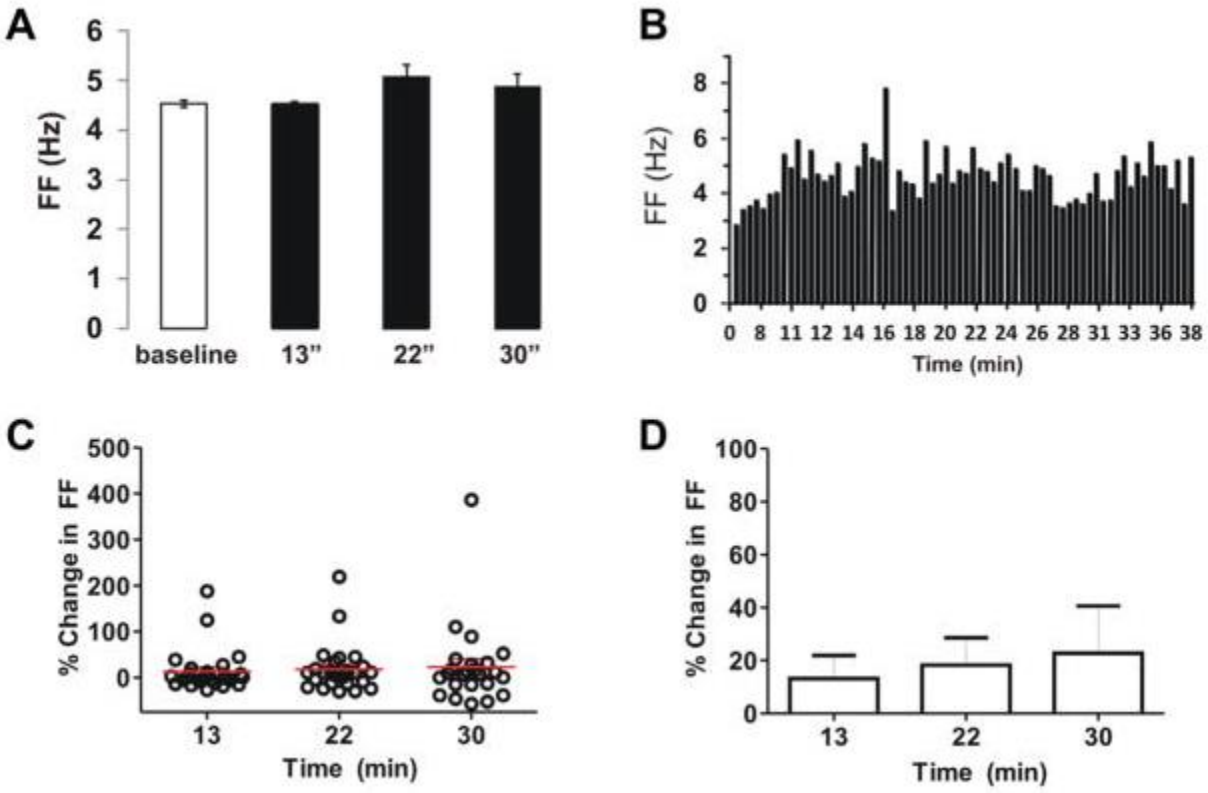

A
FF (Hz)
0
1
2
3
4
5
6
baseline
13"
22"
30"
B
FF (Hz)
0
2
4
6
8
0 8 11 12 14 16 18 20 22 24 26 28 31 33 36 38
Time (min)
C
% Change in FF
0
100
200
300
400
500
13
22
30
Time (min)
D
% Change in FF
0
20
40
60
80
100
13
22
30
Time (min)

Figure 2.3: Control Recordings Show Stability of Firing Rate in Extracellular "On-Cell" Patch Mode. (**A**) Representative trace of DA neuron in medial VTA (quinpirole-sensitive cell) during "on-cell" recording. Baseline FF was 4.5 Hz, with changes of -0.4%, 12%, and 7% after 13, 22, and 30 min, respectively. (**B**) Spike ratemeter of same cell shows FF over 40 min. (**C**, **D**) Population data of FF changes for DA neurons monitored at 13, 22, and 30 min after baseline recording. The mean increases in FF were 13%, 18%, and 23%, respectively (n = 27). These changes were not significant ($p > 0.05$). Error bars show SEM. Reproduced from Mrejeru et al., 2015.

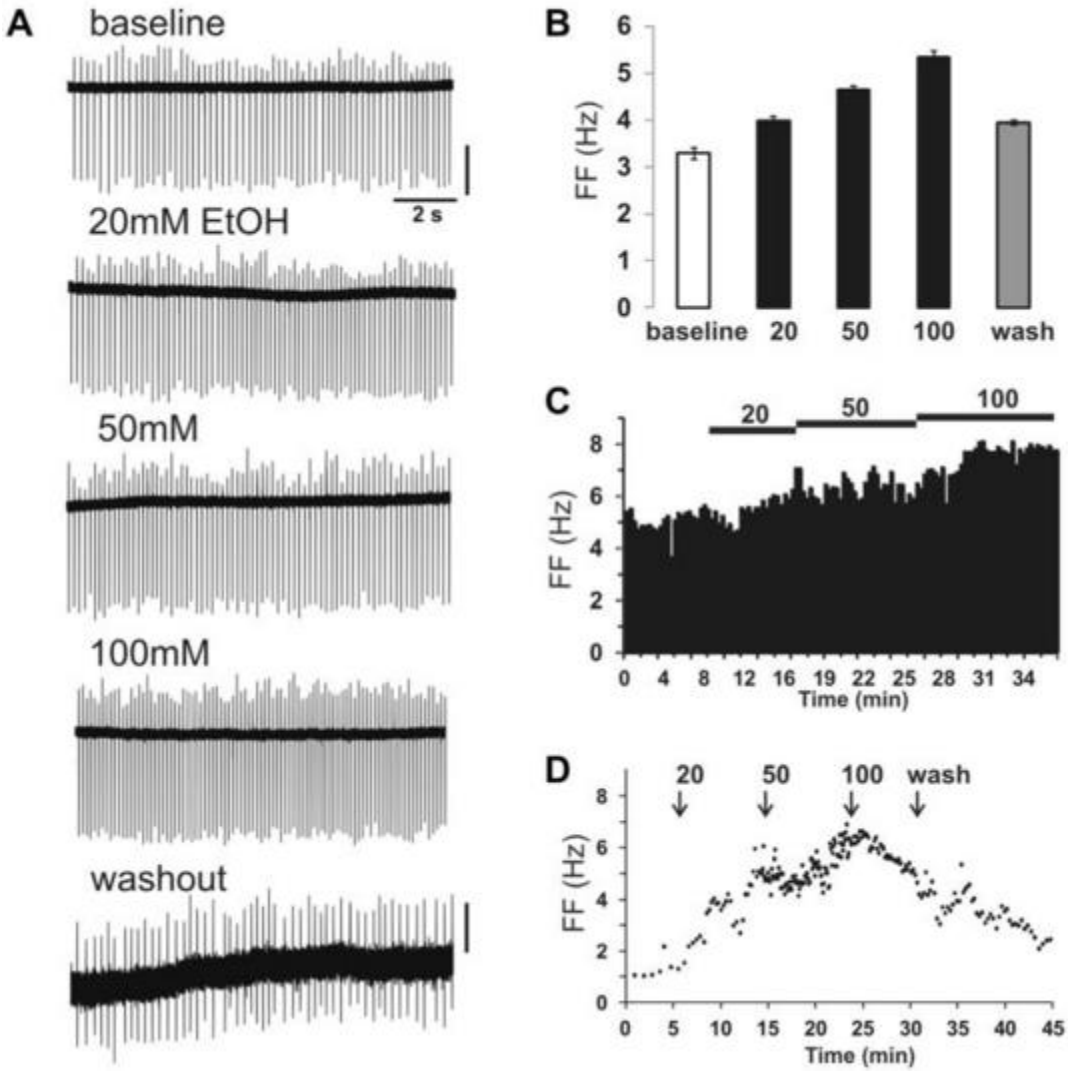
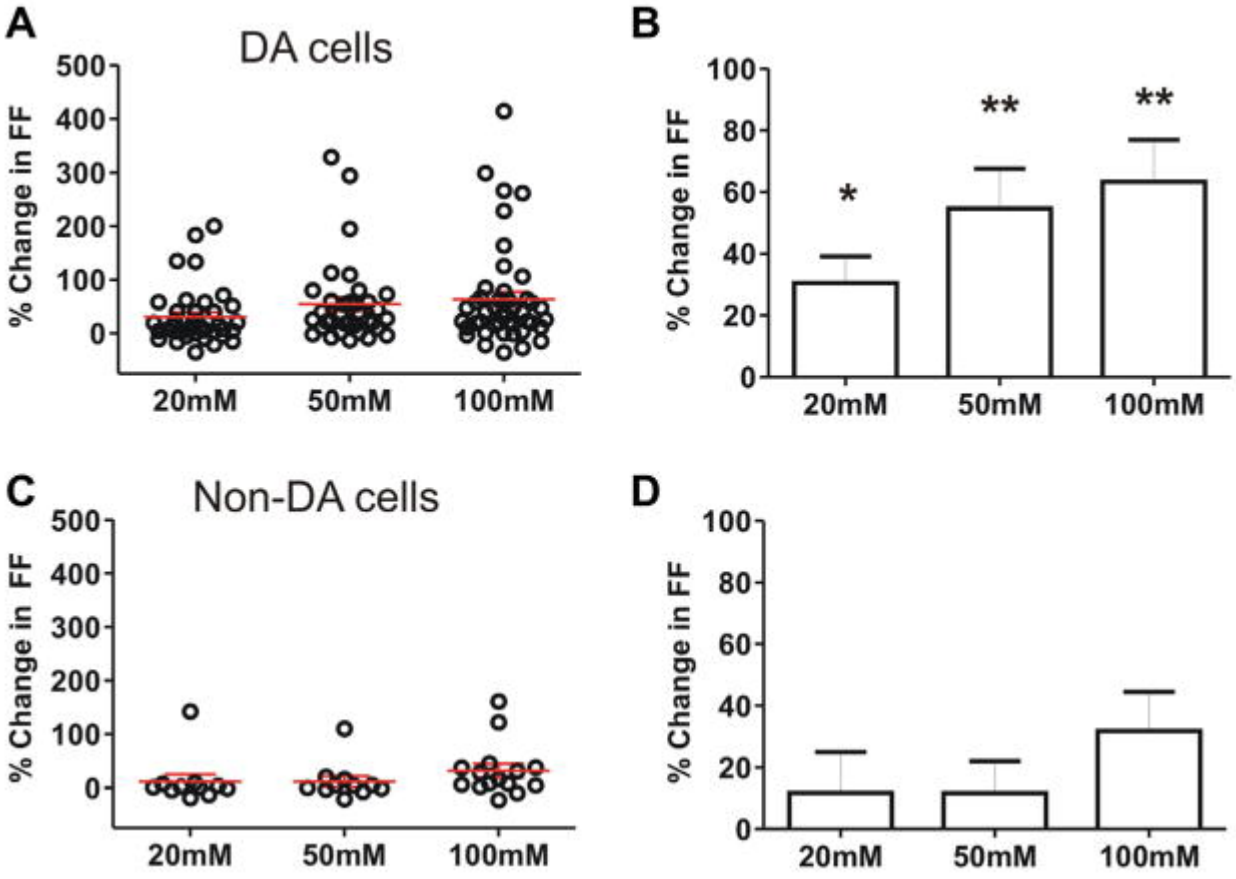

A
baseline
2 s
20mM EtOH
50mM
100mM
washout
B
FF (Hz)
baseline
20
50
100
wash
C
20
50
100
FF (Hz)
Time (min)
D
20
50
100
wash
FF (Hz)
Time (min)

Figure 2.4: Acute EtOH Responses in VTA DA Neurons. (**A**) Representative trace of extracellular recording from DA neuron in medial VTA (IF nucleus, quinpirole-sensitive cell) with a baseline FF 3.3 Hz. (Scale bars, top: 40 pA, bottom: 20 pA). (**B**) This cell had a robust concentration-dependent increase of FF (21%, 41%, and 62%) with increasing [EtOH] (20, 50, 100 mM), respectively. Washout completely reversed this effect. (**C**) Spike ratemeter showing entire recording from baseline to EtOH application. (**D**) Spontaneous firing of a medial VTA DA neuron with baseline FF of 1.3 Hz. EtOH increased the FF to 3.9 Hz at 20 mM, 5.5 Hz at 50 mM, and 5.1 Hz at 100 mM. Washout restored FF to 2.3 Hz but was incomplete after 15 min. Error bars in **B** show SEM. Reproduced from Mrejeru et al., 2015.


A
DA cells
% Change in FF
500
400
300
200
100
0
20mM
50mM
100mM
B
% Change in FF
100
80
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40
20
0
*
**
**
20mM
50mM
100mM
C
Non-DA cells
% Change in FF
500
400
300
200
100
0
20mM
50mM
100mM
D
% Change in FF
100
80
60
40
20
0
20mM
50mM
100mM

Figure 2.5: Population Data for Responses of All VTA Neurons to Acute EtOH. VTA DA and non-DA neurons were tested for EtOH responses at three concentrations (20, 50, and 100 mM). (**A, B**) For TH-positive DA cells, the mean increase in FF was 31% at 20 mM (n = 38, $p < 0.05$), 55% at 50 mM (n = 35, $p < 0.01$), and 64% at 100 mM (n = 46, $p < 0.01$). (**C, D**) TH-negative neurons had a mean increase in FF of 12% at 20 mM (n = 11), 12% at 50 mM (n = 11), and 32% at 100 mM (n = 15). Error bars show SEM. Reproduced from Mrejeru et al., 2015.

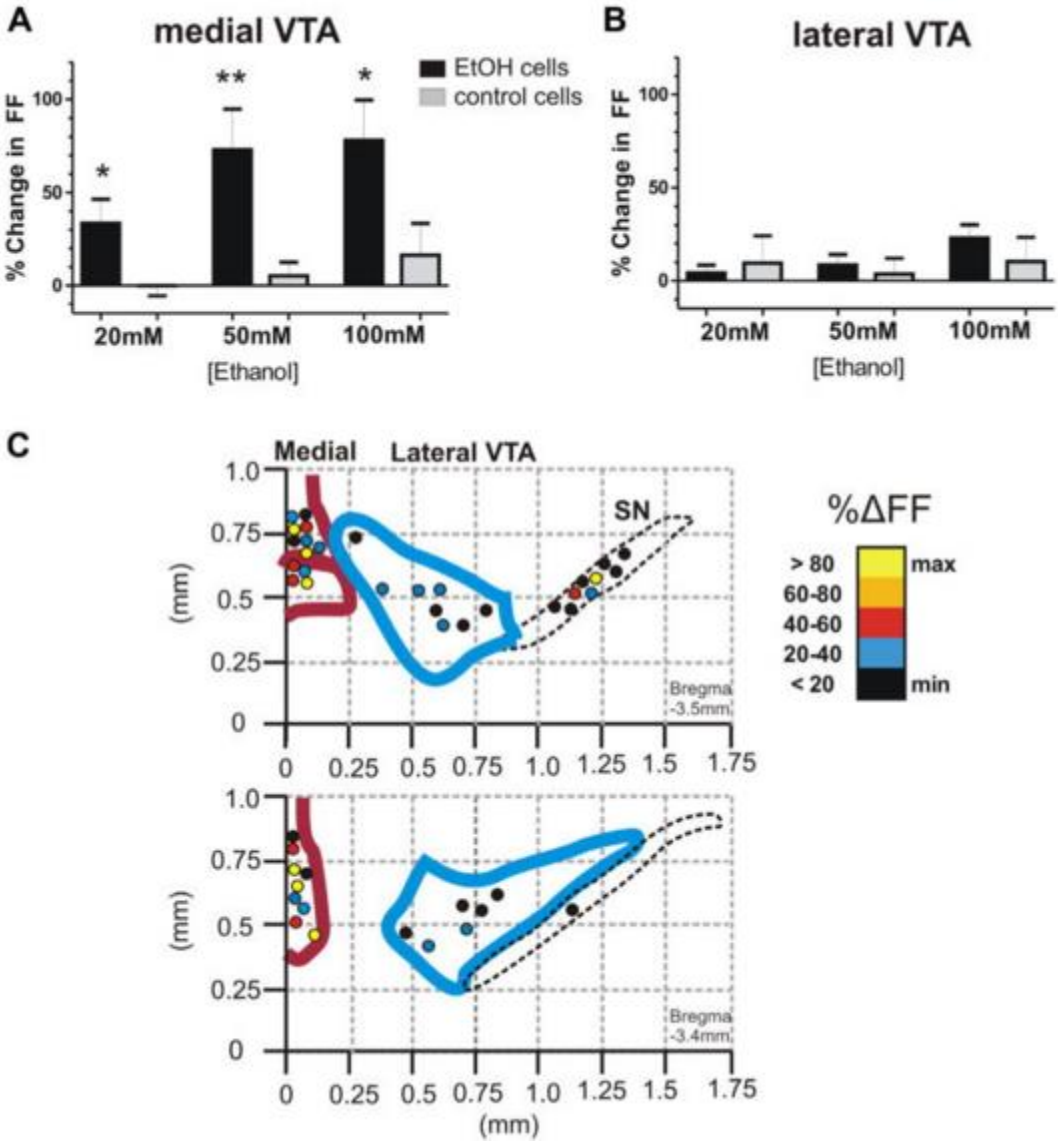

A
medial VTA
EtOH cells
control cells
% Change in FF
100
50
0
*
**
*
20mM
50mM
100mM
[Ethanol]
B
lateral VTA
% Change in FF
100
50
0
20mM
50mM
100mM
[Ethanol]
C
Medial
Lateral VTA
SN
(mm)
1.0
0.75
0.5
0.25
0
Bregma
-3.5mm
0
0.25
0.5
0.75
1.0
1.25
1.5
1.75
Bregma
-3.4mm
(mm)
%ΔFF
> 80
60-80
40-60
20-40
< 20
max
min

Figure 2.6: Medial and Lateral VTA DA Neurons Differ in Sensitivity to Ethanol. Medial and lateral VTA DA neurons were tested for responses to three concentrations of EtOH. (**A**) In the medial VTA, the mean increase in FF with EtOH (black bars) was 34 ± 13% at 20 mM, 73 ± 22% at 50 mM, and 78 ± 22% at 100 mM (n = 19). These changes were significant ($p < 0.05$ at 20 and 100 mM, $p < 0.01$ at 50 mM EtOH) as compared to medial VTA control neurons without EtOH (gray bars) at corresponding time points during the recording. (**B**) In the lateral VTA, the mean increase in FF with EtOH was 4 ± 4% at 20 mM, 8 ± 6% at 50 mM, and 23 ± 7% at 100 mM (n = 11). These changes were not significant compared to lateral VTA control neurons without EtOH (gray bars). (**C**) Map of recording locations from TH+ DA cells in the VTA and SN. Top: A caudal slice (posterior to bregma -3.5 mm). Bottom: A more rostral slice (at bregma -3.4 mm). Color scale represents the percentage change in FF in response to 50 mM EtOH. Error bars in **A** and **B** show SEM. Reproduced from Mrejeru et al., 2015.

CHAPTER 3

Adolescent Drinking Enhances Excitation of Medial VTA Dopamine Neurons by Alcohol[3]

Abstract

Enhanced DA neurotransmission from the VTA to the ventral striatum is thought to drive drug self-administration and mediate positive reinforcement. The maintenance of enhanced transmission is widely considered to be a consequence of potentiated glutamatergic synaptic input onto VTA dopamine neurons that enhances cue-invoked excitatory drive. We examined neuronal activity following adolescent binge-like alcohol drinking in mice and find that in a subset of medial VTA dopamine neurons, alcohol experience greatly increased firing activity driven by subsequent exposure to alcohol itself, without altering other measures of intrinsic excitability. NMDA receptor blockade decreased basal firing, but did not occlude the direct excitatory effects of alcohol. NMDA receptor blockade did, however, revert the magnitude of the acute alcohol excitatory response to a level indistinguishable from naïve animals. Thus, in contrast to cue-associated synaptic plasticity, drinking during adolescence induces a novel intrinsic mechanism that sensitizes dopamine neuronal response directly to a primary reinforcer.

[3] This chapter includes data collected in collaboration with other researchers in the Harrison and Sulzer labs. I am grateful to Michael Salling, Ana Mrejeru, Dan Lowes, David Sulzer, and Neil Harrison for their contributions to the ideas, text, and figures of this chapter.
The data presented in this chapter was collected by Michael Salling, Dan Lowes, and Elizabeth Avegno. Figures were prepared by Elizabeth Avegno.

Introduction

Epidemiological research points to adolescence as the critical period in the development of AUDs (Grant et al., 2001; Schuckit, 1998). Exposure of the nervous system of adolescents and young adults to alcohol initiates a process of neuroadaptation that can exacerbate heavy drinking even in the face of learned negative consequences, and increases the probability of AUDs during adulthood (Dawson et al., 2008; Hingson et al., 2006).

The self-administration of EtOH, like other drugs of abuse, is thought to be driven by enhanced neurotransmission of DA neurons from the VTA (Sulzer, 2011). More broadly, reinforcement based learning is thought to be driven by VTA DA neurons, and cues that successfully predict reinforcement can initiate a form of synaptic plasticity that subsequently enhances VTA neuron activity (Schultz, 2015). VTA DA neurons are clearly involved in mediating the rewarding properties of EtOH in rodents, including self-administration (Rodd et al., 2004) and EtOH-seeking behaviors (Hauser et al., 2011). EtOH has been shown to increase the firing rate of these neurons *in vivo* (Burkhardt & Adermark, 2014; Gessa et al., 1985) and *in vitro* (Brodie et al., 1990; Didone et al., 2014).

There is a consensus that drug self-administration is primarily driven by drug induced synaptic plasticity in excitatory pathways that impinge on the reward circuitry of the mesolimbic system (Gipson et al., 2014; Lüscher & Malenka, 2011). For EtOH, this has been suggested to occur via enhanced excitatory drive to VTA DA neurons and the expression of long-term potentiation (Stuber et al., 2008a; Wanat et al., 2009). Many studies in this field, however, have used passive rather than self-administration of EtOH to examine synaptic plasticity. Few studies have examined the relationship between drinking history or chronic alcohol exposure and DA neuron sensitivity. Studies on the effects of passive chronic i.p. administration of EtOH have

generated conflicting results (Brodie, 2002; Didone et al., 2014; Okamoto et al., 2006). To date, no studies have examined the effects of voluntary drinking on the sensitivity of VTA DA neurons to EtOH.

A major obstacle to interpreting the reports of VTA neural responses to EtOH has been that the concentration required to elicit a significant increase in firing rate *in vitro* is several times higher than that achieved through voluntary alcohol consumption (Schier et al., 2012), often corresponding to sedative or lethal concentrations in humans (Didone et al., 2014; Okamoto et al., 2006; NIAAA, 2015). We recently identified a population of medial VTA DA neurons that exhibit increased sensitivity to acute EtOH at pharmacologically relevant levels (20 mM) relative to other midbrain DA neurons (Chapter 2; Mrejeru et al., 2015). Here, we report that voluntary EtOH consumption during adolescence, in which mice learn to acquire a high preference for EtOH, enhances the physiological response of these medial DA neurons to EtOH. We further demonstrate a role of NMDA receptors in regulating the tonic firing activity of these neurons within the deafferented coronal slice; while activation of these receptors is not necessary for EtOH's acute excitatory effects on DA neurons, the enhanced response to EtOH appears to involve activation of local glutamate receptors. These results shed light on EtOH's ability to enhance DA neurotransmission downstream from excitatory drive and support a novel learning-associated enhanced response to the neuropharmacological effects of the drug itself.

Materials and Methods

All animal procedures were performed following NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. Wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). Food and water were available in the home cage ad libitum throughout the experiment. Mice were housed in cages with mild enrichment (e.g. cotton nestlets). Wild-type mice were housed on a 12-hour light-dark cycle (lights off at 7pm). TH-GFP mice, in which neuronal GFP expression showed >87% co-localization with TH immunoreactivity (Sawamoto et al., 2001), were housed on a reverse light-dark cycle (lights off at 10am).

Intermittent Access Protocol in Adolescent Male Mice

Male wild-type mice were singly housed beginning at age postnatal day 27 (p27; ± 2 days) and given 3 days to acclimate to isolation before beginning the intermittent access (IA) procedure (Melendez, 2011). Mice were given a two-bottle choice between water and 15% (vol/vol) EtOH. EtOH was substituted for water on alternate days. Each bottle and mouse was weighed daily to assess EtOH intake (as g EtOH/kg mouse/day) and percent preference for EtOH over water. EtOH presentation occurred prior to the beginning of the dark cycle (approximately 5pm). Placement of the EtOH bottle was alternated each session to control for side preference. Control "drip" cages – in which bottles were placed and weighed daily, but no mouse was housed – were run in parallel. The average drip value of water and EtOH were subtracted from data collected from each mouse, in order to better determine real fluid intake levels. The IA procedure was run for 15 drinking sessions (30 days), from p30-p60.

Handling-induced convulsion (HIC) tests were performed in a subset of mice at two or six hours after EtOH removal, corresponding to approximately 8 or 12 hours after peak BACs, at the end of the final session to assess symptoms of withdrawal. Mice were briefly lifted by the tail and lowered; if no signs of seizures were observed, mice were lifted again and gently twirled 360 degrees, then lowered. Each mouse was given a score based on evidence of seizures and facial grimace by two independent blinded observers. A set of control water-drinking mice was run in parallel.

In a separate experiment, elevated plus maze experiments were run in order to determine whether adolescent drinkers have an increased anxiety phenotype. Experiments were performed during the "extended withdrawal" phase, corresponding to 72 hours after the final drinking session. Mice (n = 15 controls, n = 16 drinkers) were placed into an elevated apparatus, consisting of two closed arms and two open arms. Locomotor activity was assessed over five minutes using AnyMAZE software (Stoelting Co., Wood Dale, IL, USA). One trial was performed per mouse. The total number of open arm entries, as well as total time spent in the open arms, was calculated.

Between one and two hours after EtOH removal at the end of the final drinking session, mice were sacrificed by cervical dislocation, and 250 μm-thick coronal sections were prepared as described in Chapter 2.

A similar series of experiments were performed in male TH-GFP mice. Mice were placed in isolation at p27, and the IA procedure was initiated at p30. EtOH bottles were presented prior to the dark cycle (approximately 9:30am) on Monday, Wednesday, and Friday; mice were offered two water bottles on Tuesday, Thursday, Saturday, and Sunday. To estimate peak blood alcohol concentrations (BACs), tail blood samples were collected at 6 hours after

alcohol presentation during the eleventh drinking session, a time point at which EtOH intake was stable across sessions. The BAC of each sample was determined using an amperometric oxygen electrode and kit (Analox Instruments Limited, London, England). Between one and two hours after removal of alcohol during the final drinking session (~p64), VTA-containing sections were prepared as described earlier, then placed in ACSF solution containing (in mM): 10 glucose, 119 NaCl, 26.2 $NaHCO_3$, 3.6 KCl, 1.2 $MgCl_2$-$6H_20$, 1.0 NaH_2PO_4-$6H_20$, and 2.4 $CaCl_2$. In a subset of mice, trunk blood was collected after cervical dislocation to ensure that mice had no detectable BACs at the time of dissection (Supplementary Figure 2).

Parallel experiments were performed in male TH-GFP mice during adulthood. Mice were placed in isolation at p87, then given intermittent access to 15% EtOH from p90-p120. After removal of the EtOH bottle at the end of the 15^{th} drinking session, mice were sacrificed, and cell-attached electrophysiological experiments were performed. The data from these experiments are presented in Supplementary Figure 3.

Electrophysiological Recordings

Neurons were visualized under a 40X water immersion objective by fluorescence and DIC optics (Olympus, Bridgeport, CT, USA). Voltage-clamp and whole cell current-clamp recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitized at 10 kHz with a Digidata 1332 (Molecular Devices). Data were acquired using Clampex 8 software (Molecular Devices). All drugs were purchased from Sigma Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Minneapolis, MN, USA).

Cell-attached recordings were performed as previously described in Chapter 2 (Mrejeru et al., 2015). Briefly, “loose-patch” recordings were performed under voltage-clamp conditions

at a command potential of 0 mV. The baseline firing rate was compared to the firing rate after application of EtOH at several concentrations. After performing a washout of EtOH, 1µM quinpirole was added to the slice, allowing D_2R-containing neurons to be hyperpolarized and tonic firing to be completely silenced. The percent change in firing rate at each concentration of alcohol was calculated.

In a separate series of experiments, whole cell recordings were performed under current-clamp conditions. A glass pipette (resistance of 4-6 MΩ) with an internal solution containing (in mM): 115 K-gluconate, 20 NaCl, 10 HEPES, 1.5$MgCl_2$, 2 Mg-ATP, 0.2 Na-GTP, 0.1 EGTA, and 10 Na-phosphocreatine, (pH = 7.3, 290 mOsm) was used. Liquid junction potentials were not corrected during recordings. RMP was measured by generating an I-V curve after applying a series of current steps from -20 pA to +20 pA (5 pA increments, 500 msec duration). Rheobase, defined as the minimum amount of current needed to elicit an action potential, was determined using a ramp protocol in current-clamp.

Confocal Imaging of TH-GFP Sections

Images of VTA-containing sections (Figure 3.2a) were obtained from a TH-GFP female mouse. The brain was removed and fixed in 4% paraformaldehyde overnight. 50 µm-thick coronal midbrain sections were collected and directly mounted onto a glass coverslip. Imaging was performed under confocal microscopy (Nikon Instruments, Tokyo, Japan) under a 10X objective. Images were acquired with NIS Elements software (Nikon Instruments).

Statistical Analysis

Statistical analysis was performed in Prism 6 (GraphPad Software, La Jolla, CA, USA). Unpaired, two-tailed *t*-tests were used to compare the percent change in firing rate between

groups at each concentration of EtOH tested (Figures 3.2 & 3.3; Supplementary Figure 3). After applying a Bonferroni correction of (α/m) = (0.05/3), a *p* value of <0.017 was considered significant. Comparison of baseline firing rate (Figure 3.2), resting membrane potentials, rheobase values, and elevated plus maze behavior (Supplementary Figure 2) was performed by two-tailed *t*-test. Two-way ANOVA was used when comparing the percent change in firing rate between groups of VTA DA neurons in response to glutamate receptor blockers (Figure 3.4b). A *p* value of <0.05 was considered significant.

Results

Intermittent Access Protocol in Adolescent Male Mice

Data were collected from C57BL/6J mice (n = 57) and congenic TH-GFP mice (Sawamoto et al., 2001) on C57BL/6J background (n = 64). As no significant differences were observed in the results between these groups of mice, the data were combined. After beginning an intermittent access (IA) two-bottle choice drinking paradigm (Figure 3.1a), within 3-5 drinking sessions, the mice achieved a stable EtOH intake of 15-20 g EtOH/kg mouse/day (Figure 3.1b), with approximately 80% preference for EtOH over water (Figure 3.1c).

Tail blood samples were taken from a subset of TH-GFP mice at 6 hours after EtOH presentation during the 11th drinking session (Figure 3.1d). The drinking behavior within each session is variable among mice, and therefore peak blood alcohol concentrations (BACs) can be difficult to estimate. Nevertheless, it is clear from these data that many of these mice are capable of achieving BACs above 80 mg/dL, considered the threshold BAC for "binge-like" drinking behavior (NIAAA, 2015).

Alcohol withdrawal symptoms were assessed in a subset of mice using handling induced convulsion (HIC) tests at 8 and 12 hours after peak EtOH consumption following the 15th drinking session. Blood samples were collected from subjects at this time point, indicating no detectable EtOH levels (Supplementary Figure 2). No evidence of withdrawal symptoms or discomfort was observed in these mice (Figure 3.1e). In addition, elevated plus maze experiments reveal that adolescent drinkers do not exhibit an increased anxiety phenotype after this level of drinking (Supplementary Figure 2). Our findings confirm previous reports, which

show that the IA drinking paradigm for this duration serves as a model of voluntary EtOH exposure, and does not model EtOH dependence (Carnicella et al., 2014).

Electrophysiological Recordings

Cell-attached loose-patch recordings were performed to measure the spontaneous firing rate of VTA neurons. In wild type mice, neurons were included for analysis if the following criteria were met: spontaneous baseline firing rate between 1-12 Hz and, once a stable seal was achieved, < 40% change in seal throughout the duration of the recording (typically up to 1 hour).

It has become clear that the physiological criteria formerly used to establish the identity of dopaminergic neurons within the VTA are insufficient, as these "classical" criteria have been shown not only to exclude some DAergic neurons but also to include non-DA neurons (Lammel et al., 2008; Margolis et al., 2006; Margolis et al., 2008b). To aid in the identification of DA neurons, most of our experiments were performed in TH-GFP mice (Sawamoto et al., 2001), providing us with a means to identify a population of medial VTA DA neurons that have previously been under-studied (Lammel et al., 2008). Neurons were identified by their fluorescence (Figure 3.2a), and their location within the slice was noted for future analysis.

We observed no significant difference in the baseline firing rate of VTA DA neurons in slices from alcohol drinking mice (3.0 ± 0.2 Hz, n = 42 cells from 27 mice), and age-matched, EtOH-naïve control mice (3.0 ± 0.4 Hz, n = 41 cells from 20 mice; $t(80) = 0.05$, $p = 0.96$, two-tailed *t*-test; Figure 3.2b). The majority (67 of 84 neurons, 80%) of the neurons recorded were inhibited by quinpirole, indicating expression of D_2 autoreceptors, coupled to GIRK channels. Both quinpirole responders and non-responders were included in our analysis. In many of the cells recorded, we observed a concentration-dependent increase in firing rate in response to

EtOH (10, 20, and 50 mM) that was partially or fully reversed upon washout (Figure 3.2c). Reversal of EtOH effects on DA neurons occurred after 15 min in 60 out of 83 (72%) washout recordings, with firing rate returning to within 30% of baseline. No difference was observed in the reversal of EtOH effects in neurons from drinking and naïve mice. A parallel set of experiments, in which no EtOH was applied but a similar time interval elapsed, provided a control for the stability of the recordings (Mrejeru et al., 2015): no systematic time-dependent change in firing rates was observed over a one hour recording period.

We observed a significant difference in the response to EtOH by VTA DA neurons in slices from EtOH-drinking mice compared to age-matched EtOH-naïve controls (Figure 3.2d). Neurons from mice that had access to EtOH during adolescence displayed a greater increase in firing rate in response to EtOH than did control animals receiving only water. A significant difference in the response of the two populations to each concentration of EtOH was observed (Table 3.1). Interestingly, similar results were not observed in mice which had intermittent access to EtOH throughout adulthood (p90-p120; Supplementary Figure 3; Supplementary Table 1). These data provide evidence that VTA DA neurons' physiological response to EtOH undergoes a form of sensitization in mice that had access to alcohol during adolescence. No significant relationship between measures of drinking behavior (such as total EtOH intake, average EtOH preference, or BAC measured) and cellular response was observed. Thus, drinking during adolescence enhances VTA DA cellular response to EtOH in a preference-independent fashion; intake behavior or preference was not correlated with cellular sensitivity to EtOH.

We recently described a form of functional heterogeneity in terms of the response of DA neurons to acutely applied EtOH, with medial VTA DA neurons showing a significantly higher

response to EtOH than cells in the lateral VTA or SN (Chapter 2; Mrejeru et al., 2015). In order to determine whether a similar pattern of EtOH response exists following the IA drinking behavior, we compared the response of DA neurons based on their location within the VTA (Figure 3.3). We observed a significant difference in the responses of medial VTA DA neurons to EtOH (Table 3.1). In the lateral VTA, we observed no significant difference between groups. We also measured the response of SN DA neurons of TH-GFP mice, and found no significant difference between groups (Figure 3.3c, Table 3.1). Therefore, this experience-related sensitization appears specific to medial VTA DA neurons. It has been suggested that the TH-GFP mice can falsely identify non-DAergic neurons (Lammel et al., 2015), particularly within the medial VTA. It is possible that this small representation of non-DA neurons contributes to the few neurons that did not respond to EtOH (1 of 15 medial VTA neurons from wild type mice, and 7 of 35 medial VTA DA neurons from TH-GFP mice, showed no response or slight inhibition to all concentrations of EtOH tested) and that the response to EtOH is thus somewhat underestimated. We found that this population of EtOH non-responders did not correlate with quinpirole response, indicating that lack of D_2 or GIRK expression does not explain EtOH insensitivity.

We then examined whether the enhanced sensitivity of VTA DA neurons in drinking individuals was the result of a change in the intrinsic neuronal excitability. We observed no change in the baseline firing rate of these cells (Figure 3.2c), indicating that drinking experience does not alter the spontaneous activity of these neurons. We performed whole cell recordings in neurons from TH-GFP mice. There was no significant difference in RMP or rheobase between medial VTA DA neurons in slices from drinkers and control mice (Table 3.2). These data

indicate that learning to drink during adolescence does not alter the intrinsic excitability of these neurons, but rather enhances their response to EtOH selectively.

DA neurons receive extensive glutamatergic input (Carr & Sesack, 2000; Geisler et al., 2007), including from local glutamatergic neurons (Dobi et al., 2010), which can influence DA neuron excitability. We next sought to determine whether the response to EtOH was mediated by glutamate. We performed a series of experiments to determine whether EtOH's effect on firing rate could be eliminated by blocking glutamatergic receptors using NBQX and AP5, antagonists of AMPA/kainate and NMDA receptors, respectively (Figure 3.4). In cell-attached experiments, addition of 10 μM NBQX alone had little effect on firing rate (-9 ± 4% drinkers, n = 20 cells, 7 mice; -10 ± 2% controls, n = 18 cells, 6 mice), suggesting a minor role of AMPA/kainate receptors in regulating tonic activity of VTA DA neurons within the deafferented slice. Addition of 50 μM AP5, however, decreased the firing rate in all neurons (-28 ± 4% drinkers; -29 ± 4% controls). No relationship between baseline firing rate and glutamate antagonist effect was observed. The effects of NBQX and AP5 did not differ in cells from drinking and naïve mice, suggesting that drinking experience does not lead to adaptations in glutamatergic activity in this preparation. Thus, it appears that even within the deafferented slice, there is sufficient local glutamatergic transmission onto NMDA receptors to influence the firing rate of VTA DA neurons.

In order to confirm that the inhibition observed in the presence of 50 μM AP5 was not due to nonspecific effects, a preliminary dose-response curve for AP5 was generated, and experiments were repeated in the presence of MK801, a non-competitive antagonist of the NMDA receptor, using EtOH-naïve TH-GFP+ mice. We observed a dose-dependent effect of AP5 on VTA DA neuron firing rate (Supplementary Figure 4a), although the concentrations of

AP5 tested (5, 20, and 50 µM) were all higher than the estimated EC50 (4.4 µM). In agreement with our results seen with AP5, application of 10 µM MK801 similarly produced a decrease in firing rate of VTA DA neurons (-12 ± 6%, n = 10 cells from 6 mice; Supplementary Figure 4c). These data provide further support that local glutamate transmission onto NMDA receptors influences VTA DA neuron activity *in vitro*.

In VTA DA neurons from naïve and drinking mice, 50 mM EtOH increased firing rate (12 ± 4% in naïve controls, n = 41; 27 ± 4% in drinkers, n = 41; Figure 3.2d). Surprisingly, in the presence of 10 µM NBQX and 50 µM AP5, EtOH was still able to enhance firing (21 ± 9% in controls, n =18 cells from 6 mice; 23 ± 5% in drinkers, n = 20 cells from 7 mice). A two-way ANOVA demonstrated a significant effect of drug application on firing rate ($p = 0.0003$), but not drinking history ($p = 0.5$); no significant interaction was observed ($p = 0.8$). Thus, EtOH drives VTA neuronal activity independently of ionotropic glutamate input (Figure 3.4c). In the presence of MK801 in EtOH-naïve mice, 50 mM EtOH still elicited an increase in firing rate, although this did not quite reach levels seen in the absence of glutamate blockers, or in the presence of NBQX/AP5 (7 ± 4%, n = 10 cells from 6 mice; Supplementary Figure 4d). The presence of NBQX/AP5, however, appeared to reverse the enhanced sensitivity to EtOH in VTA DA neurons from drinking mice compared to naïve controls (Figure 3.4c). Whereas in the absence of NBQX/AP5, VTA DA neurons exhibited a significantly greater excitation in response to EtOH compared to neurons from naïve controls (27 ± 4% drinkers, n = 41; 12 ± 4% controls, n = 41; $t(80) = 2.9$, $p = 0.005$, two-tailed *t*-test), in the presence of glutamate receptor blockers, enhanced EtOH potentiation of neuronal activity was no longer observed (23 ± 5% drinkers, n = 20; 21 ± 9% controls, n =18; $t(25) = 0.01$, $p = 0.86$, two-tailed *t*-test). No relationship was observed between baseline firing rate and EtOH effect, indicating that this lost enhanced EtOH

effect is not due to lower spontaneous activity of VTA DA neurons. The enhanced response to EtOH observed in neurons from mice which learned to drink in adolescence may therefore be due to changes in glutamate signaling in the VTA driven by a mechanism that is directly activated by EtOH.

Discussion

An enhancement of DA neurotransmission at mesoaccumbens synapses is strongly implicated as a physiological mechanism that increases the rate of executing behaviors (Olds, 1976; Schultz, 2011) including drug self-administration (Sulzer, 2011). Multiple studies indicate that experience with drugs, including alcohol (Stuber et al., 2008a), induces a long-term potentiation of glutamatergic synaptic input to VTA DA neurons (Bonci & Borgland, 2009). A similar mechanism is observed in animals trained to respond to a reward predictive cue (Stuber et al., 2008b), which suggests how cue-induced sensory stimuli produce enhanced DA neurotransmission and thereby learned drug self-administration.

In contrast to such enhanced response to cue-induced stimuli, we find that adolescent mice who have acquired a preference for EtOH develop a form of neuronal plasticity consisting of a powerful increase in the neural activity of medial VTA DA neurons to the primary reinforcer, EtOH, itself. This sensitized response is driven by alcohol self-administration during adolescence, the typical period during which alcohol dependent individuals initiate alcohol use (Gladwin et al., 2011), and occurs even in the acute brain slice preparation.

This sensitized response to EtOH self-administration has not been recognized previously in part because the specific VTA DA neurons that respond to reinforcing levels of EtOH (10-20 mM) were only recently discovered (Mrejeru et al., 2015). These medial VTA neurons, located in the rostral linear and interfascicular nuclei, are considered "atypical" of VTA DA neurons in that not all exhibit traditional DAergic physiological criteria, including expression of large I_h, presence of dopamine uptake transporter, and a low spontaneous firing frequency (Lammel et al., 2008; Neuhoff et al., 2002). We found that neither the lateral VTA DA neurons

nor SN DA neurons, which show less response to EtOH in naïve mice (Mrejeru et al., 2015) exhibited the sensitized response.

We observed a standing excitation of VTA DA neurons by local NMDA receptors in the deafferented slice preparation, revealing a previously unappreciated tonic effect of NMDA receptors on VTA DA neuron activity. The EtOH-induced increase in DA neuronal firing rate occurred in the presence of glutamate receptor blockers, demonstrating that EtOH's acute mechanism of action is glutamate receptor-independent. In contrast, the sensitization by VTA DA neurons due to adolescent EtOH self-administration was apparently absent during glutamate receptor blockade. The sensitization was not related to increased intrinsic excitability, as baseline spontaneous firing rate, rheobase, and RMP were not different between neurons of drinking and naïve mice. Rather, the process may require experience-induced adaptations in the local glutamatergic system, consistent with reports that voluntary drinking in rats increases excitatory (Stuber et al., 2008a) but not inhibitory (Margolis et al., 2008a) postsynaptic currents onto VTA neurons. These neuroadaptations could occur in synaptic inputs from glutamatergic and cholinergic drives from brainstem pedunculopontine tegmental nuclei, dorsal raphe, laterodorsal tegmentum or rostromedial tegmentum (Beier et al., 2015; Lammel et al., 2012; Omelchenko & Sesack, 2006; Watabe-Uchida et al., 2012).

The findings contrast in part with previous reports on effects of systemic involuntary administration of EtOH on the firing of VTA DA neurons, with evidence for desensitization to effects of EtOH reported in one study (Okamoto et al., 2006), but not others (Brodie, 2002; Didone et al., 2014). The present results are relevant to recent reports of a potentiation of DA release in the NAc *in vivo* in response to alcohol (Toalston et al., 2014) and other rewarding stimuli (Spoelder et al., 2015) after EtOH consumption in adolescence, although those studies

did not examine whether this was due to enhanced DA neuronal response. In light of our findings, these data support the conclusion that voluntary alcohol self-administration selectively alters the sensitivity of NAc-projecting VTA DA neurons in their response to EtOH, as the NAc is a projection target of medial VTA DA neurons (Lammel et al., 2008) implicated in reward (Lammel et al., 2011). This appears to be a consequence of adolescent drinking specifically, as neurons from mice which drank during adulthood did not exhibit increased sensitivity to EtOH. Whether this phenomenon persists after periods of abstinence remains to be determined.

As enhanced activity of mesoaccumbens DA neurons is understood to increase behaviors (Olds, 1976; Schultz, 2011), this experience-induced sensitized response to EtOH itself provides a means to "short circuit" normal learning that relies on environmental cues, including secondary reinforcement, negative reinforcement, or punishment. If increased DA neuronal activity indeed enhances the frequency of the execution of behavior, this sensitized neural response provides a mechanism that could explain why alcohol abuse can be difficult to control even in the face of severely aversive consequences.

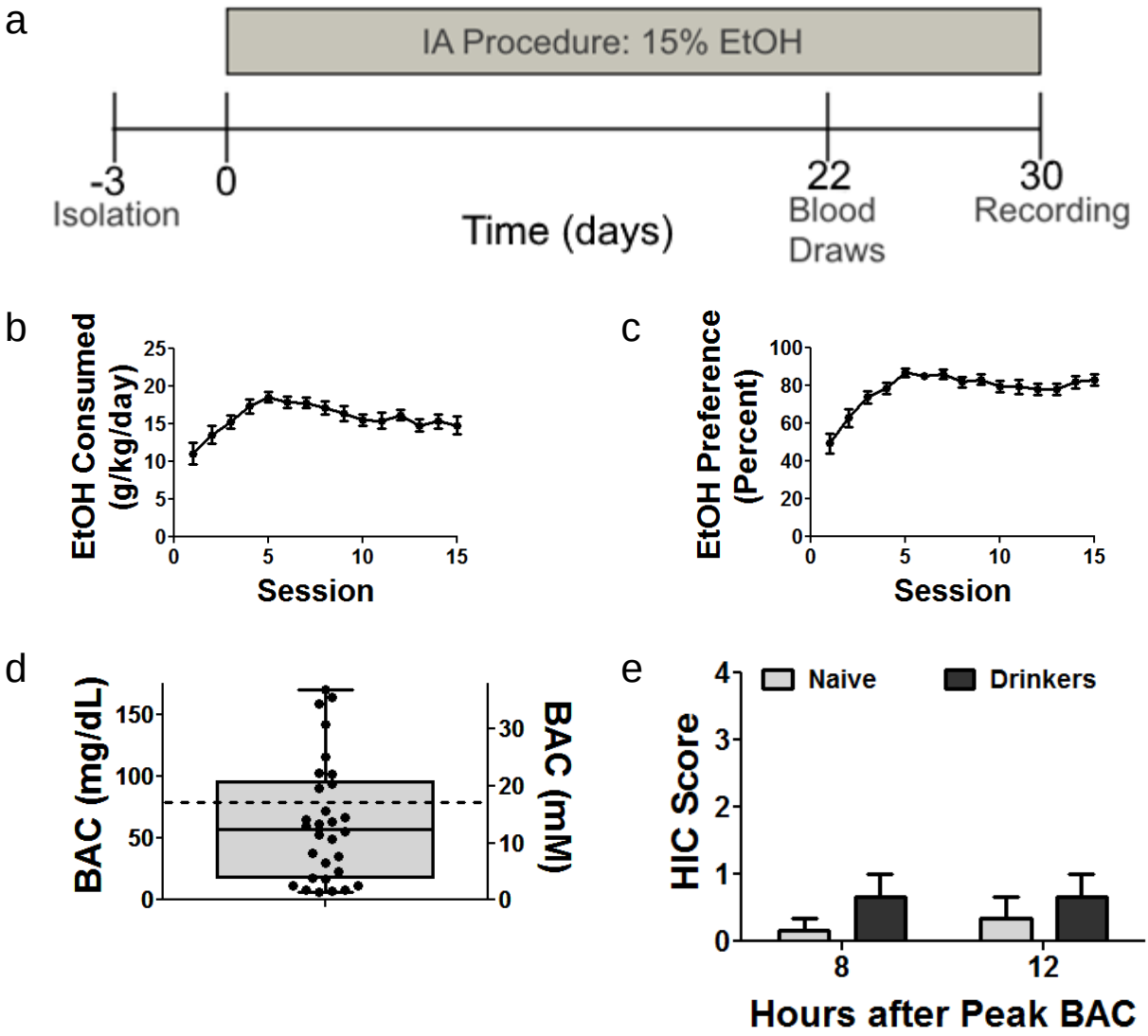
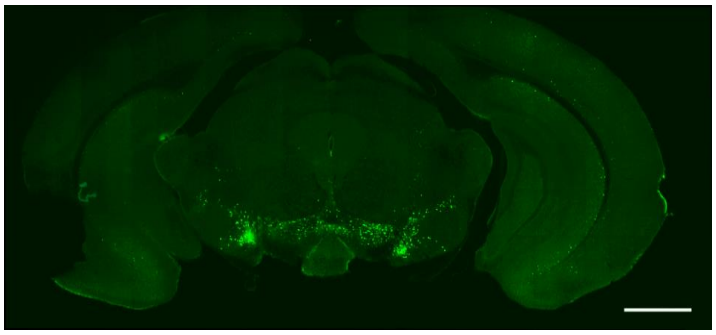
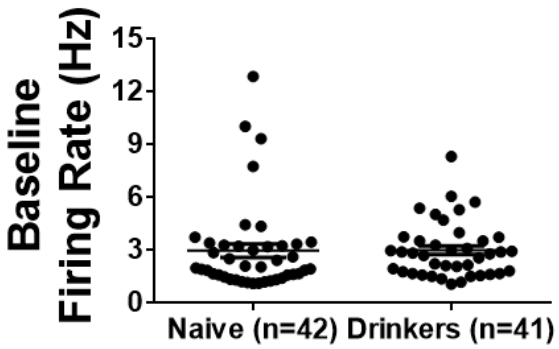

a
IA Procedure: 15% EtOH
-3
Isolation
0
22
Blood Draws
30
Recording
Time (days)
b
EtOH Consumed (g/kg/day)
Session
c
EtOH Preference (Percent)
Session
d
BAC (mg/dL)
BAC (mM)
e
Naive
Drinkers
HIC Score
Hours after Peak BAC

Figure 3.1: Intermittent Access (IA) Model Produces Escalated Binge-like Ethanol Intake in Adolescent Mice. (**a**) Timeline of the procedure. IA procedure begins at age p30 and runs for 15 drinking sessions. (**b**) Average ethanol intake per session for a group of 42 mice. (**c**) Preference for ethanol over water. (**d**) Blood alcohol concentration (BAC) values for 30 mice, measured 6 hours after EtOH presentation during the 11^{th} drinking session. (**e**) HIC scores for drinking and naïve mice (n = 6 mice per group) measured 8 and 12 hours after peak BAC (corresponding to 2 and 6 hours after removing EtOH) at the end of the 15^{th} drinking session. Error bars show SEM.

a



b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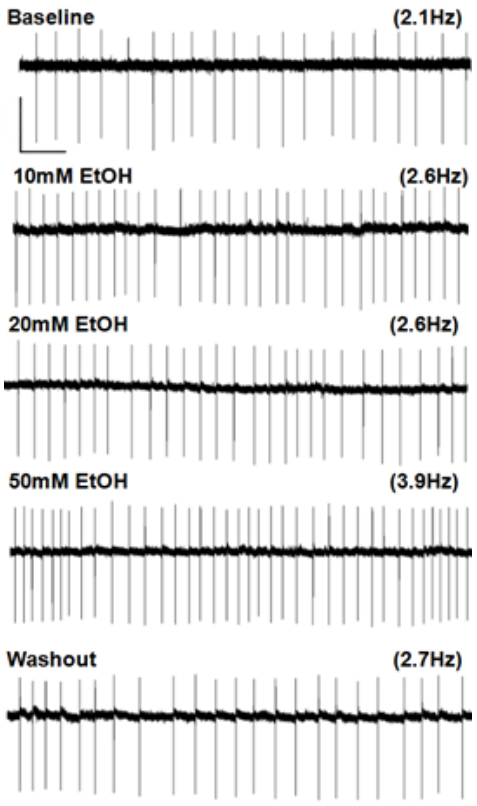
12
9
6
3
0
Naive (n=42) Drinkers (n=41)

c


Baseline
(2.1Hz)
10mM EtOH
(2.6Hz)
20mM EtOH
(2.6Hz)
50mM EtOH
(3.9Hz)
Washout
(2.7Hz)

d

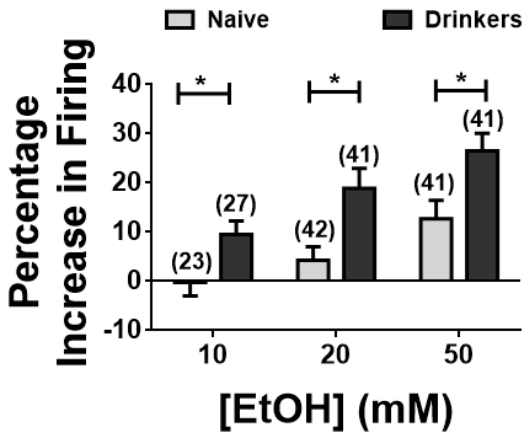
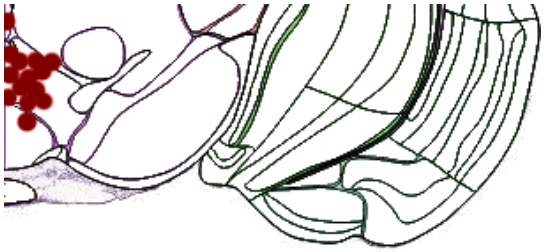
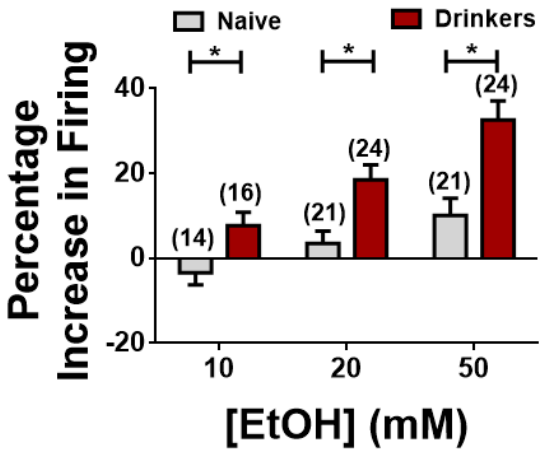

Naive
Drinkers
Percentage Increase in Firing
40
30
20
10
0
-10
*
*
*
(23)
(27)
(42)
(41)
(41)
(41)
10
20
50
[EtOH] (mM)

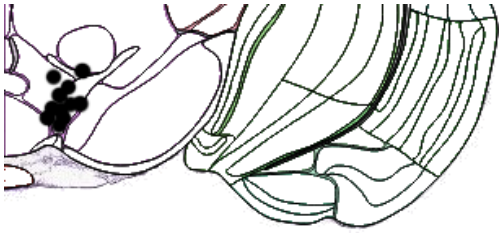
Figure 3.2: Adolescent Drinking Experience Enhances the Ethanol Sensitivity of VTA DA Neurons. (**a**) Midbrain coronal section of TH-GFP mouse. Scale bar, 1 mm. (b) Mean baseline firing rate of VTA DA neurons from drinkers (3.0 ± 0.2 Hz, n = 42 cells from 27 mice) does not significantly differ from those of age-matched controls that only drank water (3.0 ± 0.4 Hz, n = 41 cells from 20 mice; $p = 0.96$, two-tailed *t*-test). (**c**) Sample cell-attached recording of TH-GFP+ neuron. Scale bar, 100 pA, 1 s. (**d**) Percentage changes in firing rate of VTA DA neurons in alcohol drinking and water-drinking mice. Two-tailed *t*-tests reveal a significant difference in excitation induced by 10, 20, and 50 mM EtOH in DA neurons from drinking mice compared to naïve controls (see Table 3.1). Error bars show SEM.

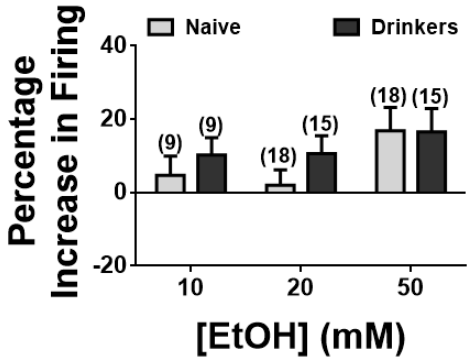
a




Naive
Drinkers
*
*
*
Percentage Increase in Firing
40
20
0
-20
(14)
(16)
(21)
(24)
(21)
(24)
10
20
50
[EtOH] (mM)

b




Naive
Drinkers
Percentage Increase in Firing
40
20
0
-20
(9)
(9)
(18)
(15)
(18)
(15)
10
20
50
[EtOH] (mM)

c

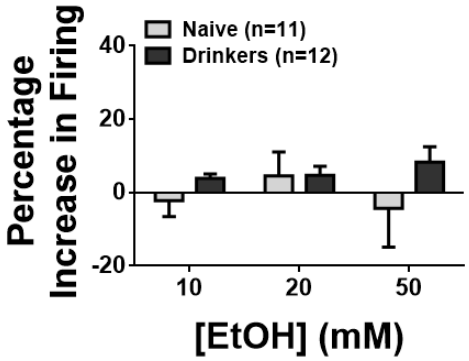

Naive (n=11)
Drinkers (n=12)
Percentage Increase in Firing
40
20
0
-20
10
20
50
[EtOH] (mM)

Figure 3.3: Adolescent Drinking Experience Selectively Enhances Ethanol Sensitivity of Medial VTA DA Neurons. Each panel contains representative image (adapted from Allen Brain Atlas) indicating approximate positions of neurons recorded in midbrain section (bregma -3.3 mm), as well as percentage change in firing rate of DA neurons from drinking mice compared to age-matched water-only controls. (**a**) Data from medial VTA DA neurons. Two-tailed *t*-tests reveal a significant difference in the response to 10, 20, and 50 mM EtOH of medial VTA DA neurons of drinking mice compared to controls (see Table 3.1). (**b**) Data from lateral VTA DA neurons. No significant difference is observed in response to EtOH of lateral VTA DA neurons of drinking mice compared to controls (Table 3.1). (**c**) Data from SN DA neurons indicates no significant difference in response to EtOH (Table 3.1). Error bars in **a**-**c** show SEM.

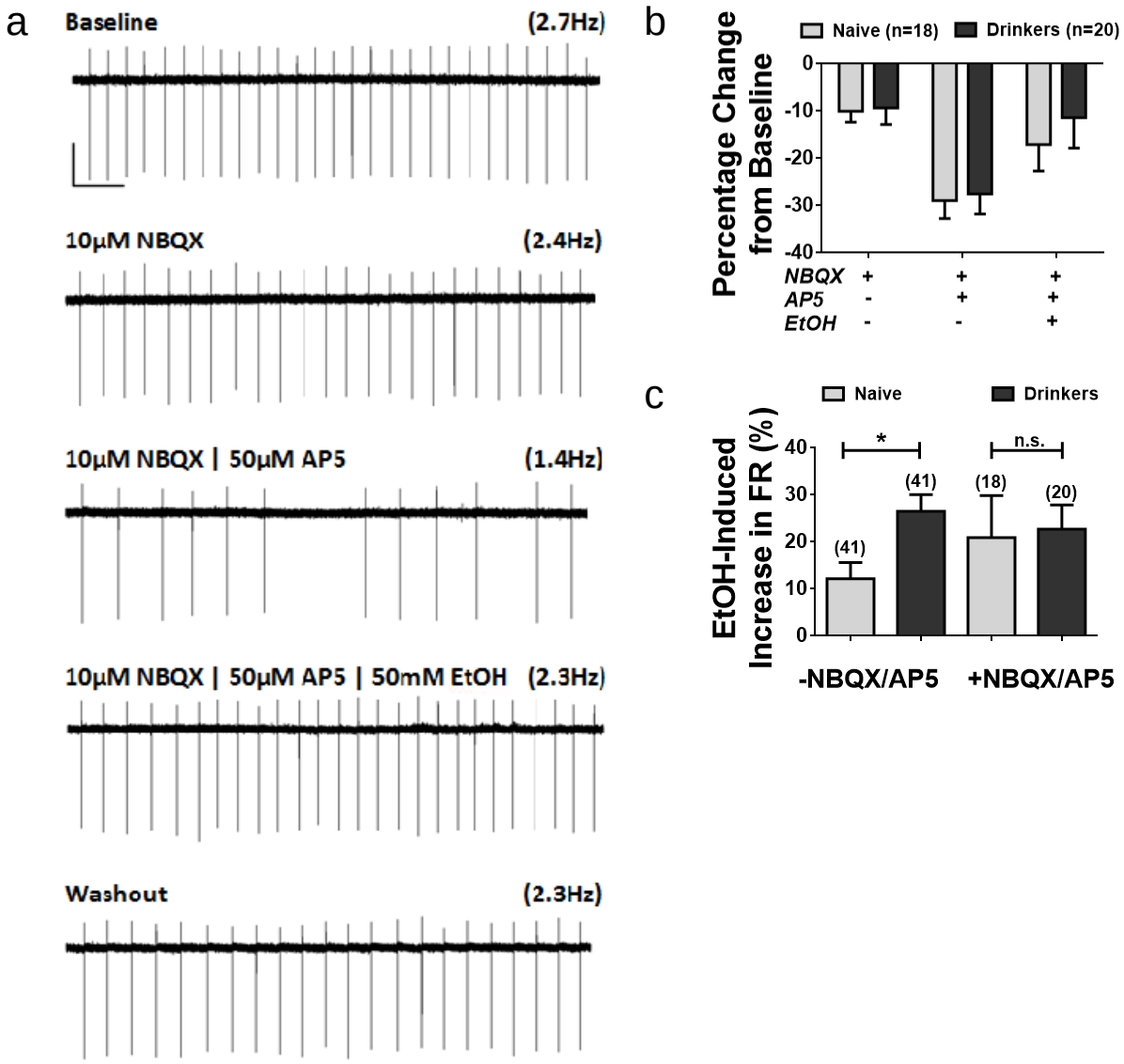

a
Baseline
(2.7Hz)
10μM NBQX
(2.4Hz)
10μM NBQX | 50μM AP5
(1.4Hz)
10μM NBQX | 50μM AP5 | 50mM EtOH
(2.3Hz)
Washout
(2.3Hz)
b
Naive (n=18)
Drinkers (n=20)
Percentage Change from Baseline
0
-10
-20
-30
-40
NBQX + + +
AP5 - + +
EtOH - - +
c
Naive
Drinkers
EtOH-Induced Increase in FR (%)
40
30
20
10
0
*
n.s.
(41)
(41)
(18)
(20)
-NBQX/AP5
+NBQX/AP5

Figure 3.4: Ethanol Excitation of VTA DA Neurons is Independent of Glutamate Receptor Activation. (**a**) Sample cell-attached recording of TH-GFP+ neuron. Scale bar, 50 pA, 1 s. (**b**) Summary of percent change in firing rate from baseline in response to 10 µM NBQX (-9 ± 4% drinkers; -10 ± 2% controls), 10 µM NBQX/50 µM AP5 (-28 ± 4% drinkers; -29 ± 4% controls), and 10 µM NBQX/50 µM AP5/50 mM EtOH (-11 ± 6% drinkers; -17 ± 6% controls). A two-way ANOVA suggests a significant effect of drug application (p = 0.0003), but not drinking history (p = 0.5); no significant interaction was observed (p = 0.8). (**c**) In the absence of NBQX/AP5, VTA DA neurons demonstrate a significantly greater excitation in response to 50 mM EtOH compared to controls (p=0.005, two-tailed t-test), whereas in the presence of glutamate receptor blockers, this difference is no longer observed (p=0.86). Error bars in **b**-**c** show SEM.

	VTA DA Neurons			Medial VTA DA			Lateral VTA DA			SN DA Neurons		
	Naive	**EtOH**	***p* value**	**Naive**	**EtOH**	***p* value**	**Naive**	**EtOH**	***p* value**	**Naive**	**EtOH**	***p* value**
Baseline FR	3.0 ± 0.4	3.0 ± 0.2	0.96	3.3 ± 0.6	3.0 ± 0.4	0.7	2.3 ± 0.4	3.0 ± 0.4	0.23	2.6 ± 0.2	3.0 ± 0.2	0.3
ISI CV	0.13± 0.01	0.16 ± 0.02	0.13	0.19 ± 0.03	0.17 ± 0.01	0.4	0.12 ± 0.01	0.10 ± 0.01	0.3	0.064 ± 0.01	0.072 ± 0.01	0.6
10mM	*-0.4 ± 2.9*	*9.5 ± 4.0*	*0.017*	*-3.4 ± 2.9*	*7.7 ± 3.1*	*0.016*	4.7 ± 5.2	10.2 ± 4.7	0.44	-2.2 ± 4.3	3.9 ± 1.2	0.17
20mM	*4.3 ± 2.6*	*18.9 ± 4.1*	*0.003*	*3.5 ± 2.9*	*19.1 ± 3.3*	*0.0012*	2.0 ± 4.2	10.7 ± 4.9	0.19	4.6 ± 6.5	4.7 ± 2.4	0.98
50mM	*12.2 ± 3.5*	*26.5 ± 3.5*	*0.0049*	*10.1 ± 4.1*	*33.1 ± 4.3*	*0.0004*	16.8 ± 6.4	16.6 ± 6.4	0.98	-4.3 ± 10.6	8.3 ± 4.3	0.27

Table 3.1: Ethanol-induced Excitation of Midbrain DA Neurons. Values presented are percent change in firing rate in response to 10, 20, and 50 mM EtOH ± SEM. Two tailed *t*-tests were used to compare the response of VTA and SN DA neurons from naïve and experienced mice; $p < 0.017$ was considered significant (italicized values). Baseline firing rates and ISI CV values are compared using two-tailed *t*-tests; $p < 0.05$ was considered significant.

	Naïve	EtOH	*p* value
Resting Membrane Potential (mV)	-64.0 ± 2.7 (n = 21)	-64.4 ± 3.5 (n = 13)	0.9
Rheobase (pA)	59.2 ± 13.0 (n = 16)	73.2 ± 15.7 (n = 11)	0.5

Table 3.2: Resting Membrane Potential and Rheobase Values of Medial VTA DA Neurons.

Values presented are average resting membrane potential and rheobase ± SEM. Data were compared using unpaired, two-tailed *t*-tests; $p < 0.05$ was considered significant.

CHAPTER 4

Medial VTA DA Neurons Projecting to the Nucleus Accumbens Medial Shell Display Enhanced Ethanol Sensitivity after Adolescent Drinking[4]

Abstract

Ethanol exerts its actions in the midbrain, in part, by eliciting an increase in the activity of dopamine neurons in the ventral tegmental area, thereby encouraging an increased local dopamine concentration in the nucleus accumbens. We have recently shown that considerable heterogeneity exists within the VTA regarding ethanol response *in vitro*, with a subset of medial VTA dopamine neurons showing high sensitivity to acute ethanol, as well as enhanced sensitivity after adolescent drinking. Given the recent research demonstrating projection-specific sensitivity of midbrain neurons to cocaine and other rewarding stimuli, we hypothesized that a population of NAc medial shell-projecting VTA DA neurons would show a uniformly high sensitivity to ethanol *in vitro*. We performed bilateral injections of retrobeads into the NAc medial shell of male TH-GFP mice, then began an intermittent access to ethanol paradigm throughout adolescence. Spontaneous activity of fluorescently labeled VTA DA neurons was measured using cell-attached recordings, and response to ethanol (50 mM) was assessed. We find evidence that NAc medial shell-projecting VTA DA neurons display an enhanced sensitivity to 50 mM EtOH after adolescent drinking, compared to alcohol-naïve controls.

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[4] This chapter includes data collected in collaboration with other researchers in the Harrison and Sulzer labs. I am grateful to Michael Salling, Anders Borgkvist, David Sulzer, and Neil Harrison for their contributions to the ideas, text, and figures of this chapter.
The data presented in this chapter was collected by Anders Borgkvist, Michael Salling, and Elizabeth Avegno. Figures were prepared by Elizabeth Avegno.

Introduction

EtOH is a commonly used drug which exerts its effects in part by increasing activity of DA neurons in the VTA and eliciting higher DA concentration in the NAc, an efferent of VTA DA neurons. The effects of EtOH within this system are not uniform, however. Evidence from cyclic voltammetry studies reveals the presence of "hot spots" in the NAc shell and core, where DA release in response to EtOH is greater than others (Robinson et al., 2009). In the VTA, recent work from our lab has demonstrated that only a subset of medially located DA neurons demonstrate considerable sensitivity to acute EtOH *in vitro*, with significant increases in firing rate in response to 20mM EtOH (Chapter 2; Mrejeru et al., 2015).

Regional heterogeneity within the VTA has been further characterized with the use of retrograde tracers, demonstrating differences in basic morphological and physiological properties (Lammel et al., 2008; Margolis et al., 2008b), as well as sensitivity to rewarding or aversive stimuli (Lammel et al., 2011; Lammel et al., 2012). To date, the data on projection-specific response of VTA DA neurons in response to EtOH is scarce. However, the evidence for a subpopulation of medial VTA DA neurons exhibiting a higher sensitivity to EtOH than lateral VTA DA neurons (Mrejeru et al., 2015) does indicate that EtOH responders may share a common projection target. Medial VTA DA neuron projection targets include the NAc medial shell, NAc core, medial prefrontal cortex (mPFC), and basolateral amygdala (BLA). Given the evidence implicating NAc shell-projecting neurons in conditioned place preference (Lammel et al., 2012), and, in particular, medial shell-projecting neurons in response to cocaine (Lammel et al., 2011), we sought to determine whether this population similarly is implicated in EtOH response. To this end, we delivered injections of red retrobeads into the medial shell of TH-GFP

mice and performed cell-attached recordings of fluorescently labeled cells in the VTA. Spontaneous firing activity and cellular response to EtOH were assessed.

After voluntary EtOH intake during adolescence, research indicates that neural response to EtOH can be enhanced. Microdialysis studies reveal higher DA concentration in the NAc shell of rats in response to EtOH after adolescent self-administration, compared to naïve animals (Toalston et al., 2014). Further, enhanced EtOH sensitivity in a subpopulation of VTA DA neurons is observed after EtOH self-administration in adolescent mice (Chapter 3), with DA neurons of drinking animals showing a greater increase in firing rate in response to 10, 20 and 50 mM EtOH, compared to the response of neurons from naïve controls. To determine whether this subpopulation of EtOH-responsive neurons shares a common projection target, we combined retrograde tracing with an intermittent access (IA) protocol in adolescent mice. We report that NAc medial shell-projecting VTA DA neurons have a low sensitivity to acute EtOH, but display enhanced sensitivity to EtOH after adolescent drinking.

Materials and Methods

All animal procedures were performed following NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. Food and water were available in the home cage ad libitum throughout the experiment. TH-GFP mice (Sawamoto et al., 2001) were housed in cages with mild enrichment (e.g. cotton nestlets). Mice were housed on a reverse light-dark cycle (lights off at 10am).

Injection of Retrobeads

Under isoflurane anesthesia and stereotactic control, male TH-GFP mice (p25) were given bilateral injections of red retrobeads (Lumafluor, Naples, FL, USA), diluted 1:4 in saline, into the NAc medial shell. Coordinates were empirically determined as bregma: +1.55 mm, lateral 0.39 mm; ventral -3.75 mm. A fine-tipped glass pipette connected to a Nanoject II (Drummond Scientific Company, Broomall, PA, USA) was used to deliver 92 nL beads per injection. Mice were allowed to recover with littermates for two days before being singly housed at age p27.

Recording of Retrobead-Containing Neurons

At age p30, mice began the chronic intermittent access protocol, after which cell-attached recordings were performed as described in Chapters 2 and 3. Baseline firing rate was compared to the firing rate after application of 50 mM EtOH. After collecting VTA-containing sections for recording, the remainder of the brain was fixed in PFA and sectioned to verify the proper injection site for each mouse.

Immunohistochemistry and Confocal Imaging

Injections of green retrobeads were performed in a subset of TH-GFP- mice for immunohistochemical experiments. Following a recovery period of two weeks, mice were transcardially perfused with saline, followed by ice cold 4% paraformaldehyde (PFA; pH 7.4). Brains were removed and placed overnight in PFA at 4°C, then stored in phosphate buffered saline (PBS). 30 µm-thick sections containing the NAc medial shell and VTA were collected and processed for immunohistochemistry to verify proper injection site and transport of beads back to the cell body.

For immunohistochemical staining of NAc medial shell-containing slices, free floating sections were washed for 10 min in 0.1% Triton X-100 in PBS, followed by 2 x 10 minute washes in PBS. Cell bodies were then stained using NeuroTrace red fluorescent Nissl stain (1:100 in PBS; Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature. Sections were transferred to 0.1% Triton X-100 in PBS for 10 min, followed by 2 x 10 minute washes in PBS, then incubated overnight at 4°C in PBS. Sections were mounted onto a glass slide and coverslipped with ProLong Antifade Mountant with DAPI (Molecular Probes).

For immunohistochemical staining of VTA-containing slices, free floating sections were washed 3 x 5 minutes in PBS at room temperature, then blocked for 1 hour in 10% Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA, USA) with 0.1% Triton X-100 in PBS. Sections were transferred to a primary antibody solution (1:1000 rabbit anti-TH (Millipore, Darmstadt, Germany) diluted in PBS with 0.01% Triton X-100 and 2% normal donkey serum) and incubated overnight at 4°C in a secondary antibody solution (1:400 Alexa Fluor 594 Donkey anti rabbit IgG (Jackson ImmunoResearch) diluted in PBS with 0.01% Triton X-100 and 1% normal donkey serum). After 3 x 10 minute washes in PBS at room temperature,

sections were mounted onto a glass slide and coverslipped with ProLong Antifade Mountant with DAPI (Molecular Probes).

Imaging was performed under confocal microscopy (Nikon Instruments, Tokyo, Japan) under 10X and 20X objectives. Images were acquired with NIS Elements software (Nikon Instruments).

Statistical Analysis

Statistical analysis was performed in Prism 6 (GraphPad Software, La Jolla, CA, USA). Unpaired, two-tailed *t*-tests were used to compare baseline firing rates and the percent change in firing rate between groups at 50 mM EtOH (Figure 4.3); a p value of < 0.05 was considered significant.

Results

A Subset of Medial VTA Neurons Project to the Nucleus Accumbens Medial Shell

Bilateral injections of red retrobeads into the NAc medial shell were performed at age p25. Immunohistochemical analysis is shown in Figure 4.1. The majority of medial shell-projecting neurons were restricted to the medial VTA (Figure 4.1b, c). These data agree with previously published results (Lammel et al., 2008).

Intermittent Access Protocol in Adolescent Male Mice

Data were collected from TH-GFP mice (Sawamoto et al., 2001) on C57BL/6J background (n = 5 drinking and 4 naïve mice). Intake behavior in retrobead-injected mice agrees with the data presented in Chapter 3. After beginning an intermittent access (IA) two-bottle choice drinking paradigm (Figure 4.2a), the mice achieved an escalated EtOH intake (Figure 4.2b) and preference for EtOH over water (Figure 4.2c), although intake levels did not reach those achieved in uninjected mice. Whereas adolescent mice in Chapter 3 achieved an average daily intake of 16.1 ± 0.37 g EtOH/kg mouse/day over the final ten drinking sessions, retrobead-injected mice consumed an average of 10.4 ± 0.45 g/kg/day. Similarly, uninjected mice had an average preference for EtOH over water of 81.7 ± 0.87%, while retrobead-injected mice had an average preference of 58.6 ± 2.7%.

Electrophysiological Recordings

Cell-attached loose-patch recordings were performed to measure the spontaneous firing rate of VTA neurons. Neurons were identified by their fluorescence (Figure 4.3b-d), and their location within the slice was noted for future analysis.

We observed no significant difference in the baseline firing rate of VTA DA neurons in slices from alcohol drinking mice (4.1 ± 1 Hz, n = 8 cells from 5 mice), and age-matched, EtOH-naïve control mice (3.1 ± 0.5 Hz, n = 7 cells from 4 mice; $t(13) = 0.86$, $p = 0.41$, two-tailed *t*-test; Figure 4.2e). The majority (14 of 15 neurons, 93%) of the neurons recorded were inhibited by quinpirole, indicating expression of D_2 autoreceptors coupled to GIRK channels. In the presence of 50mM EtOH, the firing rate of medial shell-projecting VTA DA neurons of drinking mice increased by 31.3 ± 8.6%, compared to a 6.2 ± 4.1% increase of neurons from naïve controls ($t(13) = 2.5$, $p = 0.026$, two-tailed *t*-test; Figure 4.3d). This indicates that NAc medial shell-projecting neurons of the VTA demonstrate enhanced sensitivity to EtOH after adolescent drinking.

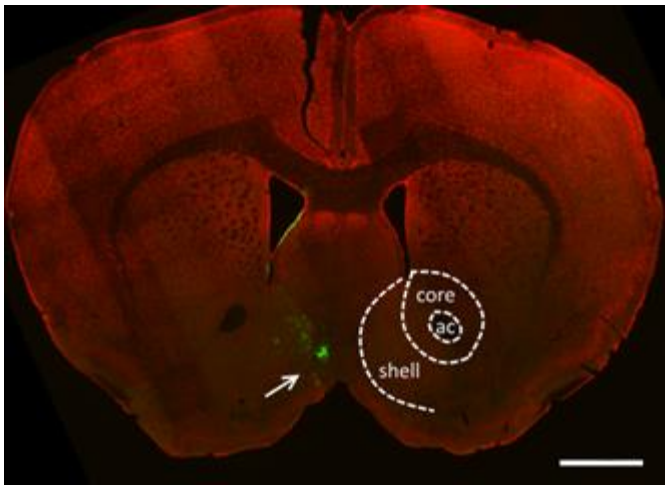
Discussion

We have previously reported regional heterogeneity within the VTA, with a subset of medial VTA DA neurons showing considerably higher sensitivity to acute EtOH *in vitro* than DA neurons in the lateral VTA or SN (Chapter 2). We subsequently reported a form of neuronal plasticity in a subset of medial VTA DA neurons in adolescent mice who have acquired a preference for EtOH (Chapter 3). Extensive heterogeneity has been reported within the VTA, both in terms of basic molecular and physiological properties (Lammel et al., 2008; Margolis et al., 2008b), as well as in terms of response to rewarding stimuli (Lammel et al., 2011; Lammel et al., 2012). Given the previously reported results implicating NAc medial shell-projecting VTA DA neurons in response to rewarding stimuli, together with our results identifying a subset of medial VTA DA neurons exhibiting a high sensitivity to EtOH, we hypothesized that NAc medial shell-projecting VTA DA neurons would show a similarly high sensitivity to EtOH *in vitro*. Our results indicate that DA neurons that project to the medial shell exhibit a low sensitivity to acute EtOH, with an average increase of only 6% in response to 50 mM EtOH. Adolescent drinking, however, results in a significantly greater response to this concentration.

We find that medial shell-projecting VTA neurons reside within the medial VTA, in areas largely restricted to the interfascicular nucleus, consistent with previous reports (Lammel et al., 2008). These locations are also the sites of EtOH-sensitive neurons observed in our previous studies (Mrejeru et al., 2015; Chapters 2 and 3). Other populations of neurons that reside in this area include those that project to the mPFC, BLA, and NAc core (Lammel et al., 2008). More detailed experiments are necessary to determine whether this NAc medial shell-projecting population demonstrates enhanced sensitivity to lower concentrations of EtOH, as well as whether this enhanced sensitivity is restricted to medial shell-projecting neurons or shared by

medial VTA DA neurons with alternate projection targets. A thorough investigation of the sensitivity of individual populations of medial VTA DA neurons based on projection target is outside the scope of this study, but could be applied to resolve these unanswered questions.

a



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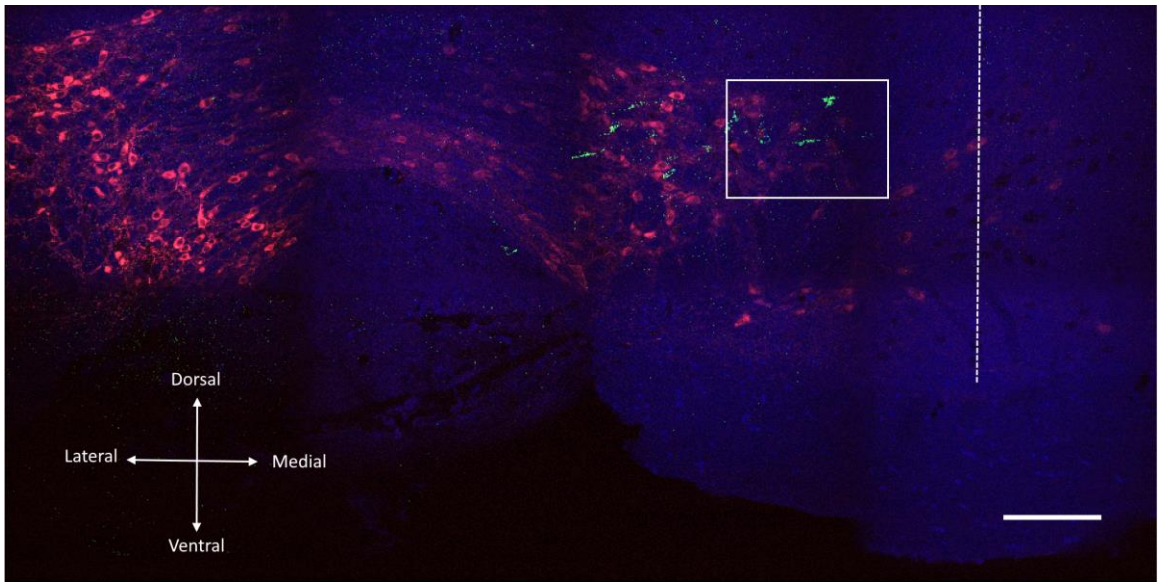

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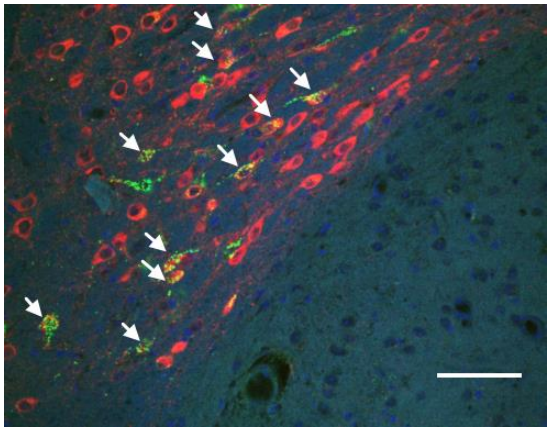

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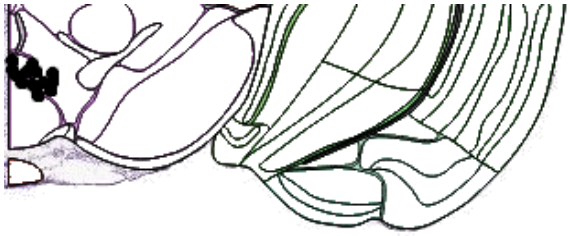
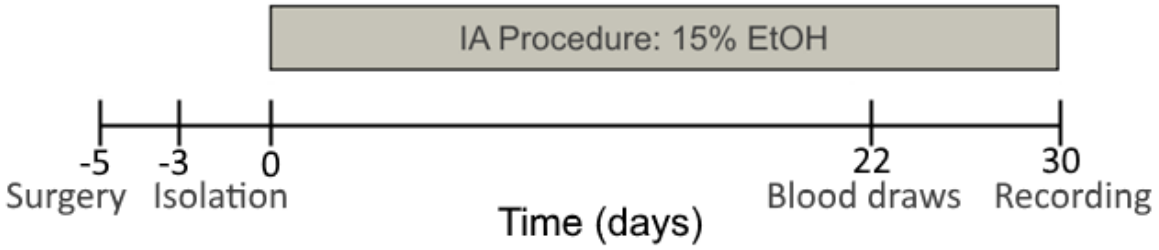


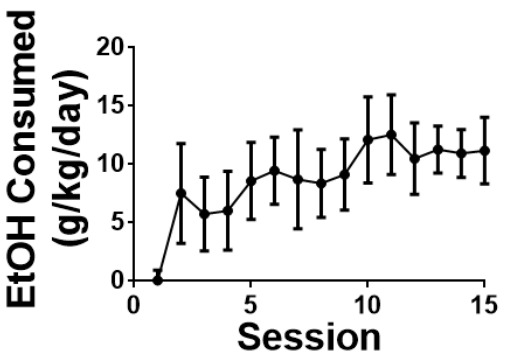
Figure 4.1: A Population of Medial VTA Neurons Projects to the NAc Medial Shell.

(**a**) Coronal section of retrobead injected mouse. Cell bodies stained with red Nissl. Location of NAc core, NAc shell, and anterior commissure (ac) indicated. Scale bar, 1 mm. (**b**) 20X tiled image of VTA. Retrobead-containing neurons shown in green; TH-expressing neurons shown in red; nuclei shown in blue. Dashed line indicates approximate location of midline. Boxed area shown in **c**. Scale bar, 200 µm. (**c**) 20X image of medial VTA. White arrows indicate position of retrobead-containing, TH-expressing neurons. Scale bar, 100 µm. (**d**) Schematic plotting location of medial shell-projecting neurons in VTA. Data averaged from 9 mice. Adapted from Allen Brain Atlas.

A


IA Procedure: 15% EtOH
-5
-3
0
22
30
Surgery
Isolation
Blood draws
Recording
Time (days)

b


EtOH Consumed (g/kg/day)
20
15
10
5
0
0
5
10
15
Session

c

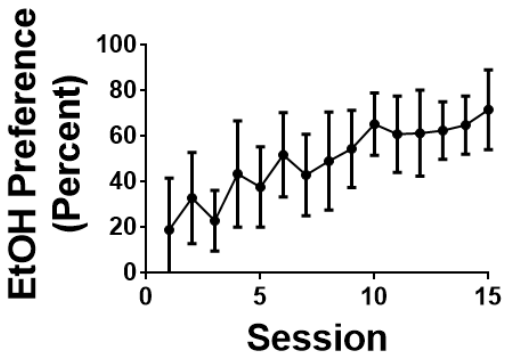
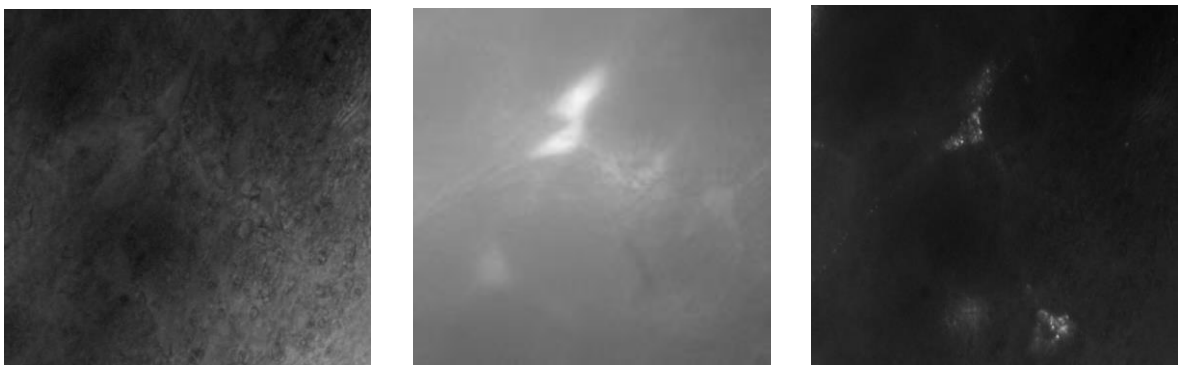
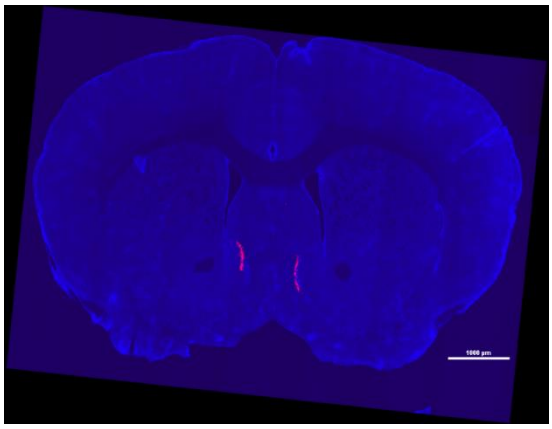

EtOH Preference (Percent)
100
80
60
40
20
0
0
5
10
15
Session

Figure 4.2: Intermittent Access (IA) Model Produces Escalated Ethanol Intake in Adolescent Mice. (**a**) Timeline of the procedure. Retrobead injections are delivered at age p25. IA procedure begins at age p30 and runs for 15 drinking sessions. (**b**) Average ethanol intake per session for a group of 5 mice. (**c**) Preference for ethanol over water. Error bars in **b-c** show SEM.

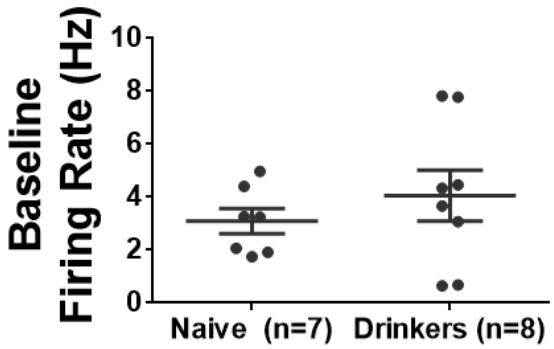
a



b



c


Baseline
Firing Rate (Hz)
10
8
6
4
2
0
Naive (n=7)
Drinkers (n=8)

d

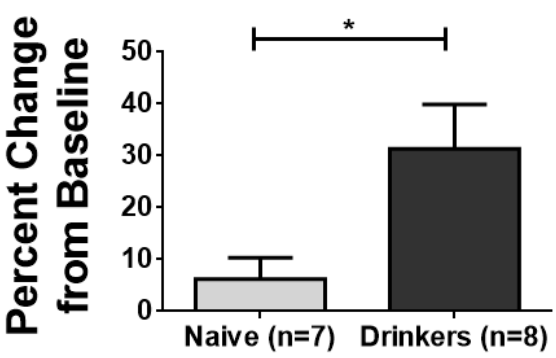

Percent Change
from Baseline
*
50
40
30
20
10
0
Naive (n=7)
Drinkers (n=8)

Figure 4.3: Adolescent Drinking Experience Enhances the Ethanol Sensitivity of Medial Shell-Projecting VTA DA Neurons. (**a**) 40X image of VTA section. Left panel, brightfield image. Middle panel, same field of view under a green filter, showing GFP-expressing (TH-containing) neurons. Right panel, same field of view under a red filter, showing retrobead-containing (NAc medial shell-projecting) neurons. (**b**) Coronal section of retrobead injected mouse. Nuclei stained in blue. Scale bar, 1mm. (**c**) Mean baseline firing rate of VTA DA neurons from drinkers (4.1 ± 1 Hz, n = 8 cells from 5 mice) does not significantly differ from those of age-matched controls that only drank water (3.1 ± 0.5 Hz, n = 7 cells from 4 mice; $t(13) = 0.86$, $p = 0.41$, two-tailed *t*-test). (**d**) Percentage changes in firing rate of VTA DA neurons in alcohol drinking and water-drinking mice. A two-tailed *t*-test reveals a significant difference in excitation induced by 50mM EtOH in DA neurons from drinking mice (31.3 ± 8.6%) compared to naïve controls (6.2 ± 4.1%; $t(13) = 2.5$, $p = 0.026$). Error bars in **c** and **d** show SEM.

CHAPTER 5

Discussion

Summary of Findings

This dissertation provides evidence that regional heterogeneity exists within the VTA in response to acute and chronic EtOH. The experiments within this dissertation were designed around one unifying question, in order to understand why considerably high concentrations, typically in the range of 50-100 mM, of EtOH are needed to produce a significant excitatory effect on VTA DA neurons *in vitro* (Brodie, 2002; Brodie et al., 1990; Didone et al., 2014; Koyama et al., 2007; Okamoto et al., 2006). Herein, we have used various electrophysiological methods to investigate the presence of regional heterogeneity in response to acute EtOH in both naïve and alcohol-experienced animals.

The data presented in Chapter 2 demonstrate evidence for regional heterogeneity within the VTA in response to acute EtOH. Specifically, we found that DA neurons within the medial VTA demonstrate significant excitation in response to EtOH, indicated by an increased firing rate from baseline. This occurs even in response to 20 mM EtOH, a concentration in that is more pharmacologically relevant than those typically tested *in vitro* (Schier et al., 2012). In contrast, all concentrations of EtOH produced a negligible effect on the firing activity of DA neurons in both the lateral VTA and SN, as well as non-DA neurons in the VTA. This demonstrates a selective effect of EtOH on a specific population of neurons in the VTA, which have, until recently, been understudied (Lammel et al., 2008; Margolis et al., 2008b), particularly within the alcohol field.

The data presented in Chapter 3 demonstrate a sensitized response of VTA DA neurons after drinking during adolescence, the period during which many individuals are vulnerable to the subsequent development of AUDs (Dawson et al., 2008; Gladwin et al., 2011). This sensitized effect was shown to be restricted to DA neurons in the medial VTA, as DA neurons in both the lateral VTA and SN show no such adaptations. The increased sensitivity to EtOH was not due to a simple increase in spontaneous activity, as baseline firing rate did not significantly differ between DA neurons from drinking and naïve mice; it was also not reflective of an increased intrinsic excitability, as baseline RMP and rheobase also did not differ between groups. While we did not exhaustively investigate the mechanism behind this phenomenon, we do report evidence that the neural adaptations that occur to cause an increased sensitivity to EtOH may involve activation of glutamate receptors; in the presence of NBQX and AP5, the increased sensitivity to EtOH was no longer observed.

The data in Chapter 4 provide more information on the subpopulations of medial VTA DA neurons by focusing on a subset of NAc medial shell-projecting medial VTA DA neurons. These were labeled by injection of retrobeads, fluorescent retrograde tracers, into the NAc medial shell. Our results suggest that NAc medial shell-projecting neurons do not display a uniformly high response to acute EtOH, although they do display an increased sensitivity after adolescent drinking. The results from this chapter do not exhaustively characterize subpopulations of VTA DA neurons, but do indicate that the enhanced EtOH sensitivity resulting from adolescent drinking occurs in a projection-specific manner. Whether the NAc medial shell is a unique target of EtOH-sensitive neurons, or whether other medial VTA neurons projecting to separate brain regions demonstrate similar experience-induced changes in sensitivity, remains to be determined.

While our results have not unambiguously defined the population of EtOH-sensitive neurons, we do find compelling evidence that heterogeneity exists within the VTA in response to EtOH applied *in vitro*. Our data are among the first to shown a significant effect of relevant concentrations of EtOH *in vitro*; we report a significant increase in firing rate in response to 10 and 20 mM EtOH, corresponding to a BAC of 0.05-0.09, within pharmacological limits readily achievable by humans (NIAAA, 2015). This is among the few studies to use voluntary intake of EtOH, as opposed to systemic passive administration of EtOH, to investigate neural adaptations that occur following "binge-like" drinking during adolescence, a time point during which the brain is still developing and individuals are particularly vulnerable to the potential of developing AUDs in adulthood (Dawson et al., 2008; Gladwin et al., 2011; Spear, 2000).

We also report a standing excitation of VTA DA neurons by transmission via NMDA receptors. While there exists evidence that blockade of NMDA receptors prevents evoked bursting of DA neurons (Deister et al., 2009), to our knowledge, inhibition of spontaneous VTA DA neuron activity by addition of NMDA receptor antagonists has not previously been reported *in vitro*. We obtained similar results using AP5, a competitive antagonist of the NMDA receptor, as well as MK801, a non-competitive antagonist of the NMDA receptor, suggesting that our *in vitro* observations are not due to off-target effects. Despite the inhibitory effect these blockers have on spontaneous activity of VTA DA neurons, addition of 50 mM EtOH did elicit an excitatory effect, similar to that produced in the absence of glutamate blockers. This indicates that the acute excitatory effects of EtOH produced *in vitro* do not involve activation of local glutamate receptors.

While the acute effects of EtOH do not involve local glutamate receptors, the enhanced sensitivity of VTA DA neurons observed after adolescent drinking is no longer observed in the

presence of AMPA and NMDA receptor blockade. This indicates that these receptors may be necessary for expression of this enhanced response. These results agree with previous experiments in rats, demonstrating an increase in AMPA:NMDA ratio after voluntary drinking (Stuber et al., 2008a), a phenomenon associated with enhanced reinforcing and activating effects of drugs of abuse (Carlezon & Nestler, 2002). Presynaptic release dynamics were found to be unaltered by drinking, indicated by a lack of change in the paired pulse ratio of VTA DA neurons. Postsynaptic effects were implicated, however; increased amplitude and frequency of miniature excitatory postsynaptic currents (mEPSCs) were observed in VTA DA neurons of drinking rats compared to controls. Together with our data, this could suggest that adolescent drinking results in postsynaptic adaptations in the glutamatergic system in the VTA, eliciting an enhanced sensitivity of medial VTA DA neurons to EtOH. However, it is possible that, rather than decreasing the enhanced response of VTA DA neurons of adolescent drinkers, NBQX and AP5 application results in an increased EtOH response of neurons from naïve controls. We emphasize that more research is needed in order to better understand the involvement of glutamate receptors in the development of this sensitization.

Potential Pitfalls and Limitations

The experiments described in this text do contain some limitations, discussed herein. In our cell-attached recordings, we selected for spontaneously active VTA DA neurons, with inactive neurons excluded. Because the information gathered using cell-attached recordings is limited to firing activity, the researcher is unable to conclusively determine whether inactivity is due to poor health of a neuron, or whether a silent neuron is healthy and simply quiescent under baseline conditions. We acknowledge this bias, which may have ignored a potentially important subset of quiescent VTA DA neurons. Using another electrophysiological technique, such as

whole cell or perforated patch recordings, would allow the researcher to better assess the health of a cell and determine whether quiescent VTA DA neurons exhibit a meaningful response to EtOH applied *in vitro*.

In Chapters 2-4, experiments were performed exclusively in male mice. Because we sought to perform a survey of a large number of neurons within a heterogeneous system, we attempted to eliminate any additional potential confounding source of heterogeneity, thus including results from neurons obtained only from male mice. Sex differences in mesolimbic DA activity, including influence of inhibitory stimuli (Melis et al., 2013), as well as differences in drinking behavior, such as EtOH intake (Schramm-Sapyta et al., 2014), response to social isolation (Varlinskaya et al., 2015), and adaptations after adolescent drinking (Varlinskaya et al., 2014), have been demonstrated. Therefore, future experiments should be repeated in female mice, to determine whether sex differences exist in this context.

In Chapters 3 and 4, subjects were singly housed for the duration of the intermittent access procedure. It is possible that social isolation of the subjects used in this our experiments, which is necessary to record individual drinking behavior, influenced our results. Indeed, social isolation has been shown to affect alcohol intake (Chappell et al., 2013; Skelly et al., 2015; Varlinskaya et al., 2015) and affect synaptic plasticity in the VTA (Whitaker et al., 2013), although recent research suggests that this may not be the case when mice are provided mild enrichment, as in this study (e.g. cotton nestlets; Lopez & Laber, 2015). Note, however, that naïve and drinking animals were housed in identical conditions, indicating that enhanced EtOH sensitivity is not simply due to social conditions.

In Chapter 4, mice underwent surgery five days before beginning the intermittent access procedure. This procedure could possibly produce confounding effects. The retrobeads are non-

toxic and should not damage neurons (Schofield, 2008). However, the stress of undergoing the surgical procedure may affect drinking behavior. Control mice did undergo the same treatment as the ethanol-drinking mice, which controlled for this issue.

Finally, it is important to discuss the limitations of using a mouse model and extrapolating our findings to humans. While the mouse serves as a reasonable system for probing EtOH's effects on the mesolimbic system (discussed in more detail in the Introduction section), caution must be taken when applying the results found in these studies to broader implications on humans. Human brains are more complex than rodents; while a human brain may contain approximately 86 billion neurons, a mouse brain contains approximately 0.1% of that number, with 71 million neurons (Herculano-Houzel, 2009). It is therefore reasonable to assume that EtOH could produce different effects in a more complicated network than those observed in the mouse.

Further, while the focus of this dissertation is on the initial effects of EtOH, and not on EtOH dependence, it is worth mentioning that several components of alcohol use, including AUDs, are too complex to sufficiently model in rodents. For example, some of the conditions included in DSM-5 that are present in an individual with AUDs can be modeled in a rodent, such as "In the past year, have you found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness…or a seizure?" (modeled with symptoms of physical withdrawal, assessed by HIC tests), or "Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?" (modeled loosely by demonstrating a preference for EtOH over other rewarding stimuli, such as sucrose or social interaction). Many of these conditions, however, cannot be modeled in any non-human study, such as "In the past year, have you more than once wanted to cut down or stop drinking,

or tried to, but couldn't?" We acknowledge that many facets of alcohol use are more complicated in a human than in a mouse and are cautious about applying our findings to explain human behavior. We do stress the similarities between the human and the mouse, both in adolescent EtOH intake behavior and effects of EtOH on the mesolimbic system (see Introduction), which indicate that our findings may prove relevant to human EtOH intake. Future experiments could replicate our findings in non-human primates, or with imaging studies in humans, to determine whether a similar regional heterogeneity exists within the VTA.

Future Directions

We have not identified the acute mechanism of action of EtOH *in vitro*, although we have demonstrated that modulation of glutamate receptor transmission is not necessary for EtOH-induced excitation of VTA DA neurons. Future research to elucidate this mechanism could involve similar experiments in the presence other synaptic blockers, to determine whether EtOH does exert a modulatory effect, perhaps by inhibiting local GABAergic transmission. There is evidence to suggest that acute EtOH can decrease GABAergic synaptic transmission onto VTA DA neurons (Xiao & Ye, 2008), which would increase DA neuron activity. However, conflicting evidence has also been reported; for example, acute EtOH has also been shown to enhance GABAergic transmission onto VTA DA neurons (Theile et al., 2008). Further research is necessary to thoroughly investigate this mechanism.

Though we have not identified molecular targets of EtOH in this study, we may speculate on potential targets within the midbrain. In addition to a modulatory effect, EtOH could act directly on DA neurons to elicit increased activity. Intrinsic targets could include voltage-gated ion channels which regulate the firing rate of VTA DA neurons. An excitatory effect on these pacemaker cells could be produced by increasing the conductance of a depolarizing channel (Na^+

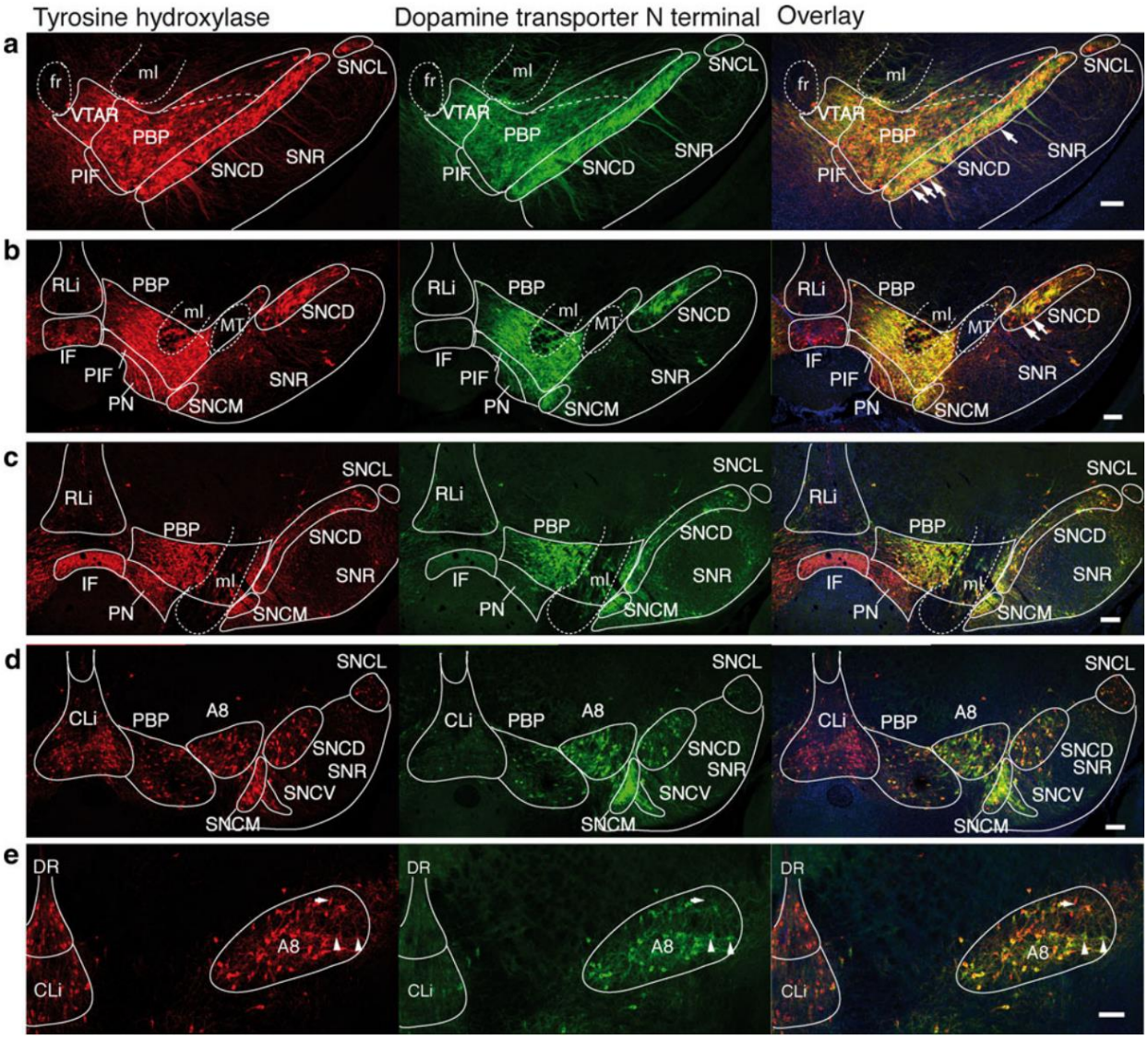
or Ca^{2+}), or decreasing the conductance of a hyperpolarizing channel (K^+). Targets that have been proposed by other labs include K^+ channels, including those responsible for I_h (Okamoto et al., 2006) and M-currents (Koyama et al., 2007), although the effects of EtOH on these currents were only observed in "traditional" DA neurons and in response to high EtOH concentrations. Additionally, expression of these potassium channels is lower in the medial VTA compared to the lateral VTA, making it unlikely that these are the primary targets of EtOH (Cooper et al., 2001; Margolis et al., 2006).

Another strategy to identify potential EtOH targets is to survey expression patterns of receptors located within the midbrain, as our evidence indicates that medial VTA DA neurons demonstrate enhanced sensitivity to EtOH compared to DA neurons in the lateral VTA or SN. Utilizing the data available in the Allen Brain Atlas to locate genes with greater expression in the medial VTA, we have identified one potential target for the purpose of this discussion. The expression of *Tacr3*, the gene which encodes tachykinin receptor 3 (NK3R), is largely limited to the medial VTA (Supplementary Figure 5). NK3Rs are found on both DAergic and GABAergic VTA neurons (Lessard et al., 2007), particularly those projecting to the mPFC and NAc (Lessard et al., 2009). These receptors have been shown to influence VTA DA neuron tonic activity; NK3R agonists increase DA neuron firing (Alonso et al., 1996). Further, activation of these receptors has been shown to influence voluntary EtOH intake (Ciccocioppo et al., 1998; Ciccocioppo et al., 1994; Slawecki & Roth, 2003). While no experiments have directly demonstrated that EtOH acts on NK3Rs to increase DA neuron activity, this is an interesting potential target which could be investigated in future experiments.

The results presented in Chapter 3 demonstrate evidence for sensitization of VTA DA neuron activity after drinking during adolescence. This is due to an increased sensitivity to the

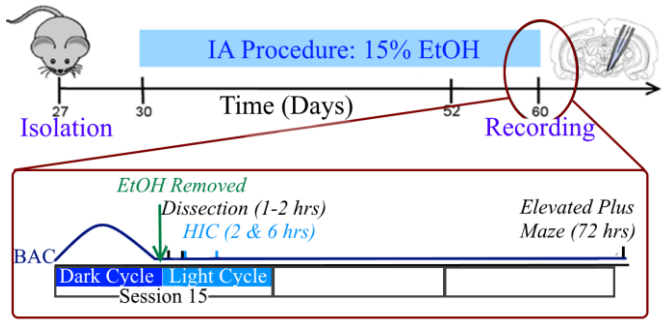
primary reinforcer itself, which to our knowledge provides a novel mechanism that may underlie the development of self-administration that has clear implications for the development of drug dependence. While we focused on effects of learning during adolescence (p30-p60), future research may focus on determining the most sensitive population of DA neurons to EtOH during different periods of maturation and uncovering the elements of these cells that dictate response to EtOH. In addition, whether the neural adaptions we observe are stable, and for how long these changes persist after withdrawal from EtOH, remain to be determined.

Finally, while we have demonstrated that medial VTA DA neurons projecting to the NAc medial shell exhibit an increased sensitivity to 50 mM EtOH after adolescent drinking, we have not exhaustively characterized the EtOH-sensitive population of medial VTA DA neurons. Future experiments to systematically compare subpopulations of medial VTA DA neurons based on projection target would reveal whether this is, indeed, a common characteristic of EtOH-responsive neurons. If EtOH response is not projection-specific, more thorough methods of post-hoc cellular characterization could be employed. For example, cells collected after electrophysiological recordings could be processed for single-cell RT-PCR, to determine whether EtOH-responsive neurons express common markers. The results of these experiments are important in understanding what aspects underlie EtOH sensitivity, both to better elucidate EtOH's acute mechanisms, as well as to potentially understand individual variability in EtOH response and susceptibility to EtOH abuse.

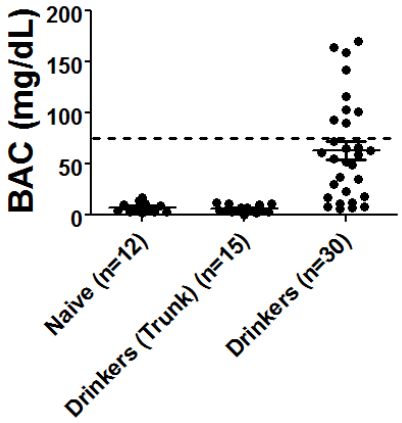

Tyrosine hydroxylase
Dopamine transporter N terminal
Overlay
a
b
c
d
e
fr
ml
VTAR
PBP
PIF
SNCL
SNCD
SNR
RLi
MT
IF
PN
SNCM
CLi
A8
SNCV
DR

Supplementary Figure 1: Architecture of the VTA in a Mouse Coronal Section. Figure reproduced from Fu et al., 2012. Co-localization of DAT and TH in A8 (retrorubral field), A9 (SN), and A10 (VTA) regions shown. Distribution of the TH shown in red (left panels) and DAT in green (middle panels), along with the overlay images (right panels). The panels **a**–**e** are arranged along the rostro-caudal axis. Positions of subregions of the VTA and SN indicated. CLi, caudal linear nucleus of the raphe; DR, dorsal raphe nucleus; fr, fasiculus retroflexus; IF, interfascicular nucleus; ml, medial lemniscus; PBP, parabrachial pigmented nucleus; PIF, parainterfascicular nucleus; PN, paranigral nucleus; RLi, rostral linear nucleus; SNCD, substantia nigra pars compacta dorsal tier; SNCL, substantia nigra pars compacta lateral tier; SNCM, substantia nigra pars compacta medial tier; SNCV, substantia nigra pars compacta ventral tier; SNR, substantia nigra pars reticulata; VTAR, rostral part of the VTA. Scale bar, 100 μm.

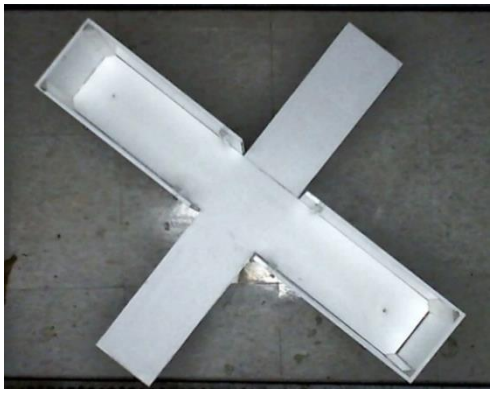
a


IA Procedure: 15% EtOH
27
30
Time (Days)
52
60
Isolation
Recording
EtOH Removed
Dissection (1-2 hrs)
HIC (2 & 6 hrs)
Elevated Plus
Maze (72 hrs)
BAC
Dark Cycle
Light Cycle
Session 15

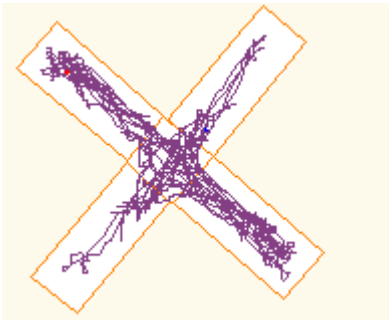
b


BAC (mg/dL)
200
150
100
50
0
Naive (n=12)
Drinkers (Trunk) (n=15)
Drinkers (n=30)

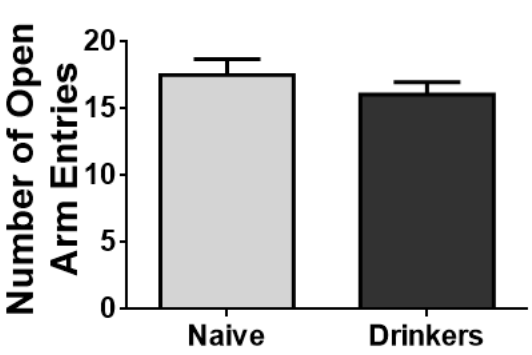
c



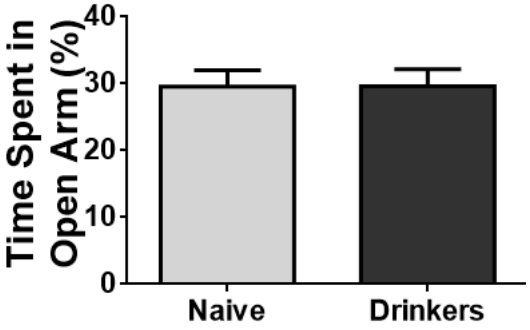
d



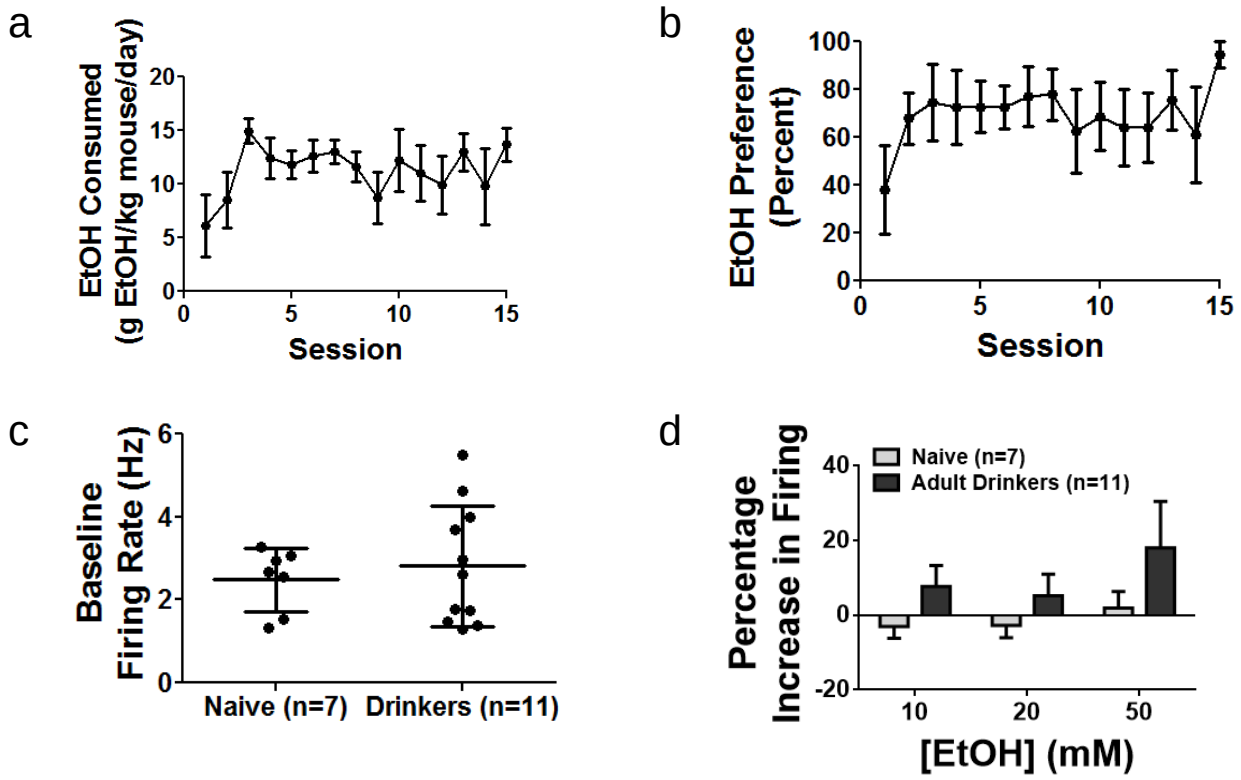
e


Number of Open Arm Entries
20
15
10
5
0
Naive
Drinkers

f


Time Spent in Open Arm (%)
40
30
20
10
0
Naive
Drinkers

Supplementary Figure 2: Intermittent Access (IA) Model Produces does not Produce Enhanced Anxiety Phenotype in Adolescent Mice. (**a**) Timeline of experimental procedures. IA procedure begins at age p30 and runs for 15 drinking sessions. Dissections for electrophysiological recordings occur 1-2 hours after removal of EtOH at the end of the 15th and final drinking session; trunk blood is collected at this time to ensure no detectable BACs. HIC tests (see Figure 3.1e) occur at 2 and 6 hours after EtOH removal, corresponding to approximately 8 and 12 hours after peak BAC, respectively. Elevated plus maze (EPM) experiments occur 72 hours after EtOH removal. (**b**) Blood alcohol concentration (BAC) values measured in EtOH-naïve controls ("Naive"), EtOH drinkers at the time of dissection ("Drinkers (Trunk)"), compared to peak BACs measured in EtOH drinkers 6 hours into the 11th drinking session ("Drinkers"; Figure 3.1d). (**c**) EPM apparatus is an elevated platform consisting of two closed arms and two open arms. (**d**) Sample locomotor behavior of an individual mouse over five minutes in the EPM. (**e**) Total number of open arm entries does not differ between a group of 16 EtOH drinkers and 15 controls. (**f**) Total amount of time spent in the open arms does not differ between EtOH drinkers and controls. Error bars in **e** and **f** show SEM.

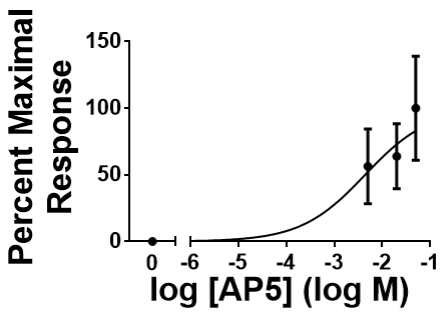


Supplementary Figure 3: Drinking During Adulthood Does Not Result in Enhanced Sensitivity of VTA DA Neurons to Ethanol. (**a**) Average ethanol intake per session for a group of 4 mice. (**b**) Preference for ethanol over water. (**c**) Mean baseline firing rate of VTA DA neurons from drinkers (2.8 ± 0.4 Hz, n = 11 cells from 4 mice) does not significantly differ from those of age-matched controls that only drank water (2.5 ± 0.3 Hz, n = 7 cells from 4 mice; $p = 0.6$, two-tailed *t*-test). (**d**) Percentage changes in firing rate of VTA DA neurons in alcohol drinking and water-drinking mice. Two-tailed *t*-tests reveal no significant difference in excitation induced by EtOH in DA neurons from drinking mice compared to naïve controls. Error bars show SEM.

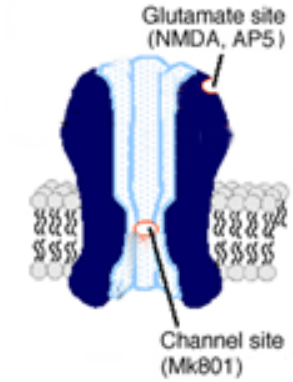
	Naïve	EtOH	*p* value
Baseline FR	2.5 ± 0.3	2.8 ± 0.4	0.6
ISI CV	0.13 ± 0.03	0.25 ± 0.06	0.15
10mM	-3.0 ± 3.1	7.7 ± 5.6	0.17
20mM	-2.7 ± 3.3	5.2 ± 5.8	0.32
50mM	1.9 ± 4.4	18.0 ± 12.5	0.33

Supplementary Table 1: Ethanol-induced Excitation of Midbrain DA Neurons from Adult Drinkers. Values presented are percent change in firing rate in response to 10, 20, and 50mM EtOH ± SEM. Two tailed *t*-tests were used to compare the response of VTA DA neurons from naïve and experienced mice. Baseline firing rates and ISI CV were compared using two-tailed *t*-tests.

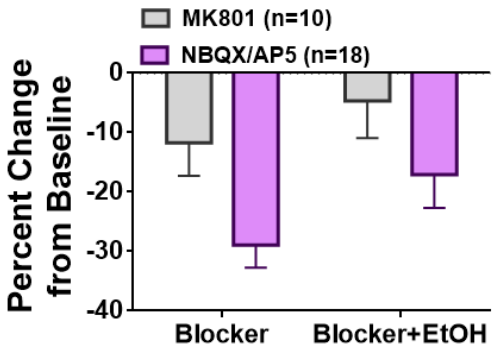
a


Percent Maximal Response
150
100
50
0
0
-6
-5
-4
-3
-2
-1
log [AP5] (log M)

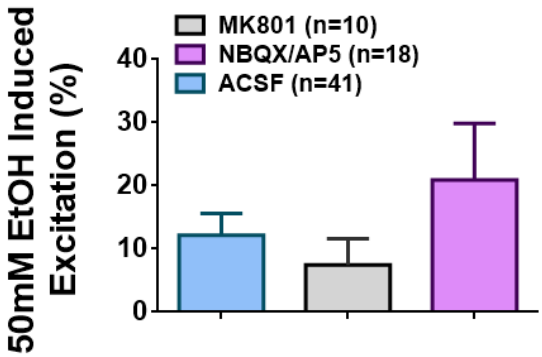
b


Glutamate site
(NMDA, AP5)
Channel site
(Mk801)

c

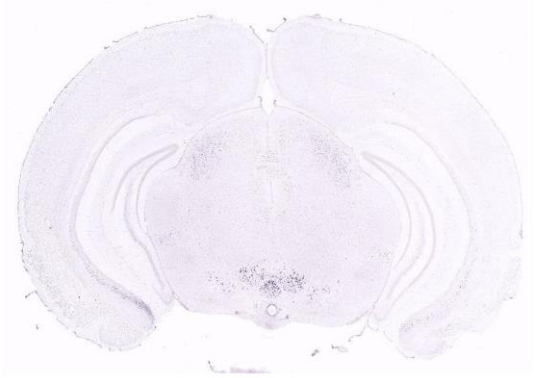

MK801 (n=10)
NBQX/AP5 (n=18)
Percent Change from Baseline
0
-10
-20
-30
-40
Blocker
Blocker+EtOH

d

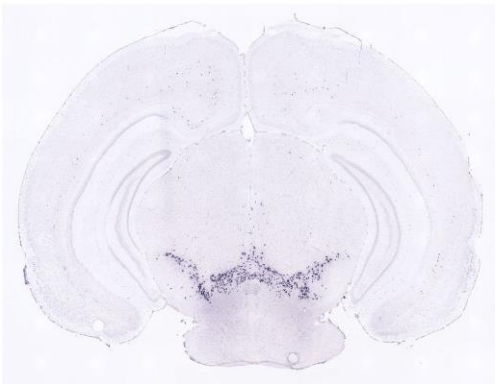

MK801 (n=10)
NBQX/AP5 (n=18)
ACSF (n=41)
50mM EtOH Induced Excitation (%)
40
30
20
10
0

Supplementary Figure 4: NMDA Receptor Antagonists Have Similar Effects on VTA DA Neuron Activity. (**a**) Preliminary dose-response curve for AP5 (n = 4-9 cells per concentration). EC50 is calculated as 4.4 µM. (**b**) Cartoon of NMDAR depicting binding site for AP5, which competes for binding at the glutamate binding site, and MK801, which binds within the pore. Figure adapted from Magnusson, 1998. (**c**) Summary of percent change in firing rate from baseline in response to 10µM MK801 (-11.7 ± 5.5%), 10µM NBQX/50µM AP5 (-29 ± 3.7%), 10µM MK801/50mM EtOH (-4.7 ± 6.3%), and 10µM NBQX/50µM AP5/50mM EtOH (-17.1 ± 5.6%). (**d**) Comparison of increase in firing rate induced by 50mM EtOH in the absence ("ACSF"; 12.2 ± 3.5%) and presence of glutamatergic blockers (20.9 ± 9% NBQX/AP5; 7.5 ± 4.1% MK801). Data collected in neurons from naïve mice. Error bars in **a, c,** and **d** show SEM.

a



b



Supplementary Figure 5: Expression of *Tacr3* is Largely Restricted to the Medial VTA. (**a**) Expression pattern of *Tacr3*, the gene encoding tachykinin receptor 3 (NK3R) in a coronal midbrain section. Image taken from the Allen Brain Atlas. (**b**) Expression pattern of *Th*, the gene encoding tyrosine hydroxylase, is shown for comparison. This gene is expressed throughout the VTA and SN. Image taken from the Allen Brain Atlas.

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