Differentially Modulating the VTA:

A Role for δ-mediated $GABA_A$ Tonic Inhibition

and the Stress Neuropeptide CRF during Ethanol Intake

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Abstract

Binge drinking is a prevalent form of short-term alcohol consumption resulting in blood alcohol levels of 0.08 g/dl. Although more frequent in men, binge drinking has become increasingly common in women. To this end, sex-specific changes are evident in underlying mesocorticolimbic circuitry, including the modulation of GABAergic inhibitory neurotransmission. Broadly speaking, extrasynaptic $GABA_A$ receptors ($GABA_ARs$) incorporating the δ subunit mediate tonic inhibition, while synaptic γ2-containing $GABA_ARs$ underlie fast, phasic inhibition. $GABA_ARs$ incorporating the δ-subunit-containing are sensitive to the low-to-moderate alcohol doses typically found in binge drinking. Critically, they can also be modulated by ovarian-derived hormones, indicating possible sex-specific differences in δ-mediated inhibition. Given this, it was hypothesized that δ-subunit-containing $GABA_ARs$ would play a sex-specific role in male and female binge-like drinking in a region critical to mesocorticolimbic circuitry—the ventral tegmental area (VTA). Initial qPCR results revealed a nearly two-fold increase in *Gabrd* transcript levels in female VTA relative to males, but no sex-specific *Gabrg2* differences. These baseline differences were also functionally evident, with increased levels of tonic inhibition in female VTA. To determine if *Gabrd* removal would sex-specifically alter binge-like drinking, both male and female floxed *Gabrd* and floxed *Gabrg2* mice were given bilateral injections into the VTA of either AAV-Cre-GFP or AAV-GFP for Cre-mediated *Gabrd* or *Gabrg2* excision. Mice were subjected to one cycle of a binge-like drinking protocol, with results indicating that female subjects with VTA-specific δ-excision had decreased binge-like alcohol intake. δ-excision had no significant effect on male binge-like drinking and there were no differences in binge-like intake in

either male or female γ2-excised subjects. While the posterior VTA had the highest viral reporter expression and is also known to contain a population of GABAergic interneurons, later cell-type-specific analysis revealed no sex-specific difference in parvalbumin-positive interneurons/δ subunit colocalization. Although these results demonstrate increased baseline δ-mediated tonic inhibition in the VTA of females is asymmetrically important in low-to-moderate binge-like drinking, a better understanding of underlying cell type will be the focus of future work.

To M.

You've put up with a lot and I owe you the world for your patience.

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List of Abbreviations

Neuroanatomy & Neurobiology
VTA, Ventral tegmental area
aVTA, Anterior ventral tegmental area
pVTA, Posterior ventral tegmental area
IF, Interfascicular nucleus
PN, Paranigral nucleus
PBP, Parabrachial nucleus
tVTA or **RMTg,** Tail of the VTA
NAc, Nucleus accumbens
SNr, Substantia nigra (including compact and reticular parts)
IPN, Interpeduncular nucleus
MM, Medial mammillary nucleus of the hypothalamus
PAG, Periaqueductal gray
PFC, Prefrontal cortex
mPFC, Medial prefrontal cortex
MSN, Medium spiny neuron
PV, Parvalbumin
HPA, Hypothalamic-pituitary-adrenal (axis)
PVN, Paraventricular nucleus of the hypothalamus

Pharmacology & Neurochemistry
GABA, γ-amino butyric acid, neurotransmitter
$GABA_A$, γ-amino butyric acid, type A receptor
$GABA_AR$, γ-amino butyric acid, type A receptor
DA, Dopamine
DAergic, Dopamine-producing neuron
THIP, 4,5,6,7-tetrahydroisoxazolo(5,4,c)pyridin-3-ol, also known as Gaboxadol
SR95531, 2-(3-Carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide, also known as Gabazine
TH, Tyrosine hydroxylase
ACTH, Adrenocorticotropic hormone
CRF, Corticotropin-releasing factor (also known as corticotropin-releasing hormone, or CRH)
CRFR1, Corticotropin-releasing factor receptor-type 1
CRFR2, Corticotropin-releasing factor receptor-type 2
CNO, Clozapine-*n*-oxide

Methods
DID, Drinking in the Dark, binge-like protocol
SHAC, Scheduled High Alcohol Consumption
IAA, Intermittent alcohol access
AAV, Adeno-associated virus
eGFP, Enhanced green-fluorescence protein
BEC, Blood ethanol concentration

CMV, Cytomegalovirus, general and non-cell-type-specific promoter
hSyn, Human synapsin 1, neuron-specific promoter
GC, Genome copies, used for viral titer reporting
PCR, Polymerase chain reaction
RT-PCR, Real-time polymerase chain reaction
qPCR, Quantitative polymerase chain reaction
C_q, Quantification cycle, for use in qPCR analysis
PBS, Phosphate-buffered saline
NGS, Normal goat serum
sIPSC, Spontaneous inhibitory post-synaptic currents
mIPSC, Miniature inhibitory post-synaptic currents
nACSF, Normal artificial cerebral spinal flui

Chapter 1
Introduction

Why study alcohol?

Alcohol consumption is a historically, societally, and socially common phenomenon. Despite the impact of religious and cultural norms on whether drinking is acceptable (*e.g.* Prohibition in the 1920s in the U.S., religious practitioners such as Baptists and Muslims), it remains one of the most commonly used—and abused—drugs in the world (Gowing *et al.* 2015). Intertwined with commonality are consequences, with alcohol use, abuse, and dependence providing no exception. To this end, data from the World Health Organization Global Status Report on Alcohol in 2004 revealed that alcohol was responsible for 3-8% of all global deaths (*e.g.* chronic illness, alcohol-related accident or injury, Rehm *et al.* 2009). The economic burdens of alcohol are no less severe, with an estimated $223.5 billion in costs stemming from excessive drinking in 2006 in the U.S. alone (Bouchery *et al.* 2011). Disturbingly, this is only another data point in an ever-increasing trend. The economic burden for alcohol in 1992 was estimated to be $148 billion (Harwood, Fountain & Livermore 1998), while in 1998 it rose to $184.6 billion (Harwood 2000). Global figures notwithstanding, the upward trajectory of the damages wrought by alcohol in the U.S. alone is hard to ignore.

1.1 Binge Drinking

While excessive drinking is the cause of a vast proportion of the economic burden resulting from alcohol consumption, other forms (*e.g.* underage drinking, drinking during pregnancy, and binge drinking) are also consequential. In particular, binge drinking was

found to have cost the U.S. approximately $170.7 billion in 2006—only 25% less than the economic costs of excessive drinking (Bochery *et al.* 2011). But what is binge drinking, exactly?

Historically, binge drinking has not been defined as it is today—that being short-term bouts of excessive alcohol consumption in individuals who may (or may not) be dependent on alcohol. In fact, it had not even been classified as a separate pattern of drinking behavior from heavy (*i.e.* dependent) drinking until fairly recently. Early work investigating clinical drinking patterns classified drinking 'binges' as those engaged in by already-dependent individuals. For instance, 'sustained-binge type patterns' (as compared to continuous drinking, see Wanberg 1969) was one pattern of behavior defined and described in alcoholics. Including binge drinking as a sub-class of drinking behavior that fell under the umbrella of alcoholism extended into the 1970s. Clinical research throughout this period continued to assess binge drinking only in alcoholics, where it was defined as "…staying high or drunk for more than one day at a time…" (Clark & Cahaln 1976; Pierce *et al.* 1976). In other words, binge drinking was defined as excessive—but measured in days rather than hours, and solely in alcoholics rather than in non-dependent individuals.

Defining binge drinking as a pattern of excessive consumption on a longer temporal scale—that is, long-term 'sprees' that were embedded in a larger pattern of excessive drinking in alcoholics—started to shift in the mid-1980s through 1990s. Risk factor surveys from the 1980s began to ask questions regarding binge drinking—specifically, whether the individual had consumed five or more drinks in a single sitting (Goldbaum *et al.* 1986;

Anda *et al.* 1990). Critically, these surveys separated binge drinking apart from categories defining the type of heavy (excessive), continuous drinking seen in dependent drinkers.

While clinical and public health research in the 1980s dissociated binge drinkers from dependent drinkers, work in the 1990s also began to shift from the adult population at large to questions specifically concerning binge drinking and young adults. Within this framework, binge drinking maintained its definition of five drinks in one sitting, but was juxtaposed against the backdrop of the increasingly prevalent college lifestyle of young adults. No different from now, binge drinking was considered to be extremely problematic and commonplace, with approximately 40% of adults 18-22 years of age engaged in it (Wechsler *et al.* 1998; SAMHSA 2015). Even more concerning, frequent binge drinkers comprised nearly 20% of those college students engaged in binge drinking (Wechsler *et al.* 1994). Importantly, the intermittency between large bouts of drinking began to be examined as a clinical component to alcoholism. The concern was binge drinking in and of itself might not be problematic, but that frequent, intermittent bouts of binge drinking would later progress to problem drinking and dependence. To this end, individuals who self-reported difficulty controlling their binge drinking were often on their way to developing alcoholism (Schuckit *et al.* 1995).

Given both the prevalence of binge drinking in certain adult populations as well as its relationship to later alcoholism in a subset of this population, developing a formal, quantitative definition for binge drinking became increasingly important. The qualitative measure of 'five drinks in a sitting' begun in the early- to mid-1980s (O'Malley, Machman

& Johnston 1984; Goldbaum *et al.* 1986) required further refinement. Early work by local chapters of Mothers Against Drunk Driving sparked national attention to the dangers of binge drinking as well as the need for stricter measures of acceptable blood alcohol concentrations (BAC, Hingson, Howland & Levenson 1988). A formal definition was finally reached by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) in the early 2000s, which stated binge drinking was a 'pattern of drinking that brings BAC levels to 0.08 g/dL' (NIAAA 2004). Using this definition in conjunction with more recent research and it becomes apparent that the many measures of who is binge drinking—and what those consequences are—have not changed. According to the 2015 National Survey on Drug Use and Health, 37.9% of college students aged 18-22 and 32.6% of non-college adults of the same age engaged in binge drinking in the past month (SAMHSA 2015). This is hardly different from the approximately 40% seen in the early 1990s (Wechsler *et al.* 1998). Moreover, evidence for the relationship between binge drinking and dependence has only strengthened (Jennison 2004).

It should be noted that while binge drinking has been largely studied in young adult populations (*e.g.* early- to mid-twenties), it is very common in other populations of adults. To this end, recent work examining the prevalence of binge drinking in adults in the US found that 69% of binge drinking episodes occurred in adults over the age of 26 (Naimi *et al.* 2003). Binge drinking is also found in middle- to older adults, but the occurrence drops significantly with increasing age (*e.g.* 20% of male respondents 50+ years of age and 6% of women respondents 50+ years of age, see Blazer & Wu 2009).

1.2 Sex Differences in Male and Female Binge Drinking

Until this point, the difference between male and female binge drinking has gone without distinction. Once a working definition of binge drinking as 'five drinks in one sitting' had been decided in the 1980s, further refinement was needed to take into account female binge drinkers. This was because female physiology—specifically differences in body mass and metabolism—resulted in higher BAC levels than men for the same amount of alcohol (Wechsler *et al.* 1995). As a result, it shifted to a five/four rule: Five drinks for men and four for women in one sitting. The current NIAAA definition of binge drinking avoids this inherent difference in body mass and metabolism by using a standard, BAC measure to define binge drinking (NIAAA 2004).

Definitional modifications aside, there are also many clinical differences that exist between the male and female binge drinkers. Historically and consistently, males have shown two- to three-times higher rates of binge drinking relative to females (Anda *et al.* 1990; Naimi *et al.* 2003). Males also show similarly elevated rates of alcohol dependence relative to women (20.1% and 8.2% respectively, see SAMHSA 2005). This difference might seem nothing more than a footnote, as men seem to have much higher problems with binge drinking—and alcohol in general—than women. However, two points belie this conclusion and underscore the differences between male and female binge drinkers. First, comparing data from the 1990s (Naimi *et al.* 2003) with more recent work from 2002-2012 has revealed the women are binge drinking at increasing rates (White *et al.* 2015). Not only is this convergence apparent in the rates of female and male binge drinking (Zilberman,

Tavares & El-Guebaly 2004; White *et al.* 2015), but it is seen in younger generational cohort relative to older ones (Keyes, Grant & Hasin 2008).

Such convergence is at least partly due to sociocultural factors (*e.g.* changing role of women in society). However, there remains the second point, which underscores more firmly the problem with female binge drinking: Their clinical trajectory. Unlike male drinkers, female drinkers have a shorter time frame from first drink to dependence—a phenomenon known as telescoping (Orford & Keddie 1985; Zilberman, Tavares & El-Guebaly 2004). While telescoping has been more convincingly shown with other drugs of abuse (*e.g.* cocaine, Kosten *et al.* 1993), this markedly shorter rate from first use to dependence indicates potential differences in how alcohol affects the underlying reward-relevant neurocircuitry between men and women. Given the role binge drinking plays in propelling a subset of individuals towards dependence, it suggests that binging—in particular—could differentially affect male and female reward-relevant neurocircuitry.

1.3 Rodent Models of Binge-like Drinking

As binge drinking began to be separated and defined differently from the intermittent, high-volume patterns of consumption found in alcoholics, questions regarding underlying neurocircuitry became more important. Concurrent with this separation, some of the first preclinical, rodent models of binge-like alcohol consumption were developed in the 1980s and 1990s. Initially, gavage-based, binge drinking models were developed solely to induce high BACs in pregnant animals like primates and rats (Clarren & Bowden 1982; Kelly, Pierce & West 1987). This was in large part due to the clinical classification of fetal alcohol

syndrome (FAS) in 1973 (Jones & Smith 1973) and the subsequent desire to better understand the craniofacial and central nervous system problems that developed from high alcohol intake during pregnancy.

However, these binge models needed to change if they were to account for (1) non-pregnant individuals and (2) the shorter-term access that was becoming the standard definition of a binge. In the 1990s, a new model of short-term binge drinking was developed for use in understanding the relationship between acute alcohol exposure and the immune system (Wu, Wolcott & Pruett 1994; Han & Pruett 1995; Carson & Pruett 1996). As previous work in this field had examined the effects of long-term alcohol exposure on immune function, researchers specifically sought to develop a short-term binge model. As such, they began by using gavage administration in mice to mimic the short-term, high alcohol doses seen in clinical populations. Measured BACs at selected time points after alcohol administration indicated that animals had BACs of approximately 0.15% 1 h after gavage of 20% alcohol (3 g/kg) and significantly higher levels after higher doses (*e.g.* 0.5%, 1 h after 7 g/kg gavage). It should be noted that while these values were significantly higher than the standard binge drinking BAC level set forth by the NIAAA in 2004 (0.08 g/dL, or 0.08%), later work with this model focused on lower gavage doses for binge drinking (*e.g.* 1 g/kg to 3 g/kg corresponding to 0.07 to 0.17%, Pruett, Collier & Wu 1998).

One of the major limitations of the protocol developed by Carson & Pruett (1996) was its involuntary nature. This was not an oversight, but merely an acknowledgement of the difficulty in getting rodents to voluntarily consume large quantities of alcohol (*e.g.* use of

sucrose fading, see Samson 1986 and Roberts *et al.* 1999; use of water restriction, see Middaugh *et al.* 1999) to reach pharmacologically relevant BAC levels (*i.e.* to intoxication). Gavage is an effective—yet forceful—introduction of alcohol and does not carry much face validity for the actual human act of binge drinking. Given this, the early 2000s saw the development of a series of voluntary access rodent model of binge drinking that induced high BACs (Scheduled High Alcohol Consumption, SHAC, Finn *et al.* 2005; Drinking in the Dark, DID, Rhodes *et al.* 2005; Sharpe, Tsivkovskaia & Ryabinin 2005). The levels approximated using both SHAC and DID saw mice consuming greater than 1 mg/mL (10 mg/dL), which corresponded to 2 g/kg consumed over 30 min for the former or 7 g/kg to 9 g/kg of alcohol consumed during a 4 h 'binge' session in the latter. While this is objectively above the limit set for by the NIAAA as the threshold for binge drinking (0.08%, or 8 mg/dL, NIAAA 2004), the high metabolic rate for mice relative to humans demanded a different—but functionally comparable—level (Crabbe, Harris & Koob 2011; Leeman *et al.* 2010; Crabbe *et al.* 2009).

Of the models developed in the mid-2000s, Rhodes' and colleagues' approach allowed for the shortest amount of time to engender pharmacologically relevant levels of binge-like drinking. To do this, they timed the introduction of a single bottle containing 20% alcohol to the natural light/dark cycle of the animal. An initial experiment revealed no g/kg differences across three different concentrations of ethanol (10%, 20%, and 30%), resulting in the use of 20% ethanol (Rhodes *et al.* 2005). Since rodents had been shown to consume more during their dark cycle (Gill, France & Amit 1986), they were able to exploit this difference in intake patterns to voluntarily induce high levels of drinking. They also limited

access to 2 h on the first three days, which was then doubled to 4 h on the final 'binge' day (Rhodes *et al.* 2005; Rhodes *et al.* 2007). It should be noted that limited alcohol access is not unknown in models of alcohol consumption. Limited (*i.e.* not continuous) access has been used and manipulated to increase alcohol consumption, both prior to the development of the Rhodes protocol (Murphy *et al.* 1986) as well as after (Hwa *et al.* 2011; Melendez 2011).

1.4 C57BL/6J Mouse Strain and Binge Drinking

The C57Bl6/J mouse strain has been a hallmark of alcohol research for decades, largely stemming from its known high preference for alcohol. As such, C57Bl6/J mice voluntarily drink without added experimental manipulations (*e.g.* sucrose fading, water restriction). Early work to this effect found that C57Bl6/J mice had a nearly 80% preference for alcohol when using a two-bottle choice procedure with water and 10% ethanol (Rodgers 1966; McLearn & Rodgers 1959) and later work has confirmed the high ethanol preference in C57Bl6/J mice relative to other strains (Yoneyama *et al.* 2008; Belknap, Crabbe & Young 1993). While the initial Rhodes binge-like drinking protocol used only C57Bl6/J and DBA2/J mice (2005), a later analysis using 12 different inbred mouse strains revealed that this protocol engendered the highest level of binge-like drinking in C57Bl6/J mice (Rhodes *et al.* 2007). Given this, C57Bl6/J mice have remained the standard mouse line used for preclinical modeling of binge-like drinking.

It is important to mention that C57Bl6/J mice are not the only rodent strain that preferentially consume high levels of alcohol—for instance, selective breeding for alcohol

preferring mice and rats began several decades ago (Erwin, McClearn & Kuse 1980) and has resulted in other high alcohol preferring lines (*e.g.* High Alcohol Preferring, HAP and Sardinian Alcohol Preferring rat lines, among others; for review, see Grahame, Li & Lumeng 1999). However, the short-term binge-like drinking protocol DID was developed using the C57Bl6/J mouse line and will be the strain of focus for the work presented here.

1.5 Sex Differences in Rodent Models of Binge-like Drinking

There are several lines of preclinical research that have examined sex differences in alcohol preference (*i.e.* two-bottle choice) using rats (Russell & Stern 1973; Lancaster *et al.* 1996) as well as mice (specifically strain C57Bl6/J due to their aforementioned high ethanol preference relative to other inbred strains, see Eriksson & Pikkarainen 1968; Melo *et al.* 1996). They have all shown that females tend to consume higher amounts of alcohol than males. Work using intermittent procedures designed to induce dependence-like drinking have also shown significantly increased female alcohol consumption in C57Bl6/J female mice relative to males (Hwa *et al.* 2011). While many of the previously mentioned animal models of binge-like drinking used female mice (Carson & Pruett 1996), the work by Rhodes and colleagues (2005, 2007) was the first to comprehensively characterize binge-like drinking in both male and female C57Bl6/J mice. More specifically, their work showed that female C57Bl6/J mice consumed approximately 2 g/kg more alcohol than males on the final 'binge' (4 h access) day (Rhodes *et al.* 2005). Later work using a multiple access, heavy binge-like drinking model (Drinking in the Dark-Multiple Scheduled Access, or DID-MSA) also showed significantly higher levels of binge-like consumption in adult female mice (approximately 11 g/kg) when compared to males (approximately 8 g/kg, see

Melón *et al.* 2013). Collectively, these past findings indicate sound differences between male and female C57Bl6/J ethanol intake, whereby females drink more than males in a variety of intake procedures. Critically, this sex difference has been extended to binge-like drinking.

These findings may seem to stand in contrast to the clinical literature, which have shown overall higher levels of binge drinking in men relative to women (Anda *et al.* 1990; Naimi *et al.* 2003). However, it is important to remember that women have been shown to escalate to dependence more quickly than men (Orford & Keddie 1985; Randall *et al.* 1999; Zilberman, Tavares & El-Guebaly 2004). In other words, the key difference is not overall percentages, but *rate*. As stated previously, this increased rate implies both fundamental differences in underlying neurocircuitry between men and women and how that neurocircuitry is affected by alcohol. While capturing the entire clinical phenomenon of telescoping in a preclinical model is impossible, there have been indications that female mice are more sensitive to the effects of alcohol than males (Grahame *et al.* 2000). Moreover, work by Melón *et al.* (2013) demonstrated that heavy binge drinking (*i.e.* using DID-MSA) yielded female mice who were more sensitive to the acute, locomotor stimulating effects of alcohol. However, the actual neurobiology underlying these sex differences remains remarkably understudied.

1.6 Dopamine and Alcohol

One intriguing finding regarding the putative sex difference in ethanol sensitivity between male and female mice has been in relation to the neurotransmitter dopamine (DA). DA is

released in response to the presence of alcohol in key areas of the brain that undergird its rewarding and reinforcing properties (Di Chiara & Imperato 1988; Stuber *et al.* 2008). In fact, DA has been shown to increase in response to other drugs of abuse (*e.g.* psychostimulants, Di Chiara & Imperato 1988) as well as natural rewards like sucrose (Hajnal, Smith & Norgren 2004). Several theories regarding DA have been developed, including its signaling role for the salience of a rewarding stimulus or drug (Robinson & Berridge 1993). While its release dynamics as well as its theoretical role is an intense topic of study, altered DA release is thought to underlie much of the neurobiological dysregulation seen in alcoholics and other drug addicts (Koob & Le Moal 1997).

To this end, there is an imbalance in the DA release dynamics between female and male mice as a function of alcohol. More specifically, acute administration (intraperitoneal, i.p) of ethanol (2 g/kg) resulted in increased dopamine (DA) release in female C57Bl6/J mice. In comparison, male mice showed nearly equivalent DA levels after ethanol injection relative to saline controls (Tang *et al.* 2003). These same results were mirrored in rats, whereby females consumed significantly more alcohol and had significantly greater accumbal (nucleus accumbens, NAc) DA release (Blanchard *et al.* 1993).

When taken in conjunction with their increased ethanol sensitivity as well as telescoping seen in clinical populations, altered DA release provides a possible mechanism for differences in male and female response to alcohol. While there are currently no studies directly examining DA release in male and female C57Bl6/J mice during binge-like drinking (*e.g.* DID), there is confirmatory evidence for DA's role using the dopamine

reuptake blocker GBR 12909. In this study, an i.p. injection of GBR 12909 significantly decreased DID drinking in male mice (Kamdar *et al.* 2007), thus validating the importance of DA signaling during binge-like drinking.

1.7 Mesocorticolimbic Circuitry and Binge-like Drinking

The effects of rewarding drugs like alcohol are seated in an interconnected web of neural substrates known as the mesocorticolimbic—or reward—circuit. DA release is situated within this network and originates from a population of DAergic neurons in the ventral tegmental area (VTA, discussed in more detail in **1.9 The Ventral Tegmental Area: Structural and Cellular Heterogeneity**). One of the major areas of release is the nucleus accumbens (NAc), which is often the site of microdialysis measurements when assessing DA release dynamics during alcohol self-administration (Weiss *et al.* 1993) or acute alcohol administration (Di Chiara & Imperato 1988). Because of this cause/effect relationship hinging on the DA production in the VTA and subsequent release in the NAc, this connection in particular is often taken as the hallmark of rewarding and reinforcing substances. However, mesocorticolimbic circuitry includes other areas of that help control both basal DA states as well as phasic release, including the prefrontal cortex (PFC), extended amygdala including the bed nucleus of the stria terminalis, hippocampus, and lateral habenula. To better understand how each of these interrelated components—including the NAc—function within reward circuitry, each will be briefly described with regards to their general neurobiology as well as their relationship to alcohol. More specifically, each structure will be discussed briefly with regards to what is currently known about its association to binge drinking. If binge drinking studies are unavailable or

sparse, other preclinical modes of alcohol intake (*e.g.* intermittent alcohol exposure) will be considered. Finally, the following descriptions are not meant to be exhaustive, but merely illustrative of the differential effects and importance each area has in binge drinking.

1.7.1 Prefrontal cortex. The PFC is well-known as the seat of higher cognitive and executive functioning. From a connectivity perspective, it summates inputs from a variety of densely interconnected cortical and subcortical regions (DeNardo *et al.* 2015). As it relates to mesocorticolimbic circuitry, the PFC receives DAergic projections from the VTA—albeit at a lower level (~30%) than those projecting to the NAc (~85%, see review by Tzschentke 2001). In particular, these projections specifically target the orbital and medial prefrontal cortices (oPFC and mPFC, Rudebeck *et al.* 2013; Lammel *et al.* 2011), areas that have strong connections to the limbic system and responds to emotional and rewarding stimuli (Rudebeck *et al.* 2013; Abernathy, Chadler & Woodward. 2010). The PFC in totality—as well as the oPFC and mPFC—are predominantly glutamatergic in nature (approximately 80% of neurons, see review by Abernathy, Chandler & Woodward 2010) and glutamatergic projections from this area are also thought to modulate DA release in the NAc (Taber, Das & Fibiger 1995). Although DAergic projections originating from the VTA have been shown to synapse with glutamatergic pyramidal cells (van Eden *et al.* 1987), work has also shown the importance of DA release on local inhibitory interneuronal populations. To this end, DA release in this region has been shown to have an inhibitory effect on oPFC and mPFC functioning, perhaps through activation of D_2 receptors on GABAergic interneurons (George *et al.* 2012; Grobin & Deutch 1998).

Of the many higher-order functions the PFC has been implicated in, perhaps the most important in the present context are impulse control and decision-making. The PFC has been thought to act as a brake on impulsivity and poor decision-making, the breakdown of which has been associated with dependence and uncontrolled drug (*e.g.* alcohol) taking (de Wit 2009). Given its higher-order level, it can be difficult to model changes in impulsivity and decision-making in rodents. It is also worth noting that the relationship between a hypofunctioning PFC (*i.e.* hypofrontality) and uncontrollable alcohol intake implies a dependent-like state. While hypofrontality is also associated with myriad other mental health disorders, its connection to alcohol naturally leads to questions regarding how much change would be evident during and after binge drinking in a non-dependent individual. Regardless, there is limited work investigating the relationship between binge-like drinking in rodent models and PFC functioning. For instance, a short-term (4 d) gavage model for binge drinking using high levels of alcohol (9 g/kg/day) showed significant cortical damage in adult animals (Crews *et al.* 2000). Behaviorally, adult rats subjected to the same heavy binge exposure also showed problems with perseveration in a reversal learning protocol (Obernier *et al.* 2002). Relatedly, this same deficit in mPFC-driven cognitive flexibility has also been shown in a chronic mouse model of alcohol consumption (Badanich, Becker & Woodward 2011). Taken together, alterations in prefrontal cortex functioning are related to binge drinking, but are more concretely implicated in heavy binge drinking and dependence-like drinking rather than short-term, non-dependent drinking.

1.7.2 Extended amygdala including the bed nucleus of the stria terminalis. The extended amygdala and bed nucleus of the stria terminalis (BNST) are included in the overall limbic

system and broadly underlie negative affective states, including stress and anxiety (Walker, Toufexis & Davis 2003). When placed within the context of binge drinking, these affective states occur predominantly after a binge has occurred and an individual is subjected to its negative consequences (*e.g.* physiological, psychological, emotional) of binge drinking. *Repeated*, negative affective states of the type seen in repeated bouts of intermittent binge drinking often help drive alterations to amygdala circuitry, rendering it more sensitive to later stress-released cues and further drinking (Sommer *et al.* 2008). A further discussion of the role of stress and intermittent, escalated alcohol consumption can be found in the second half of this **Introduction**.

As with the oPFC and mPFC, the extended amygdala receives dense DAergic projections that originate from the VTA as well as the substantia nigra (Hasue & Shammah-Lagnado 2002; Asan 1998). In particular, these projections originate from more dorsal regions of the VTA (Hasue & Shammah-Lagnado 2002). There are also reciprocal projections from this region back to the VTA, including from the BNST (Jennings *et al.* 2013). The extended amygdala also receives cortical input from the mPFC (Vertes 2004), providing a further interconnection within the mesocorticolimbic circuit as a whole. From a cellular perspective, the extended amygdala is comprised predominantly of GABAergic neurons (Hammack, Mania & Rainnie 2007; Sun & Cassell 1993). These inhibitory populations are both local GABAergic neurons as well as projection neurons (Cassell, Freedman & Shi 1999).

Amygdala and BNST dysregulation has been widely studied in the context of alcohol dependent states, particularly during periods of withdrawal (see review by Koob 2003). Subunit-specific $GABA_A$ receptor regulation in the central amygdala plays a role in chronic binge-like drinking (Liu *et al.* 2011) as do metabotropic glutamate receptors (Cozzoli *et al.* 2014). Similar long-term binge drinking protocols using alcohol-preferring rats have shown expression changes in genes underlying synaptic plasticity (McBride *et al.* 2010). More importantly, experiments conducted with the short-term DID protocol have revealed the importance of neuropeptide signaling (e.g. corticotropin releasing factor, CRF) in the amygdala to binge-like drinking (Lowery-Gionta *et al.* 2012; Sparrow *et al.* 2012; Lowery *et al.* 2010). This work also demonstrated that regulation of binge-like drinking was mechanistically different from that of low dose alcohol consumption, possibly due to the intermittency that usually accompanies such patterns of drinking.

1.7.3 Hippocampus. The hippocampus has been extensively studied with regards to its role in memory as well as its structure and cellular composition. Like the extended amygdala, it is also considered to be part of the limbic system. Briefly and broadly, the hippocampus is comprised of glutamatergic, pyramidal neurons as well as a wide variety of interneuronal subtypes (Zeisel *et al.* 2015; Klausberger *et al.* 2003). The hippocampus receives connections from cortical sites as well as direct DAergic projections from the VTA, and, in turn, projects to the NAc as well as other structures (Otmakhova *et al.* 2013). Binge drinking as it relates to hippocampal functioning remains sparse, however the structure has been implicated in the actions of chronic alcohol abuse and dependence. For instance, given its role in memory formation, it has been hypothesized to play a role in

context-induced reinstatement (see review by Heinz *et al.* 2009). Chronic alcohol consumption can result in changes to hippocampal cell populations, including hippocampal neural progenitor cells (He *et al.* 2005). The little we do know of binge drinking and its effects centers on higher doses and longer, successive cycles of exposure than those afforded by DID. For instance, experimenter-induced, binge-like drinking has also been shown to alter hippocampal neurogenesis (Nixon & Crews 2002), while sweetened alcohol solutions have resulted in depressed hippocampal neuronal activity (Ryabinin *et al.* 2003). Moderate doses of alcohol have also been shown to alter hippocampal functioning, particularly within novel contexts (Ryabinin *et al.* 1997). While these few studies provide no evidence for the importance of hippocampal functioning to short-term binge drinking, they do indicate that long-term, high dose binge drinking can lead to neuronal death and alterations in hippocampal functioning (*i.e.* Wernicke-Korsakoff syndrome).

It is worth mentioning that the mesocorticolimbic substrates described up until this point have been examined using binge-like protocols. However, while these protocols were designed to engender high levels of alcohol consumption to meet the current NIAAA definition of binge drinking, they also incorporated multiple accesses spread over days or weeks and sometimes resulted in exceedingly high BACs (*e.g.* 250 mg%). Inherent to this were repeated instances of withdrawal. Within the framework of binge drinking and its relation to dependence, this approach makes sense if the question is how does *repeated* binge drinking lead to dependence in certain individuals. Moreover, it makes sense when looking at what role substrates play in the regulation of the negative affective states engendered by a withdrawal state. Another important question falls at the earlier end of

this continuum: What about *early* binge drinking? In other words, the type that occurs before the cycle of successive, excessive binges embedded within periods of no drinking (*e.g.* binge drinking every Friday and Saturday, but abstaining during the week). To address this question, work has focused on other substrates within mesocorticolimbic circuitry, including the nucleus accumbens (NAc) and VTA.

1.7.4 Nucleus accumbens. There is neuroanatomical continuity between the extended amygdala and the nucleus accumbens (NAc), with their respective boundaries being blurry and ill-defined (Cassell, Freedman & Shi 1999). Structurally, the NAc is divided into three major regions, including the core, shell, and rostral pole (Zahm 2000; Zahm & Brog 1992). As with much of the extended amygdala (with the exception of the basolateral amygdala, see McDonald 1992), there is a high percentage (approximately 90%) of striatal GABAergic neurons (termed medium spiny neurons, MSNs) within the NAc (Taverna *et al.* 2003). From a connectivity perspective, the NAc receives glutamatergie projections from the extended amygdala (*e.g.* basolateral amygdala, see Ambroggi *et al.* 2008), hippocampus, and prefrontal cortex (Britt *et al.* 2012). There is also evidence for GABAergic connections between MSNs within the NAc (Taverna *et al.* 2003). Importantly, the NAc is densely innervated with DAergic projections originating from the VTA, leading to differential responses in D1- and D2-containing MSN populations (see review by Surmeier *et al.* 2007). From the NAc, projection targets include reciprocal connections back to the VTA that likely target local and projecting GABAergic populations (Xia *et al.* 2011).

The NAc is considered a major substrate underlying the reinforcing and rewarding aspects of drugs of abuse as well as natural rewards, in large part due to the role of these DAergic projections from the VTA (Adamantidis *et al.* 2011). Early microdialysis work revealed that experimentally administered alcohol resulted in significant increases in DA release within the NAc (Di Chiara & Imperato 1988). This work has been robustly confirmed and extended, including in operant alcohol self-administration approaches in rats (Melendez *et al.* 2002). Self-administration work was also able to better clarify the neuroanatomical region with the NAc that appeared to be most related to the actions of rewarding and reinforcing drugs—namely, the NAc shell (Rodd-Henricks *et al.* 2002). Pharmacologically, local blockade of DA receptors within the NAc has underscored the near necessity of DA release to the reinforcing effects of alcohol (Rassnick *et al.* 1992). Moreover, increasing doses of alcohol have also been shown to have corresponding increases in DA release (Yim *et al.* 2000). However, there is a limit to this linear relationship, as higher doses (*e.g.* 2 g/kg) can inhibit DA release (Yorgason *et al.* 2014). There is considerably less work that specifically investigates the effect of short-term binge-like drinking (*e.g.* DID) on the NAc as well as its specific role in same. To this end, although DA release within the NAc has not been directly measured under DID conditions, it can be inferred that such release would also occur. Finally, while it is also likely that multiple neurotransmitter and peptidergic systems (*e.g.* corticotropic releasing factor, **1.18 Corticotropin-releasing Factor and Mesocorticolimbic Circuitry**) are important in DID, so far only metabotropic glutamate receptors have been specifically examined (Cozzoli *et al.* 2012). The role of metabotropic glutamate receptors has also been confirmed

using a high-access binge model, underlying the importance of glutamatergic signaling during both short- and long-term binge-like drinking (Szumlinski *et al.* 2007).

1.7.5 Lateral habenula. The lateral habenula is an epithalamic structure that has several distinct nuclei, but is most commonly examined in terms of the medial and lateral portions (Kim & Chang 2005). While it has marked cellular heterogeneity (e.g. expression of neurotransmitters such as acetylcholine and neuropeptides like Substance P, see Grady *et al.* 2009), it is often discussed in terms of its connection to the VTA. More specifically, the reciprocal connections going from the VTA to the medial and lateral habenula (Phillopson & Pycock 1982) as well as glutamatergic projections from the lateral habenula to the VTA. Interestingly, while the former is positive for the traditional marker for DAergic neurons (*e.g.* tyrosine hydroxylase, TH), it has been shown to release predominantly GABA (Stamatakis *et al.* 2013).

The projection from the lateral habenula to the VTA has received a significant amount of interest, since initial work revealed these projections synapsed in equal amounts on DAergic projection neurons and GABAergic interneurons within the VTA (Omelchenko, Bell & Sesack 2009). However, more recent work has shown that these connections predominantly drive the inhibitory function of the latter population (*i.e.* GABAergic interneurons, Brinschwitz *et al.* 2010). This connection has been shown to have a critical role in inhibiting VTA functioning, as these neurons directly synapse onto VTA GABAergic interneurons. Intriguingly, this lateral habenula-VTA connection is found almost exclusively in the more posterior region of the VTA (tVTA or RMTg, Balcita-

Pedicino *et al.* 2011; see Section **1.9 The Ventral Tegmental Area: Structural and Cellular Heterogeneity**). Functionally, this connection has been strongly implicated in the actions of aversive stimuli. Recent work using Channelrhodpsin-2-mediated activation of glutamatergic inputs to the VTA resulted in marked behavioral avoidance (Stamatakis & Stuber 2012), indicating the induction of an aversive state. Critically, this work has also been extended to alcohol. Application of low concentrations of alcohol (10.8 mM) to slice preparations resulted in increased firing rates of lateral habenular neurons (Zuo *et al.* 2017). Moreover, animals with lesions to the lateral habenula had significantly greater voluntary alcohol consumption as well as operant alcohol self-administration. They also showed significantly less conditioned taste aversion and no evidence of pharmacologically-induced reinstatement (Haack *et al.* 2014). While there have been no direct studies examining the lateral habenula in short-term binge access, its importance as a source of inhibitory drive to VTA DAergic functioning *via* tVTA GABAergic interneurons highlights a possible role. It is likely that this pathway is an important component for coding aversive events related to binge drinking, particularly the negative physiological and affective states that can result.

1.8 Sex Differences in Mesocorticolimbic Circuitry

The majority of preclinical studies presented regarding mesocorticolimbic substrates as well as studies examining their importance to alcohol- and binge drinking-related functioning were conducted on male rodents, a notable exception being alcohol self-administration studies conducted in regions like the NAc (*e.g.* Rodd *et al.* 2004). Despite this sex asymmetry, several lines of work have pointed to important differences between

male and female mesocorticolimbic circuitry and its functioning. Much of this work investigated either the effects of maternal alcohol consumption or the role of hormones on circuit functioning and alcohol consumption. There is little work investigating basic, organizational differences between male and female mesocorticolimbic circuitry. Since the focus thus far has been on adult binge drinking, the following discussion will preclude prenatal effects and will focus on the latter two facets.

Despite the relative shortage of comprehensive studies regarding the organizational differences between male and female neuroanatomy, recent work examining sexually dimorphic gene expression in whole brain of adult male and female C57Bl/6J mice revealed 652 genes that were differentially expressed (Yang *et al.* 2006). These effects were also specifically tied to their respective chromosomes, indicating that there is a genetic basis for organizational differences in the brains of male and female rodents. This difference would obviously be complicated by the addition of hormonal influences (both organizational and activational). While this particular study did not examine specific brain areas, select studies have been done regarding prefrontal cortex, extended amygdala, hippocampus, NAc, and lateral habenula and will be presented below.

1.8.1 Sex-specific differences in prefrontal cortex. In terms of the prefrontal cortex, differences in neuronal structure and morphology have been shown between human males and females, with males showing higher neuronal density in cortical regions, but females having inferred increased neuronal processes (Rabinowicz *et al.* 1999). In rodents, a study examining intact female mice revealed significantly increased spine density in the mPFC

relative to ovariectomized females (Wallace *et al.* 2006). Other work in gonadectomized rats has also corroborated the effects of hormones (*e.g.* testosterone, estrogen) on cortical neuronal development (Kolb & Stewart 1991), thus underscoring the importance of hormonal influences in shaping patterns of development. There are also differences in between males and females in the DAergic composition of projection fibers originating from midbrain (Kritzer & Creutz 2008). While chronic alcohol consumption has been shown to cause increased neuronal loss and activation of inflammatory molecules (*e.g.* cytokines) in female mice relative to males (Alfonso-Loeches, Pascual & Guerri 2013), no preclinical studies have been conducted that examine sex-specific differences in binge-like drinking and the importance of—or the effects on—the prefrontal cortex.

1.8.2 Sex-specific differences in extended amygdala. As with the prefrontal cortex, there are morphological differences in the extended amygdala between males and females, such as overall dendrites and synapse (increased in male relative to female rats, Nishikuza & Arai 1981), excitatory synapses (increased in male relative to female rats, Cooke & Woolley 2005), and orientation of dendritic branching (Dall'Oglio *et al.* 2008). There are also differences in sex-steroid receptor expression between males and females, including the BNST (Gu, Cornea & Simerly 2003). While some of these effects can be altered by altering the hormonal milieu (*e.g.* castration or ovariectomy), many of these foundational differences are established early on in development (Morris, Jordan & Breedlove 2008; Nishikuza & Arai 1981). Although there is little work that has specifically looked at short-term binge-like drinking in female mice as it relates to the amygdala, there is evidence that withdrawal from chronic alcohol exposure is similar in male and female rats (Overstreet,

Knapp & Breese 2004). This is intriguing, given not only the known sexual dimorphism in the amygdala, but also the markedly different levels of anxiety seen in women relative to men. It is likely that there are differences in amygdala-mediated aspects of alcohol consumption (*e.g.* withdrawal effects after heavy binge drinking or after chronic consumption), but that the anxiety-like testing used is simply different between male and female rodents (Johnston & File 1991).

1.8.3 Sex-specific differences in hippocampus. Broadly speaking, there is known sexual dimorphism in hippocampal functioning at the behavioral (Williams & Meck 1991), cellular (Madeira *et al.* 1992), and synaptic (Gupta *et al.* 2001) levels. As with other regions, much of this sex-specific difference has been tied to the actions of hormones. For instance, 17β-estradiol has been shown to alter hippocampal spine density, with effects that can change according to estrus cycle (Woolley, Venczel & Schwartzkroin 1996; Woolley & McEwen 1993). Long-term (three weeks) binge-like alcohol administration has been shown to increase hippocampal glutamate levels in females, along with attendant increases in phagocytic activity (Ward *et al.* 2009). This is in line with data from male rats, which showed that chronic alcohol administration resulted in decreased neurogenesis (Nixon & Crews 2002).

Since these lines of work converge on the effects of heavy binge-like drinking (*i.e.* repeated episodes of binging followed by abstinence), it is unknown whether short-term binge-like drinking would have any effect on hippocampal cellular composition and their functioning within overall mesocorticolimbic circuitry.

1.8.4 Sex-specific differences in NAc. As the NAc is the major site of DA release for VTA-originating DAergic neurons, great effort has gone into understanding how sexually dimorphic the NAc is. Structurally, female MSNs have significantly more spines that are also significantly larger and functionally more active (Wissman *et al.* 2011; Forlano & Woolley 2010). Although no differences in TH immunoreactivity (*i.e.* DAergic innervation) have been found in the NAc of males and females (Forlano & Woolley 2010), sex-specific differences in DA receptor expression point to differences in its effects (Hruska *et al.* 1982). This has been born out in particular with psychostimulants, with administration resulting in sex-specific differences in synaptic structure and functionality as well as behavioral effects (Wissman *et al.* 2011). Although it is tempting to suggest that the same female-specific alterations are also present after alcohol administration, there is a relative lack of work examining alcohol as psychostimulants. It is known that increased DA release in the NAc—as well as sustained changes to DA levels—are evident after chronic alcohol consumption in female rats (Thielen *et al.* 2004). While this mirrors changes seen in males, any differences in short-term binge-like drinking in females are unknown.

1.8.5 Sex-specific differences in lateral habenula. As the lateral habenula is the seat of many female-centric behaviors (*e.g.* maternal behaviors), it is known for its sexual dimorphism (Matthews-Felton *et al.* 1995). This is supported at the cellular level, as fibers and neurons immunoreactive for the estrogen receptor are found specifically in the lateral habenula of females (Wagner, Silverman & Morrell 1998). Despite the recent interest in

how lateral habenular inputs to the VTA affects DAergic functioning and alcohol intake (see *Lateral Habenula*, above), no work has been done examining either sex-specific differences in baseline responsivity or female-specific effects of short-term binge-like access. However, given the connection between this region and the effects of rewarding and reinforcing substances as well as its high estrogen receptor expression, it is likely that estrogen-mediated modulation does exist.

1.9 The Ventral Tegmental Area: Structural and Cellular Heterogeneity

The focus on overall mesocorticolimbic circuitry up until this point has not been accidental; rather, it has simultaneously shown the lack of evidence for a substantive role in binge-like drinking for most regions included in the circuit (*e.g.* prefrontal cortex, lateral habenula, hippocampus). Although briefly mentioned up until this point, the VTA is a critically important area for (1) overall functioning of the mesocorticolimbic reward circuit and (2) for the actions of alcohol. More specifically, there is a significantly larger body of work detailing its singular importance to binge-like drinking. As it is the focus of the work presented in this thesis, it will be described in significantly more detail.

From a gross neuroanatomical perspective, the VTA is located in the midbrain near the midline, with the substantia nigra (compact and reticular parts) located laterally to more anterior regions of the VTA. More posterior regions feature the inclusion of the medial lemniscus lateral to the VTA and medial to the substantia nigra. As it has ill-defined borders, what has been included and excluded from the formal definition of the VTA has changed over time. This predominantly encompasses the interfascicular nucleus (IF),

which has been both included (Sanchez-Catalan *et al.* 2014) and excluded from the boundaries of the VTA (Ikemoto 2007). This minor argument notwithstanding, the VTA has been most consistently subdivided into four areas, known as the paranigral nucleus (PN), parabrachial pigmented area (PBP), parafasciculus retroflexus area, and the ventral tegmental tail (tVTA or RMTg; Ikemoto 2007). While all regions contain TH+ (DAergic) neurons, the former two have the highest TH+ cell densities. Although the VTA is often discussed in terms of TH+, DAergic populations and their projection targets, the cellular composition of this region is not homogenous. Approximately 60-70% of the neuronal population are DAergic while 30-35% are GABAergic (Olson *et al.* 2005; Ikemoto 2007; Chieng *et al*., 2011; Sanchez-Catalan *et al.* 2014). The remaining fraction (~5%) are glutamatergic. This mixed population is also inconsistently distributed throughout the VTA. A greater percentage of DAergic neurons are located in the posterior VTA (pVTA) as compared to the anterior VTA (aVTA), which is in line with the posteriorly located, denser TH+ regions of the PN and PBP (Ikemoto 2007; Sanchez-Catalan *et al.* 2014). This gradient is not as severe and is inverted for GABAergic neurons, which have a greater percentage in more anterior regions of the VTA (Chieng *et al*., 2011; Sanchez-Catalan *et al.* 2014). Importantly, there is also a dense population of GABAergic neurons in the most posterior region of the pVTA, the tVTA (Stamatakis & Stuber 2012; Olson & Nestler 2007).

Given its pivotal role in the mesocorticolimbic circuit, both neuronal populations and those populations' projection targets are of interest. Up until this point, the majority of DAergic projection targets have been indiscriminately referred to as 'going to the NAc.' While other

targets (*e.g.* prefrontal cortex, lateral habenula) have already been discussed, this projection to the NAc has been studied the most as it relates to rewarding and reinforcing drugs of abuse like alcohol. However, there are anatomical differences regarding the projection targets of a given population of DAergic neurons. To this end, dense TH+ regions like the PN and PBP predominantly target the shell of the NAc, while sparser, more lateral TH+ regions project to the core of the NAc (Ikemoto 2007; Hasue & Shammah-Lagnado 2002). In other words, more medial DAergic projections project to the more medial shell; more lateral DAergic projections project to the more lateral core. This has functional implications, as animals will self-administer drugs directly into the shell rather than the core (*e.g.* d-Amphetamine, see Ikemoto *et al.* 2005; for review, see Kelley 2004). Given the projections to the NAc shell originate in the pVTA, it is likely that this collective cell population (TH+ and relevant GABAergic interneuronal populations) are also more important to the immediate rewarding and reinforcing effects of drugs of abuse like alcohol.

The functionally-defined neuroanatomical heterogeneity that exists in the NAc is also evident in the VTA, but follows a more rostrocaudal (anterior to posterior) axis. Self-administration studies have found that not only will rats self-infuse alcohol directly into the pVTA, but that alcohol-preferring lines will do so to a greater extent than non-alcohol preferring lines (Rodd *et al.* 2004; Rodd-Henricks *et al.* 2000). Notably, self-administration did not occur in the aVTA. This functional difference is also evident with other drugs of abuse, including nicotine (Ikemoto, Qin & Liu 2006) and cocaine (Rodd *et al.* 2005). This is intriguing on two levels: One, it provides further evidence for the importance of the

pVTA and its relation to the intrinsically rewarding nature of drugs of abuse. Two, it circles back to questions regarding the cellular heterogeneity found in the pVTA. More specifically, if DAergic neurons are more commonly found in this region, is their direct activation the reason why the pVTA is a site of such generalizable self-administration? Or is it resulting from disinhibition, whereby local GABAergic neuronal firing is reduced, thus allowing for greater DAergic firing and subsequent DA release? To begin addressing this question, Ikemoto and colleagues used a set of rat self-administration experiments to ask whether or not pharmacological inhibition in either area would be reinforcing. They found that rats self-administered the $GABA_A$ receptor antagonist picrotoxin directly into the aVTA, but the $GABA_A$ agonist muscimol into the pVTA (Ikemoto, Murphy & McBride 1998; Ikemoto, Murphy & McBride 1997). Not only does this provide strong evidence for the role of disinhibition in the pVTA, but it also indicates a differentially important role for $GABA_A$ receptor expression that may be based on cell type (discussed further in Section **1.12 $GABA_A$ Receptors and Effects of Alcohol**). While this important pharmacological work pointed towards the importance of GABAergic neurons within the pVTA, its technological limitations made it impossible to fully distinguish their role. Recent advances with transgenic and viral-mediated approaches have shown that optogenetically-mediated activation of local GABAergic neurons in the VTA significantly reduced sucrose consumption (van Zessen *et al.* 2012). This behavioral effect was also evident on a circuit-level, whereby GABAergic activation led to decreased DAergic firing and NAc DA output (van Zessen *et al.* 2012). Taken together, these findings not only highlight the important role of the pVTA to reward, but also the cell-type specific role played by local GABAergic populations.

It should be noted that alcohol administration neither *exclusively* affects the pVTA nor GABAergic populations. Long-term (7 weeks) alcohol self-administration has been shown to increase AMPA/NMDA receptor ratios on DAergic neurons, indicating an overall enhancement of synaptic strength (Stuber *et al.* 2008). This is likely due to the influence of glutamatergic inputs (*e.g.* from the prefrontal cortex) and/or perhaps an imbalance in local VTA excitation and inhibition. Similarly, alcohol self-administration into the pVTA of male rats was shown to depend on D_2 receptor functioning, pointing to direct involvement of local DA release on DAergic neurons within this region (Rodd *et al.* 2004). However, these results point to either the effects of long-term alcohol self-administration (Stuber *et al.* 2008) or multiple rounds of alcohol self-infusion (Rodd *et al.* 2004). It is likely that such long-term adaptations would be less involved during short-term alcohol consumption (*i.e.* during binge-like drinking).

To this end, relatively more work has been done regarding short-term binge-like drinking and the role of the VTA than in other mesocorticolimbic regions (*e.g.* prefrontal cortex, hippocampus, lateral habenula). Of this, two major lines of work can be dissociated: (1) Binge-like drinking and the role of the stress-related neuropeptide corticotropin-releasing factor and (2) Binge-like drinking and the role of inhibitory neurotransmission. The former will be discussed in the second part of this thesis, with the current focus on the latter.

As demonstrated by the work of Ikemoto and colleagues (1997, 1998), inhibitory neurotransmission plays a critical role in the functioning of the VTA. However, an open

question remained as to the general role GABAergic inhibition within the VTA played during short-term binge-like drinking, regardless of cell population (*e.g.* DAergic or local GABAergic populations). Systemic administration of GABAergic agonists and antagonists to C57Bl/6J mice has been shown to alter DID binge-like drinking (Moore *et al.* 2007). More specific intra-VTA injection of the $GABA_B$ agonist baclofen also altered binge-like consumption using the same protocol, but only when infused into the aVTA (Moore & Boehm 2009). Collectively, these findings avoid the long-term, chronic binge-like approaches used in high access, chronic binge-like drinking and specifically interrogate short-term binging. Moreover, this work directly implicates a role for local GABAergic inhibition in the VTA during binge-like drinking.

1.10 A Sexually Dimorphic VTA?

As with other substrates within mesocorticolimbic circuitry, the question of sexual dimorphism can also be asked of the VTA. Up until this point, preclinical work investigating male and female differences in regions like the prefrontal cortex, hippocampus, and amygdala have been mixed in their depth and breadth. Fortunately, a little more is known about sex-specific differences in the VTA. To start, past immunohistochemical work has shown female rat VTA is both significantly larger than male VTA and that it contains a higher concentration of TH+ neurons (McArthur *et al.* 2005; McArthur, McHae & Gillies 2007). TH+ projections from this region also differ in male versus female rats, particularly in the density of DAergic innervation to regions of the prefrontal cortex as well as their concomitant steroid-sensitive receptor expression (Kritzer & Creutz 2008). Taken together, these data reveal that baseline organizational

differences in VTA neuroanatomy and cellular composition exist and that they likely affect VTA functioning and output (*e.g.* DA release). This is further confirmed by several preclinical studies examining baseline DA levels and release characteristics between male and females. As mentioned earlier (see Section **1.6 Dopamine and Alcohol**), there are differences in drug-induced DA release dynamics between males and females. Critically, sex-dependent differences are also seen at baseline (*i.e.* without alcohol administration). For instance, while overall DA levels are similar between males and females, females exhibit both greater DA release as well as greater DAT-mediated reuptake (Walker *et al.* 2000).

With regards to female-specific binge-like drinking, two notable studies have been conducted that directly target the VTA. Given the demonstrable effects found when manipulating GABAergic inhibition within the VTA, Melón and colleagues (2011) locally administered the $GABA_A$ alcohol antagonist Ro15-4513 into the VTA. By selectively applying Ro15-4513 to either the aVTA or pVTA, they could specifically test the role of alcohol-modulated GABAergic inhibition in either anatomical region of the VTA. Their results indicated that local administration of Ro15-4513 to the pVTA specifically reduced female binge-like alcohol consumption and had no effect on sucrose consumption. Not only did this work reassert the importance of GABAergic inhibition in the pVTA, but it also led to questions regarding specific $GABA_A$ receptor populations and the actions of alcohol within this region (*i.e.* δ-subunit containing, see Hanchar *et al.* 2006 and **1.12 $GABA_A$ Receptors: Structure, Function, and Effects of Alcohol**).

Later work used a separate $GABA_A$ receptor drug—4,5,6,7-tetrahydroisoxazolo(5,4,c)pyridin-3-ol, or THIP (also known as Gaboxadol)—that also targets benzodiazepine-insensitive $GABA_A$ receptors. In comparison to Ro15-4513, THIP is a potent allosteric modulator at δ-containing $GABA_A$ receptors and has markedly reduced affinity for benzodiazepine-sensitive $GABA_A$ receptors (γ2-containing, Meera, Wallner & Otis 2011, see **1.12 $GABA_A$ Receptors: Structure, Function, and Effects of Alcohol**). Local administration of THIP into the pVTA prior to DID binge-like drinking resulted in significant decreases in consumption in females. Notably, this effect was also dependent on estrus phase, with females in non-estrus (*e.g.* diestrus) having significantly decreased binge-like drinking relative to females in estrus (Melón *et al.* 2017).

When taken together, these findings sharply underscore the importance of the pVTA to the rewarding actions of alcohol. Given the differential effects of $GABA_A$ receptor agonism during the estrus cycle, they also provide strong support for the hypothesis that the VTA is a sexually dimorphic region. It should also be noted that upon initial assessment, these two sets of findings appear contradictory: One the one hand, using the alcohol antagonist Ro15-4513 at benzodiazepine-insensitive $GABA_A$ receptors reduced binge-like drinking. On the other hand, using a potent allosteric modulator at benzodiazepine-insensitive $GABA_A$ receptors *also* reduced binge-like drinking. While a greater discussion of $GABA_A$ receptors is presented below (**1.12 $GABA_A$ Receptors: Structure, Function, and Effects of Alcohol**), it is possible that these seemingly opposing effects were mediated by the pharmacological differences between the two compounds. More specifically, evidence indicates that Ro15-4513 competes for the same active site as alcohol on $GABA_A$ receptors

(Hanchar *et al.* 2006). Blocking this site could lead to an inability of alcohol to exert its effects, which could result in reduced DA release. Similarly, use of a potent allosteric regulator could also shift these dynamics in the other direction; too much activation could lead to excessive DA release and reduced binge-like consumption. A more in-depth discussion of this class of $GABA_A$ receptors is provided below (**1.12 $GABA_A$ Receptors: Structure, Function, and Effects of Alcohol**) and their implications on mesocorticolimbic circuit functioning in **Chapter 4: Discussion, Part A**.

1.11 Estrogen-specific Effects

Up until this point, hormonal regulation has been given cursory treatment and spoken in detail only as it relates to its early, organizational effects on neurobiological development. While the focus of this thesis does not specifically interrogate the role for sex-specific steroidal regulation of binge-like drinking, it would be remiss to not mention the specific role of estrogen-signaling as it relates to female-specific alcohol consumption.

Estrogen is a class of hormone that is present in both males and females, but is released with greater frequency and in greater amounts in sexually mature females. To this end, the most potent form of the estrogens is 17β-estradiol (Gillies & McArthur 2010) and its two cognate receptors, ERα and ERβ, are found across regions of the brain and have high expression in areas such as the ventromedial hypothalamus (Simerly *et al.* 1990). Of the two receptor types, ERα is the one predominantly found within the VTA (Shughrue, Lane & Merchenthaler 1997) and is also found on TH+ projections (Kritzer & Creutz 2008). This provides unique control over reward-relevant circuitry, particularly in modulating DA

release, and it should come as no surprise that administration of 17β-estradiol to overiectomized female rats results in increased striatal dopamine release (Pasqualini *et al.* 1995). This effect is also specific to females, as 17β-estradiol administration to castrated males does not lead to altered DA release (Becker 1990). Importantly, there is a wide body of work showing that 17β-estradiol increases drug-induced release of DA, including amphetamine (Castner, Xiao & Becker 1993), nicotine (Dluzen & Anderson 1997), and—important to this work—alcohol (Blanchard *et al.* 1993). This effect points to the role of estradiol as a 'second level' modulator for alcohol consumption. This hypothesis has been directly tested, as ovariectomized female rats have both a lower preference for alcohol and consume lower volumes in a two-bottle choice protocol relative to sham-treated females (Forger & Morin 1982; but see mouse model, Hilakivi-Clarke 1996). These findings indicate that the effects of estrogen—and specifically 17β-estradiol—are required for at least part of the observed increased alcohol consumption and preference in female rodents.

In terms of binge-like drinking, previous work by Melón and colleagues directly assessed whether estrus cycle affected binge intake. Their results did not show alterations in binge drinking as a function of estrus cycle, but rather differences that were dependent on administration of THIP (Melón *et al.* 2017). This work provides a potential dissociation between the drinking protocol used and cycling-dependent effects. In other words, perhaps longer bouts of access or a choice component are more greatly influenced by estrus cycle rather than short-term, binge bouts. Regardless, while the effects of estrogen on underlying mesocorticolimbic circuitry and alcohol intake are not disputed, its role in binge-like drinking is only beginning to be understood.

1.12 $GABA_A$ Receptors: Structure, Function, and Effects of Alcohol

The work highlighted by Moore & Boehm (2009) and Melón *et al.* (2017) focused not only on binge-like drinking, but also the specific and important role played by GABAergic inhibition. Although multiple neurotransmitter systems have been implicated in the actions of alcohol (Sprow & Thiele 2012), one that has received intense scrutiny has been $GABA_A$ receptors. This is largely because alcohol has long been known to potentiate the inhibitory effects of GABA at these receptors (Mehta & Ticku 1988) and has particular sensitivity for a subclass of $GABA_A$ receptor subunits (Wallner, Hanchar & Olsen 2003). These differences in structure and relationship to alcohol and its effects will be discussed in greater detail in the following sections.

Structurally, $GABA_A$ receptors are heteropentameric, ligand-gated ion channels that underlie much of the inhibition found in the adult central nervous system (Farrant & Nusser 2005; Macdonald & Olsen 1994; Sakmann, Hamill & Bormann 1983). Mechanistically, this occurs by the binding of two GABA molecules to sites on the extracellular side of the receptor, thereby allowing for channel opening and chloride ion influx (Baumann, Baur & Sigel 2003). Assuming a normal resting membrane potential and chloride gradient, this influx of negative charge subsequently hyperpolarizes the neuron, reducing the likelihood of firing. The heteropentameric nature of $GABA_A$ receptors indicates that their composition varies and can be comprised from a diversity of subunits. To this end, five major subunit classes have been identified (α, β, δ, γ, and ρ) and their respective family members have wide and varied distribution throughout the brain (Pirker *et al.* 2000; Fritschy & Möhler 1995; Macdonald & Olsen 1994). Incorporation of different subunits

confers different properties on a given receptor, with differences in pharmacology, location, and kinetics being most pronounced (Farrant & Nusser 2005; Sieghart 1995; Verdoorn *et al.* 1990). Critically, different subunits have also been implicated in the actions of different drugs, including alcohol. While an exhaustive treatment of each type and class of subunit is beyond the scope of this thesis, what follows will be a more detailed reckoning with what is known about some of the most relevant subunit classes as they pertain to alcohol; namely, α, δ, and γ subunits. More specifically to this point, although the β subunit family has been implicated in alcohol dependence (β1, Edenberg *et al.* 2004; Long *et al.* 1998), it will not be given any further analysis. Although there is some evidence for the role of β subunits in the actions of alcohol (Anstee *et al.* 2013; for review of β2 and β3 knockout mice, see Boehm *et al.* 2004), the evidence is more robust for other family members.

1.12.1 α subunit family. In its canonical form, two of the five subunits used to construct $GABA_A$ receptors are α subunits (Chang *et al.* 1996). There are six possible α family members and the most commonly found ones are α1, followed by α2 (Pirker *et al.* 2000; Fritschy & Möhler 1995). Importantly, these receptors tend to localize primarily to the postsynaptic surface (Somogyi *et al.* 1996). Of the remaining α family members, both α4 and α6 have been shown to exist in $GABA_A$ receptors that are located extrasynaptically (Farrant & Nusser 2005; Nusser, Sieghart & Somogyi 1998; Rossi & Hamann 1998). While it may seem trivial, receptor location has important implications for the type of inhibition that is produced—be it phasic or tonic. The difference between these two forms lies partly in the amount of time the channel is open: Phasic inhibition features short channel open

times while tonic inhibition allows for significantly longer channel open times (see review by Farrant & Nusser 2005; Salin & Prince 1996). This affects the amount of chloride ion influx that is allowed and subsequent effects on neuronal excitability. In other words, shorter channel open times afforded by phasic inhibition allow for the precision most often associated with synaptic inhibition. However, other factors in addition to channel open time can ultimately influence both forms of inhibition, including the amount of presynaptic release as well as GABA clearance time from the synaptic cleft. To this end, quick diffusion of GABA away from the synaptic cleft (approximately 100 μs, see Farrant & Nusser 2005) is an important factor to the precision of phasic inhibition (Overstreet, Westbrook & Jones 2002). Simultaneously, diffusion is also an underlying component of tonic inhibition, as diffusion of GABA to extrasynaptic sites allows for activation of extrasynaptic $GABA_A$ receptors and corresponding persistent form of tonic inhibition.

With regards to the α family, both α1- and α2-containing $GABA_A$ receptors have been shown to underlie fast, phasic inhibition, while their α4- and α6-containing counterparts are important for tonic inhibition (Somogyi *et al.* 1996). The association of certain α subunits to a given type of inhibition is not merely chance; rather, it is due to a number of factors including affinity for GABA. In a ranking assessment of all six α subunit members, both α4- and α6-containing $GABA_A$ receptors have relatively higher affinities for GABA when compared with α1 and α2 (Bohme, Rabe & Luddens 2004). In other words, the lower EC_{50} values for α4 and α6—relative to α1 and α2—indicate that lower concentrations of GABA are needed to reach the 50% receptor population threshold for GABA binding. When interwoven with other factors affecting phasic and tonic inhibition (*i.e.* diffusion

time from the synaptic cleft), it is clear that receptors with higher affinity that are located outside the site of presynaptic release respond to lower—even ambient—levels of GABA. It is this combined set of characteristics that result in tonic inhibition. It should also be mentioned that differences in inhibition (*i.e.* phasic versus tonic) are not *solely* due to one kind of subunit family; the inclusion of either a γ2 or δ subunit is also of critical importance and will be described in more detail in subsequent subsections.

In addition to important locational differences and associations one of the two forms of inhibition, α subunits have been specifically implicated in the actions of drugs of abuse, including the propensity to develop alcoholism (*e.g. Gabra2*, see Edenberg *et al.* 2004). Relatedly, the differential sedative and anxiolytic effects of benzodiazepines are mediated by α1 and α2 subunits, respectively (Vollenweider *et al.* 2011; Kralic *et al.* 2002; Rudolph *et al.* 1999). Interestingly, work by Rudolph and colleagues (1999) also revealed that some of the effects of alcohol were mediated by α2- rather than α1-containing receptors. This work was extended with mouse genetic deletion models, which demonstrated the importance of both the α1 and α2 subunits to ethanol-mediated potentiation and recovery from acute intoxication as well as alcohol consumption and preference under two-bottle choice (Werner *et al.* 2006; Blednov *et al.* 2013; Blednov *et al.* 2011). Perhaps more relevant, α2-mutant mice that have a histidine to arginine substitution are benzodiazepine-insensitive and were found to have altered binge-like consumption (Newman *et al.* 2016). Taken together, it is clear that synaptically-located α subunits are important to some of the behavioral effects of alcohol. It is also worth mentioning that several of these α1- and α2-

mediated effects have been seen in both females and males, with some being sex-specific (Boehm *et al.* 2004).

In terms of extrasynaptically located α subunits, *in vitro* work revealed that both α4- and α6-containing $GABA_A$ receptors are potentiated by alcohol (Wallner, Hanchar & Olsen 2003). Intriguingly, this ethanol-induced potentiation occurred at low ethanol concentrations (10-30 mM). This work has also been extended to clinical observations, whereby *Gabra6* polymorphisms were linked to low-level effects of alcohol (*e.g.* 0.75 mg/kg, see Hu *et al.* 2005, but also see Sander *et al.* 1999). From an *in vivo* perspective, α4 global knockout mice also have significantly reduced ethanol-mediated potentiation of tonic current in the hippocampus (Liang *et al.* 2008), further implicating this receptor in the actions of alcohol. Finally, chronic, intermittent alcohol consumption has also been shown to increase transcript levels of the α4 subunit (Mahmoudi *et al.* 1997) and that this effect can vary by sex (Devaud & Alele 2004). In contrast, α6 knockout mice have relatively little alterations on a variety of alcohol-related behavioral measures (Homanics *et al.* 1998), indicating that they may not be as integral to how alcohol affects extrasynaptic $GABA_A$ receptor functioning (but see Hanchar *et al.* 2005). Recent work has also implicated α5-containing $GABA_A$ receptors as important to underlying tonic inhibition (Glykys, Mann & Mody 2008). However, this particular subunit has low expression in the brain (Pirker *et al.* 2000) and there is limited evidence as to its importance to alcohol.

While there is robust work demonstrating a role for α1- and α2-containing $GABA_A$ receptors, there is equally intriguing evidence for those extrasynaptically-located subunits

that mediate tonic inhibition. Upon closer inspection of this receptor population, two points stand out: (1) α4 knockout studies have highlighted the importance of the apparent necessary partner to α4—the δ subunit (Glykys *et al.* 2007) and (2) Concentration-dependent differences are evident in how $GABA_A$ receptors respond to alcohol. To this end, both lines of questions point towards a pivotal role for the δ subunit.

1.12.2 δ subunit. As with α4- and α6-containing $GABA_A$ receptors, those receptors that contain the δ subunit are also found in the extrasynaptic space and are thought to be one of the primary subunits responsible for tonic inhibition (Glykys, Mann & Mody 2008; Hanchar *et al.* 2006; Farrant & Nusser 2005; Wei *et al.* 2003). δ-containing $GABA_A$ receptors also have significantly higher affinity for GABA than those typically found at the synapse (*e.g.* γ2-containing receptors), as evidenced by their EC_{50} values (0.5 μM and 2.6 μM respectively, Brown *et al.* 2002). As briefly mentioned, extrasynaptically-located receptors (*e.g.* α4 and α6-containing $GABA_A$ receptors) have been implicated in the actions of low concentrations of alcohol. However, unlike members of the α family, no genetic relationship between *Gabrd* and alcoholism have yet been described. Despite this, the impact of such low alcohol concentrations has been explored most thoroughly and convincingly in δ-containing $GABA_A$ receptors. For instance, *in vitro* work using recombinant receptors expressed in oocytes showed that 10-30 mM concentrations of ethanol potentiated $GABA_A$ receptor inhibition when compared to (1) higher concentrations (>30 mM) and (2) γ2-containing $GABA_A$ receptors (see following subsection *γ subunit family*) (Hanchar *et al.* 2005; Wallner, Hanchar & Olsen 2003, but see Borghese *et al.* 2006). Importantly, this work translated to slice electrophysiological

studies, which demonstrated that the same ethanol concentration range potentiated δ-mediated tonic inhibition in a region containing high levels of δ-subunit containing $GABA_A$ receptors (dentate gyrus, see Wei, Faria & Mody 2004). Collectively, these findings provided strong evidence for not only the critical importance of δ-mediated tonic inhibition, but the potentiated tonic current that results from application of low concentrations of alcohol.

In terms of *in vivo* work, the creation of a global δ-knockout mouse line allowed for yet further confirmation of these findings, as knockout mice had decreased alcohol consumption and preference in a two-bottle choice protocol (Mihalek *et al.* 2001; Mihalek *et al.* 1999). Other testing also revealed some changes to dependence-like measures (*e.g.* withdrawal-induced hyperexcitability), while no changes to others (*e.g.* tolerance as measured by sleep-time assays). While this work underscored the *in vivo* contribution of δ-containing $GABA_A$ receptors, it failed to account for their effect in short-term, binge-like drinking. From a technical perspective, global knockout lines also carry the inherent risk of compensatory changes that could complicate data interpretation (*i.e.* perhaps changes to $GABA_A$ receptor subunit composition resulted in alterations to voluntary alcohol intake, rather than an effect of the δ subunit itself). To this end, additional work using site-specific shRNA knockdown of δ in the dorsomedial NAc shell of rats revealed that the δ subunit specifically contributed to intermittent alcohol consumption (Nie *et al.* 2011). In other word, that reduction of δ subunit levels in this region—rather than the lateral or intermediate regions of the shell—significantly reduced voluntary, intermittent drinking (Nie *et al.* 2011). This is in keeping with the more critical role played by the NAc

shell in the rewarding and reinforcing actions of drugs (Rodd-Henricks *et al.* 2002) as well as DAergic efferents from the pVTA (Ikemoto 2007). However, while site-specific importance of δ has now been established, questions regarding its role in other areas of mesocorticolimbic circuitry remain open, as do those regarding short-term binge-like consumption.

1.12.3 γ subunit family. In contrast to δ-containing $GABA_A$ receptors, γ2-containing $GABA_A$ receptors are expressed at significantly higher levels throughout the brain (Pirker *et al.* 2000). It is also notable that $GABA_A$ receptors harboring a γ2 subunit tend to be localized to the synaptic space (Somogyi *et al.* 1996), thereby allowing them to participate in phasic inhibition. As a worthwhile side note, that the most common forms of $GABA_A$ receptors include either a δ or γ2 subunit; there is no evidence that a naturally occurring receptor will contain both (Sieghart & Sperk 2002; Saxena & Macdonald 1994).

Clinically, there is some evidence that alterations to *Gabrg2* is related to the propensity to develop alcoholism (Buck *et al.* 1997, but see Sander *et al.* 1999). Unlike the δ subunit, two known splice variants of the γ2 subunit have been identified that have an 8-amino acid difference (short and long variants, or γ2S and γ2L). While there is some work showing that the latter (γ2L) might be implicated in the actions of alcohol (Wafford & Whiting 1992), later *in vivo* studies revealed no differences in γ2L null mice responses to alcohol (Homanics *et al.* 1999). Critically, extensive work has shown γ2-subunit containing receptors are responsive to high alcohol concentrations (>30-40 mM, Wallner Hanchar &

Olsen 2003). This indicates that γ2-containing receptors are less sensitive to alcohol, particularly when compared to their δ-containing counterparts.

1.13 Sex-specific Considerations for δ Subunit

Up until this point, $GABA_A$ receptors have been considered homogenous with regards to males and females. An added level of complexity is the known sex difference in GABAergic inhibition that begins early in development (Davis *et al.* 1996). From a protein perspective, δ has been shown to have high expression in several areas of the brain, including the cerebellum, thalamus, and hippocampus (*e.g.* dentate gyrus, Pirker *et al.* 2000). More moderate and diffuse expression was found in the VTA and NAc (Pirker *et al.* 2000). These findings were also true at the transcript level (Wisden *et al.* 1992). However, it is stressed that these levels were all determined from adult male rat brain—no conclusive studies have been conducted regarding $GABA_A$ subunit expression levels or localization in female brain.

Although a comprehensive comparison of $GABA_A$ receptor subunit expression between males and females is lacking, subunit levels have been shown to fluctuate in females. This is particularly true for the δ subunit. For instance, protein and mRNA analysis of the δ subunit revealed increased levels in the hippocampus and VTA, respectively, during non-estrus (Maguire *et al.* 2005; Melón *et al.* 2017). While direct male comparisons were not made in either of these studies, it leaves an open question as to whether or not female expression patterns are comparable to males. Given the known role δ-containing $GABA_A$ receptors play in tonic inhibition, expression differences would naturally lead to questions

regarding sex-specific functional alterations (*i.e.* increased or decreased baseline tonic inhibition).

While the question of whether or not there are sex-specific baseline differences in δ subunit expression remains relatively unexplored, there are tantalizing *in vivo* data that suggest there might be. Using generated global *Gabrd* mice, Mihalek *et al.* (1999, 2001) revealed differences in both male and female intermittent alcohol consumption and preference. While females had greater levels of consumption and preference relative to males, both sexes had decreased consumption and preference after global *Gabrd* deletion. From a pharmacological perspective, the use of the drug 4,5,6,7-tetrahydroisoxazolo(5,4,c)pyridin-3-ol (also known as Gaboxadol or THIP) has become a crucial tool in targeting δ-containing $GABA_A$ receptors (Drasbek & Jensen 2006). Work by Melón *et al.* used THIP to directly examine whether pharmacological modulation of δ-containing $GABA_A$ receptors in the aVTA or pVTA would differentially affect binge-like drinking in female mice. Intriguingly, only intra-pVTA THIP modulation in non-estrus females significantly reduced binge-like drinking. Given the higher expression of the δ subunit found in the VTA during non-estrus, it is likely that direct modulation of a higher percentage of δ-containing $GABA_A$ receptors altered VTA functioning and increased subsequent DA release. It is probable that these putatively higher levels of DA release led to the observed reduction in binge-like drinking.

1.14 Shifting the focus from binge drinking to later, escalated alcohol intake

An understanding of how alcoholism develops is predicated on teasing apart the intricacies at each stage of the cycle—from the drinking behavior characterized by early use (*e.g.* binge-like drinking) to the increased consumption and escalated behavior seen in later stages (see **Figure 1.1**). While many people engage in the former, it is only a subset of those who will escalate their intake and progress to later dependence drinking. A critical question to ask is why this transition occurs and what neurobiological factors might influence it. As the first half of this **Introduction** focused on binge drinking, the work discussed now will focus on the middle ground between these two points and will emphasize escalated intake.

1.15 Rodent Models of Escalated Drinking

The term 'escalated intake' is more commonly used in conjunction with rodent models of alcohol consumption and it is used to define the period when a rodent is voluntarily consuming increasingly large amounts of alcohol (**Figure 1.1**). It should be noted that while successive cycles of this type of intake often lead to dependence-like drinking (Hwa *et al.* 2011), dependence will not be the primary focus of the present discussion.

Many approaches have been used to engender escalated drinking and most have featured some component of deprivation or intermittency (for review see Becker 2012). To this end, an early approach used an alternating period of every-other-day access to alcohol and resulted in increased levels of consumption (Wise 1973). This method has been refined in the intervening decades to incorporate shorter time frames (*e.g.* 30 min access periods

every 24 h, Szumlinski *et al.* 2007; Finn *et al.* 2005) as well as more frequent periods of short alcohol access designed to model heavy binge-like drinking (Melón *et al.* 2013). A third modification has been termed intermittent alcohol access (IAA) and was developed within the past decade. In this two-bottle approach, mice are given alternating 24 h access to a bottle of water as well as a bottle of 20% ethanol (w/v) or just water. When continued for three weeks, this method results in reliably escalated intake as well as increased ethanol preference in both male and female mice (Hwa *et al.* 2011).

1.16 Stress Effects on Alcohol Intake

The induction of escalated intake in rodents allows for a better neurobiological understanding of what factors might influence its progression. From a clinical perspective, many factors can affect the drinking patterns of individuals, including genetic predisposition (Edenbeg *et al.* 2004) as well as environmental conditions like context and cue exposure (Vollstädt-Klein *et al.* 2011). One such factor that has received heavy focus has been that of stress.

The term 'stress' can lead to a multitude of definitions, including from sociological, environmental, psychological, and biological perspectives. At its most biological, stress is most commonly defined as a physiological event or stimulus that engages the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (McEwen & Wingfield 2003; Tsigos & Chrousos 2002). This peripheral response readies the animal for action—the typical 'flight or fight' that is often associated with autonomic activation. More broadly, stress in clinical populations also includes adverse life events (*e.g.* financial

insecurity, relationship problems, legal trouble) that intersect and interact with these same biological systems. From the perspective of alcohol drinkers, stress is related to the level of drinking, with abstainers having reported less overall stress and heavy drinkers reporting higher levels of stress (Cole, Tucker & Friedman 1990). In fact, the consumption of alcohol—regardless of drinker status—has been associated with the reduction of stress (so-called tension-reduction hypothesis, but see Cooper, Russel & Frone 1990 for conflicting findings). This connection between both alcohol consumption, drinking, and stress indicates that similar neurobiological systems are interacting.

1.17 HPA Axis and Alcohol

To this end, the HPA axis has become a focus of research interested in investigating the interaction between alcohol and stress. Briefly, at its 'start', the paraventricular nucleus (PVN) of the hypothalamus contains a population of neurons that produce and release the hormone corticotropic releasing factor (CRF) in response to a given stressor. CRF crosses to the anterior pituitary *via* the hypophyseal portal and subsequently stimulates release of the hormone adrenocorticotropic hormone (ACTH). ACTH then travels systemically and results in release of the stress hormone cortisol (humans) or corticosterone (most rodent species) from the adrenal glands (Stephens & Wand 2012). Increased or decreased corticosterone (or cortisol) release is taken a as a measure of HPA axis activation and can be behaviorally correlated with measures of stress responsivity. The regulation of HPA functioning will not be a topic of discussion here, but operates through negative feedback from the interaction of released glucocorticoids (*e.g.* cortisol, corticosterone) and cognate receptors in the central nervous system (*e.g.* hypothalamus and other regions).

While many factors can influence HPA axis functioning, the effects of alcohol have been a focus of study in both clinical populations and preclinical models. Clinically, the responsivity of the HPA axis to alcohol has had mixed findings. Hyporesponsive functioning has been noted in family members with a history of alcoholism (Schuckit, Gold & Risch 1987) and acute increases in ACTH have been found in non-dependent subjects (Lukas & Mendelson 1988). When taken together, it is likely that the effects of alcohol on HPA axis functioning are intrinsically related to the drinker status of the individual—with a blunted response being more typical of actively dependent individuals or those with a history of dependence. Preclinical work has also investigated the effects of alcohol on the HPA axis and have found similar results, with increased levels of ACTH after acute alcohol administration in alcohol-naïve rats, but more blunted HPA responses in rats that have a history of alcohol consumption (Richardson *et al.* 2008; Ogilvie, Lee & Rivier 1997). Taken together, it is clear that alcohol can have differential effect on underlying stress neurocircuitry, with longer histories of alcohol consumption (*e.g.* dependence and dependence-like drinking) being tied to significant HPA axis dysregulation.

1.18 Corticotropin-releasing Factor and Mesocorticolimbic Circuitry

Although HPA activation is useful as a measure of stress (Marin, Cruz & Planeta 2007; Sillaber & Henniger 2004), its activation is not isolated to peripheral effects. To this end, extrahypothalamic sites within the central nervous system are also responsive to key components of the HPA axis, with one of the most well-studied and important being CRF. Structurally, CRF is a 41-amino acid neuropeptide that has two corresponding receptors—corticotropin-releasing factor receptor-type 1 (CRFR1) and –type 2 (CRFR2, Dautzenberg

& Hauger 2002). Critically, both CRFR1 and CRFR2 are differentially expressed throughout the brain at both the transcript (van Pett *et al.* 2000) and protein (Chen *et al.* 2000) levels. Areas of expression include those that are traditionally associated with HPA axis functioning (*e.g.* hypothalamus), which is critical to negative feedback regulation of HPA axis functioning.

Importantly, CRFR1 and CRFR2 expression is also found in regions of the brain not traditionally associated with stress-relevant neurocircuitry, including the mesocorticolimbic circuit. This overlap in CRF signaling and reward-centric regions marks CRF as a neurochemical mediator between stress- and reward-relevant behaviors. A thorough discussion of important neuroanatomical substrates in mesocorticolimbic circuitry and their organization can be found in **1.7 Mesocorticolimbic Circuitry and Binge-like Drinking**. The following will focus specifically on the role of CRF within both the extended amygdala and VTA.

1.18.1 Extended amygdala. The extended amygdala is important to limbic system functioning and has long been associated with negative behavioral states such as anxiety and stress (Yang *et al.* 2008; Walker, Toufexis & Davis 2003). Given its neuronal connections with regions important to overall reward circuitry (*e.g.* VTA), it should come as no surprise that it plays a role in alcohol consumption. When considered within this context, the amygdala appears to be important during periods of withdrawal from alcohol and subsequent abstinence. This is particularly relevant when considering the intermittent nature of most escalated alcohol consumption. Perhaps most intriguingly, the actions of

CRF within this region have been specifically tied to these effects. To this end, CRF levels have been shown to increase in the amygdala during alcohol withdrawal (Merlo Pich *et al.* 1995); high CRF levels in withdrawing rats can also be reduced if the rats are given alcohol (Olive *et al.* 2002). Changes in receptor expression can also be seen after a history of alcohol consumption (Sommer *et al.* 2008). Critically, these changes in CRF signaling have been implicated in the transition (*i.e.* kindling) from non-dependent drinking to dependence (Breese, Overstreet & Knapp 2005). In other words, repeated cycles of alcohol access followed by abstinence engender changes in CRF-derived amygdala signaling that may allow for the progression to dependence.

1.18.2 VTA. Given the prominence of CRF signaling to the amygdala, one might assume that its primary function is within this region and solely relevant to the negative affective states encountered during withdrawal and abstinence. However, local CRF signaling within the VTA has also been shown to be critical to both stress and alcohol drinking. To this end, a simple stressor like footshock can result in increased local DA release, which is blocked by local, intra-VTA infusion of the CRF antagonist α-helical CRF (Wang *et al.* 2005). Moreover, site-specific CRFR1 knockdown in the VTA results in significantly reduced stress-induced drug seeking (Chen *et al.* 2014). Intermittent alcohol access using DID also resulted in enhanced, CRF-mediated excitatory neurotransmission on DAergic neurons within the VTA (Sparta *et al.* 2013). Taken together, these results provide significant evidence for (1) a role for local CRF signaling in the VTA signaling during stress and (2) a potential causal relationship between CRF modulation in the VTA and alcohol intake.

Perhaps most crucially, the importance of CRF signaling within the VTA during escalated, intermittent alcohol consumption has also been demonstrated. To this end, Hwa and colleagues (2013) subjected rats and mice to either IAA or a continuous access protocol. Subsequent intra-VTA administration of a CRFR1 antagonist resulted in significant decreases in alcohol intake across both IAA and continuous access groups (Hwa *et al.* 2013). While this study certainly underscored the importance of CRF signaling within the VTA during drinking, it did not directly tie the effect of stress, CRF signaling, and intake. Later work specifically examined this intersection. Here, Hwa *et al.* (2016) subjected mice to a stressor known as social defeat stress. It should first be noted that social defeat stress alters DA release in a similar way to other stressors (Wang *et al.* 2005; Tidey & Miczek 1996), but is considered a more ethologically valid stressor. The application of social defeat stress has also been shown to escalate alcohol consumption during IAA (Hwa *et al.* 2016; Norman *et al.* 2015). However, whether this social defeated-mediated escalation is the direct result of altered CRF signaling within the VTA remained an open question. Intra-VTA microinjection of a CRFR1 antagonist in defeated mice specifically decrease IAA intake as well as preference, with no effect on continuous access intake. Critically, CRFR1 antagonism was also shown to increase DA release in the NAc (Hwa *et al.* 2016).

When taken together, these findings recast the VTA as an area where local CRF signaling can influence (1) VTA functioning, (2) mesocorticolimbic circuit output (*e.g.* DA release), and (3) escalated, intermittent alcohol consumption. As such, it lends significant weight to the role of CRF in this region during escalated alcohol intake.

1.19 Is CRF endogenous to the VTA?

One open question regarding the role of CRF in the VTA during stress-induced, escalated alcohol intake is whether the source is from within the VTA itself, outside, or both. To this end, work by Grieder *et al.* (2014) provided convincing evidence for the presence of *Crf* mRNA in DAergic neurons within the VTA. However, projections from other regions with CRF+ populations have also been shown. For instance, retrograde approaches in conjunction with immunohistochemistry using CRF co-label revealed projections into the VTA originating from the central nucleus of the amygdala, BNST, and PVN (Rodaros *et al.* 2007). Later work confirmed a subset of these projections, particularly from the extended amygdala and BNST (Rinker *et al.* 2017; Dabrowska *et al.* 2016). This work also found CRF+ neurons within the VTA, but their functional relevance remained contested. Regardless, the presence of both intra-VTA CRF production as well as projection-specific CRF release indicates that CRF release in the VTA is modulated on multiple levels.

1.20 Conclusion

1.20.1 Female binge-like drinking. From a clinical perspective, it is clear that female binge drinking is becoming an increasingly larger problem. Despite this, the exact mechanisms behind binge drinking alone—let alone female-specific binge-drinking—are relatively unknown. Given its preeminent role in the effects of rewarding and reinforcing behavior, an obvious point of focus has been the mesocorticolimbic circuit. To this end, past preclinical work examining the neurocircuitry and modulation of binge-like drinking has focused heavily on higher doses and longer cycles (*e.g.* gavage approaches, Scheduled High Access Consumption, or SHAC). The use of these models has had both a technical

and theoretical component. On the one hand, it is extremely difficult to get animals to consume enough alcohol to engender pharmacologically relevant levels. As such, gavage and other high-access schedules have been the only approaches able to induce drinking at higher levels (**Figure 1.1, lower left**). This is also excluding other models of drinking (*e.g.* vapor chambers, intermittent alcohol access, or IAA) that are used to model escalated and dependence-like drinking (**Figure 1.1, ascending region**).

On the other hand, binge drinking has been taken as the initial phase on the road to dependence (**Figure 1.1, lower left**). As such, there is great interest in understanding the transition from binge drinking to uncontrolled drinking (**Figure 1.1, ascending region**). To best understand this process, longer cycles of intermittent bouts of binge drinking have also been used, in the form of chronic intermittent alcohol consumption, vapor chamber, or IAA (**Figure 1.1, ascending region**). To both of these points, it is only recently that the DID model has been developed. Not only has this protocol allowed mice to achieve pharmacologically relevant blood alcohol levels voluntarily, but the time course to such drinking is short (4 d total). While multiple DID cycles can (and have) been used, the short-term and voluntary nature of this approach allows for the means to examine the initial phases of the addiction cycle (**Figure 1.1, lower left**). More specifically, the kind of early binge drinking that occurs before (1) escalating to a cycle of intermittent, heavy bouts of binge drinking and (2) progressing to dependence (**Figure 1.1, ascending region**). In short, this phase sets the stage, as early alterations to underlying mesocorticolimbic circuitry will likely be involved in later progression to dependence.

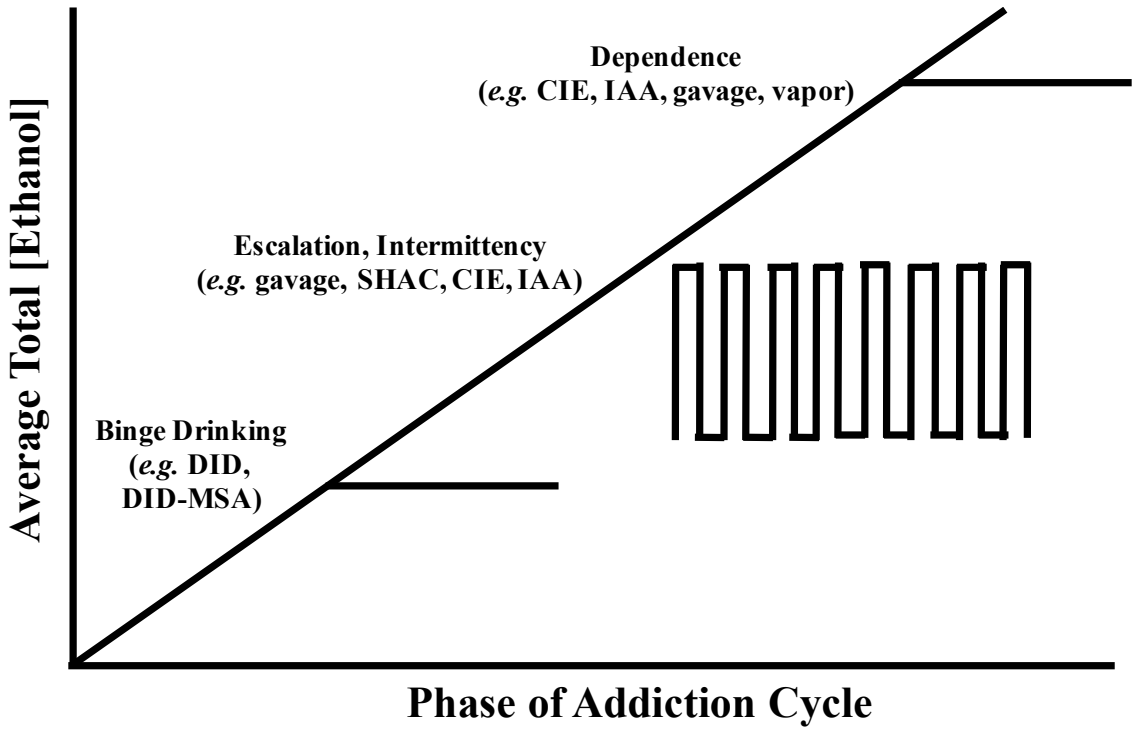


Figure 1.1: Overview of preclinical drinking models and phase of addiction cycle. *On a relative scale, binge drinking in its initial stages occurs at lower levels than other types of drinking, including dependence. While some populations plateau at this level (as indicated by horizontal line), others proceed to longer cycles of heavy drinking followed by withdrawal and abstinence (as indicated by cycling lines). Bouts of heavy drinking followed by periods of abstinence can eventually lead to dependent drinking, with higher alcohol consumption indicative of other factors (e.g. tolerance).*

Given the solidification of the DID protocol as a method for the short-term induction of pharmacologically relevant alcohol consumption, the question of male and female differences is more easily addressed. The importance of DAergic projection fibers from the VTA and subsequent DA release in the NAc cannot be understated. While other areas are certainly important to overall mesocorticolimbic circuitry (*e.g.* prefrontal cortex, lateral

habenula), release of DA in the NAc is strongly related to the rewarding actions of alcohol (Di Chiara & Imperato 1988). Critically, there is a difference in sensitivity of this release between males and females (*i.e.* greater release in females than in males in response to alcohol). This difference puts a large target on the VTA as a key area of possible difference between males and females, as differences in cellular composition and volume attest to (see **1.10: A Sexually Dimorphic VTA?**).

Although the VTA is a heterogeneous structure, the mix of DAergic and local (as well as projection) GABAergic neurons has revealed that certain areas seem to be more important to the directly rewarding actions of drugs than others. As such, the pVTA has become a focused target. While control of this unofficial subregion within the VTA is certainly influenced by projections from other regions (*e.g.* glutamatergic projections from the prefrontal cortex, GABAergic projections from the NAc), a good body of work using binge drinking approaches has centered on the importance of local GABAergic control.

While $GABA_A$ receptors themselves have multiple subunits from which they can be comprised, the δ subunit provides an intriguing target. Its expression is regulated in female mice, both in the VTA and other regions, and it is known to provide the basis for tonic inhibition. Perhaps most critically, low-to-moderate concentrations of alcohol have been shown to preferentially interact with δ-containing $GABA_A$ receptors. This nexus of the VTA, female-specific regulation, and the effects of lower concentrations of alcohol provide a prime target for study as it relates to mechanisms that may underlie the neurobiology behind female binge drinking.

With this in mind, I have asked three specific questions in the following work: (1) whether there are sex-specific baseline differences in δ subunit-mediated tonic inhibition in the VTA, (2) whether *Gabrd* excision in the pVTA would lead to sex-specific alterations in binge-like drinking, and (3) whether δ subunit-containing $GABA_A$ receptors are localized to a particular cellular population within the pVTA.

1.20.2 Stress-escalated alcohol intake. As with GABAergic signaling and binge-like drinking, the role of CRF signaling in the VTA has been pharmacologically tied to escalated alcohol intake. This is particularly after application of a stressor. However, an outstanding question has been whether direct activation of local CRF release within the VTA can alter later escalated drinking. In other words, was the stress-escalated alcohol intake observed by Hwa and colleagues (Hwa *et al.* 2016; Norman *et al.* 2015) caused *specifically* by local CRF release within the VTA?

To address this question, recent advances in cell-type-specific manipulation have offered an avenue of opportunity, including the development of the G-protein coupled receptor-based designer receptors exclusively activated by designer drugs (DREADDs, Armbruster *et al.* 2007). Two point mutations within the acetylcholine receptor render it inactive to its natural ligand acetylcholine, but active to the exogenously applied drug clozapine-*n*-oxide (CNO, for review, see Rogan & Roth 2011). CNO-activation of excitatory DREADDs (*e.g.* hM3Dq) can lead to burst firing (Armbruster *et al.* 2007), while CNO administration to inhibitory DREADDs (*e.g.* hM4Di) leads to neuronal hyperpolarization (Ferguson *et al.* 2011). When used in conjunction with Cre-technology, DREADD receptors can be

selectively expressed in a cell-type-specific manner, thereby allowing for chemogenetic control over different neuronal subtypes.

While this technology has been used in a wide variety of cell types thus far, the one of most relevance is CRF+ neuronal populations. To this end, Rinker *et al.* (2017) used bilateral, site-specific administration of AAV8-hSyn-DIO-hM4d-mCherry to the BNST in conjunction with CRF-Cre mice. Local CNO administration to the VTA was used to alter later alcohol consumption. Similarly, excitatory and inhibitory DREADD approaches have also been successfully used in CRF+ populations of the extended amygdala to manipulate pain (Andreoli, Marketkar & Dimitrov 2017).

Given the success of using chemogenetic stimulation on CRF+ neurons, it was hypothesized that the same approach would be able to successfully modulate local CRF release in the VTA. The singular question was the following: Is local CRF release in the VTA a critical, mechanistic component to stress-escalated alcohol consumption? As the data provided from this set of studies was preliminary in nature, further information regarding their (1) materials, (2) design, and (3) pilot data can be found in **Chapter 6: Appendix**.

Chapter 2
Materials and Methods

2.1 Transgenic Animal Lines

Floxed *Gabrd* mice were previously generated and characterized by our lab (Lee & Maguire 2013). Briefly, loxP sites were designed to flank exons 2 to 6b of the *Gabrd* gene. Conditional, Cre-mediated excision results in an unstable mRNA product and subsequent degradation. All floxed *Gabrd* mice were maintained on a C57Bl/6J background. In-house genotyping was performed at weaning and assessed for the presence of integrated loxP sites using the following primer sequences: 5'-AGC AAC TTT GCT TGC GCT G-3'and 5'-TTG ATA GCT GAA GCC CGT GG-3'

Floxed *Gabrg2* mice were obtained from The Jackson Laboratory (#016830, Bar Harbor, ME, USA). LoxP sites were designed to flank exon 8 of the *Gabrg2* gene. This site is important for synaptic anchoring and localization and excision leads to loss of $GABA_A$ γ2-containing receptors (Schweizer *et al.* 2003). All floxed *Gabrg2* mice were maintained on a C57Bl/6J background. In-house genotyping was performed at weaning and assessed for the presence or absence of Cre using the following primers: 5'-GCC TGA TTG TGG AAA TAA AA-3' and 5'-CAT CCC CTT ACT CTA TGT C-3'.

All floxed *Gabrd* and floxed *Gabrg2* mice were same-sex, group housed at the Tufts University School of Medicine, Division of Laboratory Animal Medicine, in clear, plastic cages in a temperature- and humidity-controlled environment with a 12:12 h light/dark cycle and *ad libitum* access to food and water. All mice were handled according to

protocols approved by the Tufts University Institutional Animal Care and Use Committee. and remained group housed until one week prior to binge-like drinking.

2.2 Adeno-associated Viruses

Two recombinant, adeno-associated viruses (AAVs) were used in this study: AAV-eGFP (control) and AAV-Cre-eGFP (experimental). AAV-eGFP (#7004, Vector Biolabs, Malvern, PA, USA) was serotype 2 and expressed enhanced green fluorescent protein (eGFP). AAV-Cre-eGFP (#7016, Vector Biolabs, Malvern, PA, USA) was also serotype 2 and contained Cre recombinase with a nuclear localization signal along with eGFP as a fluorescent marker. Both viruses were under control of a non-cell-type-specific CMV promoter and titers were approximately $1x10^{13}$ GC/mL.

2.3 *In vivo*, Stereotaxic Surgery

Adult (8- to 14-weeks of age) male and female floxed *Gabrd* and *Gabrg2* mice were randomly assigned to either AAV-eGFP (control) or AAV-Cre-eGFP (experimental) groups. Groups were balanced across litters to ensure equal and random assignment of control and experimental conditions. Mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine (i.p.) and tested for responsivity using both toe and tail pinches. A longitudinal incision was made through the shaved scalp to reveal the skull. The head was then securely fastened into a stereotaxic apparatus and manually leveled according to anterior/posterior and medial/lateral planes. A dental drill was used to make bilateral burr holes above the VTA according to the following coordinates: anterior/posterior (-3.6mm), and medial/lateral (±0.5mm). All coordinates were made relative to bregma and adjusted

using a ratio derived from each mouse and a standard bregma-to-lamda distance (4.2mm). A 0.5 µL Neuros Syringe (Neuros Model 7000.5, Hamilton Company, Reno, NV, USA) was lowered separately into each burr hole to a dorsal/ventral coordinate of -4.5mm. Dorsal/ventral distance was measured from the surface of the brain. After needle insertion, 250nL of either AAV-eGFP or AAV-Cre-eGFP was slowly injected over the course of three minutes. The syringe remained in place for an additional 5-7 minutes to (1) allow for complete diffusion of the virus and (2) prevent off-target transduction up the needle tract. Bilateral injection proceeded in an identical fashion. Total infusion time per side was approximately 12-15 min. Once ambulatory, mice were again group-housed and allowed to recover for three weeks to ensure efficient viral transduction.

2.4 Drinking in the Dark

Approximately two weeks after surgery, mice were individually housed and placed in a separate room under reverse light/dark housing conditions (12:12 h, lights off at 0700). One week later, they were subjected to the four-day Drinking in the Dark (DID), binge-like drinking protocol established by Rhodes *et al.* (2005). For the first three days of the protocol, mice were given one bottle of 20% (v/v) ethanol for a total of two, continuous hours. Introduction of the drinking tube was timed to occur three hours after the start of the dark cycle. On the fourth and final 'binge' day, mice were given 4 h of continuous access. No water was present during any ethanol access period. Volume differences across each access period were converted to individual g/kg values for each mouse based on mouse weights obtained on the day prior to the start of DID.

2.4.1 Blood ethanol concentration measurements. Blood ethanol concentration (BEC) measurements were conducted on a subset of *Gabrg2* and *Gabrd* mice used for the DID studies. Submandibular blood samples were obtained immediately after the end of the 4 h binge session. Samples were immediately centrifuged for 15 min at 14,000 rpm, plasma removed, and stored at -80ºC until later use. An AM1 Alcohol Analyzer (Analox Instruments, Stourbridge, UK) was used to obtain all BEC values. Samples were run in duplicate and averaged to obtain a final value.

2.5 Preference and Aversion Testing

One to two weeks after the end of DID, mice were subjected to successive sucrose preference and quinine aversion testing. Briefly, two, 50 mL plastic bottles with removable, double ball bearing, stainless steel sipper tubes were filled with either 2% (w/v) sucrose (Sigma-Aldrich, Natick, MA, USA) or normal drinking water were placed in two-bottle access metal tops fitted to each mouse's cage. Bottle weights were obtained every 24 h (1200 each day) for four, consecutive days. Bottles were alternated after each 24 h period to avoid the development of a side preference. Differences in consumption were used to calculate preference scores according to the following formula: ($g_{sucrose}/[g_{sucrose} + g_{water}]$). Volume (mL) of water consumption was also calculated for each individual mouse ($1g_{H2O} = 1mL_{H2O}$).

Subsequently, mice were then subjected to quinine aversion testing. Two, 50 mL plastic bottles with removable, double ball bearing, stainless steel sipper tubes were filled with either 0.3 mM quinine monohydrochloride (Sigma-Aldrich, Natick, MA, USA) or normal

drinking water and placed in two-bottle access metal tops fitted to each mouse's cage. Bottle weights were obtained every 24 h (1200 each day) for four, consecutive days. Bottles were alternated after each 24 h period to avoid the development of side preference. Differences in consumption were calculated as above, with aversion scores and total water intake (mL) obtained for each mouse over the four-day period.

2.6 Immunohistochemistry

After preference and aversion testing, mice were deeply anesthetized with isoflurane and rapidly decapitated. Brains were rapidly removed and immediately placed in 4% (w/v) paraformaldehyde at 4^{o}C for overnight fixation. Brains were then cryoprotected in a series of sucrose concentrations (10-30%), snap frozen in isopentane, frozen at -80^{o}C, and free-floating (PBS), 40 μM sections taken using a Leica CM1900 cryostat (Buffalo Grove, IL, USA).

2.6.1 eGFP. Three anterior VTA (aVTA, coordinate range -2.8mm to -3.4mm) and three posterior VTA (pVTA, coordinate range -3.52mm to -4.04mm) slices were selected for placement analysis from each animal used for behavioral data (drinking, sucrose preference, and quinine aversion). Sections were blocked with 10% normal goat serum (NGS, Vector Labs, Burlingame, CA, USA) and 0.3% Triton in PBS for 1 h at room temperature and incubated with a polyclonal anti-GFP primary antibody (rabbit, 1:1000, Invitrogen, Waltham, MA, USA) overnight at 4^{o}C. Sections were washed three times with PBS and incubated with a polyclonal AlexaFluor 488 secondary antibody (rabbit, 1:200, Invitrogen, Waltham, MA, USA) for 2 h at room temperature. After three more washes

with PBS, sections were briefly rinsed in dH_2O, mounted on slides, coverslipped the following day using VECTASHIELD mounting medium with DAPI (Vector Labs, Burlingame, CA, USA). Slides were imaged on a Keyence BZ-X700 (Keyence, Itasca, IL, USA), and analyzed using NIH Fiji software.

2.6.2 Parvalbumin and $GABA_A$ δ-subunit co-label. Adult (8- to 14-weeks of age) male (n=5) and female (n=5) C57Bl/6J mice were deeply anesthetized, decapitated, and tissue harvested and processed according to the above methods. Six hippocampal slices, three aVTA, and three pVTA slices were obtained from each animal. Slices were subjected to antigen retrieval by incubating in 0.05M citrate buffer for 30 min at room temperature, 45 min at 80°C, and allowed to cool down for an additional 20 min. Slices were washed three times with PBS then blocked in 10% NGS and 0.3% Triton in PBS for 1 h at room temperature. Slices were co-incubated with a polyclonal anti-δ primary antibody (rabbit, 1:250, EMD Millipore, Billerica, MA, USA) and a monoclonal anti-parvalbumin primary antibody (mouse, 1:1000, Sigma Life Sciences, St. Louis, MO, USA) for four days at 4°C. Slices were then washed three times with PBS and incubated with a biotinylated goat anti-rabbit IgG secondary (1:1000, Vector Labs, Burlingame, CA, USA) and a polyclonal AlexaFluor 647 secondary antibody (mouse, 1:200, Invitrogen, Waltham, MA, USA) overnight at 4°C. Slices were washed three times with PBS and incubated for 2 h at 4°C with streptavidin-conjugated AlexaFluor 488 secondary antibody (rabbit, 1:1000, Invitrogen, Waltham, MA, USA). After three more washes with PBS, sections were briefly rinsed in dH_2O, mounted on slides, and coverslipped the following day using VECTASHIELD mounting medium with DAPI (Vector Labs, Burlingame, CA, USA).

Slides were imaged on a Keyence BZ-X700 (Keyence, Itasca, IL, USA) and analyzed using NIH Fiji software. Hippocampal slices and VTA slices were processed separately.

2.7 Real-time quantitative PCR (qPCR)

Separate cohorts of *Gabrd* and *Gabrg2* mice were used to assess: (1) Sex-specific, baseline *Gabrd* and *Gabrg2* levels and (2) Effectiveness of Cre-mediated *Gabrd* and *Gabrg2* excision. Briefly, subjects for experiment (1) were WT littermates and were ethanol naïve. Subjects were deeply anesthetized using isoflurane, rapidly decapitated, and brains harvested under RNAse-free conditions. Whole brain was submerged in RNAlater (Invitrogen, Waltham, MA, USA) for 1 min and then sectioned into 1mm coronal brain slices using an adult mouse brain matrix (Zivic Instruments, Pittsburgh, PA, USA). Slices containing the VTA were selected and incubated in RNAlater for an additional 24 h at 4^{o}C. Slices were then removed and stored at -80^{o}C until later use. Subjects for experiment (2) were first bilaterally infused with either AAV-eGFP or AAV-Cre-eGFP according to previously described method. Three-weeks post-op, tissue was harvested in an identical manner as for experiment (1) and stored at -80^{o}C until later use.

Tissue slices were then further isolated for one of two regions of interest, VTA or hippocampus. Bilateral hippocampal tissue was visually dissected. VTA tissue punches were obtained using a Leica MZ6 stereoscope (Buffalo Grove, IL, USA) and a 0.5mm tissue punch (Stoelting Co., Wood Dale, IL, USA). RNA isolation was then performed on all tissue using a QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (Frederick, MD, USA). During RNA extraction, genomic DNA was digested using an

RNase-free DNase kit according to the manufacturer's instructions (QIAGEN, Frederick, MD, USA). Resulting RNA was analyzed for concentration and integrity using an Agilent Genomics 2100 Bioanalyzer (Santa Clara, CA, USA). All samples that had RNA integrity numbers (RIN) above 8.0 indicated highly intact RNA and were used to generate cDNA. Reverse transcription was performed using SuperScript III First-Strand Synthesis System according to the manufacturer's instructions and using a 1:1 mixture of randomers and oligomers (ThermoFisher Scientific, Cambridge, MA, USA). Resulting cDNA was stored at -20^{o}C until later use.

qPCR primers used are listed in Table 2.1. Two separate primers sets were used to assess WT baseline levels and Cre excision relative to GFP controls for both *Gabrd* and *Gabrg2* cDNA. Separate sets of *Gabrd and Gabrg2* primers were designed to assess either (1) baseline levels or (2) effectiveness of Cre-mediated excision. Those primers assessing baseline levels were located outside of either the *Gabrd* or *Gabrg2* excision regions, whereas those assessing excision were separately generated to be within the excised region. All primers were generated using Primer-BLAST (NCBI, NIH) and designed to span exon-exon junctions to avoid genomic DNA amplification. SYBR-green-based qPCR was performed using a Stratagene Mx3000P (Agilent Genomics, Santa Clara, CA, USA) and 3 ng of each VTA cDNA template. The thermocycling protocol began with a 10min ramp-up cycle to 95^{o}C. This was followed by 40 cycles of the following: 30sec at 95^{o}C, 1 min at 55^{o}C, and 30 s at 72^{o}C. After 40 cycles, a final dissociation curve was obtained (95^{o}C for 1 min, 55^{o}C for 30sec, and 95^{o}C for 30 sec) for each reaction to verify primer specificity. Relative transcript levels were calculated according to the $2^{-\Delta\Delta ct}$ method (Livak &

Schmittgen 2001), normalized first to the reference gene β-actin and then to either female WT (baseline qPCR) or female GFP (Cre-excision qPCR). Relative rather than absolute quantification was performed since samples did not contain a single, pure species of RNA. All reactions were performed in triplicate and a final, averaged C_q value used for analysis. A C_q value was excluded only if it was greater or less than 1 cycle relative to the remaining C_q values.

Gene	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
β-actin	GGC TGT ATT CCC CTC CAT CG	CCA GTT GGT AAC AAT GCC ATG T
Gabrd (WT)	ATT GGG GAC TAC GTG GGC T	CCA CAT TCA CAG GAG CAC C
Gabrd (Cre excision)	GGC GCC AGG GCA ATG AAT	CTG GAT GAT GTA GAC GCC CC
Gabrg2 (Variant 2, WT)	AGT TCG CCA AAT ACA TGG AGC	GTA GAG CGA TAG CAG GAG CA
Gabrg2 (Cre excision)	ACC ATG ACA ACT TTA AGC ACC A	CTT GCT TGG CTT CCG GTT G

Table 2.1: qPCR primer list. Gabrd *(WT) indicates primers used to assess male and female baseline* Gabrd *transcript levels.* Gabrd *(Cre excision) indicates primers used to assess Cre-mediated excision of* Gabrd *between AAV-eGFP and AAV-Cre-eGFP.* Gabrg2 *(Variant 2) indicates primers used to assess male and female baseline* Gabrg2 *transcript levels.* Gabrg2 *(Cre excision) indicates primers used to assess Cre-mediated excision between AAV-eGFP and AAV-Cre-eGFP. β-actin was used as a reference gene for both* Gabrd *and* Gabrg2*.*

2.8 Electrophysiology

Adult (8–14-weeks of age) *Gabrd* and *Gabrg2* mice were bilaterally injected with either AAV-eGFP or AAV-Cre-eGFP. Three- to five-weeks post-op, mice were anesthetized with isoflurane and decapitated, and the brain was rapidly removed and placed immediately in ice-cold, oxygenated normal artificial cerebrospinal fluid [nACSF; containing (in mM) 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 CaCl2, 2 $MgCl_2$, and 10 dextrose (300–310mosM)] containing 3mM kynurenic acid and bubbled with 95% O_2-5% CO_2. Coronal slices (350mm thick) containing the VTA were prepared using a Leica VT1000S vibratome. For voltage-clamp recordings, slices were maintained in oxygenated nACSF containing 3mM kynurenic acid. For current-clamp recordings, slices were maintained in oxygenated nACSF. After a 1 hour minimum recovery period, VTA-containing slices were placed into a recording chamber maintained at 33°C (in-line heater; Warner Instruments) and perfused at a high flow rate (4ml/min) throughout the experiment. SR95531 (200M) was added to the extracellular solution where indicated.

2.8.1 Voltage-clamp recordings. For voltage-clamp recordings, the intracellular recording solution contained (in mM) 140 CsCl, 1 MgCl2, 10 HEPES, 4 NaCl, 0.1 EGTA, 2 Mg-ATP, and 0.3 Na-GTP (pH 7.25, 280–290mosM). Electrodes were used with DC resistance of 5–8M. Following stabilization of the holding current and series resistance and

capacitance measurements, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in GFP+ cells from both AAV-eGFP and AAV-Cre-eGFP slices over a five-minute period at a holding potential of -70 mV. The frequency, peak amplitude, and weighted decay (w) of sIPSCs were measured using MiniAnalysis software (version 6.0.3, Synaptosoft Inc., Fort Lee, NJ, USA). Tonic GABAergic currents were measured as previously described (Maguire *et al.* 2005, 2009; Maguire and Mody 2007; Sarkar *et al.* 2011; Stell *et al.* 2003) in visually identified GFP+ cells from both AAV-eGFP and AAV-Cre-eGFP slices. The mean current was measured during 10ms epochs collected every 100ms throughout the experiment. A Gaussian was fit to these points to determine the mean holding current in nACSF and following bath application of SR95531 (200 nM) (Stell and Mody 2002). The difference in the holding current in the presence or absence of SR95531 was determined to be a measure of tonic GABAergic inhibition. Series resistance and whole cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by 20%.

2.9 Statistical Analyses

All data were collected and organized using Microsoft Excel (v. 15.39). All statistical analyses were conducted using Prism 7 (GraphPad, La Jolla, CA, USA). Repeated measures, two-way ANOVA was used to analyze DID 2 h drinking data, with AAV (GFP versus Cre) as the between-subjects factor and time as the within-subjects factor. Repeated measures, two-way ANOVAs were used to analyze both the sucrose preference and quinine aversion experiments, with AAV as the between-subjects factor and time as the within-

subjects factor. An unpaired, two-tailed Student's t test was used to analyze DID binge, 4 h drinking data (GFP versus Cre). Unpaired, two-tailed Student's t tests were also used to analyze baseline and Cre-excision qPCR data (male versus female, GFP versus Cre, respectively) as well as differences in PV+ and δ+ co-expression between males and females. A Pearson's correlation was used to analyze the relationship between AAV transduction (optical density) and binge drinking as well as intake (g/kg) and BECs. A p-value of $p<0.05$ was considered statistically significant for all analyses.

Chapter 3
Results

3.1 There is greater baseline $GABA_A$ **Gabrd** *expression and corresponding tonic inhibition in the VTA of females than males.*

Global *Gabrd* mRNA expression has been examined in male rodent brain, with high transcript levels in select areas such as cerebellum, several thalamic nuclei, dentate gyrus, and select basal nuclei (Wisden *et al.* 1992). Later work using immunohistochemical approaches confirmed these findings at the protein level (Fritschy & Möhler 1995). While a complete understanding of global *Gabrd* expression in females is still unknown, estrus cycle-dependent differences in transcript and protein levels of δ-subunit expression have both been shown in the hippocampus, VTA, and periaqueductal gray (PAG, Melón *et al.* 2017; Griffiths & Lovick 2005; Maguire *et al.* 2005). To better understand whether there were underlying sex differences in baseline δ subunit expression within the VTA, *Gabrd* transcript levels as well as δ-mediated tonic inhibition in C57Bl/6J males and females were analyzed. As cycling is known to be instigated by the presence of male cues such as urine and other odorants (Whitten 1956; Jemiolo, Harvey & Novotny 1986), tissue was obtained from same-sex housed animals maintained under separate, individual ventilation. Under these conditions, females remained naïve to the cycle-triggering cues presented by males and have been shown to be acyclic.

C57Bl/6J WT males and females were sacrificed under RNAse-free conditions and 1 mm slices containing the VTA were obtained. From these, bilateral VTA punches (0.5mm in diameter) were harvested and total RNA was extracted (**Figure 3.1a, top)**. VTA cDNA

was generated and *Gabrd* WT primers were used to assess baseline levels. Due to their higher levels, female *Gabrd* transcript levels were used to normalize male transcript levels (see **Chapter 2: Materials and Methods, Part A**). An unpaired, two-tailed Student's t-test was then conducted to compare baseline *Gabrd* transcript levels in the VTA of males and females. There was a significant difference in baseline VTA *Gabrd* levels between males (0.6508±0.157) and females (1.232±0.1383, $t(6)=2.775$, $p=0.0322$, **Figure 3.1a, bottom)**. These results indicated that in a baseline state (*i.e.* acyclic and ethanol-naïve), female mice have significantly increased *Gabrd* transcript levels in the VTA relative to males.

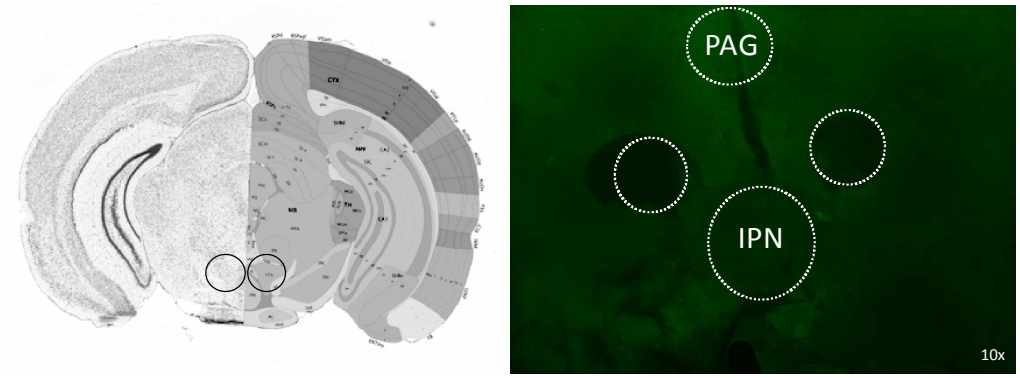
Given this significant increase, it was next determined if there was a functional effect of increased *Gabrd* levels on underlying inhibition. δ subunit-containing $GABA_A$ receptors have higher GABA affinity and are also extrasynaptically located. These twin properties allow for a persistent—or tonic—inhibitory tone on a given neuron (Stell *et al.* 2003; Wei *et al.* 2003; Glykys, Mann & Mody 2008). To determine whether there were baseline differences in tonic inhibition in the VTA, male and female floxed *Gabrd* mice were bilaterally injected with AAV-eGFP into the VTA. GFP+ cells within the VTA were then assessed for alterations in tonic inhibition. An unpaired, two-tailed Student's t-test was conducted, revealing a significant increase in tonic inhibitory currents in female VTA (27.23±4.957) relative to male VTA (8.554±4.291, $t(16)=2.849$, $p=0.0116$, **Figure 3.1b**). Collectively, these results show increased δ-mediated, tonic inhibition in the VTA of female mice relative to males.

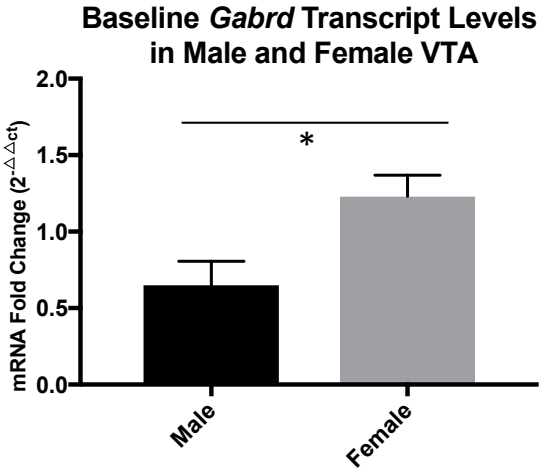
3.2 Cre-mediated **Gabrd** *excision in VTA results in decreased* **Gabrd** *transcript levels and reduced tonic inhibition.*

The observed increase in both *Gabrd* expression and δ-mediated tonic inhibition in females (**Figure 3.1**) indicated that there were fundamental, sex-specific differences in how tonic inhibition modulated VTA functioning. Given this, it was next hypothesized that *Gabrd* excision would not only decrease δ-mediated tonic inhibition, but that this decrease would be more significant in the VTA of females relative to males.

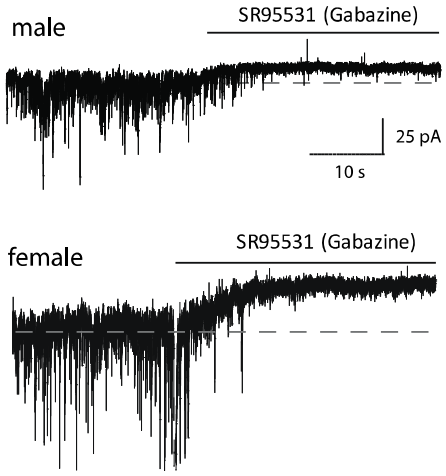
Adult male and female floxed *Gabrd* mice were generated (Lee & Maguire 2013) with loxP sites flanking exons 2 to 6b of the *Gabrd* gene (**Figure 3.2a**). Two cohorts of male and female mice were bilaterally injected into the VTA with AAV-Cre-eGFP to allow for site-specific *Gabrd* excision. Controls were bilaterally injected with AAV-eGFP, leaving the *Gabrd* gene intact. The first cohort was sacrificed three weeks post-op and 1 mm slices containing the VTA were obtained under RNAse-free conditions. VTA punches were then taken from both AAV-eGFP and AAV-Cre-eGFP males and females (**Figure 3.2b, top**) and validated for at least unilateral location of the virus (Cre or GFP) in the VTA as well as correct placement of at least one punch that included the virus (**Figure 3.2b, bottom**). RNA was extracted and subsequent male and female VTA cDNA assessed using *Gabrd* primers specific for the floxed *Gabrd* excision site. Male and female *Gabrd* levels were measured according to the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001), normalized first to the reference gene β-actin and second to female AAV-eGFP *Gabrd* levels.

a


PAG
IPN
10x


Baseline Gabrd Transcript Levels
in Male and Female VTA
mRNA Fold Change (2-△△ct)
2.0
1.5
1.0
0.5
0.0
*
Male
Female

b


male
SR95531 (Gabazine)
25 pA
10 s
female
SR95531 (Gabazine)

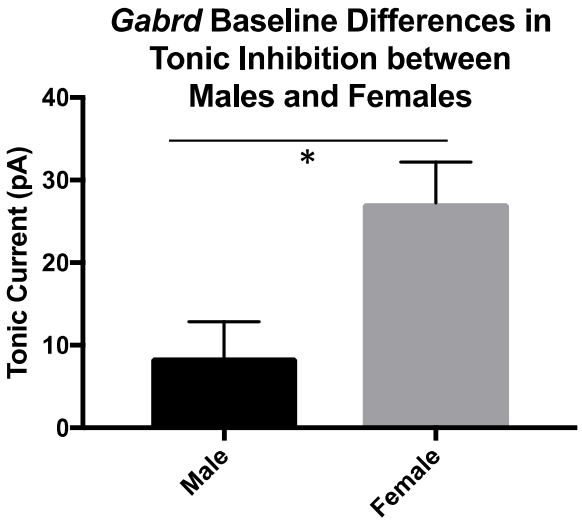

Gabrd Baseline Differences in
Tonic Inhibition between
Males and Females
Tonic Current (pA)
40
30
20
10
0
*
Male
Female

Figure 3.1: Increased baseline VTA $GABA_A$ Gabrd transcript levels and tonic inhibition in females. *(a) Bilateral VTA punches were obtained from C57Bl/6J male and female mice, as indicated by the representative image (40 μm). qPCR analysis revealed a significant increase in* Gabrd *transcript levels in female VTA relative to males. ($p=0.0322$, $n=4$ per group). (b) A separate cohort of mice received bilateral, AAV-eGFP injections into the VTA and were assessed for baseline differences in tonic current three weeks post-op. Representative trace (top) illustrating the effect on tonic current after application of the $GABA_A$ antagonist Gabazine in male and female mice. (below) Addition of Gabazine resulted in a significant increase in tonic current in female VTA relative to male. ($p<0.01$). Coronal image courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing, grayscale, and indicated regions of interest.*

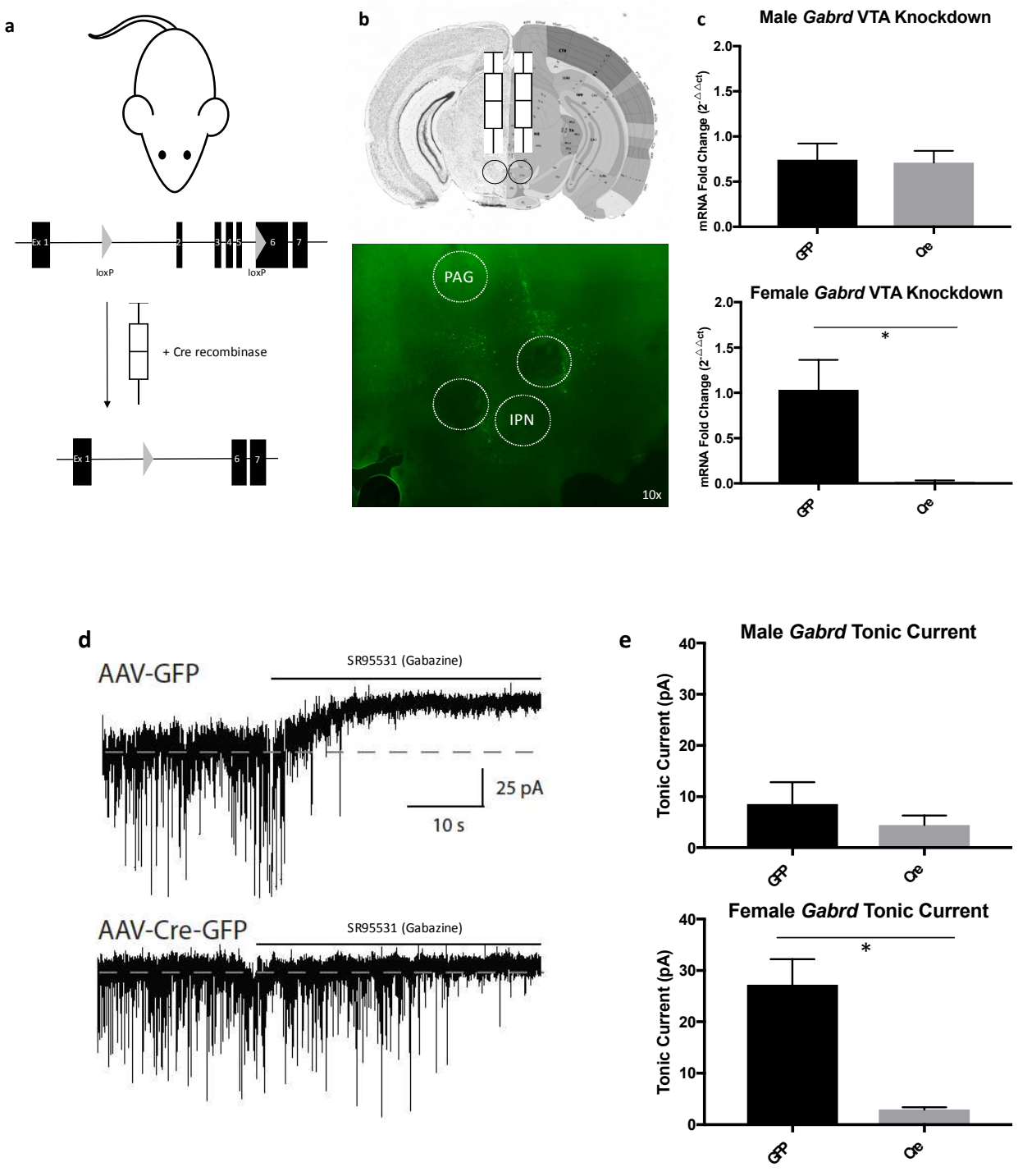

a
Ex 1
loxP
2
3
4
5
loxP
6
7
+ Cre recombinase
Ex 1
6
7
b
PAG
IPN
10x
c
Male Gabrd VTA Knockdown
mRNA Fold Change (2^-△△ct)
2.0
1.5
1.0
0.5
0.0
GFP
Cre
Female Gabrd VTA Knockdown
mRNA Fold Change (2^-△△ct)
2.0
1.5
1.0
0.5
0.0
*
GFP
Cre
d
AAV-GFP
SR95531 (Gabazine)
25 pA
10 s
AAV-Cre-GFP
SR95531 (Gabazine)
e
Male Gabrd Tonic Current
Tonic Current (pA)
40
30
20
10
0
GFP
Cre
Female Gabrd Tonic Current
Tonic Current (pA)
40
30
20
10
0
*
GFP
Cre

Figure 3.2: Cre-mediated* Gabrd *knockdown in VTA results in decreased* Gabrd *transcript levels and reduced tonic inhibition only in females. *(a) Floxed* Gabrd *mice were generated to allow for excision of exons 2 through 6b of the* Gabrd *gene. (b) 0.5mm VTA punches were obtained from floxed Gabrd male and female mice bilaterally injected with either AAV-eGFP or AAV-Cre-eGFP. (c) qPCR analysis revealed a significant decrease in* Gabrd *transcript in the VTA of (bottom) female AAV-Cre-eGFP (n=3) relative to AAV-eGFP (n=3) controls (p=0.006), but no significant difference (top) between male AAV-Cre-eGFP (n=3) and AAV-eGFP (n=4) controls. (d) Representative traces from the VTA of female AAV-eGFP (top) and female AAV-Cre-eGFP (bottom). Change in holding current after application of Gabazine is nearly absent in slices obtained from females with Cre-mediated excision of* Gabrd*, indicating near ablation of δ-mediated tonic inhibition. (e) Significant decrease in tonic inhibition was seen (bottom) between female AAV-Cre-eGFP and AAV-eGFP (p<0.0001), but not between male AAV-Cre-eGFP and AAV-eGFP (top). Coronal image courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing, grayscale, indicated regions of interest, and bilateral neurosyringes.*

Female AAV-eGFP levels were chosen for normalization since they were approximately two-fold higher than baseline levels in males (**Figure 3.1**). An unpaired, two-tailed Student's t-test was conducted to compare female AAV-eGFP VTA *Gabrd* levels with those from female AAV-Cre-eGFP. There was a significant decrease in *Gabrd* levels in female AAV-Cre-eGFP (0.01594±0.01077) relative to female AAV-eGFP (1.032±0.1921, $t(4)=5.282$, $p=0.0062$, **Figure 3.2c, bottom**). Similarly, an unpaired, two-tailed Student's t-test was conducted to compare male AAV-eGFP VTA *Gabrd* levels with those from male AAV-Cre-eGFP. There was no significant difference in *Gabrd* levels between male AAV-Cre-eGFP (0.5137±0.06122) and male AAV-eGFP (0.7064±0.2474, $t(5)=0.8785$, $p=0.4199$, **Figure 3.2c, top**).

To assess whether there was a functional effect of Cre-mediated *Gabrd* excision, the second cohort of bilaterally injected (AAV-Cre-eGFP or AAV-eGFP) male and female mice were sacrificed three weeks post-op and changes in tonic inhibition under voltage

clamp were recorded from GFP+ cells as previously described (see Materials and Methods). An unpaired, two-tailed Student's t-test was conducted to compare tonic inhibitory currents from the VTA of female AAV-eGFP mice with those from female AAV-Cre-eGFP. One female AAV-eGFP cell was a statistical outlier and was removed from the final analysis. There was a significant decrease in tonic inhibitory currents from the VTA in female AAV-Cre-eGFP (2.902±0.4899) relative to female AAV-eGFP (27.23±4.957, $t(17)=5.159$, $p<0.0001$), **Figure 3.2d, e, top**). Similarly, an unpaired, two-tailed Student's t-test was conducted to compare tonic inhibitory currents from the VTA of male AAV-eGFP with those from male AAV-Cre-eGFP. There was no significant difference in tonic inhibition between either male AAV-Cre-eGFP or male AAV-eGFP (4.462±1.846 and 8.554±4.291, respectively, $t(16)=0.8761$, $p=0.3940$, **Figure 3.2e, bottom**).

Collectively, these results indicate that Cre-mediated excision can effectively decrease both *Gabrd* transcript levels and has functionally impact on tonic inhibition within the VTA. While this effect was only notable in females, its apparent ineffectiveness in males is due to the floor effect of low baseline *Gabrd* expression (**Figure 3.1,** male AAV-eGFP in **Figure 3.2e, top**). In other words, *Gabrd* is expressed at such low levels in the VTA of males that Cre-mediated excision has no noticeable effect on either transcript levels or tonic inhibition.

3.3 VTA **Gabrd** *excision results in decreased binge-like drinking in females, but not males.*

In addition to underlying the difference between phasic and tonic inhibition, there are also subunit-specific effects that are fundamental to the actions of certain drugs (e.g. benzodiazepines, see Rudolph *et al.* 1999), including alcohol. More specifically, low concentrations (<30 mM) like those seen during binge drinking (Wallner, Hanchar & Olsen 2006) interact with δ subunit-containing $GABA_A$ receptors (Wallner Hanchar & Olsen 2003; Hanchar *et al.* 2005). Given the fundamental importance of the VTA to the effects of alcohol (Di Chiara & Imperato 1988; Stuber *et al.* 2008), it is likely that the baseline and Cre-mediated sex differences seen in δ subunit-containing $GABA_A$ receptors would alter lower doses of alcohol consumption. As such, it was next determined whether δ subunit-containing $GABA_A$ receptors had a sex-dependent effect on binge drinking.

To address this question, adult male and female floxed *Gabrd* mice were bilaterally injected into the VTA with either AAV-eGFP or AAV-Cre-eGFP. Three weeks post-op, mice were subjected to one cycle (4 d) of the DID binge-like drinking protocol (Rhodes *et al.* 2005). During the first three days, mice were allowed 2 h of access to 20% (v/v) ethanol that was timed to occur three hours after the start of the dark cycle. The fourth and final day was the 'binge' day, where animals had 4 h of access rather than 2 h.

A repeated, two-way ANOVA was conducted to compare 2 h drinking for the first three days of access between female AAV-Cre-eGFP and AAV-eGFP. There were no significant main effects of day ($F(2,28)=1.93$, $p=0.1643$) or virus ($F(1,14)=2.08$, $p=0.1712$) on alcohol

drinking (**Figure 3.3b, top**), which corroborate the lower intake seen during the first three days of 2 h access in the DID protocol (Rhodes *et al.* 2005). An unpaired, two-tailed Student's t-test was then conducted to compare female AAV-Cre-eGFP and AAV-eGFP binge-like drinking on the final, 4 h access day. There was a significant decrease in binge-like drinking in AAV-Cre-eGFP (0.9694±0.5473) relative to AAV-eGFP (3.899±1.1.446, p=0.0369, **Figure 3.3b, bottom**). A repeated, two-way ANOVA was also conducted to compared 2 h drinking for the first three days of access between male AAV-Cre-eGFP and AAV-eGFP. Similar to female 2 h intake, there were no significant main effects of day ($F(2,46)=0.07$, $p=0.9292$) or virus ($F(1, 23)=0.000212$, $p=0.9885$) on alcohol drinking (**Figure 3.3a, top**). An unpaired, two-tailed Student's t-test was conducted to compare male AAV-Cre-eGFP and AAV-eGFP binge-like drinking on the final, 4 h access day. There was no significant difference in binge-like drinking between AAV-Cre-eGFP (3.667±0.865) and AAV-eGFP (3.877±0.843, p=0.8683, **Figure 3.3a, bottom**). Collectively, these findings indicate that selective *Gabrd* excision from the VTA results in dramatically decreased binge-like drinking; critically, that this effect is only evident in females.

To validate g/kg intake values against measureable BECs, a subset of the male and female mice subjected to DID had submandibular bloods taken immediately after the 4 h 'binge' session. BEC measurements were obtained and correlated with g/kg 'binge' intake values. There was a significant correlation across subjects and AAV group between BEC and g/kg intake ($r=0.9762$, $n=12$, $p<0.0001$, **Figure 3.3c**), verifying that g/kg values were an accurate reflection of g/kg alcohol consumed.

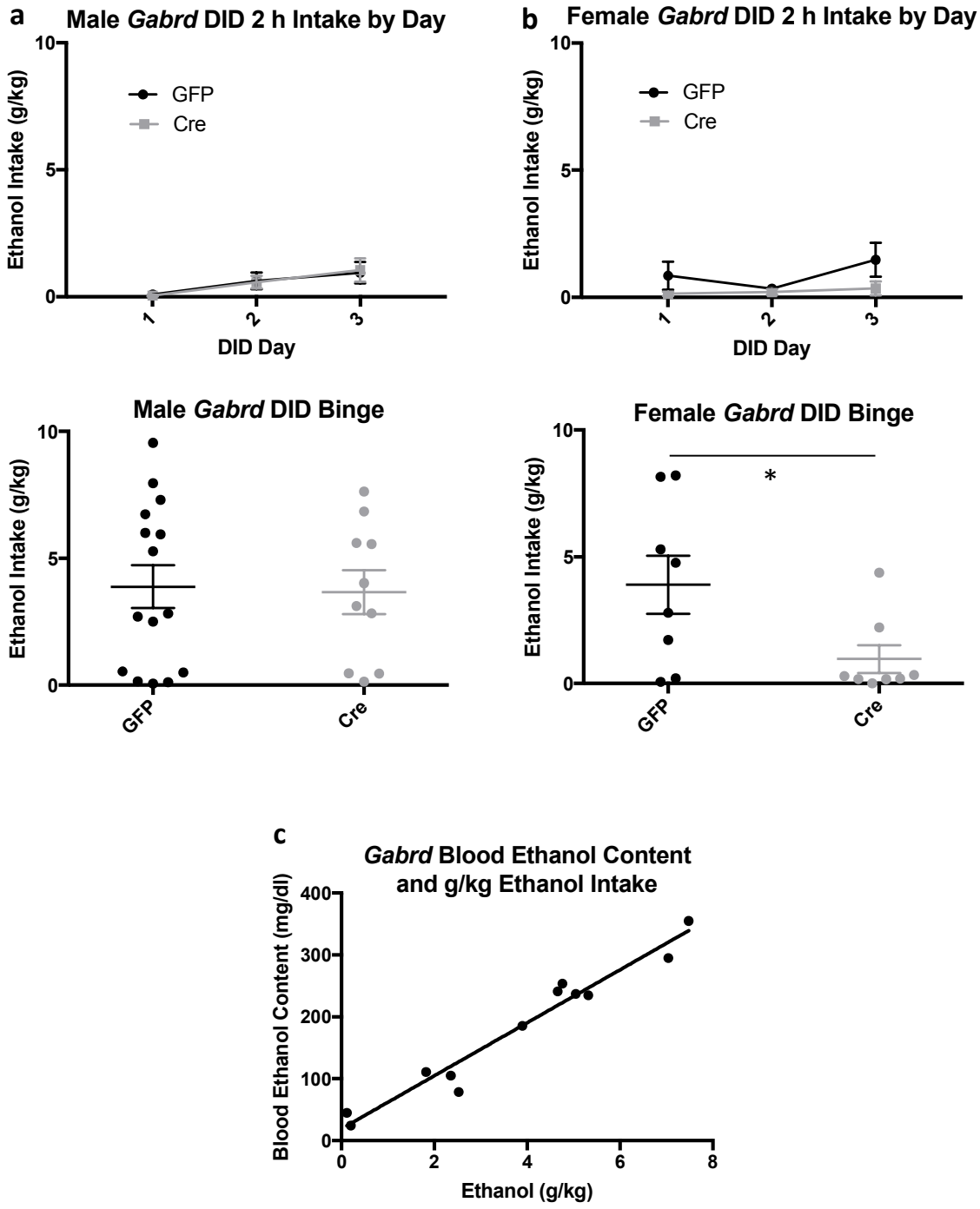

a
Male *Gabrd* DID 2 h Intake by Day
Ethanol Intake (g/kg)
GFP
Cre
DID Day
b
Female *Gabrd* DID 2 h Intake by Day
Male *Gabrd* DID Binge
Female *Gabrd* DID Binge
*
c
Gabrd Blood Ethanol Content and g/kg Ethanol Intake
Blood Ethanol Content (mg/dl)
Ethanol (g/kg)

Figure 3.3:* Gabrd *excision from the VTA selectively reduces female binge-like drinking. Gabrd *excision resulted in a significant difference in female binge-like drinking with no change in males. (a) VTA* Gabrd *excision had no effect on male 20% alcohol drinking over the first three days of 2 h access (top) and no effect on binge-like drinking (bottom). (b) VTA* Gabrd *excision had no effect on female 20% alcohol drinking across the first three days of 2 h access (top), but led to a significant decrease in binge-like drinking (bottom, $p=0.0369$). (c) To verify that calculated g/kg intake translated to measurable BEC values, a subset of male and female* Gabrd *mice subjected to DID had submandibular blood samples collected immediately after the 4 h 'binge' session. BEC measurements revealed a significant correlation between final g/kg binge intake values and BEC ($r=0.9762$, $p<0.0001$).*

Alcohol contains both a sweet and bitter component to its flavor profile. Differences in intake could be due to alterations in the chemosensory properties of the drug rather than its pharmacological effects, although this has been repeatedly shown to not be the case (Nachman, Larue & Magnen 1971; Kiefer & Lawrence 1988). Barring that, differences in intake might also be specific to the pharmacological properties of alcohol or generalizable to other naturally rewarding stimuli like sucrose (Pfaffmann 1982). Given that, it was important to ascertain whether there were alterations in either sucrose preference or quinine aversion in a subset of animals used for DID. One to two weeks after DID, animals were sequentially subjected to tests of sucrose preference (4 d) followed by quinine aversion (4 d). Animals had a 3 d break in between tests, during which time they had two bottles of water.

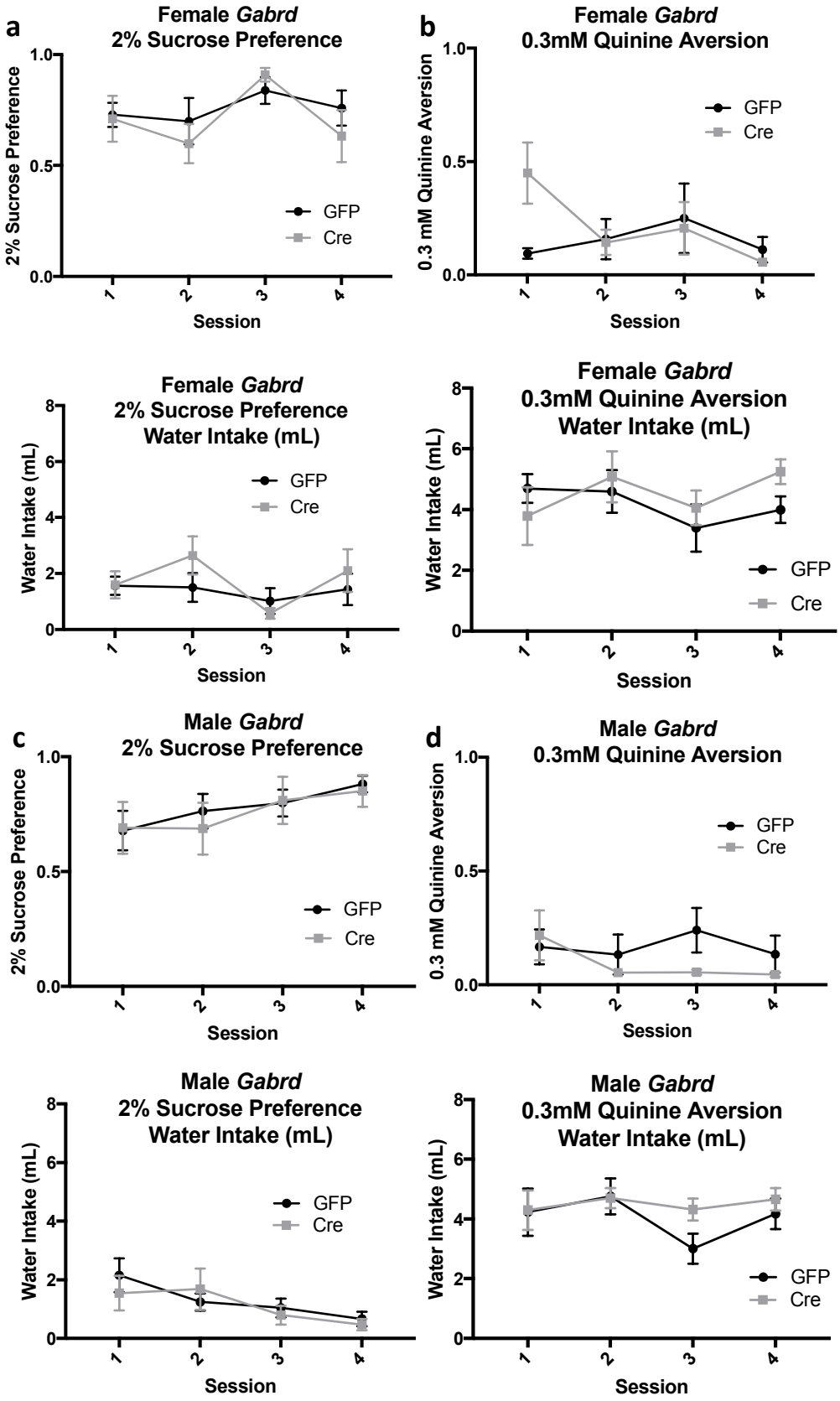

a
Female *Gabrd*
2% Sucrose Preference
2% Sucrose Preference
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
b
Female *Gabrd*
0.3mM Quinine Aversion
0.3 mM Quinine Aversion
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
Female *Gabrd*
2% Sucrose Preference
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
Female *Gabrd*
0.3mM Quinine Aversion
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
c
Male *Gabrd*
2% Sucrose Preference
2% Sucrose Preference
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
d
Male *Gabrd*
0.3mM Quinine Aversion
0.3 mM Quinine Aversion
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
Male *Gabrd*
2% Sucrose Preference
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
Male *Gabrd*
0.3mM Quinine Aversion
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session

Figure 3.4: No significant* effect of Gabrd *excision on sucrose preference or quinine aversion. *One to two weeks after finishing DID, male and female AAV-Cre-eGFP and AAV-eGFP mice were subjected to 4 d of sucrose (2%, w/v) preference. After a 3 d break, they were then subjected to 4 d of 0.3 mM quinine aversion. A repeated, two-way ANOVA was used to analyze all preference, aversion, and water intake data. (a) (top) There were no significant main effects of either day (F(3,42)=0.62, p=0.6091) or virus (F(1,14)=0.42, p=0.5287) for female* Gabrd *sucrose preference. (bottom) There were no significant main effects of either day (F(3,42)=0.92, p=0.4395) or virus (F(1,14)=0.79, p=0.3883) for female* Gabrd *water intake during sucrose preference testing. (b) (top) There were no significant main effects of either day (F(3,42)=2.57, p=0.0668) or virus (F(1,14)=0.59, p=0.4548) for female* Gabrd *quinine aversion. (bottom) There were no significant main effects of either day (F(3,42)=1.35, p=0.2708) or virus (F(1,14)=0.32, p=0.5817) for female* Gabrd *water intake during quinine aversion testing. (c) (top) There were no significant main effects of either day (F(2,46)=0.07, p=0.9292) or virus (F(1,23)=0.000212, p=0.9885) for male* Gabrd *sucrose preference. (bottom) There were no significant main effects of either day (F(3,54)=0.76, p=0.5236) or virus (F(1,18)=0.12, p=0.7287) for male* Gabrd *water intake during sucrose preference testing. (d) (top) There were no significant main effects of either day (F(3,51)=1.08, p=0.3673) or virus (F(1,17)=0.95, p=0.3445 for male* Gabrd *quinine aversion). (bottom) There were no significant main effects of either day (F(3,51)=1.13, p=0.3452) or virus (F(1,17)=0.49, p=0.4940) for male* Gabrd *water intake during quinine aversion testing.*

Results indicated no significant differences between female AAV-Cre-eGFP or AAV-eGFP groups in their preference for 2% (w/v) sucrose or their aversion to 0.3 mM quinine (**Figure 3.4a, b**). Similarly, there were no significant differences between male AAV-Cre-eGFP or AAV-eGFP groups in their sucrose preference or quinine aversion (**Figure 3.4c, d**). Although there were no differences in sucrose preference—and, therefore, no caloric considerations to control for—a subset of these animals were also tested for saccharin preference. There was a significant main effect for day for female saccharin preference, but there were no significant preference differences between either AAV-Cre-eGFP or AAV-eGFP groups for either males or females (see **Figure 9.1**).

Taken together, these results indicate that female binge-like drinking is significantly affected by the absence of δ subunit-containing $GABA_A$ receptors in the VTA.

Comparatively, male binge-like drinking is unchanged by *Gabrd* excision from the VTA. Furthermore, this effect is specific to the pharmacological properties of alcohol, as it neither affects animals' taste responsivity (e.g. sweet versus bitter) nor does it generalize to other rewarding substances (e.g. sucrose or saccharin). It should also be noted that this effect does not alter general water consumption, indicating that excision does not affect general fluid consumption.

3.4 No differences in either baseline VTA Gabrg2 *transcript levels or phasic inhibition between males and females.*

As discussed previously, there are structural differences underlying $GABA_A$-mediated phasic and tonic inhibition that are driven partly by either the δ or γ2 subunit, respectively (Farrant & Nusser 2005). Given the δ-subunit-mediated effects we observed in female binge drinking, it was next determined if they were specific to δ-mediated tonic inhibition or apply to GABAergic inhibition more broadly.

Unlike *Gabrd, Gabrg2* transcript is widely expressed in the male rat brain and is abundant in the VTA (Wisden *et al.* 1992; Fritschy & Möhler 1995). Although its overall expression patterns have not been extensively studied in females, cycling-dependent effects on hippocampal γ2 levels have (protein levels, Maguire *et al.* 2005) and have not (transcript levels, Melón *et al.* 2017) been observed. However, since γ2 is the most commonly found γ subunit family member, it is likely that it also has high, non-cyclic-dependent expression in females. Given this, we first sought to determine whether there were baseline differences in *Gabrg2* transcript levels. The same C57Bl/6J VTA cDNA used to assess baseline *Gabrd*

levels was used (see previous description, **Figure 3.1**), but with primers specific to *Gabrg2*. An unpaired, two-tailed Student's t-test was conducted to compare baseline *Gabrg2* transcript levels in the VTA of males and females. There was no significant difference in baseline *Gabrg2* transcript levels in the VTA between males (2.428±0.1073) and females (2.136±0.1737; t(6)=1.431, p=0.2025, **Figure 3.5a**).

To verify that the equivalent *Gabrg2* levels seen corresponded to equivalent phasic inhibition, a cohort of male and female floxed *Gabrg2* mice were bilaterally injected with AAV-eGFP into the VTA. Three weeks post-op, mice were sacrificed and coronal sections were obtained to assess relative differences in sIPSC frequency and amplitude between male and females. Introduction of AAV-eGFP did not alter the *Gabrg2* gene and only GFP+ cells within the VTA were recorded. An unpaired, two-tailed Student's t-test was conducted to compare sIPSC frequency differences in the VTA of males and females. There was no significant difference in baseline sIPSC frequency differences between male VTA (7.000±0.7071) and female VTA (5.744±1.046, t(18)=1, p=0.3304, **Figure 3.5b, left**). Using a separate, unpaired, two-tailed Student's t-test, we compared sIPSC peak amplitude between male and female VTA. There was no significant difference in baseline peak amplitude between male VTA (69.49±6.715) and female VTA (59.54±6.49, t(18)=1.05, p=0.3075, **Figure 3.5b, right**).

Taken together, these results indicate that there are similar levels of *Gabrg2* transcript in the VTA of both males and females. Moreover, there are no functional, sex-specific

differences in phasic inhibition within the VTA, as indicated by similar sIPSCs and peak amplitude between males and females.

3.5 Cre-mediated excision of **Gabrg2** *results in decreased* **Gabrg2** *transcript levels as well as decreased sIPSC frequency and peak amplitude.*

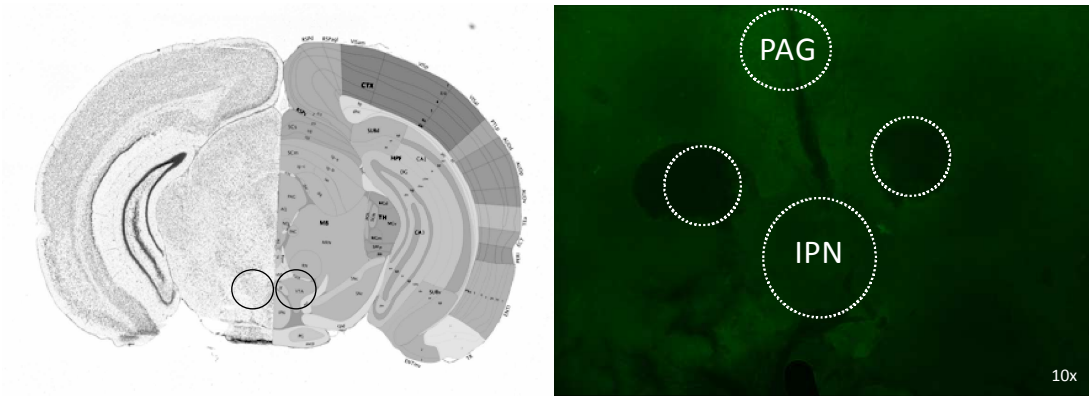
The sex-specific baseline differences in *Gabrd* expression observed in the VTA resulted in the sex-specific decrease seen in females subjected to Cre-mediated, *Gabrd* excision. Since no differences in either *Gabrg2* transcript levels or phasic inhibition were observed, it was hypothesized that Cre-mediated *Gabrg2* excision would be equivalently effective in both males and females.

Adult male and female floxed *Gabrg2* mice were generated (Schweizer *et al.* 2003) and maintained on a C57Bl/6J background (**Figure 3.6a**) Two cohorts were selected and bilaterally injected with either AAV-Cre-eGFP or AAV-eGFP into the VTA. Three weeks post-op, the first cohort was sacrificed and 1 mm slices containing the VTA were harvested under RNase-free conditions. VTA tissue punches were obtained, total RNA extracted, and cDNA generated. Primers specific to the excised exon 8 of *Gabrg2* were selected to assess *Gabrg2* levels (**Figure 3.6a**). AAV and punch placement were verified (**Figure 3.6b**) and only punches that were at least unilateral viral and neuroanatomical hits were included in the final analysis (**Figure 3.6b, below**). *Gabrg2* levels were assessed according to the $2^{-\Delta\Delta ct}$ method as previously described, standardized first the reference gene β-actin and then to female AAV-eGFP *Gabrg2* levels. An unpaired, two-tailed Student's t-test was conducted to compare female AAV-eGFP VTA *Gabrg2* levels with those from female

AAV-Cre-eGFP. There was a near significant decrease in *Gabrg2* levels in female AAV-Cre-eGFP (0.7342±0.0645) relative to female AAV-eGFP (1.000±0.01271, t(5)=2.459, p=0.0573, **Figure 3.6c, bottom**). Similarly, an unpaired, two-tailed Student's t-test was conducted to compare male AAV-eGFP VTA *Gabrg2* levels with those from male AAV-Cre-eGFP. There was a significant difference in *Gabrg2* levels between male AAV-Cre-eGFP (1.000±0.0312) and male AAV-eGFP (0.7003±0.02797, t(3)=6.989, p=0.0060, **Figure 3.6c, top**).

Coronal slices from the second cohort of stereotaxically injected mice were next obtained to determine whether Cre-mediated excision of *Gabrg2* affected phasic inhibition as previously described (see Materials & Methods). An unpaired, two-tailed Student's t-test was conducted to compare sIPSC frequency from the VTA of female AAV-eGFP mice with those from female AAV-Cre-eGFP. There was a significant decrease in sIPSC frequency in female AAV-Cre-eGFP (0.5444±0.2039) relative to female AAV-eGFP (5.774±1.046, t(19)=5.63, p<0.0001), **Figure 3.6e, bottom left**). Similarly, an unpaired, two-tailed Student's t-test was conducted to compare sIPSC peak amplitude from the VTA of female AAV-eGFP mice with those from female AAV-Cre-eGFP. There was a significant decrease in peak amplitude in female AAV-Cre-eGFP (39.19±6.982) relative to female AAV-eGFP (59.54±6.49, t(19)=2.068, p=0.0526), **Figure 3.6e, bottom right**).

a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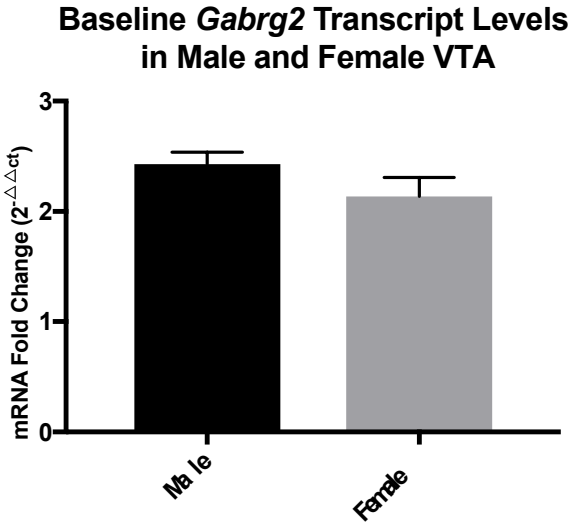
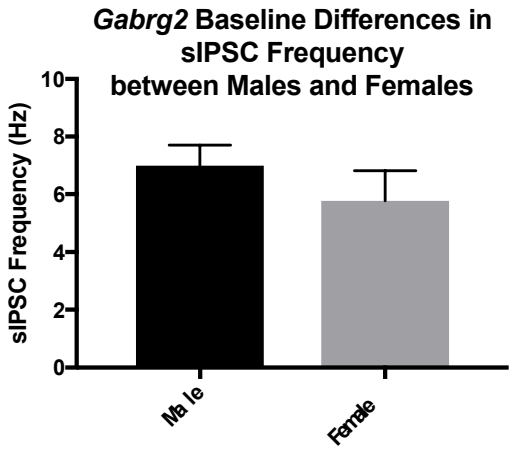

Baseline Gabrg2 Transcript Levels
in Male and Female VTA
mRNA Fold Change (2-△△ct)
3
2
1
0
Male
Female

b


Gabrg2 Baseline Differences in
sIPSC Frequency
between Males and Females
sIPSC Frequency (Hz)
10
8
6
4
2
0
Male
Female

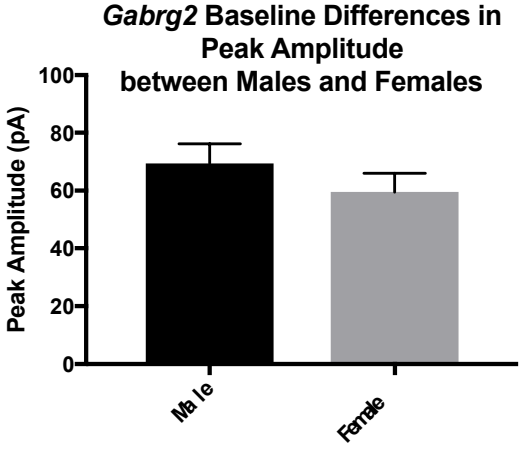
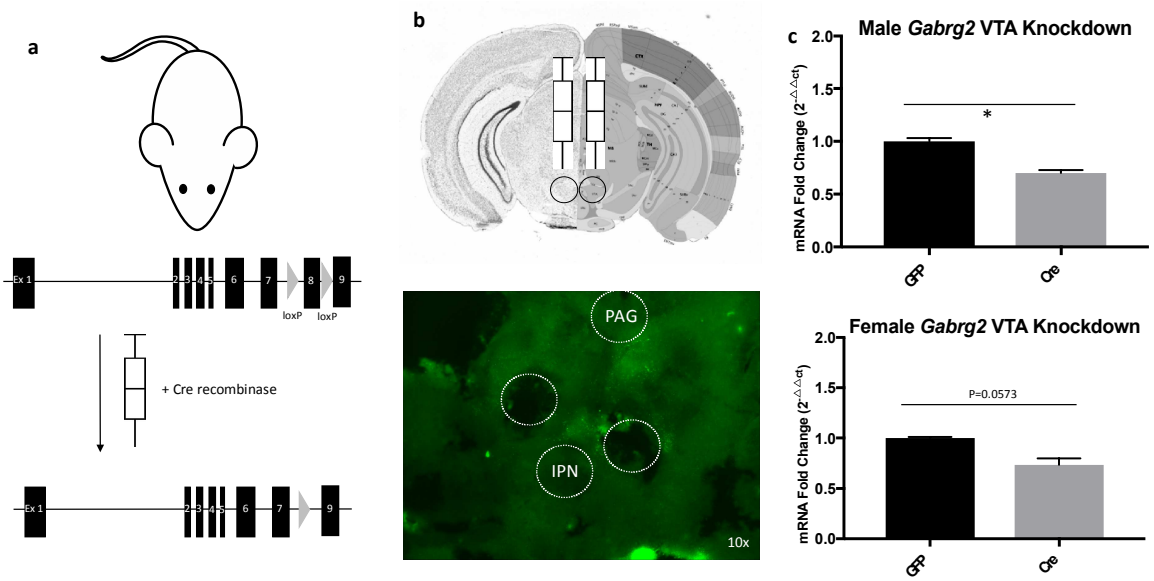

Gabrg2 Baseline Differences in
Peak Amplitude
between Males and Females
Peak Amplitude (pA)
100
80
60
40
20
0
Male
Female

Figure 3.5: No baseline differences in VTA* Gabrg2 *transcript levels, sIPSC frequency, or sIPSC peak amplitude between male and females. *(a) (top) Bilateral VTA punches were taken from C57Bl/6J male and female mice as described in* ***Figure 3.1****. Resulting cDNA was assessed using* Gabrg2*-specific primers for baseline transcript levels. (bottom) There were no significant differences between male and female* Gabrg2 *level (p=0.2025). (b) There were no significant functional differences in phasic inhibition between male and female VTA, as indicated by the lack of difference in both sIPSC frequency and peak amplitude (p=0.3304 and p=0.3075, respectively). Coronal image courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing, grayscale, and indicated region of interest.*

The effectiveness of Cre-mediated *Gabrg2* excision in male AAV-eGFP and AAV-Cre-eGFP mice was next assessed. An unpaired, two-tailed Student's t-test was conducted to compare sIPSC frequency from the VTA of male AAV-eGFP mice with those from male AAV-Cre-eGFP. There was a significant decrease in sIPSC frequency in male AAV-Cre-eGFP (1.5±0.6118) relative to female AAV-eGFP (7.000±0.7071, $t(18)=5.735$, $p<0.0001$), **Figure 3.6e, top left**). Finally, an unpaired, two-tailed Student's t-test was conducted to compare sIPSC peak amplitude from the VTA of male AAV-eGFP mice with those from male AAV-Cre-eGFP. There was a significant decrease in peak amplitude in male AAV-Cre-eGFP (38.73±8.754) relative to male AAV-eGFP (69.49±6.715, $t(18)=2.836$, $p=0.0110$), **Figure 3.6e, top right**). Collectively, these findings demonstrate that Cre-mediated excision decreases *Gabrg2* transcript levels, which results in a functional decrease in phasic inhibition in both male and female VTA.


a
Ex 1
2 3 4 5
6
7
8
9
loxP
loxP
+ Cre recombinase
b
PAG
IPN
10x
c
Male Gabrg2 VTA Knockdown
mRNA Fold Change (2^-△△ct)
2.0
1.5
1.0
0.5
0.0
*
GFP
Cre
Female Gabrg2 VTA Knockdown
P=0.0573

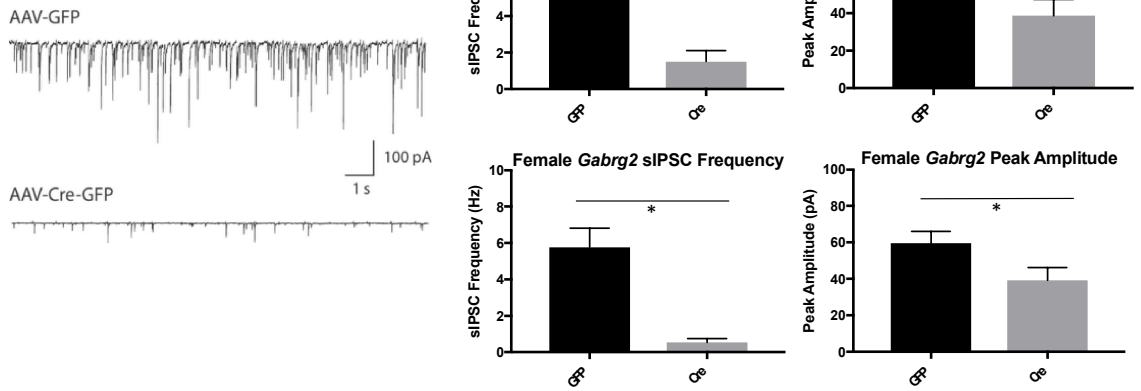

d
AAV-GFP
100 pA
1 s
AAV-Cre-GFP
e
Male Gabrg2 sIPSC Frequency
sIPSC Frequency (Hz)
Male Gabrg2 Peak Amplitude
Peak Amplitude (pA)
Female Gabrg2 sIPSC Frequency
Female Gabrg2 Peak Amplitude
GFP
Cre

Figure 3.6: Cre-mediated* Gabrg2 *excision in VTA results in decreased* Gabrg2 *transcript levels and phasic inhibition. *(a) Floxed* Gabrg2 *mice were previously generated (Schweizer* et al. *2003) and maintained on a C57Bl/6J background. loxP sites flanked exon 8 of the* Gabrg2 *gene, which interferes with normal trafficking and subsequently reduced levels. (b) Tissue punches were obtained from 1 mm thick sections containing the VTA. AAV targeting and neuroanatomical punch placement was verified for at least a unilateral hit. (c) (top)* Gabrg2 *transcript levels were significantly decreased in male AAV-Cre-eGFP VTA relative to AAV-eGFP. (bottom)* Gabrg2 *transcript levels were near significantly (p=0.0573) decreased in female AAV-Cre-eGFP VTA relative to AAV-eGFP. (d) Representative trace demonstrating decreased phasic inhibition after Cre-mediated excision of* Gabrg2*. (e) (top left, top right) Cre-mediated excision resulted in significant decreases in both sIPSC frequency and peak amplitude in GFP+ cells from the VTA of male AAV-Cre-eGFP relative to AAV-GFP ($p<0.0001$ and $p=0.0110$, respectively). (e) (bottom left, bottom right) Cre-mediated excision resulted in significant decreases in both sIPSC frequency and peak amplitude in GFP+ cells from the VTA of female AAV-Cre-eGFP relative to AAV-eGFP ($p<0.0001$ and $p=0.0526$, respectively). Coronal image courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing, grayscale, indicated regions of interest, and bilateral neurosyringes.*

3.6 VTA Gabrg2 *excision results in no change to binge-like drinking in either females or males.*

While low-to-moderate alcohol concentrations have been shown to interact with δ subunit-containing $GABA_A$ receptors (Wallner Hanchar & Olsen 2003; Hanchar *et al.* 2005; Wallner, Hanchar & Olsen 2006), γ2-containing $GABA_A$ containing receptors are thought to partly mediate the effects of higher concentrations (>30-40 mM, Wallner Hanchar & Olsen 2003). Given this, it was important to investigate any potential *Gabrg2* contribution to binge-like drinking. Since binge-like consumption engenders physiologically relevant, but low (<30mM) blood alcohol concentrations, we hypothesized that its excision would have no effect—sex-specific or otherwise.

To this end, floxed *Gabrg2* male and female mice were bilaterally injected into the VTA with either AAV-Cre-eGFP or AAV-eGFP and subjected to one cycle of binge-like drinking as previously described. A repeated, two-way ANOVA was conducted to compare 2 h drinking for the first three days of access between female AAV-Cre-eGFP and AAV-eGFP. Three AAV-eGFP and two AAV-Cre-eGFP mice were removed from this analysis due to faulty bottle readings on day 2. However, their data were included for the final, binge-like drinking. There were no significant main effects of day ($F(2,22)=1.10$, $p=0.3502$) or virus ($F(1,11)=1.87$, $p=0.1992$) on alcohol drinking (**Figure 3.7b, top**). An unpaired, two-tailed Student's t-test was conducted to compare female AAV-Cre-eGFP and AAV-eGFP binge-like drinking on the final, 4 h access day. There was no significant difference in binge-like drinking in AAV-Cre-eGFP (4.423±0.4003) relative to AAV-eGFP (3.626±0.7708, $p=0.4080$, **Figure 3.7b, bottom**). A repeated, two-way ANOVA was also conducted to compared 2 h drinking for the first three days of access between male AAV-Cre-eGFP and AAV-eGFP. There were no significant main effects of day ($F(2,28)=1.97$, $p=0.1582$) or virus ($F(1, 14)=0.28$, $p=0.6073$) on alcohol drinking in males (**Figure 3.7a, top**). An unpaired, two-tailed Student's t-test was conducted to compare male AAV-Cre-eGFP and AAV-eGFP binge-like drinking on the final, 4 h access day. There was also no significant different in binge-like drinking between AAV-Cre-eGFP (3.094±0.4181) and AAV-eGFP in males (3.854±0.6436, $p=0.3392$, **Figure 3.7a, bottom**). Submandibular bloods were obtained immediately after the 4 h 'binge' in a subset of *Gabrg2* mice to verify the accuracy of g/kg intake measures. There was a significant correlation between BEC measures and g/kg intake ($r=0.5786$, $n=26$, $p=0.0020$), demonstrating the accuracy of using g/kg as a drinking measure.

Gabrg2 mice were then subjected to consecutive 2% sucrose preference and 0.3mM quinine aversion testing in an identical fashion to *Gabrd* mice. There were no significant differences in either 2% sucrose preference or 0.3 mM quinine aversion in males or females as a result of *Gabrg2* excision from the VTA (**Figure 3.8**). Although the lack of difference in sucrose preference indicated there was no need to control for caloric contributions, a subset of *Gabrg2* AAV-Cre-eGFP and AAV-eGFP mice were also tested for saccharin preference. No significant saccharin preference differences were found (**Figure 9.4**).

3.7 AAV-Cre-eGFP transduction efficiency in the pVTA of female **Gabrd** *mice is correlated with larger decreases in binge-like drinking.*

The cellular heterogeneity of the VTA can be divided into two major populations: dopaminergic (DAergic) neurons, which account for roughly 60% of the total cell population and local GABAergic neurons, which account for approximately 30-35% (Chieng *et al.* 2011; Morales & Margolis 2017). These two populations are not equally dispersed throughout the VTA, but rather are differentially distributed along several dimensions including the anterior/posterior or rostrocaudal axis (Olson *et al.* 2005; Ikemoto 2007; Chieng *et al*., 2011; Sanchez-Catalan *et al.* 2014).

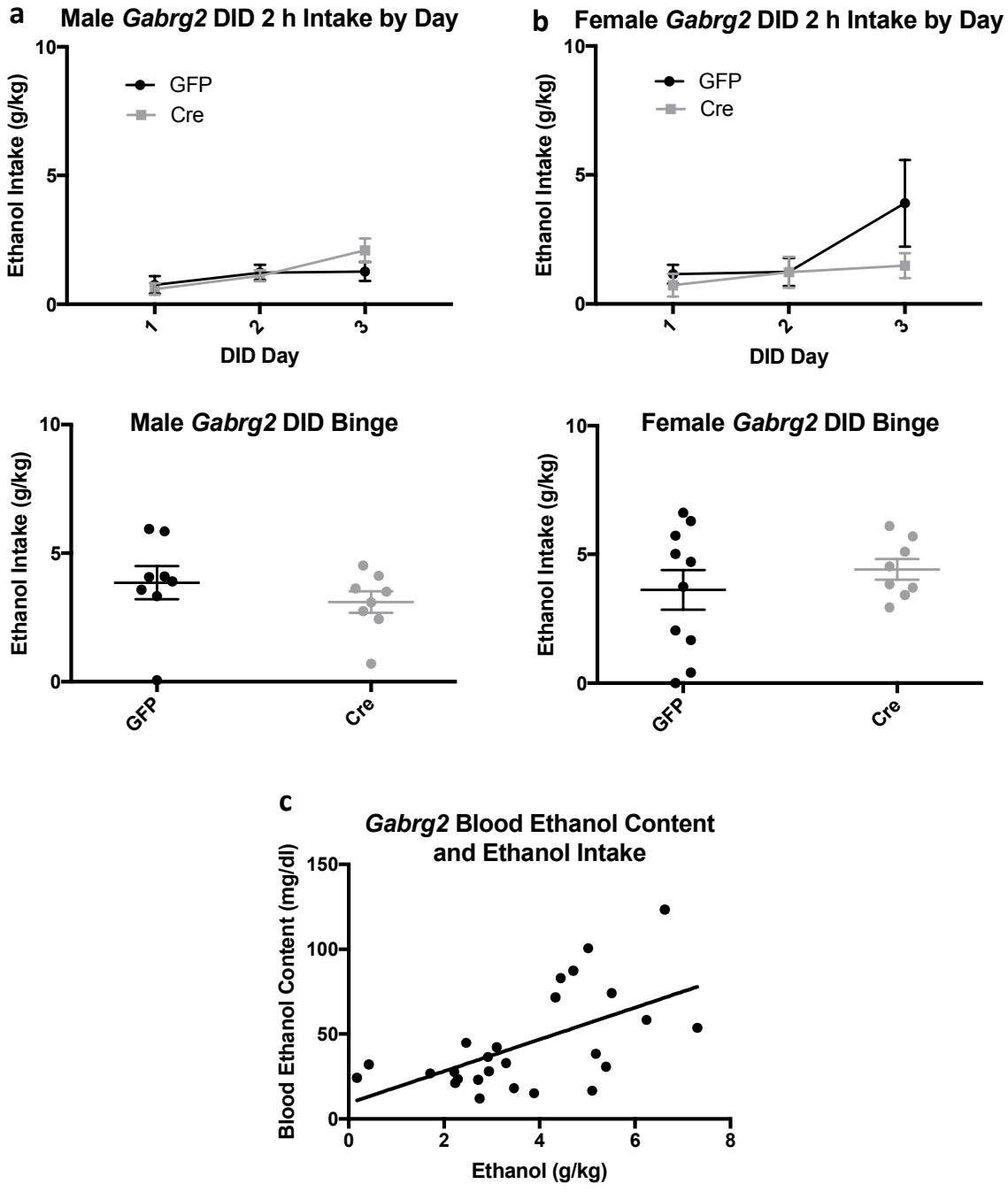

a
Male *Gabrg2* DID 2 h Intake by Day
Ethanol Intake (g/kg)
GFP
Cre
DID Day
b
Female *Gabrg2* DID 2 h Intake by Day
Male *Gabrg2* DID Binge
Female *Gabrg2* DID Binge
c
Gabrg2 Blood Ethanol Content and Ethanol Intake
Blood Ethanol Content (mg/dl)
Ethanol (g/kg)

Figure 3.7: **Gabrg2** ***excision from the VTA has no effect on either female or male binge-like drinking****. (a) VTA* Gabrg2 *excision had no effect on male 20% alcohol drinking over the first three days of 2 h access (top) and no effect on binge-like drinking (bottom). (b) VTA* Gabrg2 *excision had no effect on female 20% alcohol drinking across the first three days of 2 h access (top) and no effect on binge-like drinking (bottom). (c) BEC measurements revealed a significant correlation between final g/kg binge intake values and BEC (r=0.5786, p=0.0020), validating g/kg intake measures as an accurate assessment of binge-like drinking.*

Behavioral pharmacological work has shown that the effects of GABAergic inhibition is dependent on this distributional difference; namely, that rats will self-administer $GABA_A$ antagonists into the anterior VTA (aVTA), but agonists into the posterior VTA (pVTA; Ikemoto, Murphy & McBride. 1998; Ikemoto 2010). Recently, the subunit-specific importance of GABAergic inhibition between the aVTA and pVTA was explored using a drug specific for δ subunit-containing $GABA_A$ receptors (4,5,6,7-tetrahydroisoxazolo(5,4,c)pyridin-3-ol, also known as Gaboxadol or THIP). These results revealed that intra-pVTA—but not intra-aVTA—THIP administration reduced binge-like drinking in non-estrus female mice (Melón *et al.* 2017).

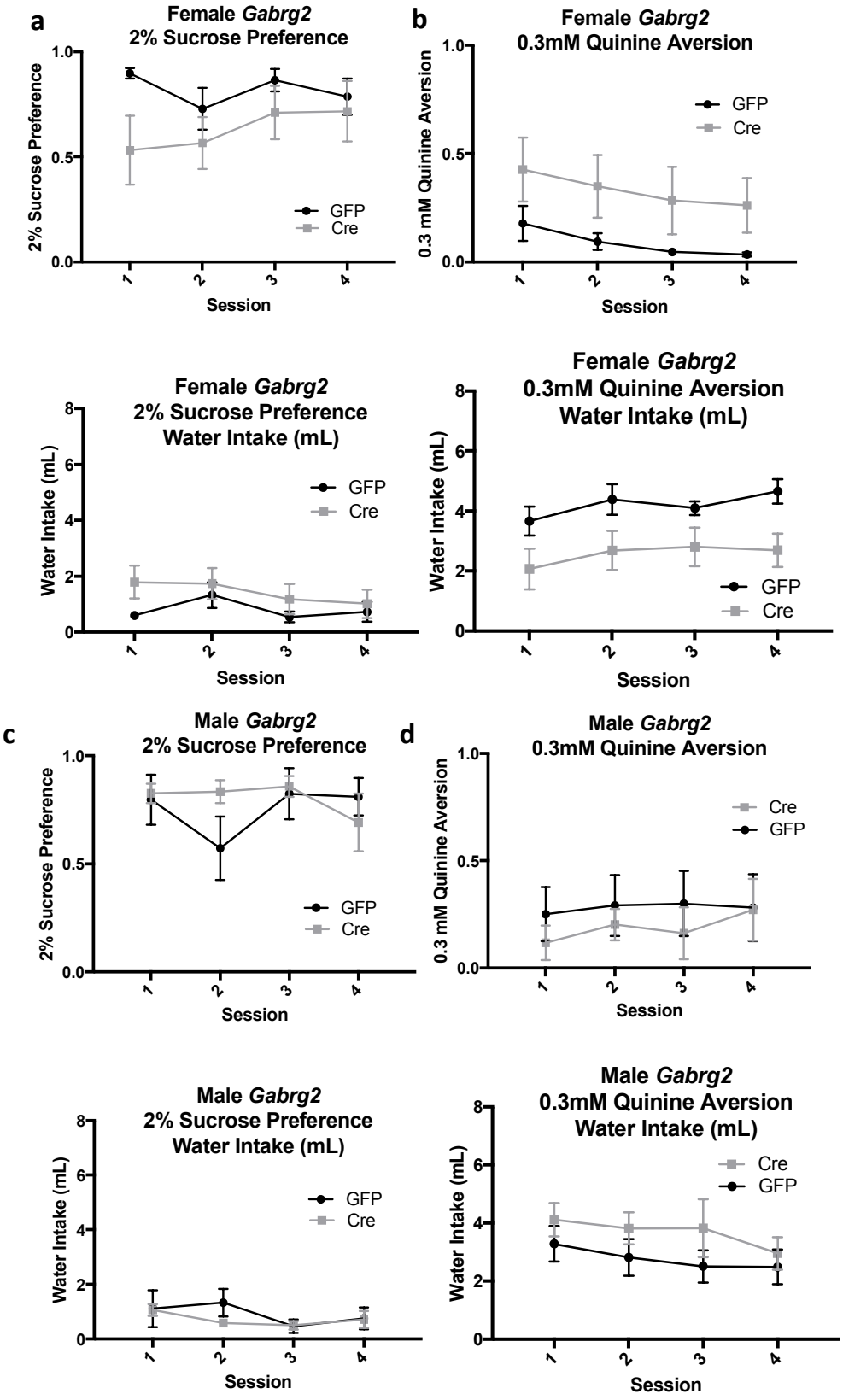

a
Female *Gabrg2*
2% Sucrose Preference
2% Sucrose Preference
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
b
Female *Gabrg2*
0.3mM Quinine Aversion
0.3 mM Quinine Aversion
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
Female *Gabrg2*
2% Sucrose Preference
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
Female *Gabrg2*
0.3mM Quinine Aversion
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
c
Male *Gabrg2*
2% Sucrose Preference
2% Sucrose Preference
1.0
0.5
0.0
GFP
Cre
1
2
3
4
Session
d
Male *Gabrg2*
0.3mM Quinine Aversion
0.3 mM Quinine Aversion
1.0
0.5
0.0
Cre
GFP
1
2
3
4
Session
Male *Gabrg2*
2% Sucrose Preference
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
GFP
Cre
1
2
3
4
Session
Male *Gabrg2*
0.3mM Quinine Aversion
Water Intake (mL)
Water Intake (mL)
8
6
4
2
0
Cre
GFP
1
2
3
4
Session

Figure 3.8: No significant effect of* Gabrg2 *excision on sucrose preference or quinine aversion. *Immediately after DID, male and female AAV-Cre-eGFP and AAV-eGFP mice were subjected to 4 d of sucrose (2%, w/v) preference. After a 3 d break, they were then subjected to 4 d of 0.3 mM quinine aversion. A repeated, two-way ANOVA was used to analyze all preference, aversion, and water intake data. (a) (top) There were no significant main effects of either day (F(3,45)=1.01, p=0.3971) or virus (F(1,15)=0.3.06, p=0.1006) for female* Gabrg2 *sucrose preference. (bottom) There were no significant main effects of either day (F(3,45)=0.75, p=0.5300) or virus (F(1,15)=1.89, p=0.1898) for female* Gabrg2 *water intake during sucrose preference testing. (b) (top) There were no significant main effects of either day (F(3,45)=0.02, p=0.9950) or virus (F(1,15)=3.71, p=0.0731) for female* Gabrg2 *quinine aversion. (bottom) There was no significant main effect of day (F(3,45)=0.25, p=0.8609), but a significant effect or virus (F(1,15)=8.42, p=0.0109) for female* Gabrg2 *water intake during quinine aversion testing. (c) (top) There were no significant main effects of either day (F(3,42)=1.72, p=0.1780) or virus (F(1,14)=0.28, p=0.6052) for male* Gabrg2 *sucrose preference. (bottom) There were no significant main effects of either day (F(3,42)=0.72, p=0.5483) or virus (F(1,14)=0.30, p=0.5938) for male* Gabrg2 *water intake during sucrose preference testing. (d) (top) There were no significant main effects of either day (F(3,42)=0.73, p=0.5404) or virus (F(1,14)=0.30, p=0.5950 for male* Gabrg2 *quinine aversion). (bottom) There were no significant main effects of either day (F(3,42)=0.43, p=0.7313) or virus (F(1,14)=1.30, p=0.2728) for male* Gabrg2 *water intake during quinine aversion testing.*

Given the importance of δ subunit-containing $GABA_A$ receptors in the pVTA to female binge-like drinking (Melón *et al.* 2017), it was important to examine whether there was a neuroanatomical relationship within in the pVTA between AAV transduction efficiency and binge-like drinking in females and males. An initial placement analysis revealed that both AAV-Cre-eGFP and AAV-eGFP transduction were predominantly targeted to the pVTA (**Figure 3.9a, bottom row**), with little viral spread to the aVTA (**Figure 3.9a, top row**). A fine-grained placement analysis was then conducted to better assess viral transduction and binge-like drinking. Regions of interest (ROIs) were selected to include both the aVTA and pVTA as well as spillover areas that had evidence of viral transduction (e.g. substantia nigra, SNr; interpeduncular nucleus, IPN, see **Figure 9.5**). ImageJ masks were then made using Allen Brain Atlas as a template; this allowed for consistent anatomical measurements for each ROI between animals. Each masked area was then

measured to obtain an integrated density value, which was taken as a measure of AAV transduction efficiency. Integrated density values were calculated using ImageJ and pixel values were inverted prior to measuring (e.g. black to white; lower values indicate higher viral transduction).

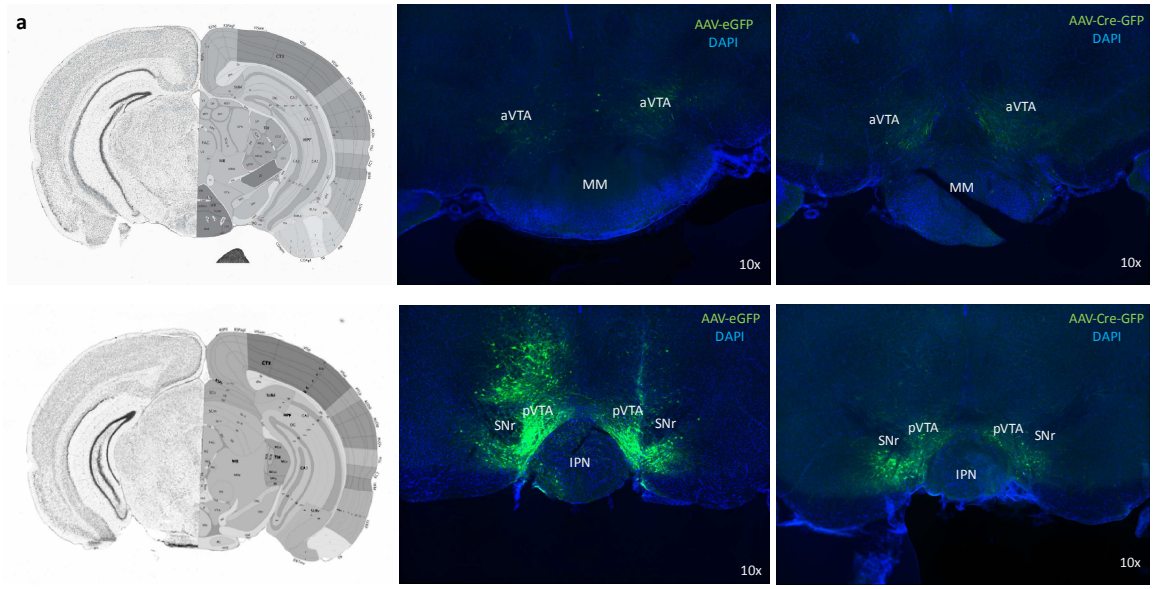
As shown in **Figure 3.9b (bottom)**, there was a significant correlation between pVTA viral transduction in female AAV-Cre-eGFP *Gabrd* mice and binge-like drinking (r=0.6828, p=0.0144), but not between pVTA viral transduction in female AAV-eGFP *Gabrd* mice and binge-like drinking (r=0.5353, p=0.2738). There was no significant relationship in the aVTA of female AAV-Cre-eGFP *Gabrd* mice (r=-0.2445, p=0.5260) or female AAV-eGFP (r=0.1244, p=0.8143) (**Figure 3.9b, top**). Despite having similar integrated density values (**Figure 3.9, b versus c)**, there was no significant relationship between pVTA viral transduction in either male AAV-Cre-eGFP (r=-0.05075, p=0.9140) or male AAV-eGFP (r=-0.4186, r=0.2287) *Gabrd* mice and binge-like drinking (**Figure 3.9c, bottom**). There was also no significant relationship between aVTA viral transduction in either male AAV-Cre-eGFP (r=-0.2121, r=0.6480) or male AAV-eGFP (r=-0.01672, r=0.9634) *Gabrd* mice and binge-like drinking (**Figure 3.9c, top**). Analysis of relevant spillover areas (e.g. SNr, IPN) also revealed no significant correlations between either female or male aVTA or pVTA and binge-like drinking (see **Figure 9.5**).

Although correlative, these results indicate a significant relationship between efficiency of viral transduction in the pVTA of females and later binge-like drinking. In other words,

that increased AAV-Cre-eGFP viral transduction in the pVTA was significantly related to decreased binge-like drinking in females.

3.8 There was no relationship between AAV transduction efficiency in AAV-Cre-eGFP **Gabrg2** *mice and binge-like drinking.*

Given the correlation between AAV transduction in the pVTA and female *Gabrd* binge-drinking, it was next ascertained whether there was a relationship between AAV transduction efficiency and binge-like drinking in *Gabrg2* mice. Since there was no significant effect of *Gabrg2* excision on binge-like drinking in either males or females, it was hypothesized that there would be no significant relationship between viral transduction in any anatomical region and binge-like drinking. Similar to the initial *Gabrd* placement analysis, both AAV-Cre-eGFP and AAV-eGFP transduction was localized predominantly to the pVTA (**Figure 3.10, bottom row**). There was little evident spillover transduction in the aVTA (**Figure 3.10, top row**). Further analysis evaluating ROIs and their corresponding integrated densities revealed no significant relationship between pVTA viral transduction in female AAV-Cre-eGFP (r=-0.01381, p=0.9741) or female AAV-eGFP (r=0.6766, p=0.0950) *Gabrg2* mice and binge-like drinking (**Figure 3.10b, bottom**). There was no relationship between aVTA AAV transduction in female AAV-Cre-eGFP (r=0.212, p=0.5565) or female AAV-eGFP (r=0.1024, p=0.8094) *Gabrg2* mice and binge-like drinking (**Figure 3.10b, top**). Similarly, there was no relationship between pVTA viral transduction and male AAV-Cre-eGFP (r=0.2194, p=0.6365) or male AAV-eGFP (r=-0.6276, p=0.1822) and binge-like drinking (**Figure 3.10b, bottom**).


a
AAV-eGFP
DAPI
aVTA
aVTA
MM
10x
AAV-Cre-GFP
DAPI
aVTA
aVTA
MM
10x
AAV-eGFP
DAPI
SNr
pVTA
pVTA
SNr
IPN
10x
AAV-Cre-GFP
DAPI
SNr
pVTA
pVTA
SNr
IPN
10x

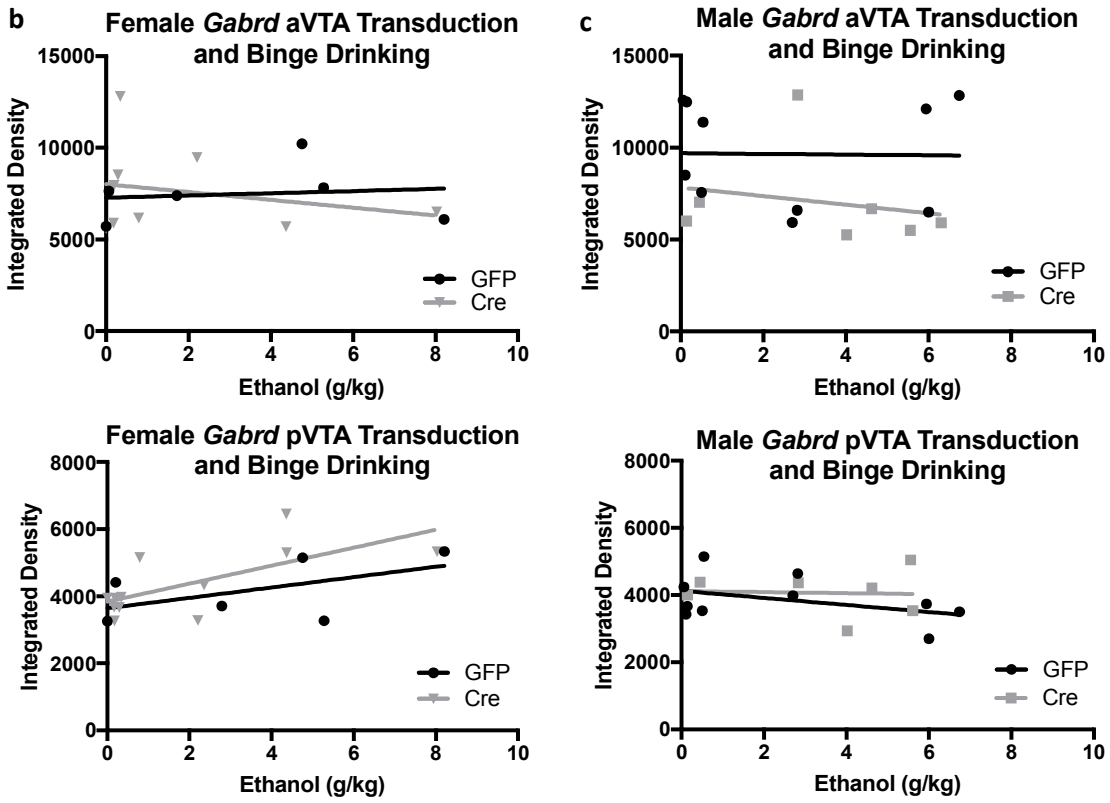
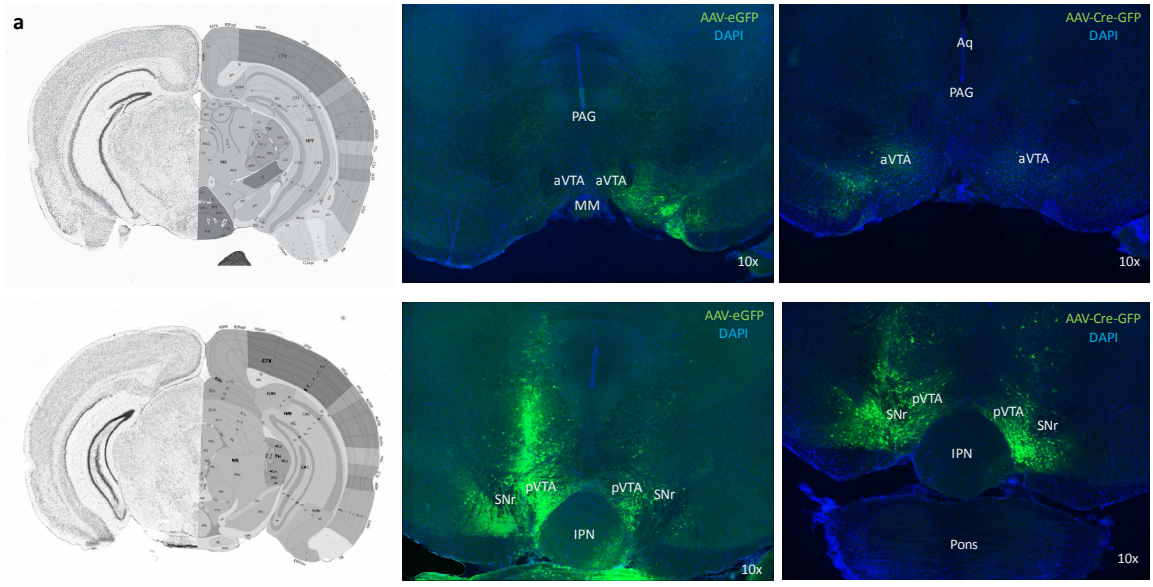

b
Female *Gabrd* aVTA Transduction and Binge Drinking
Integrated Density
15000
10000
5000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
c
Male *Gabrd* aVTA Transduction and Binge Drinking
Integrated Density
15000
10000
5000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
Female *Gabrd* pVTA Transduction and Binge Drinking
Integrated Density
8000
6000
4000
2000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
Male *Gabrd* pVTA Transduction and Binge Drinking
Integrated Density
8000
6000
4000
2000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre

Figure 3.9: Significant correlation between female* Gabrd *AAV-Cre-eGFP transduction efficiency in the pVTA and binge-like drinking. *Both aVTA and pVTA slices were selected for placement analysis and were spread throughout the rostrocaudal axis (-2.8mm to -3.88mm, A/P, relative to bregma). (a) There was only minor AAV transduction in the aVTA of both AAV-Cre-eGFP and AAV-eGFP mice. (b) Significantly higher levels of eGFP reporter expression were noted in the pVTA of both AAV-Cre-eGFP and AAV-eGFP mice, indicating higher AAV transduction in this region. Minor, spillover viral transduction was observed in regions outside of the pVTA (see* ***Figure 9.5****). (b) There was a significant correlation between female AAV-Cre-eGFP binge-like drinking and AAV transduction in the pVTA (top, p=0.0144), but not the aVTA (bottom, p= r=-0.2445, p=0.5260). (c) There was no significant relationship between male AAV-Cre-eGFP binge-like drinking and AAV transduction in either the pVTA or aVTA. N.B. Integrated density values were measured after inversion of pixilation. Low density values indicate higher levels of GFP reporter expression. Coronal images courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing and grayscale.*

There was also no relationship between aVTA viral transduction and male AAV-Cre-eGFP (r=0.1149, p=0.7864) or male AAV-eGFP (r=0.07492, p=0.8732) and binge-like drinking (**Figure 3.10c, top**). Additional analyses of spillover regions with visible viral transduction (e.g. IPN, SNr) also showed no relationship with binge-like drinking (see **Figure 9.6**). In short, AAV transduction in *Gabrg2* mice was unrelated to later binge-like drinking.


a
AAV-eGFP
DAPI
PAG
aVTA aVTA
MM
10x
AAV-Cre-GFP
DAPI
Aq
PAG
aVTA
aVTA
10x
AAV-eGFP
DAPI
SNr
pVTA
pVTA
SNr
IPN
10x
AAV-Cre-GFP
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pVTA
SNr
pVTA
SNr
IPN
Pons
10x

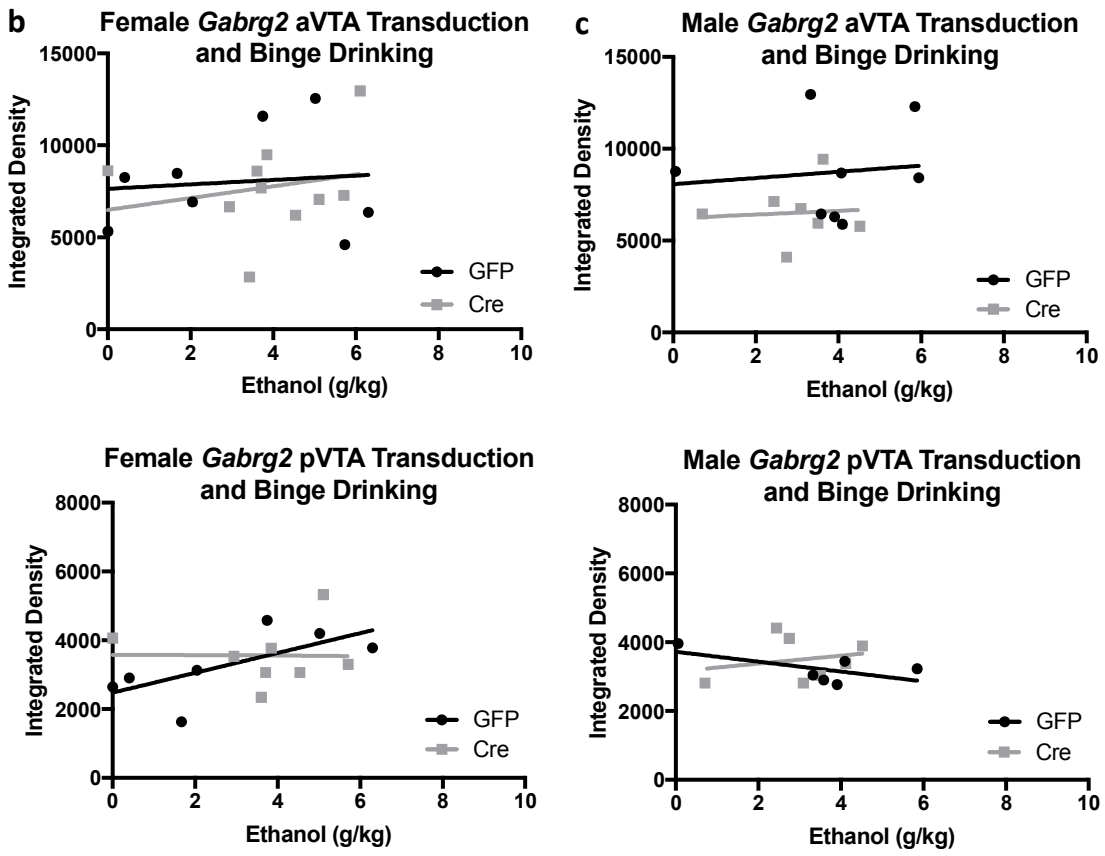

b
Female Gabrg2 aVTA Transduction and Binge Drinking
Integrated Density
15000
10000
5000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
c
Male Gabrg2 aVTA Transduction and Binge Drinking
Integrated Density
15000
10000
5000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
Female Gabrg2 pVTA Transduction and Binge Drinking
Integrated Density
8000
6000
4000
2000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre
Male Gabrg2 pVTA Transduction and Binge Drinking
Integrated Density
8000
6000
4000
2000
0
0
2
4
6
8
10
Ethanol (g/kg)
GFP
Cre

Figure 3.10: No significant correlation between AAV transduction efficiency in either male or female* Gabrg2 *and binge-like drinking. *Slices containing both aVTA and pVTA were selected for placement analysis along the rostrocaudal axis of the VTA. (a) There was only minor AAV transduction in the aVTA of both AAV-Cre-eGFP and AAV-eGFP mice. Atlas image from Allen Brain Atlas. (b) Significantly higher levels of eGFP reporter expression were noted in the pVTA of both AAV-Cre-eGFP and AAV-eGFP mice, indicating higher AAV transduction in this region. Minor, spillover viral transduction was observed in regions outside of the pVTA, but was also unrelated to binge-like drinking (see* ***Figure 9.6****). N.B. Integrated density values were measured after inversion of pixilation. Low density values indicate higher levels of GFP reporter expression. Coronal images courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing and grayscale.*

3.9 There are no sex-specific differences in δimmunoreactivity on PV+ cells in the pVTA

The significant relationship between AAV-Cre-eGFP viral transduction in the pVTA of female *Gabrd* mice and decreased binge-like drinking is intriguing in light of the current understanding of alcohol's effects in this region. For instance, not only do female alcohol-preferring Wistar rats self-administer alcohol into the VTA (Gatto *et al.* 1994), but they do so specifically in the pVTA (Rodd-Henricks *et al.* 2000). When taken in conjunction with the known self-administration of $GABA_A$ agonists in the pVTA (Ikemoto, Murphy & McBride. 1998; Ikemoto 2010), it becomes clear that the posterior region of the VTA is not only a site of specific alcohol action, but one where increased GABAergic inhibition is reinforcing. In fact, the interactions between the pVTA, alcohol, and GABAergic inhibition in females has been investigated, which showed that intra-pVTA administration of the alcohol antagonist Ro15-4513 reduced binge-like drinking (Melón & Boehm 2011).

As both DAergic and GABAergic cell populations are found in the pVTA (Chieng *et al.* 2011; Sanchez-Catalan *et al.* 2014), it is possible that the site-specific effects of alcohol as well as $GABA_A$ receptors agonists and antagonists are more significant on local

GABAergic populations. Potentiation of $GABA_A$ inhibition on these cells would ultimately allow for disinhibition of local DAergic populations, increasing overall mesocorticolimbic DA release. As the current findings give strong evidence for increased δ-mediated tonic inhibition in the VTA of females, we next sought to determine whether there were sex-specific differences in underlying δ subunit immunoreactivity on pVTA GABAergic populations.

To assess this, 40 μm thick slices containing the VTA were harvested from a new cohort of adult male and female C57Bl/6J mice. Slices were co-incubated with anti-parvalbumin (PV) and anti-δ primary antibodies followed by incubation with appropriate secondary antibodies (see Materials & Methods). PV+ GABAergic interneurons are just one class of interneurons, but were specifically chosen since their fast-spiking nature exerts tight control over neuronal firing (Galarreta & Hestrin 2002). A preliminary assessment using CA3-containing hippocampal tissue revealed significantly more PV+/δ+ cells in females compared to males (**Figure 9.7**). Fine-grained analysis of co-labeled neurons in the VTA revealed no significant difference in PV+/δ+ cells in the pVTA between males (1.000±0.4472) and females (2.8±1.114, $t(8)=1.5$, $p=0.1720$, **Figure 3.11**). There were also no significant differences in PV+/δ+ cells in the aVTA between males (0.8±0.5831) and females (0.2±0.2, $t(8)=0.9733$) or in the VTA as a whole (males, 1.8±0.5831, females, 3±1.304, $t(8)=0.8402$, $p=0.4252$, **Figure 3.11**).

The lack of PV+ interneurons in both male and female pVTA confirms earlier findings in male rats showing the near absence of PV+ interneurons in the VTA (Olson & Nestler

2007). PV+ interneurons were specifically chosen due to their outsized role in controlling and coordinating neuronal firing, particularly in areas like the hippocampus (Lee & Maguire 2013; Glykys, Mann & Mody 2008). Given the behavioral pharmacological work presented by Ikemoto and colleagyues (1998, 2010), it is still likely that non-PV, GABAergic interneuronal control plays a critical role in VTA functioning and will need to be explore further.

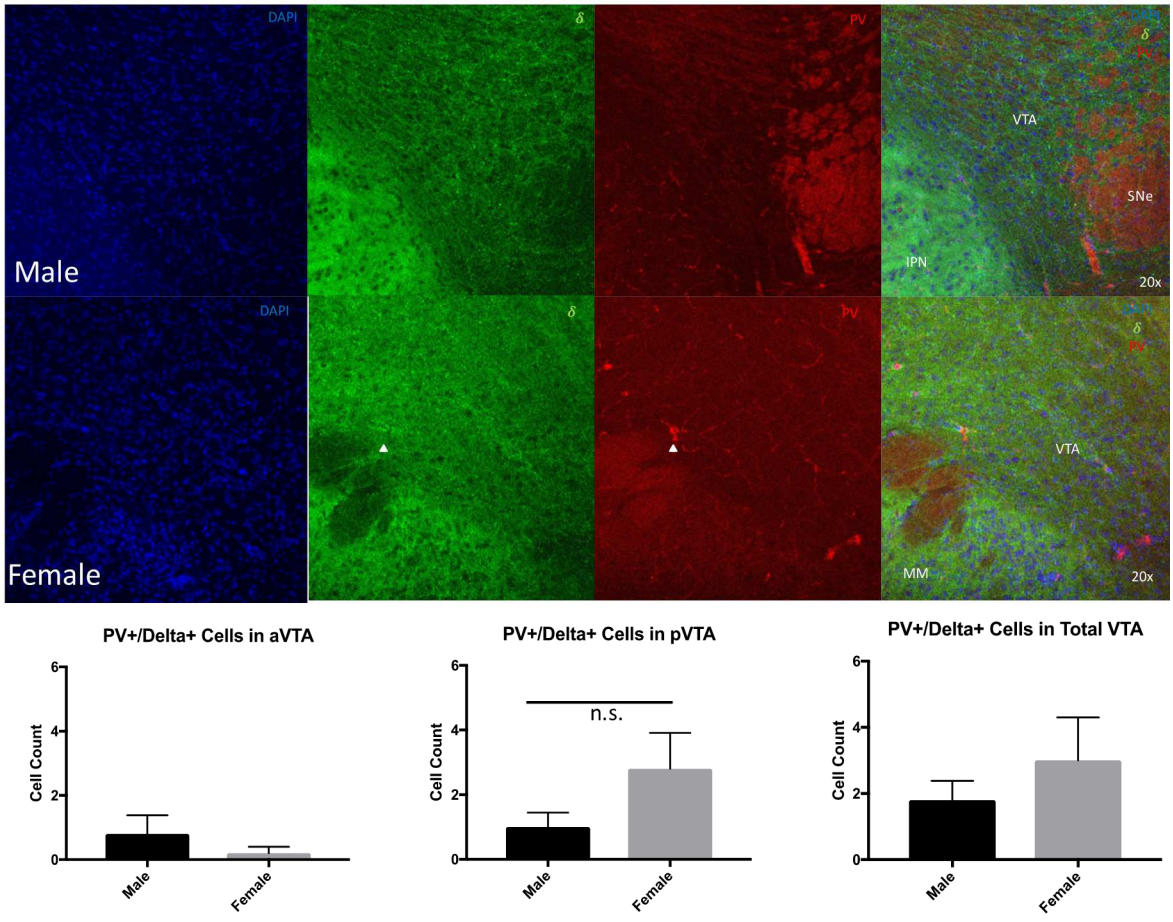


Figure 3.11: No significant differences in PV+/δ+ populations between males and females in the VTA. *Analysis of* PV+/δ+ *cells in both the aVTA and pVTA revealed no significant differences between males and females. There were also no significant differences in either PV+/δ- cells or PV-/δ+ cells. Average cell counts were taken from 4-8 slices per animal and used in final analysis. Male representative image indicates pVTA and female representative image indicates aVTA.*

Chapter 4
Discussion

The results presented here show increased baseline *Gabrd* transcript levels as well as δ-mediated tonic inhibition in the VTA of female mice. This sex-specific difference was missing in both *Gabrg2* transcript levels as well as γ2-mediated phasic inhibition. Subsequent Cre-mediated *Gabrd* excision primarily from the pVTA significantly reduced female binge-like drinking, with Cre-mediated *Gabrg2* excision having no effect in males. Taken together, these results indicate that while δ-mediated tonic inhibition is not unique to females, it is more important to the interactions of alcohol in the VTA during female binge drinking.

4.1 *Gabrd* Baseline Levels and Estrus Cycle Importance

While there is limited evidence regarding female $GABA_A$ receptor subunit expression patterns, past work has demonstrated cycling-dependent changes in *Gabrd* transcript levels in the VTA, PAG, and hippocampus (Melón *et al.* 2017; Griffiths & Lovick 2005; Maguire 2005). Specifically, *Gabrd* levels in non-estrus females were found to be approximately 80% greater than those in estrus. In the current study, all mice were maintained in same-sex housing conditions under separate ventilation, which would theoretically avoid estrus cycle-inducing male cues. However, this is no guarantee that all female mice were acyclic. As such, preliminary attempts were made to verify that cyclicity was not a factor in binge-like consumption using a subset of *Gabrd* females (**Figure 9.2**). Although statistical comparison was impossible due to low n, there were no clear differences in binge-like consumption between AAV-eGFP females in either non-estrus or estrus mice (**Figure 9.2**). These preliminary findings were in agreement with previous work demonstrating no

significant difference in female binge-like consumption across estrus cycle (Melón *et al.* 2017). Even if a large number of females were cycling, the effects on subsequent binge-like consumption would be negligible.

Although cycling was present in at least a subset of the tested female *Gabrd* mice, its influence on Cre-mediated *Gabrd* excision would also be inconsequential. Our highest relative *Gabrd* female Cq value (1.54) was approximately 50% greater than our lowest relative *Gabrd* female Cq value (0.89). This variation is markedly lower than that seen between estrus and non-estrus states as reported in Melón *et al.* (2017), indicating a fairly stable overall *Gabrd* expression and a likely non-important role for estrus cycle in these females. In other words, some female mice may have been cycling, but the overwhelming majority may have been acyclic. Perhaps most importantly, Cre-mediated excision reduced VTA *Gabrd* levels in females to almost negligible relative *Gabrd* Cq levels (0.0037-0.0054). Even if there were significant cycling-dependent variability in *Gabrd* expression, Cre-mediated excision would ensure that any expression (high or low) would be knocked down. In short, Cre recombinase activity is independent of baseline *Gabrd* levels and any cycling-dependent effects on subunit expression for *Gabrd* mice would be irrelevant once Cre-mediated excision had occurred.

4.2 Influence of Neurosteroids

It should be noted that the potential effect of cycling on *Gabrd* transcript levels is not the only matter of consequence. Increases in 17β-estradiol (*e.g.* beginning in proestrus and decreasing through estrus) as well as corresponding changes in progesterone (*e.g.* increased

during metestrus and diestrus phases) have been functionally tied to differences in δ-mediated tonic inhibition (Maguire *et al.* 2005). More specifically, these differences are at least partly attributable to the increased presence of neurosteroids like 3α-hydroxy- 5α-pregnan-20-one (3α,5α-THP or allopregnanolone), which is produced as a metabolite of progesterone as well as *de novo* in the brain (Mellon, Griffin & Compagnone 2001). Although derived from cholesterol in conjunction with the work of stereoidogenic enzymes, neurosteroids can have non-genomic effects on neuronal firing by positively and allosterically modulating $GABA_A$ receptor functioning (Belelli & Lambert 2005). While recent work has revealed the neurosteroid binding site is situated between α and β subunits (Hosie *et al.* 2009), they also increase δ-mediated tonic inhibition (Spigelman *et al.* 2003; Stell *et al.* 2003; Brown *et al.* 2002; Wohlfarth *et al.* 2002). Collectively, these findings indicate that δ subunit inclusion allows for potentiation of the original neurosteroid binding effect.

Relevant to this discussion, it is unsurprising that neurosteroids have been implicated in the actions of ethanol (Ford *et al.* 2008; Kumar, Fleming & Morrow 2004). Plasma levels of neurosteroids are increased after ethanol administration (Barbaccia *et al.* 1999), but show sex-specific differences (Finn *et al.* 2004). Importantly, the mutual site of action at $GABA_A$ receptors provides a nexus for synergistic activity between neurosteroids and alcohol. To this end, co-administration of alcohol and the neurosteroid ACN potentiates GABA-mediated inhibition (Akk & Steinbach 2003). Other work has indicated enhanced GABA-mediated inhibition through neurosteroid-enhancement of presynaptic GABA release (Helms, Rossi & Grant 2012). Whatever the mechanism, it is likely that binge-like

drinking led to altered neurosteroid levels. While this may have increased GABA-mediated, phasic inhibition in the VTA, the cumulative effects were not enough to mitigate the loss of δ-mediated tonic inhibition.

As a final note on neurosteroids, steroid-based organizational effects are significant throughout development (Schumacher *et al.* 2003; Belelli & Lambert 2005). Thus, it is likely that the difference seen in δ subunit-containing $GABA_A$ receptors within the VTA (**Figure 3.1**) was established early on and not the function of estrus-based rhythms. Future work will need to investigate this possibility.

4.3 *Gabrg2* Baseline Levels and Estrus Cycle Importance

As for *Gabrg2*, conflicting reports have both shown (Maguire *et al.* 2005) and not shown (Melón *et al.* 2017) fluctuating *Gabrg2* transcript levels as a function of estrus cycle. Importantly, work specifically investigating VTA *Gabrg2* levels has not shown any cycling-dependent expression differences (Melón *et al.* 2017). γ2 subunit-containing $GABA_A$ receptors mediate phasic inhibition and are markedly more common than those that contain the δ subunit (Chang *et al.* 1996). This is evident in the increased *Gabrg2* transcript levels in both male and female VTA relative to *Gabrd* levels (**Figure 3.5a** and **Figure 3.1a**, respectively). Even if cycling-dependent fluctuations occurred in the VTA, the high level of expression and lack of observed difference between males and females would indicate they are too low to quantify. This lack of difference was also evident in baseline electrophysiological measures, which showed no change between males and

females in either sIPSC frequency or amplitude. This provided further confirmatory evidence for the functional similarity in phasic inhibition between males and females.

Finally, we noted a greater than one-fold decrease in *Gabrg2* levels in AAV-eGFP control mice relative to baseline C57Bl/6J mice. This difference was not unexpected since two sets of primers were used—one set annealing early in the *Gabrg2* gene at exon 2 and the second annealing much later to assess the *Gabrg2* excision at exon 8. Annealing to exon 2 allowed the primer set that was used to assess C57Bl/6J levels to be specific for the γ2S variant rather than γ2L. While both variants are present in adult mouse brain, the former is more abundant (Baer *et al.* 2000). Since the primers used to assess Cre-mediated *Gabrg2* excision would have indiscriminately assessed *both* γ2S and γ2L variants, one might expect to see the reverse pattern (*i.e.* increased levels in AAV-eGFP mice relative to C57Bl/6J controls). However, the *Gabrg2* excision site was only 206 base pairs long and primers were designed to anneal within the sequence rather than flanking it. Given this, it is likely that the reduced AAV-eGFP levels seen were a technical issue, due primarily to the small excision site rather than any biological factors.

4.4 Possible Inclusion of the tVTA

Assessments of both *Gabrd* and *Gabrg2* transcript levels were based on tissue punches obtained from slices that included the pVTA. However, tissue punches have limited anatomical specificity, making aVTA and pVTA subdivisions along the anterior-to-posterior axis functionally impossible. Electrophysiological recordings also precluded this same assessment; since only GFP+ cells from within the VTA were recorded, locational information along the anterior/posterior axis was impossible to determine. In the future, a

more fine-grained approach (*e.g.* laser-capture microdissection) will be needed to better assess sex-specific, baseline levels of *Gabrd* and *Gabrg2* along the anterior/posterior axis. A consequence of using tissue punches is the possible inclusion of the tVTA (or RMTg) in the tested samples. As previously described (see *1.7.5 Lateral habenula*), this region is predominantly comprised of local GABAergic interneurons (Olson & Nestler 2007) and merges into regions of the pVTA (Bourdy & Barrot 2012). On a circuit-level, it is thought to provide strong, tonic inhibition (*i.e.* consistent inhibition and not necessarily δ-mediated tonic inhibition) on DAergic projection neurons (Jhou *et al.* 2009; Stamatakis & Stuber 2012). At baseline, this would provide strong, inhibitory drive on DA release. An intriguing question is whether the measured *Gabrd* levels included this region and to what extent. If transcript levels and/or electrophysiological measures of tonic inhibition were obtained predominantly from this area, it would strongly indicate that δ-mediated tonic inhibition was a more critical component to tVTA functioning in females than males.

4.5 *Gabrd* Excision and Female Binge-like Drinking

The inclusion or exclusion of the tVTA notwithstanding, the effectiveness on female binge-like drinking of reducing δ-mediated tonic inhibition in the VTA was significant. While there have been some conflicting reports (see Borghese *et al.* 2006), there is substantial evidence that low-to-moderate alcohol concentrations interact with δ-containing $GABA_A$ receptors in recombinant receptor systems as well as in slice (Wallner Hanchar & Olsen 2003; Hanchar *et al.* 2005; Wallner, Hanchar & Olsen 2006). Further underscoring this interaction, globally *Gabrd* deleted male and female mice were found to have significant decreases in both alcohol consumption and preference (Mihalek *et al.* 2001). In contrast to

this previous work, the present study allowed for site-specific *Gabrd* deletion within the VTA. Given this, it is possible that *Gabrd* excision in other areas of mesocorticolimbic circuitry (*e.g.* dorsomedial shell of the nucleus accumbens, see Nie *et al.* 2011) would be more effective in males. One interesting finding in the 'binge' data was the lack of difference in drinking between males and females. This lack of difference was also apparent in *Gabrg2* males and females. Along with other models of alcohol intake (*e.g.* intermittent access, see Hwa *et al.* 2011), binge-like drinking using the DID model yields increased female drinking relative to males (Rhodes *et al.* 2005). However, there are reports of equivalent levels of drinking between males and females. For instance, in a separate experiment examining strain differences and binge-like drinking, Rhodes *et al.* (2007) found no significant difference in male and female DID g/kg values when using a lickometer apparatus. In addition, all DID experiments were conducted in a shared facility. It is possible that disruptions from husbandry staff and/or other labs during the time of testing resulted in the dampened control female drinking levels that were observed. The likelihood of outside disturbances is even more probable given that both transgenic strains lacked a difference in male and female drinking.

4.6 *Gabrg2* Excision and Female Binge-like Drinking

Considering the *Gabrg2* mice, it is notable that the level of reduction in *Gabrg2* levels was not as significant as that seen in Cre-mediated *Gabrd* excision. Although the same AAV-Cre virus containing the same non-cell-type-specific promoter CMV was used in both floxed lines, the assumption of 100% Cre-mediated excision efficiency cannot necessarily be assumed (Bao *et al.* 2013). Moreover, the *Gabrg2* line was constructed to specifically

remove the synaptic clustering and localization ability of γ2-containing $GABA_A$ receptors (Schweizer *et al.* 2003), rather than the robust genetic inactivation inherent to the floxed *Gabrd* line (Lee & Maguire 2013). These divergent strategies alone could result in different levels of excision effectiveness. Despite this, it is important to note that although the excision strategy featured in the *Gabrg2* line may not be as effective as that in the *Gabrd* line, the functional outcome was significant: Both male and female mice had reductions in both sIPSC frequency and peak amplitude. In short, phasic inhibition was significantly reduced on GFP+ cells within the VTA, even if transcript levels were not nearly as low as those seen with Cre-mediated *Gabrd* excision.

One concern is that the remaining γ2 subunit-containing $GABA_A$ receptors might counterbalance any effect of the knockdown. To this end, γ2 subunit-containing $GABA_A$ receptors have been implicated the actions of alcohol, but only at higher concentrations (>30-40 mM, Wallner Hanchar & Olsen 2003). While accurate assessment of the alcohol concentration within the VTA is nearly impossible, *Gabrg2* mice drank an average of 3.5-4.5 g/kg for females (Cre and eGFP) and 3.0-3.8 g/kg for males (Cre and eGFP), corresponding to approximate BECs of 40 mg/dL immediately after the 'binge.' While these values are lower than have been reported for DID (Rhodes *et al*. 2005; Rhodes *et al.* 2007), they approximate to alcohol concentrations of 10-15 mM. While this range is a rough calculation and does not include metabolic effects and other physiological parameters that might affect brain alcohol concentrations, it is far lower than the threshold for reported γ2 subunit activation.

4.7 $GABA_A$ receptor compensatory mechanisms

One concern with any global or site-specific knockout strategy is the potential introduction of compensatory mechanisms. This is an even more salient consideration given the multiple subunit members within each subunit family of $GABA_A$ receptors as well as their high homology (~70% within a family, but only ~30% across families, Belelli & Lambert 2005). From the δ perspective, it is possible—and likely—that site-specific reduction would also decrease expression of α subunit family members that partner exclusively with δ-subunit containing $GABA_A$ receptors (*e.g.* α4 and α6; perhaps α5).

Work using global α6 knockout mice revealed corresponding decreases in δ subunit expression (Jones *et al.* 1997). Similarly, global δ knockdown resulted in decreased forebrain α4 expression, but increased γ2 subunit expression (Korpi *et al.* 2002; Peng *et al.* 2002). However, these methods used global knockdowns, leading to alterations in underlying receptor composition throughout development. A more targeted approach might be the use of a site-specific, conditional knockdown. To this end, work by Rewal *et al.* (2009) used shRNA-mediated, site-specific knockdown of α4 and found no change in either δ or γ2 subunit protein expression 18 d after surgery. Given that the former is critical to α4-containing $GABA_A$ receptors, it is likely that a similar reduction would also be seen in the Cre-mediated *Gabrd* excision presented here. However, this does not contradict the present findings. Rather, it underscores that α4 and α6 subunits are critical partners for δ subunit-containing $GABA_A$ receptors. More importantly, that site-specific *Gabrd* excision likely resulted in a complete decrease in overall number of $GABA_A$ receptors responsible for mediating tonic inhibition (*e.g.* δ-, α4-, and α6-containing receptors). It is also unlikely

that γ2 subunit compensation (*e.g.* upregulation) plays a significant role. Even if γ2 increases were seen, the low alcohol concentrations achieved during binge-like consumption would make it unlikely that this receptor type would contribute any functional contribution to these results. An interesting future direction for this work would be to perform a similar knockdown using α4- or α6-directed approaches to see if similar effects on binge-like drinking were observed.

In comparison, global γ2 knockout mice do not survive into adulthood (Chandra *et al.* 2005; Günther *et al.* 1995) and any examination of compensatory changes after γ2 manipulation has been done with conditional or mutational approaches (*e.g.* for review see Reynolds 2008). These approaches have allowed for avoidance of both lethality as well as compensatory mechanisms; as such, little is known of up- (or down) regulation of extrasynaptic subunits in response to site-specific *Gabrg2* knockdown. Given this, it is clear that compensation by a different γ family member (*e.g.* γ1 or γ3) did not occur, as there was a robust decrease in sIPSC measures in AAV-Cre-eGFP *Gabrg2* mice and no evidence of functional compensation. However, alterations in *Gabrg2* tonic conductance was not determined and changes to transcript levels were also not assessed; as such, it is possible that tonic inhibitory, compensatory changes resulted from site-specific Gabrg2 excision.

In short, while compensation was not directly examined, it is unlikely to have played a significant role in these findings. The site-specific, conditional nature of the approach avoided many of the long-term regulatory changes seen in global knockout models. It is

likely that the excision of *Gabrd* and subsequent reduction in δ-mediated tonic inhibition had a simultaneous effect on α family members that partner with δ subunit-containing $GABA_A$ receptors. If anything, this only further implicates extrasynaptically located $GABA_A$ receptors in the VTA in the effects of low-to-moderate doses of alcohol in females. Extrasynaptic receptor compensation was not directly assessed in *Gabrg2* mice and will need to be verified in the future.

4.8 Zeroing in on GABAergic Interneurons: Cellular Culprit?

Cre-mediated *Gabrd* excision specifically targeted the pVTA had limited transduction in either the aVTA or in surrounding areas. While not causal, a correlational analysis provided a relationship between reporter expression and binge-like consumption—the more reporter expression that was present, the lower the female binge-like drinking. While this relationship will need to be specifically correlated with knockdown efficiency (*i.e.* is it also the case that more reduction in *Gabrd* expression was correlated with decreased binge-like drinking), the causal question remains. To better assess this directly, the cell-type-specific expression patterns of δ subunit will need to be better understood.

Interneurons are a broad group of GABAergic cells that are largely responsible for coordinating pyramidal and projection cell output. While they can be divided into a wide range of classes, one of note are parvalbumin-positive (PV+) interneurons (Hu, Gan & Jonas 2014). Importantly, PV+ interneurons are known to be fast-spiking in nature and have a dense morphology; this allows for their putative coordination of neuronal firing, including in the generation of oscillatory behavior (Ferando & Mody 2014). Perhaps most

importantly for the present discussion, δ-mediated tonic inhibition has been demonstrated for GABAergic interneurons as well as primary neurons (Milenkovic *et al.* 2013; Glykys, Mann & Mody 2008). When coupled with behavioral pharmacological work demonstrating the reinforcing properties of pVTA administration of $GABA_A$ agonists (*e.g.* muscimol, see **1.9 The Ventral Tegmental Area: Structural and Cellular Heterogeneity**), interneurons become an even likelier target.

As such, it was interesting to find no sex-specific differences in PV+ interneuronal populations within the pVTA. Given the tight control they would be expected to have on TH+ neuronal firing, any sex-specific differences in δ-mediated tonic inhibition would have a large effect on VTA functioning. However, this finding was enlightening in several ways. For one, it replicated characterization work done previously (albeit in males, see Olson & Nestler 2007), indicating that PV+ interneurons might not be as important to VTA functioning as they are to areas like the hippocampus. Second, it does not negate previous behavioral pharmacological work, but hints at the involvement of a different subclass (or subclasses) of interneurons. Third—and most interestingly—it asks whether δ-subunit expression is exclusive to GABAergic interneurons. Although this has been a consistent finding in the hippocampus (Lee & Maguire 2013; Glykys, Mann & Mody 2008), it could be a different story in the VTA. However, if δ-mediated inhibition is not exclusive to GABAergic interneurons, it is still likely it would have an outsized effect on VTA functioning (see **4.9 Proposed Model**).

4.9 Proposed Model

When examined holistically, our data revealed two major conclusions: (1) higher, sex-specific baseline *Gabrd* levels in females and (2) female-specific reductions in binge-like drinking after *Gabrd* excision from the VTA. But what does this mean for overall differences in VTA circuit functioning between males and females? Given the current lack of cell-type specific expression data, there are two possibilities.

(1) In a baseline state, female mice have a larger amount of δ-mediated tonic inhibition on neurons within the VTA, whether localized exclusively to the pVTA or more broadly to include the tVTA. While a small percent of GABAergic neurons within the VTA are thought to project to the nucleus accumbens (NAc), the majority synapse onto DAergic projection neurons (van Zessen *et al.* 2012). Given this, the larger tonic inhibition on this population relative to those in males would result in greater GABAergic inhibition, ultimately disinhibiting DAergic activity and allowing for greater activity (e.g. more DA release) in a basal state (**Figure 4.1**). To this end, female Sprague-Dawley rats have been shown to have increased, basal DA release relative to males that is unaffected by estrus phase (Walker *et al.* 2000).

After binge-like drinking, low amounts of alcohol allosterically interact with δ subunit-containing $GABA_A$ receptors, thereby enhancing tonic inhibition. Further disinhibition of local GABAergic populations would putatively allow for even greater DA release in the NAc. To this end, female Wistar rats trained to lever press for alcohol were shown to have greater DA release after low dose (0.25 and 0.5 g/kg) alcohol administration relative to

males (Blanchard *et al.* 1993). Removal of *Gabrd* from this population of GABAergic interneurons through Cre-mediated excision would not only reduce baseline tonic inhibition, but would also eliminate a key allosteric site for low-dose alcohol binding. This would putatively decrease DA release and the reward associated with it (**Figure 4.1**). It should be noted that DA release was not specifically assessed as a function of Cre-mediated *Gabrd* excision and is an avenue for future investigation.

(2) A second possibility is that δ-mediated tonic inhibition is present to a higher degree on TH+ neurons within the pVTA. It is likely that a larger population of TH+ neurons were affected relative to interneurons, particularly in light of the large viral spread within this region and the equally large cell population affected (**Figures 3.9** and **3.10**) as well as the noted low number of GABAergic interneurons within the VTA (**Figure 3.11**; Chieng *et al.* 2011). Under these conditions, low-to-moderate levels of alcohol would differentially inhibit DAergic projection neurons in females relative to males; similarly, one would expect GABA agonists to be less effective in this region than in the aVTA. *Gabrd* excision would remove this site of action for low-to-moderate doses of alcohol, influencing the excitability of DAergic projection neurons.

However, these conclusions are opposite to what the current body of literature reports and indicate that even if TH+ neurons have a component of δ-mediated tonic inhibition, GABAergic interneurons either (1) express more or (2) are functionally more relevant to overall VTA control (*e.g.* refer to **4.8 Zeroing in on GABAergic Interneurons: Cellular Culprit?**). In further support of this conclusion, recent work by Vashchinkina and

colleagues (2014) has shown that neurosteroids activation of δ-mediated tonic inhibition specifically targets GABAergic interneurons rather than DAergic projection neurons.

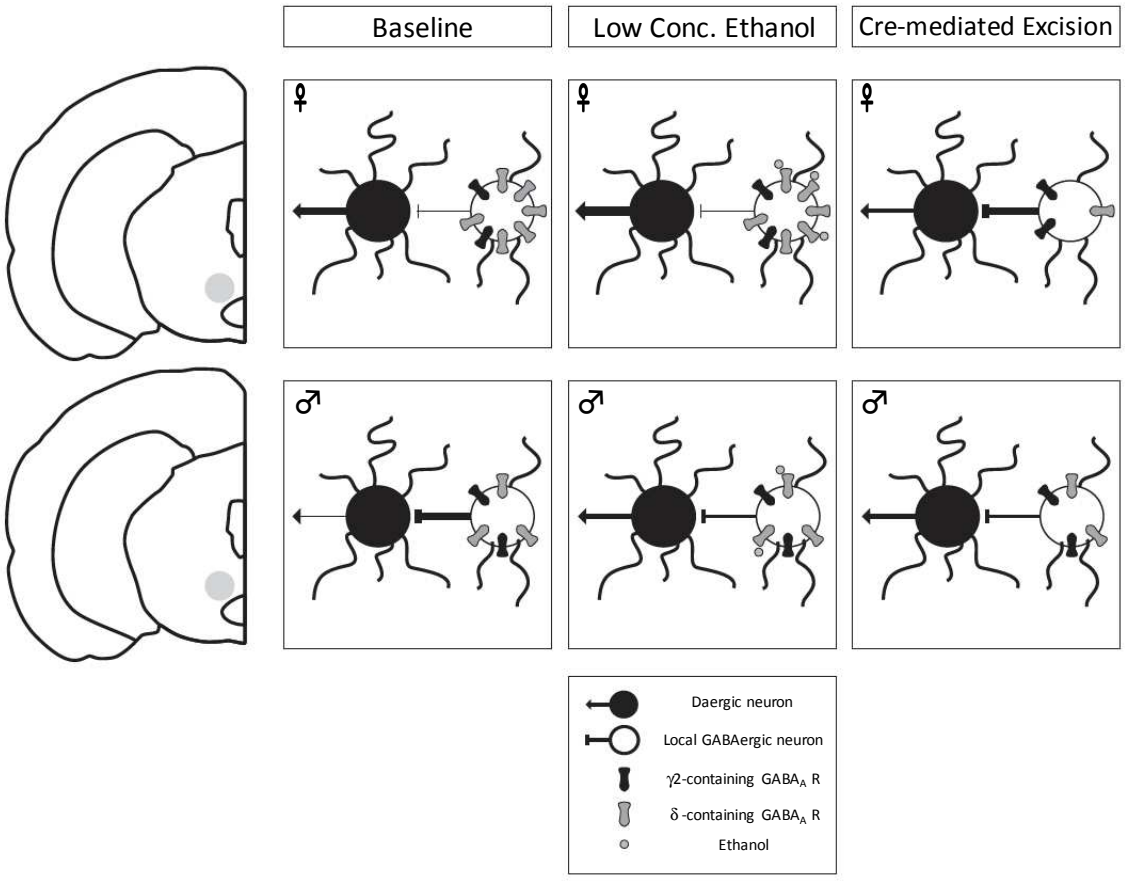


Figure 4.1: Increased baseline δ-mediated tonic inhibition in the VTA of females has implications for overall mesocorticolimbic excitability and effects of low dose drinking. *(top row, left to right) Female VTA has increased baseline levels of δ-subunit containing $GABA_A$ receptor in the VTA, resulting in decreased inhibition on DAergic neurons and increased DA release. Low concentrations of alcohol interact with δ-subunit containing $GABA_A$ receptors on this population, further increasing DA release. Cre-mediated excision markedly reduces* Gabrd *expression and δ-mediated tonic inhibition, reducing overall DA release. (bottom row, left to right) Male VTA has decreased baseline δ-subunit containing $GABA_A$ receptor in the VTA, resulting in increased inhibition on DAergic projection neurons. While low concentrations of alcohol disinhibit DAergic neurons and increase DA release, the effect is not as significant as in females. Due to the low baseline* Gabrd *levels, Cre-mediated excision has limited effect on both* Gabrd *levels, δ-mediated tonic inhibition, and resulting DA release. In all cases, γ2-containing $GABA_A$ receptors are present at equal levels between males and females and are not affected by low concentrations of alcohol.*

4.10 Future Directions

Collectively, this work demonstrates the sex-specific importance of δ subunit-containing $GABA_A$ receptors in the VTA of female mice during binge-like drinking. Given this, two major questions need to be addressed to gain a fuller picture of the role it plays in overall VTA and mesocorticolimbic circuit functioning. First, the open question of what cell type predominantly expresses the δ subunit remains unknown. While pharmacological work certainly points to a disinhibitory effect mediated by a local GABAergic population, quantitative evidence to this effect remains missing. Second, a larger question remains as to the effect of *Gabrd* excision on overall mesocorticolimbic functioning. Pilot work with animals from this study has indicated a trend towards increased sensitivity to the acute effects of alcohol (**Figure 9.3**), but this effect remains relatively unexplored. A critical future experiment would be to examine whether *Gabrd* excision alters DAergic functioning and output using microdialysis or fast scan cyclic voltammetry. Ultimately, cell-type specific manipulation of δ subunit expression should be envisioned, either through transgenic approaches (*e.g.* crossing Gad67-Cre with floxed *Gabrd* mice) or through site-specific manipulation of local interneuronal populations in female versus males (*e.g.* DREADD delivery to pVTA of Gad67-Cre mice). This would allow the most clear-cut, causal relationship between δ-mediated tonic inhibition in females to be established and offer a potential avenue forward for therapeutic options.

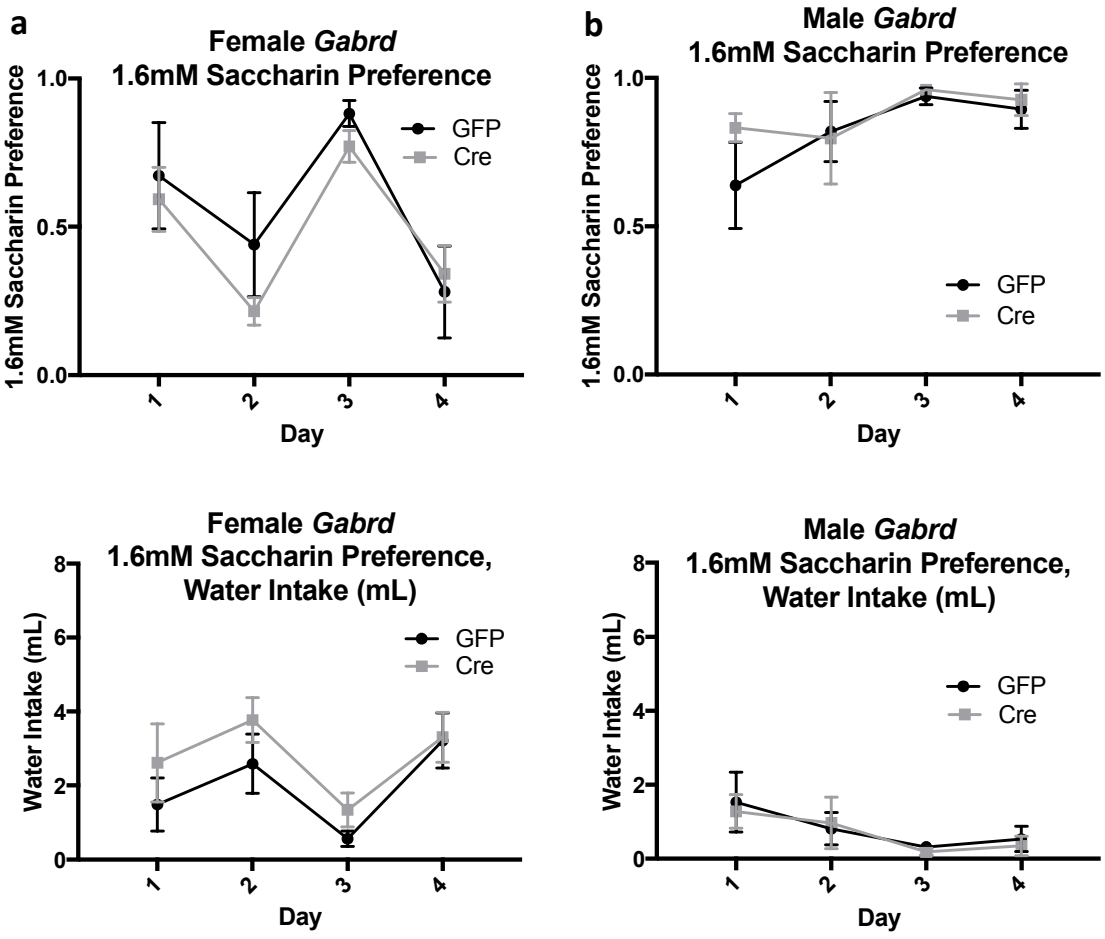


Figure 5.1: No significant effect of* Gabrd *excision on saccharin preference or quinine aversion. *A subset of the male and female AAV-Cre-eGFP and AAV-eGFP mice that had undergone sucrose preference and quinine aversion testing were also subjected to 4 d of saccharin (1.6 mM) preference. Testing occurred 3 d after the conclusion of quinine aversion testing. A repeated, two-way ANOVA was used to analyze both saccharin preference and water intake data. (a) (top) There was a significant main effect of day ($F(3,27)=9.35$, $p=0.0002$), but no significant main effect of virus ($F(1,9)=1.32$, $p=0.2808$) for female* Gabrd *saccharin preference. (bottom) There was a significant main effect of day ($F(3,27)=8.61$, $p=0.0004$), but no significant main effect of virus ($F(1,9)=1.07$, $p=0.3290$) for female* Gabrd *water intake during saccharin preference testing. (b) (top) There were no significant main effects of either day ($F(3,27)=2.38$, $p=0.0918$) or virus ($F(1,9)=0.63$, $p=0.4481$) for male* Gabrd *saccharin preference. (bottom) There was a significant main effect of day ($F(3,27)=3.12$, $p=0.0426$), but not for virus ($F(1,9)=0.8239$, $p=0.8239$) for male* Gabrd *water intake during saccharin preference testing.*

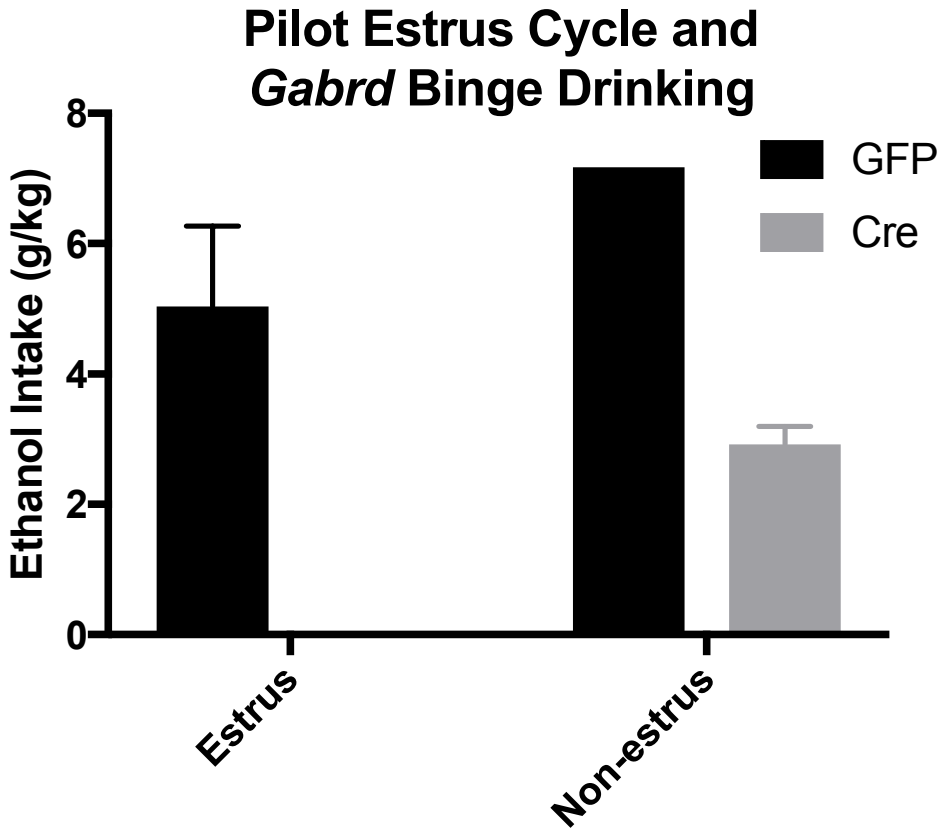


Figure 5.2: Pilot data on relationship between estrus cycle and binge-like drinking in* Gabrd *female mice. *To habituate to the procedure, female* Gabrd *mice were vaginally smeared for 5 d leading up to the start of DID. Smearing occurred daily after each 2 h drinking session (data not shown) as well as after the final, 4 h 'binge' session. Briefly, a small, plastic transfer pipette was inserted into the vaginal canal. Warmed, 1x PBS was gently expelled and withdrawn 3-5 times to allow for cytological sampling. Samples were placed on a glass slide and examined using brightfield microscopy. Cytological assessment was made according to previously described cellular appearance during estrus and non-estrus (proestrus, metestrus, and diestrus, see Caligioni 2009). Placement analysis revealed that the two AAV-Cre-eGFP mice in estrus during the 'binge' session were neuroanatomical misses. They were included as additional control subjects for the purpose of analysis. Although statistics were not calculated due to low n (AAV-eGFP, estrus n=3; AAV-eGFP, non-estrus n=1, AAV-Cre-eGFP, non-estrus n=2), the effects of Cre-mediated excision of* Gabrd *are noticeable.*

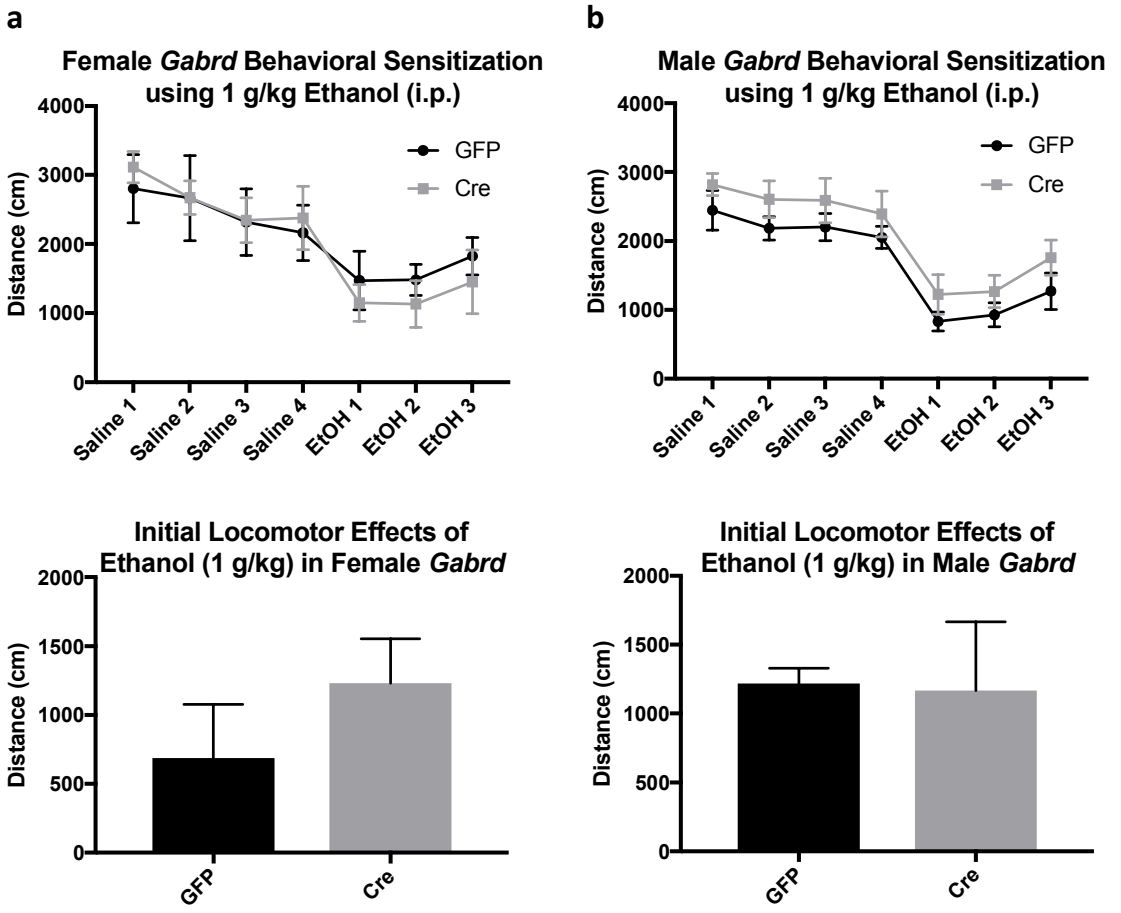


Figure 5.3: Behavioral sensitization using 1 g/kg ethanol after* Gabrd *excision in females and males. *Behavioral sensitization is typically engendered after several administrations of a drug (e.g. psychostimulants, alcohol) and results in increased locomotion (Melón & Boehm 2011; Itzhak & Martin 1999). To test whether* Gabrd *male and female mice had altered sensitivity to acute alcohol treatment, a subset of animals was subjected to a 1 g/kg (i.p.) ethanol challenge after conclusion of preference and aversion testing. Testing was conducted approximately six weeks post-op. Briefly, animals were relocated to the testing room, which was maintained in reverse light/dark housing conditions. Mice were allowed to habituate for at least 1 h prior to the start of testing. An open field apparatus was cleaned with 70% alcohol and the mouse was injected i.p. with 0.9% saline at an equal volume to subsequent 1 g/kg ethanol injection. The mouse was immediately placed in the open field and baseline locomotion was recorded using Kinder Scientific MotorMonitor software (Poway, CA, USA) for a total of 20 min. The mouse was removed, the open field was again cleaned with 70% ethanol, and the mouse injected with 1 g/kg ethanol (20% v/v in 0.9% saline). Locomotion was recorded for the next 15 min. All locomotion was analyzed in 5 min bins.*

Figure 5.3 (cont.): *Since the motor-activating effects of ethanol have been shown to occur shortly after administration of the drug (Melón & Boehm 2011), data were analyzed as the difference in locomotion between the last 5 min of saline habituation and the first 5 min after ethanol administration. Later placement analysis revealed that two AAV-Cre-eGFP female mice and two AAV-Cre-eGFP male mice were neuroanatomical misses. They were included as additional control subjects for the purpose of analysis. Results indicated no significant differences seen between female AAV-eGFP (689±388.4) and AAV-Cre-eGGP (1232±321.1, t(8)=1.077, p=0.3130) or between male AAV-GFP (1220±111.6) and AAV-Cre-eGFP (1167±498.8, t(12)=0.1517, p=0.8819).*

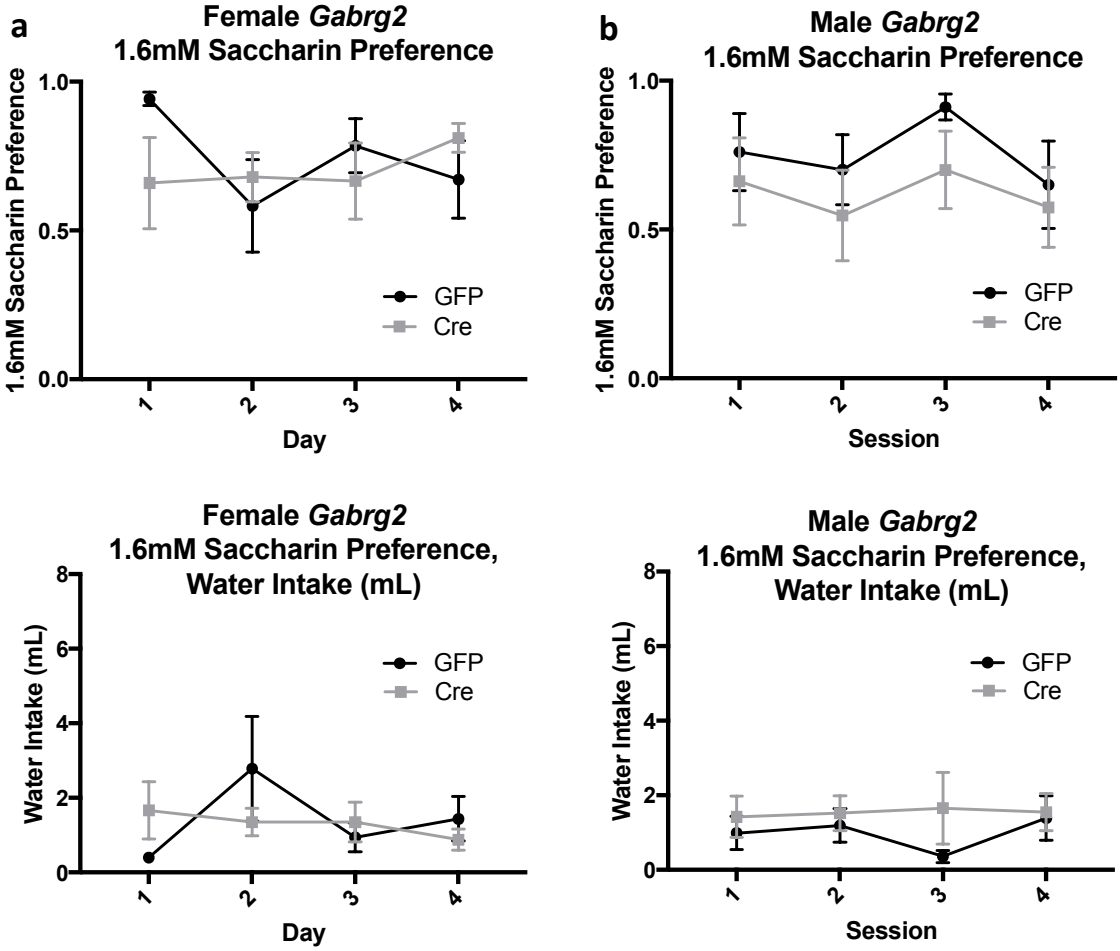


Figure 5.4: No significant effect of* Gabrg2 *excision on saccharin preference or quinine aversion. *A subset of the male and female AAV-Cre-eGFP and AAV-eGFP mice that had undergone sucrose preference and quinine aversion testing were also subjected to 4 d of saccharin (1.6 mM) preference. Testing occurred 3 d after the conclusion of quinine aversion testing. A repeated, two-way ANOVA was used to analyze both saccharin preference and water intake data. (a) (top) While there were no significant main effects for either day ($F(3,27)=1.51$, $p=0.2333$) or virus ($F(1,9)=0.11$, $p=0.7481$) for female* Gabrd *saccharin preference, there was a significant interaction between time and virus ($F(3,27)=2.97$, $p=0.0494$). (bottom) There were no significant main effects of either day ($F(3,27)=1.76$, $p=0.1789$ or virus ($F(1,9)=0.01$, $p=0.9077$) for female* Gabrd *water intake during saccharin preference testing. (b) (top) There were no significant main effects of either day ($F(3,39)=1.86$, $p=0.1532$) or virus ($F(1,13)=0.84$, $p=0.3766$) for male* Gabrd *saccharin preference. (bottom) There were no significant main effects of either day ($F(3,39)=0.70$, $p=0.5557$) or virus ($F(1,13)=0.80$, $p=0.3882$) for male* Gabrd *water intake during saccharin preference testing.*

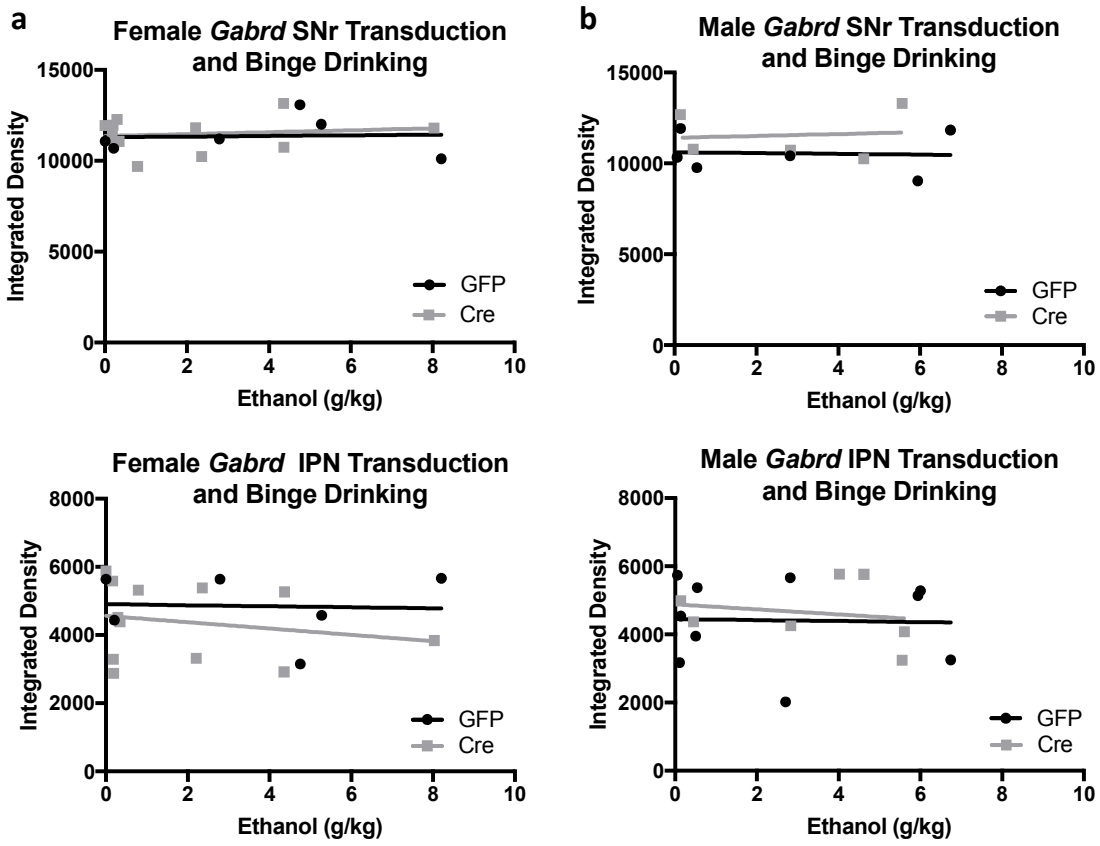


Figure 5.5: No significant relationship between AAV transduction spillover in either* Gabrd *male or female mice and binge-like drinking. *(a) (top) There was no significant correlation between substantia nigra (SNr) viral transduction in either female AAV-Cre-eGFP (r=0.1396, p=0.6651) or female AAV-eGFP (r=0.0494, p=0.9260) and binge-like drinking. (a) (bottom) There was no significant correlation between interpeduncular nucleus (IPN) viral transduction in either female AAV-Cre-eGFP (r=-0.2098, p=0.5128) or female AAV-eGFP (r=-0.04673, p=0.9300) and binge-like drinking. (b) (top) There was no significant correlation between SNr viral transduction in either male AAV-Cre-eGFP (r=0.09351, p=0.8811) or male AAV-eGFP (r=-0.05579, p=0.9164) and binge-like drinking. (b) (bottom) There was no significant correlation between IPN viral transduction in either male AAV-Cre-eGFP (r=-0.181, p=0.6977) or male AAV-eGFP (r=-0.03116, p=0.9316) and binge-like drinking. N.B. Since the only significant relationship was in the pVTA of females, all spillover ROIs were selected from pVTA-containing slices.*

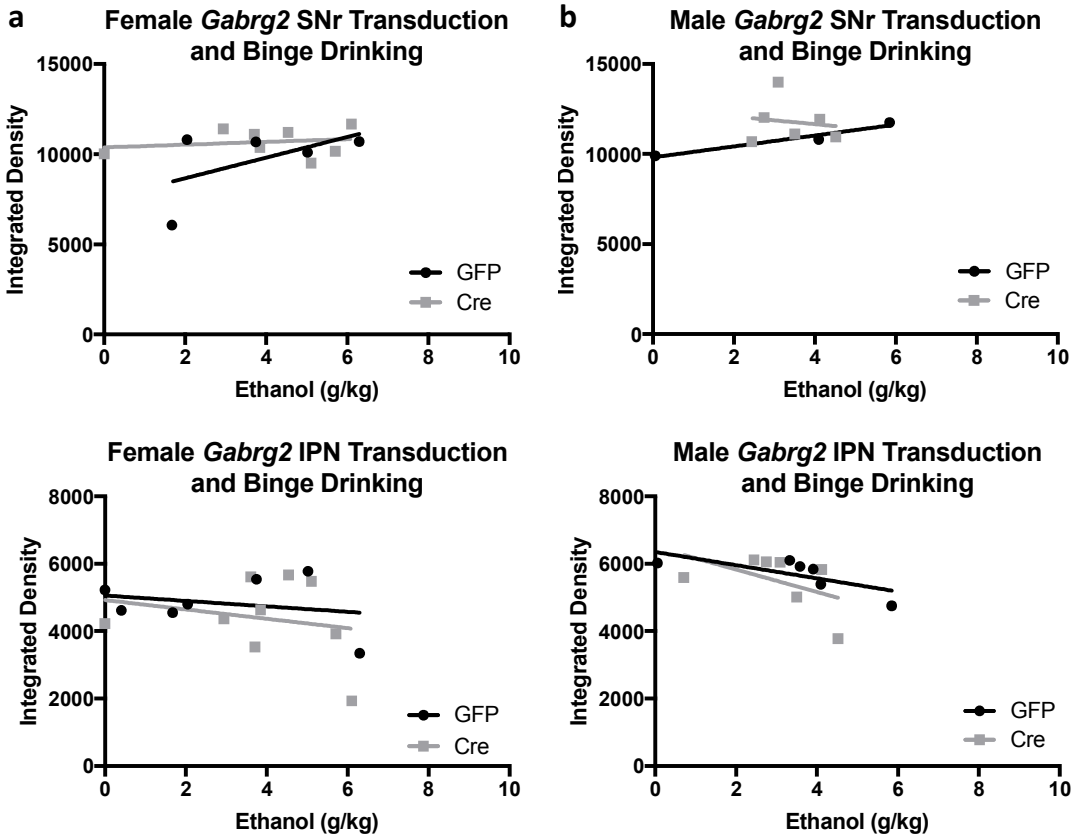


Figure 5.6: No significant relationship between AAV transduction spillover in either* Gabrg2 *male or female mice and binge-like drinking. *(a) (top) There was no significant correlation between substantia nigra (SNr) viral transduction in either female AAV-Cre-eGFP (r=0.1916, p=0.6495) or female AAV-eGFP (r=0.0.5497, p=0.3372) and binge-like drinking. (a) (bottom) There was no significant correlation between interpeduncular nucleus (IPN) viral transduction in either female AAV-Cre-eGFP (r=-0.2103, p=0.5870) or female AAV-eGFP (r=-0.2358, p=0.6107) and binge-like drinking. (b) (top) There was no significant correlation between SNr viral transduction in either male AAV-Cre-eGFP (r=-0.1403, p=0.7909) or male AAV-eGFP (r=0.9724, p=0.1499) and binge-like drinking. (b) (bottom) There was no significant correlation between IPN viral transduction in either male AAV-Cre-eGFP (r=-0.494, p=0.2598) or male AAV-eGFP (r=-0.7228, p=0.1046) and binge-like drinking. N.B. All spillover ROIs were selected from pVTA-containing slices.*

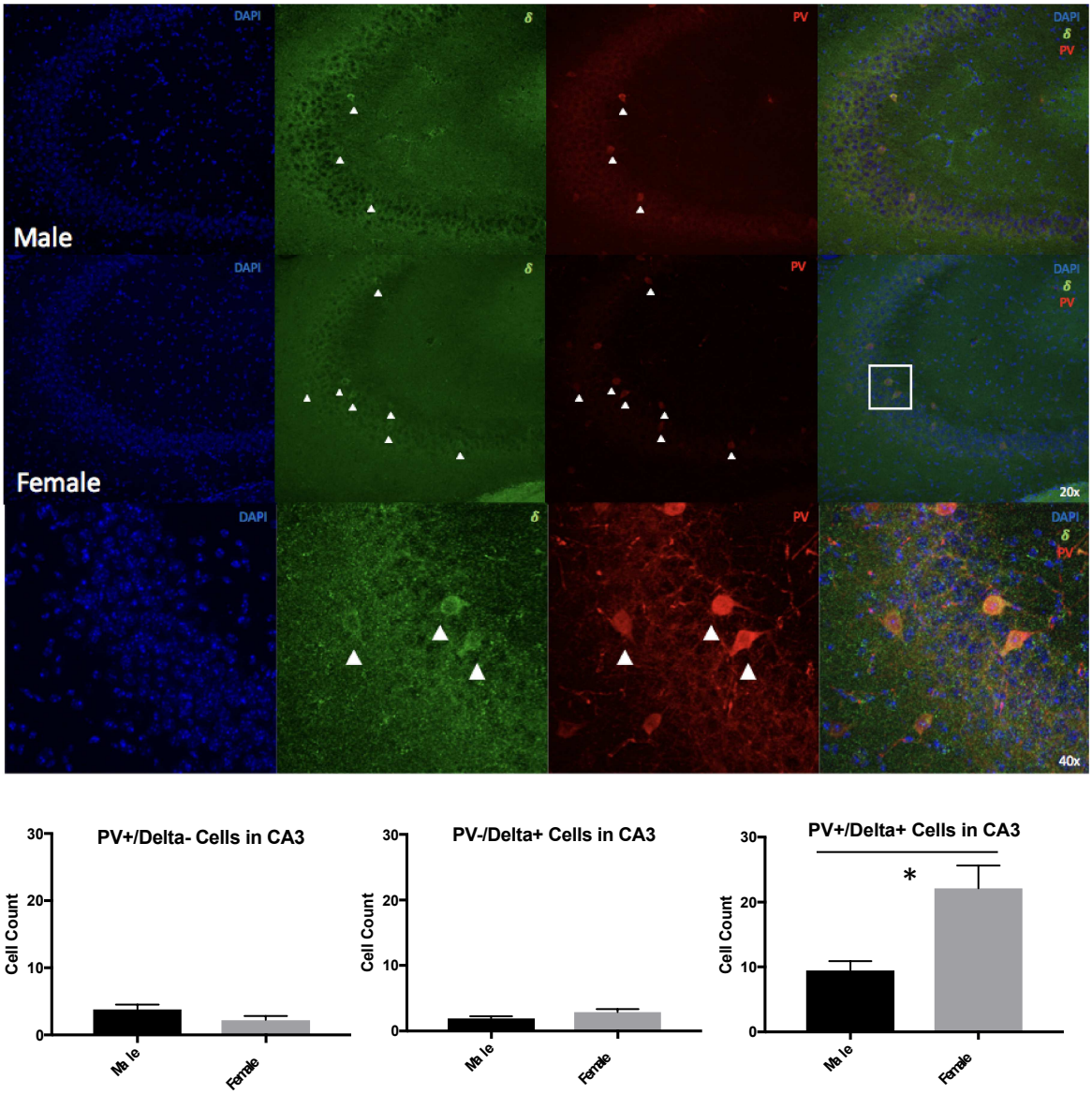


Figure 5.7: Significantly more PV+/δ+ cells in female hippocampus relative to male. *Hippocampal slices from male and female C57Bl/6J mice were co-labeled with anti-PV and anti-δ primary antibodies (see Materials & Methods) and analyzed for resulting immunopositive cells. Results indicated that there were significantly more PV+/δ+ cells in the female CA3 region relative to males ($t(8)=7.511$, $p<0.0001$). There were no significant differences seen in PV+/δ- ($t(8)=1.681$, $p=0.1313$) or PV-/δ+ ($t(8)=1.891$, $p=0.0952$) cell populations. Average cell counts were taken from 4-8 slices per animal. White arrows indicate PV+/δ+ co-labeled cells.*

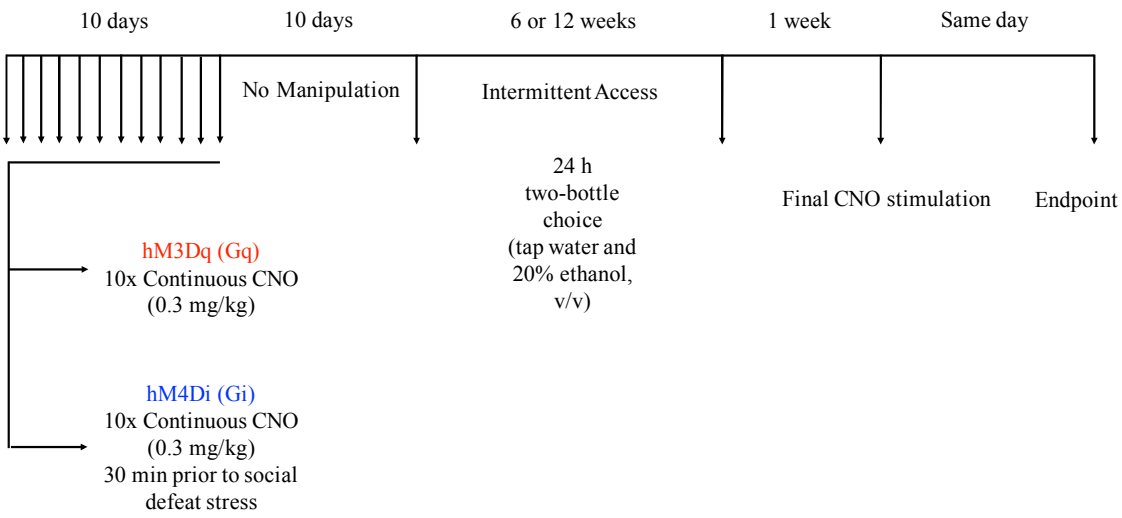


Figure 5.8: Gq and Gi DREADD materials, methods, and experimental timeline. *CRF-IRES-Cre mice were obtained from The Jackson Laboratory (#012704, Bar Harbor, ME, USA) and has been previously characterized (Huang et al. 2011). All CRF-IRES-Cre mice were bred and maintained in-house on a C57Bl/6J background. All mice were same-sex, group-housed at either the Tufts University School of Medicine, Division of Laboratory Animal Medicine (Gq DREADD experiment) or in the Tufts University, Department of Psychology (Gi DREADD experiment). Regardless of location, all mice were housed in clear, plastic cages in a temperature- and humidity-controlled environment with a 12:12 h light/dark cycle and ad libitum access to food and water. All mice were handled according to protocols approved by the Tufts University Institutional Animal Care and Use Committee and remained group housed until the time of intermittent alcohol access.*

Three AAVs were used: AAV-hSyn-DIO-hM3D4-mCherry (excitatory Gq DREADD), AAV-hSyn-DIO-hM4Di-mCherry (inhibitory Gi DREADD), and AAV-eGFP (control). Both viruses were made and produced at the UNC Viral Vector Core and are now available at Addgene (#50474, pAAV-hSyn-hM3D(Gq)-mCherry; #50475, pAAV-hSyn-hM4D(Gi)-mCherry, Cambridge, MA, USA). These AAVs were also serotype 8 and expressed either hM3D4 or hM4Di under control of the neuron-specific promoter human synapsin 1 (hSyn). The inclusion of double-floxed inverse orientation (DIO) sequences allows for Cre-specific expression in cells that are also transcribing CRF. AAV-eGFP (#7004, Vector Biolabs, Malvern, PA, USA) was serotype 2 and expressed enhanced green fluorescent protein (eGFP). It was under control of a non-cell-type-specific CMV promoter and had a titer of approximately $1x10^{13}$ *GC/mL.*

In vivo *stereotaxic surgeries were performed as described in* **Chapter 2: Materials and Methods**. *To investigate the specific role of local CRF signaling in the VTA, two opposing experiments were conducted to answer the following questions: (1) Does CNO-mediated stimulation of CRF release within the VTA result in increased intermittent alcohol intake and (2) Does CNO-mediated inhibition of local CRF release within the VTA inhibit stress-induced intermittent alcohol intake.*

(1) Mice that had been bilaterally injected with either AAV-eGFP (control) or Gq DREADD (experimental) were subjected to 10 d of continuous CNO stimulation (0.3mg/kg, i.p.).

(1, cont.) CNO administration would allow for site-specific activation of Gq DREADD receptors in CRF+ cells of the VTA, putatively mimicking the pattern of CRF release seen during social defeat stress.
(2) Mice that had been bilaterally injected with either AAV-eGFP (control) or Gi DREADD (experimental) were subjected to 10 d of social defeat stress according to previously published methods (Norman et al. 2015). Briefly, adult, male Swiss-derived Carworth Farms Webster (CFW, Charles River Laboratories International, Inc., Wilmington, MA, USA) were used as resident mice and either AAV-eGFP or Gi DREADD mice were used as intruders. CFW resident mice remained pair-housed until the time of social defeat, at which point the female was removed from the home cage. The intruder mouse was then introduced into the CFW resident home cage and aggressive behavior (e.g. tail rattling, biting) ensued almost immediately. All intruder mice were subjected to either 15 bites or 5 min of social defeat (whichever came first) by the resident CFW male before being returned to their respective home cages. Female mice were also subjected to social defeat stress, but used a lactating dam in place of a resident male (Holly et al. 2012). Critically, both AAV-eGFP and Gi DREADD mice were administered CNO (0.3 mg/kg, i.p.) 30 min prior to the start of social defeat stress. CNO administration would allow for site-specific activation of the inhibitory Gi DREADD receptors in only CRF+ cells of the VTA, putatively decreasing CRF release during each episode of defeat. A second group of AAV-eGFP and Gi DREADD mice were administered CNO as described, but were not subjected to social defeat stress and remained in their home cages for the duration of the experiment.

After 10 d of CNO-mediated CRF release or CNO-mediated inhibition of CRF release, all mice were given 10 d of no manipulation to avoid the acute effects of stress. CRF stimulation (Gq DREADD) or CNO-mediated, CRF inhibition (Gi DREADD), mice were subjected to either 6 or 12 weeks of the two-bottle choice procedure intermittent alcohol access (IAA), respectively. IAA was conducted as previously described (Hwa et al. 2016; Hwa, DeBold & Miczek 2013; Hwa et al. 2011). Briefly, mice were individually housed and allowed alternating, 24 h access to either 20% ethanol (v/v) and water or only water. Tubes were alternated every 24 h to avoid the development of a side preference. All fluids were delivered in 50 mL, double-ball bearing plastic tubes. Volume differences were converted to individual g/kg values for each mouse for each 24 h access period. Mouse weights were obtained weekly.

After IAA was completed, all mice were given a final administration of CNO 1 h before sacrifice. Tissue was collected for later placement and c-Fos, neuronal activation assessments. All data were collected and organized using Microsoft Excel (v. 15.39). All statistical analyses were conducted using Prism 7 (GraphPad La Jolla, CA, USA). Multiple, repeated measures, two-way ANOVAs were used to analyze IAA drinking data, with AAV (GFP versus Gq DREADD) as the between-subjects factor and time as the within-subjects factor or defeat as the between-subjects factor and time as the within-subjects factor. An unpaired, two-tailed Student's t test was used to analyze DID binge, 4 h drinking data (GFP versus Cre). A p-value of $p<0.05$ was considered statistically significant for all analyses.

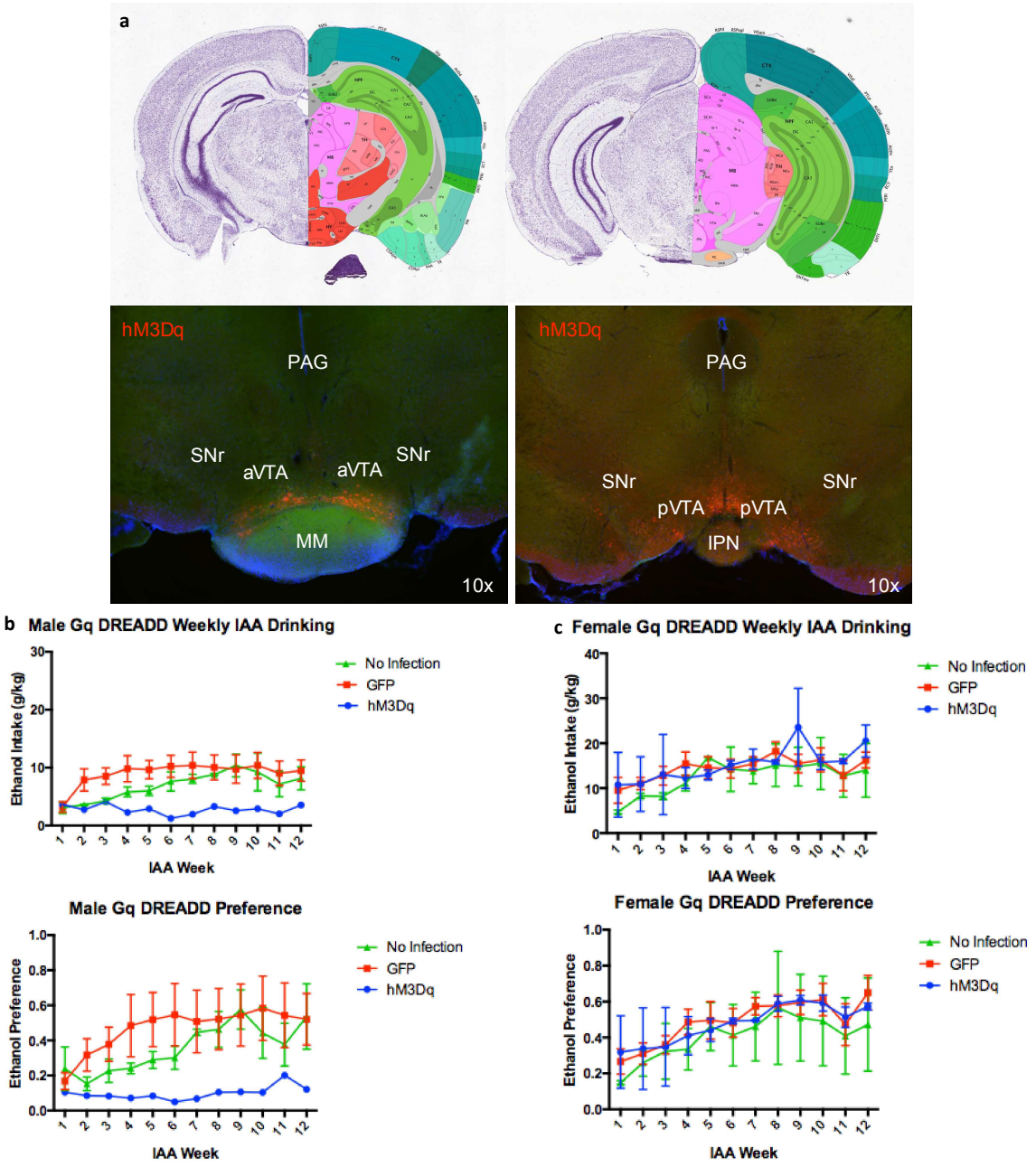


Figure 5.9: No significant effect on IAA drinking or preference after CNO-stimulated CRF release. *The functional importance of local CRF signaling within the VTA has been robustly demonstrated using pharmacological approaches (Hwa et al. 2016). However, social defeat stress is a complex stressor that engages multiple circuits within the brain (Hwa et al. 2016; Krishnan et al. 2007); as such, the causal role of local CRF in the VTA to later escalated intake has yet to be definitively verified.*

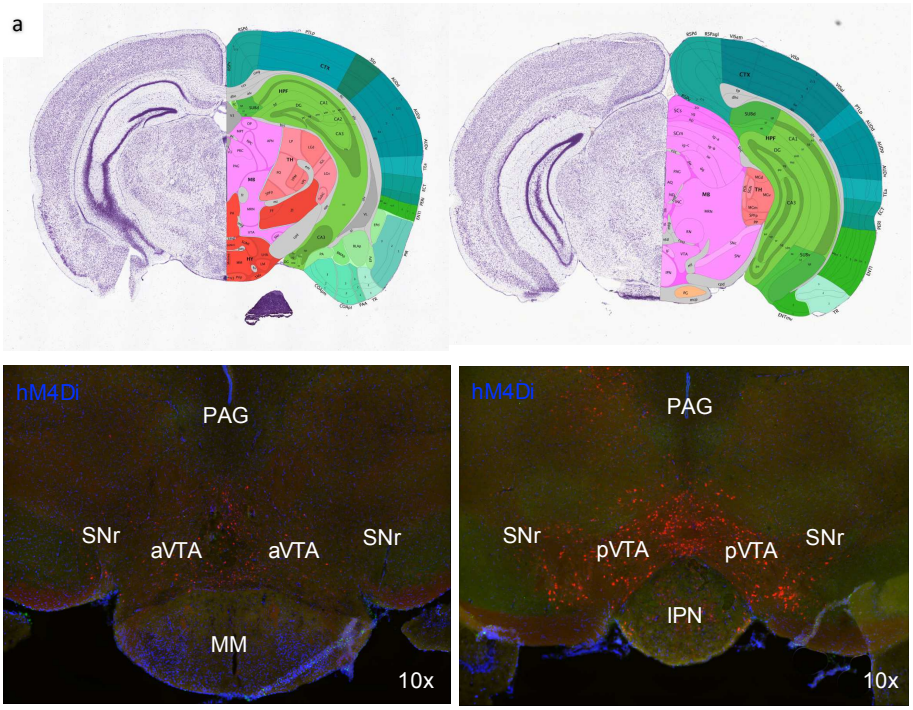
Given this, cohorts of male and female mice were microinjected with either AAV-eGFP or Gq DREADD into the VTA and administered CNO for 10 continuous days. All mice were then subjected to 12 weeks of IAA. Weekly intake (g/kg) and alcohol preference scores were

*calculated. Later placement analysis allowed a further subdivision of mice in the Gq DREADD group, to separate those animals who had clear signs of viral transduction (**Figure 5.9a**) versus those that did not. These groups were then analyzed separately. Results revealed a significant main effect of time for male IAA intake, with all male mice (AAV-eGFP, Gq DREADD, and No Infection) drinking more alcohol over the duration of IAA ($F(11,44)=2.45$, $p=0.0175$, **Figure 5.9b, top**). However, there was no significant main effect of virus and IAA intake ($F(2,4)=1.66$, $p=0.2983$) and no significant interaction between time and virus ($F(22,44)=1.31$, $p=0.2161$). There were also no significant main effects of time ($F(11,44)=1.82$, $p=0.0787$) or virus ($F(2,4)=0.93$, $p=0.4671$) for male alcohol preference during IAA (**Figure 5.9b, bottom**).*

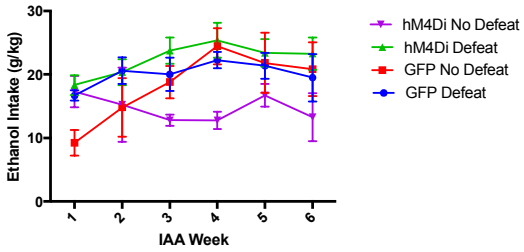
*Female mice also had a significant main effect of time for IAA intake, with all mice (AAV-eGFP, Gq DREADD, and No Infection) drinking more alcohol over the duration of IAA ($F(11,44)=3.27$, $p=0.0025$, **Figure 5.9c, top**). There was no significant main effect of virus ($F(2,4)=0.39$, $p=0.6990$) and no significant interaction between time and virus ($F(22,44)=0.54$, $p=0.9371$). There was also a significant main effect of time for alcohol preference during IAA ($F(11,44)=7.65$, $p<0.0001$, **Figure 5.9c, bottom**). Similar to intake, there was no significant main effect of virus for IAA preference ($F(2,4=0.19$, $p=0.8354$) and no significant interaction between time and virus ($F(22,44)=0.25$, $p=0.9995$).*

Taken together, both male and female mice showed escalated IAA intake and preference over the 12 weeks of testing. It is worth noting that there was only one male Gq DREADD subject that had successful viral transduction in the VTA. Drinking and preference data from this mouse were markedly lower than in either the AAV-eGFP or No Infection groups; however, the low n prohibit any conclusive statements.

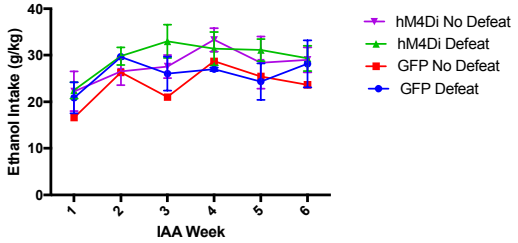
(a) Viral placement images showing Gq DREADD reporter expression in the VTA of both males and females CRF-IRES-Cre mice. (b) While there were no significant differences in either IAA intake or preference in male control mice (AAV-eGFP, n=4), Gq DREADD (n=1), or Gq DREADD mice who had no clear signs of transduction (n=2), the low n make interpretation difficult. It is notable that the Gq DREADD mouse had notably lower intake and preference (n=1). (c) There was also no significant difference in intake or preference between female AAV-eGFP controls (n=3), Gq DREADD (n=2), or Gq DREADD mice with no clear signs of transduction (n=2). Coronal images courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing.



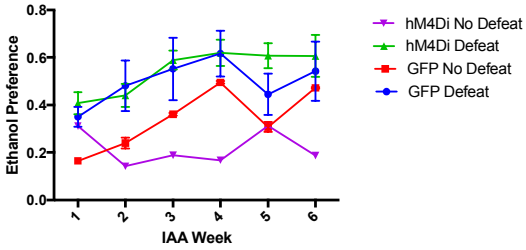
b CNO-silenced CRF Signaling and Weekly Ethanol Intake in Socially Defeated Male Mice



c CNO-silencing CRF Signaling and Weekly Ethanol Intake in Socially Defeated Female Mice



CNO-silenced CRF Signaling and Weekly Ethanol Preference in Socially Defeated Male Mice



CNO-silencing CRF Signaling and Weekly Ethanol Preference in Socially Defeated Female Mice

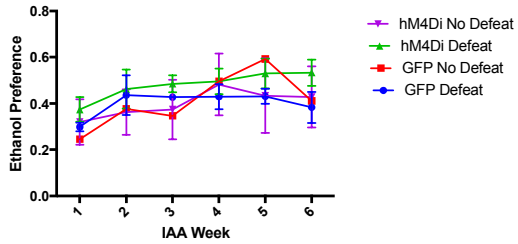


Figure 5.10: Silencing CRF release during social defeat stress has no significant effect on IAA drinking or preference. *Stimulating local CRF release in the VTA is one of two approaches to determine if such release is important to stress-escalated alcohol intake. The second would be inhibition of local CRF release during social defeat stress. To this end, cohorts of male and female mice were microinjected with either AAV-eGFP or Gi DREADD into the VTA. Critically, a subset of these mice also received daily CNO injections prior to 10 d of social defeat stress, thereby allowing for local inhibition of CRF in the VTA during stress. All mice were then subjected to 6 weeks of IAA and weekly intake (g/kg) and alcohol preference scores were calculated. Placement analysis was conducted after behavioral testing, which indicated good viral localization to the VTA (**Figure 5.10a**).*

*Male AAV-eGFP mice were divided into two groups, those that received social defeat stress and those that did not. There was a significant main effect of time ($F(5,15)=10.56$, $p=0.0002$), but no significant effect of defeat ($F(1,3)=0.25$, $p=0.6492$). There was also a significant interaction between time and defeat ($F(5,15)=3.21$, $p=0.0360$). Male Gi DREADD mice were divided into social defeat stress and no defeat stress; there were no significant main effects of either time ($F(5,30)=0.49$, $p=0.7788$) or virus ($F(!,6)=4.43$, $p=0.0801$). There were also significant main effects of time for weekly IAA preference in AAV-eGFP mice ($F(5,15)=6.36$, $p=0.0023$), but not for Gi DREADD mice ($F(5,25)=1.3$, $p=0.2955$). There was a significant effect of virus for Gi DREADD mice and alcohol preference ($F(1,5)=7.08$, $p=0.0448$, **Figure 5.10b**).*

*Female AAV-eGFP mice were also divided into the same groups and there was no significant main effect of defeat on either IAA intake ($F(1,1)=0.29$, $p=0.6866$) or preference ($F(5,5)=2.91$, $p=0.1328$). Female Gi DREADD mice also had a significant main effect of time on IAA intake ($F(5,20)=10.28$, $p<0.0001$) as well as IAA preference ($F(5,20)=3.56$, $p=0.0183$, **Figure 5.10c**).*

Taken together, Gi DREADD intake and preference showed a similar increase over the course of IAA as with Gq DREADD. However, there was no clear indication of previously demonstrated stress-escalated IAA intake (Norman et al. 2015). There was also no effect of CNO silencing during social defeat on escalated IAA intake or preference.

(a) Viral placement images indicating Gi DREADD reporter expression in the VTA of both males and females CRF-IRES-Cre mice. (b) There were no significant main effects of either social defeat or virus in male mice on later IAA intake or preference (AAV-eGFP defeat, n=3; AAV-eGFP no defeat, n=2; Gi DREADD defeat, n=6; Gi DREADD no defeat, n=2). (c) There were also no significant main effects of either social defeat or virus in female mice on later IAA intake or preference (AAV-eGFP defeat, n=2; AAV-eGFP no defeat, n=1; Gi DREADD defeat, n=4; Gi DREADD no defeat, n=2). Coronal images courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing.

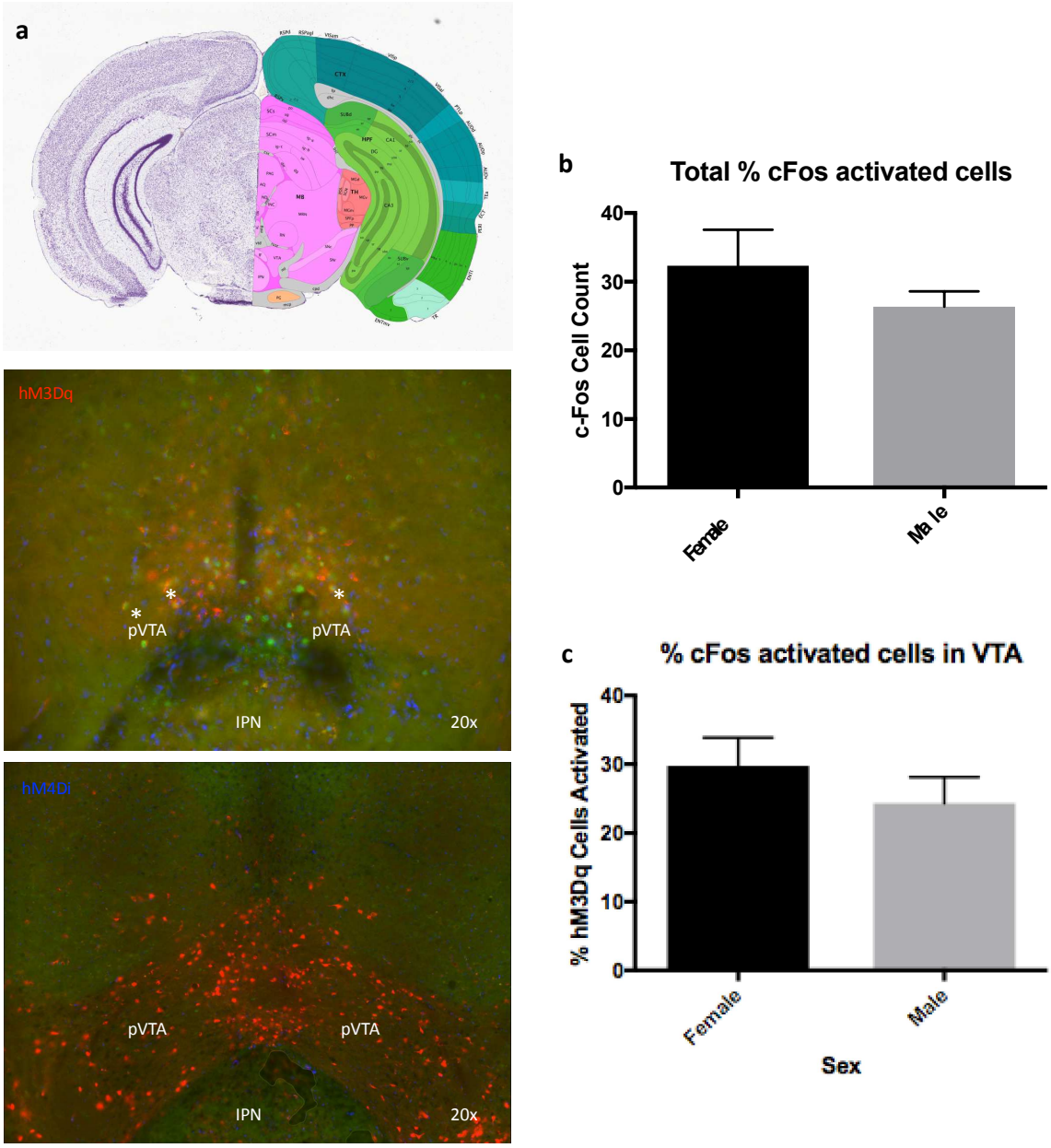


Figure 5.11: CNO-mediated neuronal activation using Gq DREADDs, but inhibition with Gi DREADDs. *After IAA, mice were deeply anesthetized with isoflurane and rapidly decapitated. Brains were rapidly removed and immediately placed in 4% (w/v) paraformaldehyde at 4°C for overnight fixation. Brains were then cryoprotected in a series of sucrose concentrations (10-30%), snap frozen in isopentane, frozen at -80°C, and free-floating (PBS), 40 µM sections taken using a Leica CM1900 cryostat (Buffalo Grove, IL, USA).*

Three anterior VTA (aVTA, coordinate range -2.8mm to -3.4mm) and three posterior VTA (pVTA, coordinate range -3.52mm to -4.04mm) slices were selected for placement analysis from each animal. Sections were blocked with 10% normal goat serum (NGS, Vector Labs,

Burlingame, CA, USA) and 0.3% Triton in PBS for 1 h at room temperature and incubated with a polyclonal anti-c-Fos primary antibody (rabbit, 1:5000, MilliporeSigma, Burlington, MA, USA) for 72 h at 4°C. Sections were washed three times with PBS and incubated with a biotinylated goat anti-rabbit IgG secondary (1:200, Vector Labs, Burlingame, CA, USA) overnight at 4°C. Slices were washed three times with PBS and incubated for 2 h at 4°C with streptavidin-conjugated AlexaFluor 488 secondary antibody (rabbit, 1:1000, Invitrogen, Waltham, MA, USA). After three more washes with PBS, sections were briefly rinsed in dH_2O, mounted on slides, coverslipped the following day using VECTASHIELD mounting medium with DAPI (Vector Labs, Burlingame, CA, USA). Slides were imaged on a Keyence BZ-X700 (Keyence, Itasca, IL, USA), and analyzed using NIH Fiji software. Unpaired, two-tailed Student's t tests were used to analyze differences in c-Fos expression. A p-value of $p<0.05$ was considered statistically significant for all analyses.

*The lack of difference seen in both Gq and Gi DREADD experiments could be due to insufficient neuronal activation (Gq) or inhibition (Gi). While the CNO dose used has been previously reported to display significant behavioral effects (Boender et al. 2014), it is possible that this population of CRF-expressing neurons had a different dose-response range. To assess whether virally transduced cells were effectively activated or inhibited, animals were given a final injection of CNO (0.3 mg/kg) and sacrificed one hour later. After tissue harvesting and slice selection, cells were incubated with a primary antibody to c-Fos to assess for neuronal activation. Both male and female mice had significant populations that were positive for both mCherry (reporter) and c-Fos (**Figure 5.11a, middle**). Importantly, neuronal activation was seen at equivalent levels between males (26.35±2.273) and females (33.34±5.034) for overall number of c-Fos+ cells ($t(17)=1.119$, $p=0.2789$, **Figure 5.11b, top**). It was also equivalent between males (24.32±3.838) and females (30.35±4.257) in neurons that were positive for mCherry and c-Fos ($t(17)=1.008$, $p=0.3227$, **Figure 5.11b, bottom**). While not quantified, a preliminary examination of Gi DREADD tissue indicated almost no neuronal activation in successfully transduced cells (**Figure 5.11a, bottom**).*

(a) Representative images indicating increases c-Fos activation in the VTA of Gq DREADD mice (top) relative to Gi DREADD mice (bottom). (b) There was no significant difference between percent of total c-Fos activated cells in the VTA between males and females. (c) There was no significant difference between percent of c-Fos activated Gq DREADD cells (mCherry) between males and females. Coronal image courtesy of © 2017 Allen Institute for Brain Science. Adapted with Permission from Allen Brain Reference Atlas. Changes include resizing.

Chapter 6
Bibliography

Abernathy K, Chandler LJ & Woodward JJ. Alcohol and the Prefrontal Cortex. *Int Rev Neurobiol* 91: 289-320, 2010.

Adamantidis AR, Tsai H-C, Boutrel B, Zhang F, Stuber GD, Budygin EA, Touriño C, Bonci A, Deisseroth K & de Lecea L. Optogenetic Interrogation of Dopaminergic Modulation of the Multiple Phases of Reward-Seeking Behavior. *J Neurosci* 31(30): 10829-10835, 2011.

Akk G & Steinbach JH. Low doses of ethanol and a neuroactive steroid positively interact to modulate rat GABA(A) receptor function. *J Physiol* 546: 641-646, 2003.

Akk G, Li P, Manion BD, Evers AS & Steinbach JH. Ethanol modulates the interaction of the endogenous neurosteroid allopregnanolone with the alpha1beta2gamma2L $GABA_A$ receptor. *Mol Pharmacol* 71: 461-472, 2007.

Alfonso-Loeches S, Pascual M & Guerri C. Gender differences in alcohol-induced neurotoxicity and brain damage. *J Toxicol* 311(1-2): 27-34, 2013.

Allen Institute for Brain Science, Allen Adult Mouse Brain Reference Atlas. 2016, available from http://atlas.brain-map.org/.

Ambroggi F, Ishikawa A, Fields HL & Nicola SM. Basolateral amygdala neurons facilitate reward-seeking behavior by exciting nucleus accumbens neurons. *Neuron* 59: 648-661, 2008.

Anda RF, Waller MN, Wooten KG, Mast EE, Escobedo LG & Sanderson LM. Behavioral Risk Factor Surveillance, 1988. Source: *Morbidity and Mortality Weekly Report: Surveillance Summaries,* Vol. 39, No. SS-2, June 1990, 1-21. Published by Centers for Disease Control and Prevention.

Andreoli M, Marketkar T & Dimitrov E. Contribution of amygdala CRF neurons to chronic pain. *Exp Neurol* 298(A): 1-12, 2017.

Anstee QM, Knapp S, Maguire EP, Hosie AM, Thomas P, Mortensen M, Bhome R, Martinez A, Walker SE, Dixon CI, Ruparelia K, Montagnese S, Kuo Y-T, Herlihy A, Bell JD, Robinson I, Guerrini I, McQuillin A, Fisher EMC, Ungless MA, Gurling HMD, Morgan MY, Brown SDM, Stephens DN, Belelli D, Lambert JJ, Smart TG & Thomas HC. Mutations in the *Gabrb1* gene promote alcohol consumption through increased tonic inhibition. *Nat Commun 4*: 2816, 2013.

Armbruster BN, Li X, Pausch MH, Herlitze S & Roth BL. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an intert ligand. *Proc Natl Acad Sci* 104(12): 5163-5168, 2007.

Asan E. The catecholaminergic innervation of the rat amygdala. *Adv Anat Embryol Cell Biol* 142: 1-118, 1998.

Atasoy D, Betley JN, Su HH & Sternson SM. Deconstruction of a neural circuit for hunger. *Nature* 488: 172-177, 2012.

Badanich KA, Becker HC & Woodward JJ. Effects of chronic intermittent ethanol exposure on orbitofrontal and medial prefrontal cortex-dependent behaviors in mice. *Behav Neurosci* 125(6): 879-891.

Baer K, Essrich C, Balsiger S, Wick MJ, Harris RA, Fritschy JM & Lüscher B. Rescue of γ2 subunit-deficient mice by transgenic overexpression of the $GABA_A$ receptor γ2S or γ2L subunit isoforms. *Eur J Neurosci*, 12: 2639-2643, 2000.

Balcita-Pedicino JJ, Omelchenko N, Bell R & Sesack SR. The Inhibitory Influence of the Lateral Habenula on Midbrain Dopamine Cells: Ultrastructural Evidence for Indirect Mediation via the Rostromedial Mesopontine Tegmental Nucleus. *J Comp Neurol* 519: 1143-1154, 2011.

Bao J, Ma H-Y, Schuster A, Lin Y-M & Yan W. Incomplete Cre-mediated excision pleads to phenotypic differences between *Stra8-iCre; Mov10l1*$^{lox/lox}$ and *Stra8-iCre; Mov10l1lox/Δ mice*. *Genesis* 51(7): 481-490, 2013.

Barbaccia ML, Affricano D, Trabucchi M, Purdy RH, Colombo G, Agabio R & Gessa GL. Ethanol markedly increases "GABAergic" neurosteroids in alcohol-preferring rats. *Eur J Pharmacol* 384: R1-R2, 1999.

Baumann S, Baur R & Sigel E. Individual properties of the two functional agonist sites in $GABA_A$ receptors. *J Neurosci* 23: 11158-11166, 2003.

Becker JB. Direct Effect of 17β-Estradiol on Striatum: Sex Differences in Dopamine Release. *Synapse* 5: 157-164, 1990.

Becker JB. Gender Differences in Dopaminergic Function in Striatum and Nucleus Accumbens. *Pharmacol Biochem Behav* 64(4): 803-812, 1999.

Becker H.C. (2012) Animal Models of Excessive Alcohol Consumption in Rodents. In: Sommer W., Spanagel R. (eds) Behavioral Neurobiology of Alcohol Addiction. Current Topics in Behavioral Neurosciences, vol 13. Springer, Berlin, Heidelberg

Becker JB, Perry AN & Westenbroek. Sex differences in the neural mechanisms mediating addiction: a new synthesis and hypothesis. *Biol Sex Differ* 3(14): 1-35, 2012.

Belelli D & Lambert JJ. Neurosteroids: Endogenous Regulators of the $GABA_A$ Receptor. *Nature Rev Neurosci* 6: 565-575, 2005.

Belknap JK, Crabbe JC & Young ER. Voluntary consumption of ethanol in 15 inbred mouse strains. *Psychopharmacology* 112(4): 503-510, 1993.

Blanchard BA, Steindorf S, Wang S & Glick SD. Sex Differences in Ethanol-induced Dopamine Release in Nucleus Accumbens and in Ethanol Consumption in Rats. *Alcohol Clin Exp Res* 17(5): 968-973, 1993.

Blazer DG & Wu L-T. The Epidemiology of At-Risk and Binge Drinking Among Middle-Aged and Elderly Community Adults: National Survey on Drug Use and Health. *Am J Psychiatry* 166: 1162-1169, 2009.

Blednov YA, Borghese CM, McCracken ML, Benavidez JM, Geil CR, Osterndorff-Kahanek E, Werner DF, Iyer S, Swihart A, Harrison NL, Homanics GE & Harris RA. Loss of Ethanol Conditioned Taste Aversion and Motor Stimulation in Knockin Mice with Ethanol-Insensitive α2-Containing $GABA_A$ Receptors. *J Pharmacol Exp Ther* 336(1): 145-154, 2011.

Blednov YA, Benavidez JM, Black M, Chandra D, Homanics GE, Rudolph U & Harris RA. Linking GABAA receptor subunits to alcohol-induced conditioned taste aversion and recovery from acute alcohol intoxication. *Neuropharmacology* 67: 46-56, 2013.

Boehm II, SL, Ponomarev I, Jennings AW, Whiting PJ, Rosahl TW, Garrett EM, Blednov YA & Harris RA. γ-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol action. *Biochem Pharmacol* 68(8): 1581-1602, 2004.

Boender AJ, de Jong JW, Boekhoudt L, Luijendijk MCM, van der Plasse G, & Adan RAH. Combined Use of the Canine Adenovirus-2 and DREADD-Technology to Activate Specific Neural Pathways *In Vivo*. *PLOS ONE*, 9(4): 1-6, 2014.

Bohme I, Rabe H & Luddens H. Four amino acids in the α subunits determine the γ-aminobutyric acid sensitivities of $GABA_A$ receptor subtypes. *J Biol Chem* 279, 35193-35200, 2004.

Borghese CM, Stórustovu Sí, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA & Harris RA. The δ Subunit of γ-Aminobutyric Acid Type A Receptors Does Not Confer Sensitivity to Low Concentrations of Ethanol. *J Pharmacol Exp Ther* 316(3): 1360-1368, 2006.

Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ & Brewer RD. Economic Costs of Excessive Alcohol Consumption in the U.S., 2006. *Am J Prev Med*, 41(5): 516-524, 2011.

Bourdy R & Barrot M. A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci* 35(11): 681-690, 2012.

Breese GR, Overstreet DH & Knapp DJ. Conceptual framework for the etiology of alcoholism: A "kindling"/stress hypothesis. *Psychopharmacology* 178: 367-380, 2005.

Brinschwitz K, Dittgen A, Madai VI, Lommel R, Geisler S & Veh RW. Glutamatergic Axons from the Lateral Habenula Mainly Terminate on GABAergic Neurons of the Ventral Midbrain. *Neuroscience* 168: 463-476, 2010.

Britt JP, Benaliouad F, McDevitt RA, Stuber GD, Wise RA & Bonci A. Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens. *Neuron* 76(4): 790-803, 2012.

Brown N, Kerby J, Bonnert TP, Whiting PJ & Wafford KA. Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br J Pharmacol* 136: 965-974, 2002.

Buck KJ, Metten P, Belknap JK & Crabbe JC. Quantitative Trait Loci Involved in Genetic Predisposition to Acute Alcohol Withdrawal in Mice. *J Neurosci* 17(10): 3946-3955, 1997.

Caligioni C. Assessing Reproductive Status/Stages in Mice. *Curr Protoc Neurosci*, Published online July 2009 in Wiley Interscience, 2009.

Carillo J & Gonzales RA. A single exposure to voluntary ethanol self-administration produces adaptations in ethanol consumption and accumbal dopamine signaling. *Alcohol* 45: 559-566, 2011.

Carson EJ & Pruett SB. Development and Characterization of a Binge Drinking Model in Mice for Evaluation of the Immunological Effects of Ethanol. *Alcohol Clin Exp Res* 20(1): 132-138, 1996.

Cassel MD, Freedman LJ & Shi C. The Intrinsic Organization of the Central Extended Amygdala. *Ann N.Y. Acad Sci* 877: 217-241, 1999.

Castner SA, Xiao L & Becker JB. Sex differences in striatal dopamine: in vivo microdialysis and behavioral studies. *Brain Res* 610: 127-134, 1993.

Chandra D, Korpi ER, Miralles CP, De Blas AL & Homanics GE. $GABA_A$ receptor γ2 subunit knockdown mice have enhanced anxiety-like behavior but unaltered hypnotic response to benzodiazepines. *BMC Neuroscience* 6(30): 1-13, 2005.

Chang Y, Wang R, Barot S & Weiss DS. Stoichiometry of a Recombinant $GABA_A$ Receptor. *J Neurosci* 16(17): 5415-5424, 1996.

Chen NA, Jupp B, Sztainberg Y, Lebow M, Brown RM, Kim JH, Chen A & Lawrence AJ. Knockdown of CRF1 Receptors in the Ventral Tegmental Area Attenuates Cue- and Acute Food Deprivation Stress-Induced Cocaine Seeking in Mice. *J Neurosci* 34(35): 11560-11570, 2014.

Chen Y, Brunson KL, Müller MB, Cariaga W & Baram TZ. Immunocytochemical Distribution of Corticotropin-Releasing Hormone Receptor Type-1 (CRF_1)-Like Immunoreactivity in the Mouse Brain: Light Microscopy Analysis Using an Antibody Directed Against the C-Terminus. *J Comp Neurol* 420: 305-323, 2000.

Chieng B, Azriel Y, Mohammadi S & Christie MJ. Distinct cellular properties of identified dopaminergic and GABAergic neurons in the mouse ventral tegmental area. *J Physiol* 589.15: 3775-3787, 2011.

Clark WB & Cahalan D. Changes in problem drinking over a four-year span. *Addict Behav* 1: 251-259, 1976.

Clarren SK & Bowden DM. Fetal alcohol syndrome: A new primate model for binge drinking and its relevance to human ethanol teratogenesis. *J Pediatr* 101(5): 819-824, 1982.

Cole G, Tucker L & Friedman GM. Relationships Among Measures of Alcohol Drinking Behavior, Life-Events, and Perceived Stress. *Psychol Rep* 67: 587-591, 1990.

Cooke BM & Woolley CS. Sexually Dimorphic Synaptic Organization of the Medial Amygdala. *J Neurosci* 25(46): 10759-10767, 2005.

Cooper ML, Russell M & Frone MR. Work Stress and Alcohol Effects: A Test of Stress-Induced Drinking. *J Health Soc Behav* 31(3): 260-276, 1990.

Cozzoli DK, Courson J, Caruana AL, Miller BW, Greentree DI, Thompson AB, Wroten MG, Zang P-W, Xiao B, Hu J-H, Klugmann M, Metten P, Worley PF, Crabbe JC & Szumlinski. Nucleus Accumbens mGluR5-Associated Signaling Regulates Binge Alcohol Drinking Under Drinking-in-the-Dark Procedures. *Alcohol Clin Exp Res* 36(9): 1623-1633, 2012.

Cozzoli DK, Courson J, Wroten MG, Greentree DI, Lum EN, Campbell RR, Thompson AB, Maliniak D, Worley PF, Jonquieres G, Klugmann M, Finn DA & Szumlinski. Binge Alcohol Drinking by Mice Requires Intact Group 1 Metabotropic Glutamate Receptor Signaling Within the Central Nucleus of the Amygdala. *Neuropsychopharmacology* 39(2): 435-444, 2014.

Crabbe JC, Metten P, Rhodes JS, Yu CH, Brown LL, Phillips TJ & Finn DA. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biol Psychiatry* 65: 662-670, 2009.

Crabbe JC, Harris RA & Koob GF. Preclinical studies of alcohol binge drinking. *Ann NY Acad Sci*, 1216: 24-40, 2011.

Crews FT, Braun CJ, Hoplight B, Switzer III RC & Knapp DJ. Binge Ethanol Consumption Causes Differential Brain Damage in Young Adolescent Rats Compared with Adult Rats. *Alcohol Clin Exp Res* 24(11): 1712-1723.

Crippens D, White ML, George MA, Jaworski JN, Brunner LJ, Lancaster FE & Gonzales RA. Gender Differences in Blood Levels, but not Brain Levels, of Ethanol in Rats. *Alcohol Clin Exp Res* 23(3): 414-420, 1999.

Cui C & Koob GF. Titrating Tipsy Targets: The Neurobiology of Low-Dose Alcohol. *Trends Pharmacol Sci* 38(6):556-568, 2017.

Dabrowska J, Martinon D, Moaddab M & Rainnie DG. Targeting Corticotropin-Releasing Factor Projections from the Oval Nucleus of the Bed Nucleus of the Stria Terminalis Using Cell-Type Specific Neuronal Tracing Studies in Mouse and Rat Brain. *J Neuroendocrinol* 28(12): 1-15, 2016.

Dall'Oglio A, Gehlen G, Achaval M & Rasia-Filho AA. Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: Further data based on the Golgi method. *Neurosci Lett* 2(10): 151-156, 2008.

Dautzenberg FM & Hauger RL. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol Sci* 23(2): 71-77, 2002.

Davis AM, Grattan DR, Selmanoff M & Mccarthy MM. Sex Differences in Glutamic Acid Decarboxylase mRNA in Neonatal Rat Brain: Implications for Sexual Differentiation. *Horm Behav* 30(4): 538-552, 1996.

de Wit H. Impulsivity as a determinant and consequence of drug use: a review of underlying processes. *Addict Biol* 14: 22-31, 2009.

DeNardo LA, Berns DS, DeLoach K & Luo L. Connectivity of Mouse Somatosensory and Prefrontal Cortex Examined with Trans-synaptic Tracing. *Nat Neurosci* 18(11): 1687-1697, 2015.

Devaud LL, Smith FD, Grayson DR & Morrow AL. Chronic ethanol consumption differentially alters the expression of gamma-aminobutyric acid A receptor subunit mRNAs in rat cerebral cortex: competitive reverse transcriptase-polymerase chain reaction analysis. *Mol Pharmacol* 48: 861-868, 1995.

Devaud LL & Alele P. Differential Effects of Chronic Ethanol Administration and Withdrawal on γ-Aminobutyric Acid Type A and NMDA Receptor Subunit Proteins in Male and Female Rat Brain. *Alcohol Clin Exp Res* 28(6): 957-965, 2004.

Di Chiara G & Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci* 85: 5274-5278, 1988.

Dluzen DE & Anderson LI. Estrogen differentially modulates nicotine-evoked dopamine release from the striatum of male and female rats. *Neurosci Lett* 230(2): 140-142, 1997.

Drasbek KR & Jensen K. THIP, a Hypnotic and ANtinociceptive Drug, Enhances an Extrasynaptic $GABA_A$ Receptor-mediated Conductance in Mouse Neocortex. *Cereb Cortex* 16: 1134-1141, 2006.

Edenberg HJ, Dick DM, Xuei X, Tian H, Almasy L, Bauer LO, Crowe RR, Goate A, Hesselbrock V, Jones K, Kwon J, Li T-K, Nurnberger Jr. JI, O'Connor SJ, Reich T, Rice J, Schuckit MA, Porjesz B, Foroud T & Begleiter H. Variations in *GABRA2* Encoding the α2 Subunit of the $GABA_A$ Receptor, Are Associated with Alcohol Dependence and with Brain Oscillations. *Am J Hum Genet* 74: 705-714, 2004.

Eriksoon K. & Pikkarainen PH. Differences between the sexes in voluntary alcohol consumption and liver ADH-activity in inbred strains of mice. *Metabolism* 17: 1037-1042, 1968.

Erwin VG, McClearn GE & Kuse AR. Interrelationships of alcohol consumption, actions of alcohol, and biochemical traits. *Pharmacol Biochem Behav* 13(Suppl 1): 297-302, 1980.

Essrich C, Lorez M, Benson JA, Fritschy JM & Lüscher B. Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gepyrin. *Nat Neurosci* 1: 563-571, 1998.

Ferando I & Mody I. Interneuronal $GABA_A$ receptors inside and outside of synapses. *Curr Opin Neurobiol* 26: 57-63, 2014.

Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PEM, Dong Y, Roth BL & Neumaier JF. Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. *Nat Neurosci* 14(1): 22-24, 2011.

Finn DA, Sinnott RS, Ford MM, Long SL, Tanchuck MA & Phillips TJ. Sex differences in the effect of ethanol injection and consumption on brain allopregnanolone levels in C57Bl/6 mice. *Neuroscience* 123: 813-819, 2004.

Finn DA, Belknap JK, Cronise K, Yoneyama N, Murillo A & Crabbe JC. A procedure to produce high alcohol intake in mice. Psychopharmacology (Berl) 178:471–80, 2005.

Floresco SB, West AR, Ash B, Moore H & Grace AA. Afferent modulation of dopamine neuron firing differentially regulates tonic and phase dopamine transmission. *Nat Neurosci* 6: 968-973, 2003.

Forlano PM & Woolley CS. Quantitative Analysis of Pre- and Postsynaptic Sex Differences in the Nucleus Accumbens. *J Comp Neurol* 518: 1330-1348, 2010.

Forger NG & Morin LP. Reproductive state modulates ethanol intake in rats: Effects of ovariectomy, ethanol concentration, estrous cycle, and pregnancy. *Pharmacol Biochem Behav* 17: 323-331, 1982.

Ford MM, Eldridge JC & Samson HH. Microanalysis of Ethanol Self-Administration: Estrous Cycle Phase-Related Changes in Consumption Patterns. *Alcohol Clin Exp Res* 26(5): 635-643, 2002.

Ford MM, Beckley EH, Nickel JD, Eddy S & Finn DA. Ethanol intake patterns in female mice: Influence of allopregnanolone and the inhibition of its synthesis. *Drug Alcohol Depend* 97: 73-85, 2008.

Fritschy JM & Möhler H. $GABA_A$-Receptor Heterogeneity in the Adult Rat Brain: Differential Regional and Cellular Distribution of Seven Major Subunits. *J Comp Neurol* 359:154-194, 1995.

Galarreta M & Hestrin S. Electrical and chemical synapses among parvalbumin fast-spiking GABAergic interneurons in adult mouse neocortex. *Proc Natl Acad Sci* 99(19): 12438-12443, 2002.

Gatto GJ, McBride WJ, Murphy JM, Lumeng L & Li TK. Ethanol Self-Infusion Into the Ventral Tegmental Area by Alcohol-Preferring Rats. *Alcohol* 11(6):557-564, 1994.

George O, Sanders C, Freiling J, Grigoryan E, Vu Shayla, Allen CD, Crawford E, Mandyam CD & Koob GF. Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. *Proc Natl Acad Sci* 109(44): 18156-18161, 2012.

Gill K, France C & Amit Z. Voluntary ethanol consumption in rats: an examination of blood/brain ethanol levels and behavior. *Alcohol Clin Exp Res* 10(4): 457-462, 1986.

Gillies GE & McArthur S. Estrogen Actions in the Brain and the Basis for Differential Action in Men and Women: A Case for Sex-Specific Medicines. *Pharmacol Rev* 62(2): 155-198, 2010.

Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR & Mody I. A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci* 10(1):40-48, 2007.

Glykys J, Mann EO & Mody I. Which $GABA_A$ Receptor Subunits Are Necessary for Tonic Inhibition in the Hippocampus? *J Neurosci* 28(6):1421-1426, 2008.

Goldbaum GM, Remington PL, Powell KE, Hogelin GC & Gentry EM. Failure to Use Seat Belts in the United States: The 1981-1983 Behavioral Risk Factor Surveys. *JAMA* 255 (18): 2459-2462, 1986.

Gowanlock D, Tervo R, Hwang B-Y, Viswanathan S, Gaj T, Lavzin M, Ritola KD, Lindo S, Michael S, Kuleshova E, Ojala D, Huang C-C, Gerfen CR, Schiller J, Dudman JT, Hantman AW, Looger LL, Schaffer DV & Karpova AY, A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. *Neuron* 92: 1-11, 2016.

Gowing LR, Ali RL, Allsop S, Marsden J, Turf EE, West R & Witton J. Global statistics on addictive behaviours: 2014 status report. *Addiction* 110(6): 904-919, 2015.

Grahame NJ, Rodd-Henricks K, Li TK & Lumeng L. Ethanol locomotor sensitization, but not tolerance correlates with selection for alcohol preference in high- and low-alcohol preferring mice. *J Psychopharmacol* 151: 252-260, 2000.

Grady SR, Moretti M, Zoli M, Marks MJ, Zanardi A, Pucci L, Clementi F & Gotti C. Rodent habenulo-interpeduncular pathway expresses a large variety of uncommon AChR subtypes, but only the $\alpha3\beta4$ and $\alpha3\beta3\beta4$ subtypes mediate acetylcholine release. *J Neurosci.* 29, 2272–2282, 2009.

Grahame NJ, Li T-K & Lumeng L. Selective Breeding for High and Low Alcohol Preference in Mice. *Behav Genet* 29(1): 47-57, 1999.

Grieder TE, Herman MA, Contet C, Tan LA, Vargas-Perez H, Cohen A, Chwalek M, Maal-Bared G, Freiling J, Schlosburg JE, Clarke L, Crawford E, Koebel P, Repunte-Canonigo V, Sanna PP, Tapper AR, Roberto M, Kieffer BL, Sawchenko PE, Koob GF, van der Kooy D & George O. VTA CRF neurons mediate the aversive effects of nicotine withdrawal and promote intake escalation. *Nat Neurosci* 17: 1751-1758, 2014.

Griffiths JL & Lovick TA. GABAergic neurones in the rat periaqueductal grey matter express $\alpha4$, $\beta1$, and δ $GABA_A$ receptor subunits: Plasticity of expression during the estrous cycle. *Neuroscience* 136(2): 457-466, 2005.

Grobin AC & Deutch AY. Dopaminergic Regulation of Extracellular γ-Aminobutyric Acid Levels in the Prefrontal Cortex of the Rat. *J Pharmacol Exp Ther* 285(1): 350-357, 1998.

Gu G, Cornea A& Simerly RB. Sexual differentiation of projections from the principal nucleus of the bed nuclei of the stria terminalis. *J Comp Neurol* 460(4): 542-562, 2003.

Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M & Lang Y. Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci* 92(17): 7749-7753, 1995.

Gupta RR, Sen S, Diepenhorst LL, Rudick CN & Maren S. Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats. *Brain Res* 888(2): 356-365, 2001.

Haack AK, Sheth C, Schwager AL, Sinclair MS, Tandon S & Taha SA. Lesions of the Lateral Habenula Increase Voluntary Ethanol Consumption and Operant Self-Administration, Block Yohimbine-Induced Reinstatement of Ethanol Seeking, and Attenuate Ethanol-Induced Conditioned Taste Aversion. *PLOS ONE* 9(4): 1-14, 2014.

Hajnal A, Smith GP & Norgren R. Oral sucrose stimulation increases accumbens dopamine in the rat. *Am J Physiol* 286(1): R31-R37, 2004.

Haller J, Fuchs E, Halász H & Makara GB. Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats. *Brain Res Bull* 50(1): 33-39, 1999.

Hammack SE, Mania I & Rainnie DG. Differential Expression of Intrinsic Membrane Currents in Defined Cell Types of the Anterolateral Bed Nucleus of the Stria Terminalis. *J Neurophysiol* 98: 638-656, 2007.

Han Y-C & Pruett SB. Mechanisms of Ethanol-Induced Suppression of a Primary Antibody Response in a Mouse Model for Binge Drinking. *J Pharmacol Exp Ther* 275(2): 950-957, 1995.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS & Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic $GABA_A$ receptor activity. *Nat Neurosci* 8(3):339-345, 2005.

Hanchar H, Chutsrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M & Olsen RW. Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to $\alpha_{4/6}\beta_3\delta$ $GABA_A$ receptors. *Proc Natl Acad Sci* 103(22): 8546-8551, 2006.

Harwood H, Fountain D & Livermore G. The economic costs of alcohol and drug abuse in the U.S., 1992. Report prepared for the National Institute on Drug Abuse and The National Institute on Alcohol Abuse and Alcoholism, NIH, DHHS. Rockville MD: NIH, 1998. NIH Publication No. 98-4327.

Harwood H. Updating estimates of the economic costs of alcohol abuse on the U.S.: estimates, update method methods and data. Report prepared by the Lewin Group for the National Institute on Alcohol Abuse and Alcoholism, 2000.

Hasue RH & Shammah-Lagnado SJ. Origin of the Dopaminergic Innervation of the Central Extended Amygdala and Accumbens Shell: A Combined Retrograde Tracing and Immunohistochemical Study in the Rat. *J Comp Neurol* 454: 15-33, 2002.

He J, Nixon K, Shetty AK & Crews FT. Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. *Eur J Neurosci* 21: 2711-2720, 2005.

Heinz A, Beck A, Grüsser SM, Grace AA & Wrase J. Identifying the neural circuitry of alcohol craving and relapse vulnerability. *Addict Biol* 14: 108-118, 2008.

Helms CM, Rossi DJ & Grant KA. Neurosteroid influences on sensitivity to ethanol. *Front Neuroendocrinol* 3(10): 1-19, 2012.

Hilakivi-Clarke L. Role of Estradiol in Alcohol Intake and Alcohol-Related Behaviors. *J Stud Alcohol* 57(2): 162-170, 1996.

Hingson RW, Howland J & Levenson S. Effects of Legislative Reform to Reduce Drunken Driving and Alcohol-related Traffic Fatalities. *Public Health Rep* 103(6): 659-667, 1988.

Hnasko TS, Chuhma N, Zhang H, Goh GY, Sulzer D, Palmiter RD, Raport S & Edwards RH. Vesicular glutamate transport promotes dopamine storage and glutamate co-release in vivo. *Neuron* 65: 643-656, 2010.

Hodge CW, Haraguchi M, Chappelle AM & Samson HH. Effects of Ventral Tegmental Microinjections of the GABAA Agonist Muscimol on Self-Administration of Ethanol and Sucrose. *Pharmaol Biochem Behav* 53(4): 971-977, 1996.

Holly EN, Shimamoto A, DeBold JF & Miczek KA. Sex differences in behavioral and neural cross-sensitization and escalated cocaine taking as a result of episodic social defeat stress in rats. *Psychopharmacology* 224: 179-188, 2012.

Homanics GE, Le NQ, Kist F, Mihalek R, Hart AR & Quinlan JJ. Ethanol Tolerance and Withdrawal Responses in $GABA_A$ Receptor Alpha 6 Subunit Null Allele Mice and in Inbred C57BL/6J and Strain 129/SvJ Mice. *Alcohol Clin Exp Res* 22(1): 259-265, 1998.

Homanics GE, Harrison NL, Quinlan JJ, Krasowski MD, Rick CEM, de Blas AL, Mehta AK, Kist F, Mihalek RM, Aul JJ & Firestone LL. Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the γ2 subunit of the γ-aminobutyrate type A receptor. *Neuropharmacology* 38: 253-255, 1999.

Hosie AM, Clarke L, da Silva H & Smart TG. Conserved site for neurosteroid modulation of $GABA_A$ receptors. *Neuropharmacology* 56(1): 149-154, 2009.

Hruska RE, Ludmer M, Pitman KT, de Ryck M & Silbergeld EK. Effects of Estrogen on Striatal Dopamine Receptor Function in Male and Female Rats. *Pharmacol Biochem Behav* 16: 285-291, 1982.

Hu H, Gan J & Jonas P. Fast-spiking, parvalbumin+ GABAergic interneurons: From cellular design to microcircuit function. *Science* 345(6196): 529-543, 2014.

Hu X, Oroszi G, Chun J, Smith TL, Goldman D & Schuckit MA. An Expanded Evaluation of the Relationship of Four Alleles to the Level of Response to Alcohol and the Alcoholism Risk. *Alcohol Clin Exp Res* 29(1): 8-16.

Hwa LS, Chu A, Levinson SA, Kayyali TM, DeBold JF & Miczek KA. Persistent Escalation of Alcohol Drinking in C57Bl/6J Mice with Intermittent Access to 20% Ethanol. *Alcohol Clin Exp Res* 35(11): 1938-1947, 2011.

Hwa LS, DeBold JF & Miczek KA. Alcohol in excess: CRF1 receptors in the rat and mouse VTA and DRN. *Psychopharmacology* 225: 313-327, 2013.

Hwa LS, Holly EN, DeBold JF & Miczek KA. Social stress-escalated intermittent alcohol drinking: modulation by CRF-R1 in the ventral tegmental area and accumbal dopamine release in mice. *Psychopharmacology* 233: 681-690, 2016.

Ikemoto S, Murphy JM & McBride WJ. Self-infusion of GABAA antagonists directly into the ventral tegmental area and adjacent regions. *Behav Neurosci* 111: 369-380, 1997.

Ikemoto S, Murphy JM, & McBride WJ. Regional differences within the rat ventral tegmental area for muscimol self-infusions. *Pharmacol Biochem Behav* 61:87-92, 1998.

Ikemoto S, Qin M & Liu Z-H. The Functional Divide for Primary Reinforcement of d-Amphetamine Lies between the Medial and Ventral Striatum: Is the Division of the Accumbens Core, Shell, and Olfactory Tubercle Valid? *J Neurosci* 25(20: 5061-5065, 2005.

Ikemoto S, Qin M, Liu Z-H. Primary Reinforcing Effects of Nicotine are Triggered from Multiple Regions Both Inside and Outside the Ventral Tegmental Area. *J Neurosci* 26(3): 723-730, 2006.

Ikemoto S. Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Rev* 56: 27-78, 2007.

Ikemoto S. Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory. *Neurosci Biobehav Rev* 35:129-150, 2010.

Itzhak Y & Martin JL. Effects of cocaine, nicotine, dizocipline and alcohol on mice locomotor activity: cocaine-alcohol cross-sensitization involves upregulation of striatal dopamine transporter binding sites. *Brain Res* 818(2): 204-211, 1999.

Jacob TC, Bogdanov YD, Magnus C, Saliba RS, Kittler JT, Haydon PG & Moss SJ. Gephyrin Regulates the Cell Surface Dynamics of Synaptic $GABA_A$ Receptors. *J Neurosci* 25(45): 10469-10478, 2005.

Jacob TC, Moss SJ & Jurd R. $GABA_A$ receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci* 9: 331-343, 2008.

Jemiolo B, Harvey & Novotny M. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proc Natl Acad Sci* 83(12): 4576-4579, 1986.

Jennings JH, Sparta DR, Stamatakis AM, Ung RL, Pleil KE, Kash TL & Stuber GD. Distinct extended amygdala circuits for divergent motivational states. *Nature* 496: 224-228, 2013.

Jennison KM. The Short-Term Effects and Unintended Long-Term Consequences of Binge Drinking in College: A 10-Year Follow-Up Study. *Am J Drug Alcohol* 30(3): 659-684, 2004.

Jhou TC, Geisler S, Marinelli M, Degarmo BA & Zahm DS. The Mesopontine Rostromedial Tegmental Nucleus: A Structure Targeted by the Lateral Habenula That Projects to the Ventral Tegmental Area of Tsai and Substantia Nigra Compacta. *J Comp Neurol* 513: 566-596, 2009.

Johnston AL & File SE. Sex Differences in Animal Tests of Anxiety. *Physiol Behav* 49: 245-250, 1991.

Jones A, Korpi ER, McKernan RM, Pelz R, Nusser Z, Mäkelä R, Mellor JR, Pollard S, Bahn S, Stephenson FA, Randall AD, Sieghart W, Somogyi P, Smith AJH & Wisden W. Ligand-Gated Ion Channel Subunit Partnerships: GABAA Receptor α6 Subunit Gene Inactivation Inhibits δ Subunit Expression. *J Neurosci* 17(4): 1350-1362, 1997.

Jones KL & Smith DW. Recognition of the Fetal Alcohol Syndrome in Early Infancy. *Lancet* 302(7836): 999-1001, 1973.

June HL, Harvey SC, Foster KL, McKay PF, Cummings R, Garcia M, Mason D, Grey C, McCane S, Williams LS, Johnson TB, He X, Rock S & Cook JM. $GABA_A$ Receptors containing α5 Subunits in the CA1 and CA3 Hippocampal Fields Regulate Ethanol-Motivated Behaviors: An Extended Ethanol Reward Circuitry. *J Neurosci* 21(6): 2166-2177, 2001.

Kamdar NK, Miller SA, Syed YM, Bhayana R, Gupta T & Rhodes JS. Acute effects of Naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *J Psychopharmacol* 192: 207-217, 2007.

Kelley AE. Ventral striatal control of appetitive motivation: role of ingestive behavior and reward-related learning. *Neurosci Biobehav Rev* 27: 765-776, 2004.

Kelly SJ, Pierce DR & West JR. Microencephaly and hyperactivity in adult rats can be induced by neonatal exposure to high blood alcohol concentrations. *Exp Neurol* 96(3): 580-593, 1987.

Keyes KM, Grant BF & Hasin DS. Evidence for a closing gender gap in alcohol use, abuse, and dependence in the United States population. *Drug Alcohol Depend* 93: 21-29, 2008.

Kiefer SW & Lawrence GJ. The sweet-bitter taste of alcohol: Aversion generalization to various sweet-quinine mixtures in the rat. *Chem Senses* 13:633-641, 1988.

Kim U & S-Y Change. Dendritic morphology, local circuitry, and intrinsic electrophysiology of neurons in the rat medial and lateral habenular nuclei of the epithalamus. *J Comp Neurol*, 483(2): 236-250, 2005.

Klausberger T, Magill PJ, Márton LF, Roberts JD, Cobden PM, Buzsáki G & Somogyi P. Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. *Nature* 421: 844-848, 2003.

Kolb B & Stewart J. Sex-Related Differences in Dendritic Branching of Cells in the Prefrontal Cortex of Rats. *J Neuroendocrinol* 3(1): 95-99, 1991.

Koob GF & Le Moal M. Drug Abuse: Hedonic Homeostatic Dysregulation. *Science* 278(5335): 52-58, 1997.

Koob GF. Neuroadaptive mechanisms of addiction: studies on the extended amygdala. *Eur Neuropsychopharmacol* 13(6): 442-452, 2003.

Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homaincs GE, Sieghart W & Lüddens H. Altered receptor subtypes in the forebrain of GABAA receptor δ subunit-deficient mice: recruitment of γ2 subunits. *Neuroscience* 109(4): 733-743, 2002.

Kosten TA, Gawin FH, Kosten TR & Rounsaville BJ. Gender differences in cocaine use and treatment response. *J Subst Abuse Treat* 10: 63-66, 1993.

Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE & Morrow AL. GABAA receptor alpha-1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacology* 43: 685-694, 2002.

Krishnan V, Han M-H, Graham DL, Berton B, Renthal W, Russo SJ, LaPlant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Rannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK & Nestler EJ. Molecular Adaptations Underlying Susceptibility and Resistance to Social Defeat in Brain Reward Regions. *Cell* 131(2): 391-404, 2007.

Kritzer MF & Creutz LM. Region and Sex Differences in Constituent Dopamine Neurons and Immunoreactivity for Intracellular Estrogen and Androgen Receptors in Mesocortical Projections in Rats. *J Neurosci* 28(38): 9525-9535, 2008.

Kroener S, Mulholland PJ, New NN, Gass JT, Becker HC & Chandler LJ. Chronic Alcohol Exposure Alters Behavioral and Synaptic Plasticity of the Rodent Prefrontal Cortex. *PLOS ONE* 7(5): 1-2, 2012.

Kumar S, Fleming RL & Morrow AL. Ethanol regulation of γ-aminobutyric acid$_A$ receptors: genomic and nongenomic mechanisms. *Pharmacol Ther* 101: 211-226, 2004.

Lammel S, Ion DI, Roeper J & Malenka RC. Projection-Specific Modulation of Dopamine Neuron Synapses by Aversive and Rewarding Stimuli. *Neuron* 70(5): 855-862, 2011.

Lancaster FE, Brown TD, Coker KL, Elliot JA & Wren SB. Sex Differences in Alcohol Preference and Drinking Patterns Emerge during the Early Postpubertal Period in Sprague-Dawley Rats. *Alcohol Clin Exp Res* 20(6): 1043-1049, 1996.

Lee V & Maguire J. Impact of inhibitory constraint of interneurons on neuronal excitability. *J Neurophysiol* 110: 2520-2535, 2013.

Leeman RF, Heilig M, Cunningham CL, Stephens DN, Duka T & O'Malley SS. Ethanol Consumption: How Should We Measure It? Achieving Consilience between Human and Animal Phenotypes. *Addict Biol*, 15(2): 109-124, 2010.

Li X, Qi J, Yamaguchi T, Wang HL & Morales M. Heterogeneous composition of dopamine neurons of the rat A10 region: molecular evidence for diverse signaling properties. *Brain Struc Funct* 218: 1159-1176, 2013.

Liang J, Suryanarayanan A, Chandra D, Homanics GE, Olsen RW & Spigelman I. Functional Consequences of GABA$_A$ Receptor α4 Subunit Deletion on Synaptic and Extrasynaptic Currents in Mouse Dentate Granule Cells. *Alcohol Clin Exp Res* 32(1): 19-26, 2008.

Liu J, Yang AR, Kelly T, Puche A, Espga C, June Jr. HL, Elnabawi A, Merchenthaler I, Sieghart W, June Sr. HL & Aurelian L. Binge alcohol drinking is associated with GABA$_A$ α2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala. *Proc Natl Acad Sci* 108(11): 4465-4470, 2011.

Livak KJ & Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4): 402-8, 2001.

Long JC, Knowler WC, Hanson RL, Robin RW, Urbanek M, Moore E, Bennett PH & Goldman D. Evidence for genetic linkage to alcohol dependence on chromosome 4 and 11 from an autosome-wide scan in an American Indian population. *Am J Med Genet* 81: 216-221, 1998.

Lowery EG, Spanos M, Navarro M, Lyons AM, Hodge CW & Thiele T. CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57Bl/6J mice independent of the HPA axis. *Neuropsychopharmacology* 35: 1231-1252, 2010.

Lowery-Gionta EG, Navarro M, Li C, Pleil KE, Rinker JA, Cox BR, Sprow GM, Kash TL & Thiele TE. Corticotropin Releasing Factor Signaling in the Central Amygdala is Recruited during Binge-Like Ethanol Consumption in C57Bl/6J Mice. *J Neurosci* 32(10): 3405-3413, 2012.

Lukas SE & Mendelson JH. Electroencephalographic activity and plasma ACTH during ethanol-induced euphoria. *Biol Psychiatry* 23: 141-148, 1988.

Lynch WJ, Roth ME & Carroll ME. Biological basis of sex differences in drug abuse: preclinical and clinical studies. *Psychopharmacology* 164: 121-137, 2002.

Macdonald RL & Olsen RW. GABA$_A$ Receptor Channels. *Annu Rev Neurosci* 17: 569-602, 1994.

Madeira MD, Sousa N, Lima-Andrade MT, Calheiros F, Cadete-Leite A & Paula-Barbosa MM. Selective vulnerability of the hippocampal pyramidal neurons to hypothyroidism in male and female rats. *J Comp Neurol* 322: 501-518, 1992.

Maguire JL, Stell BM, Rafizadeh M, & Mody I. Ovarian cycle-linked changes in $GABA_A$ receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nat Neurosci* 8(6): 797-804, 2005.

Mahmoudi M, Kang M-H, Tillataratne N, Tobin AJ & Olsen RW. Chronic Intermittent Ethanol Treatment in Rats Increases GABAA Receptor α4-Subunit Expression: Possible Relevance to Alcohol Dependence. *J Neurochem* 68(6): 2485-2492, 1997.

Marin MT, Cruz FC & Planeta CS. Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weights in rats. *Phsiol Behav* 90(1): 29-35, 2007.

Matthews-Felton TM, Corodimas KP, Rosenblatt JS & Morrell JI. Lateral habenula neurons are necessary for the hormonal onset of maternal behavior and postpartum estrus in naturally parturient female rats. *Behav Neurosci* 109: 1172-1188, 1995.

McArthur S, McHale E, Dalley JW, Buckingham JC & Gillies GE. Altered Mesencephalic Dopaminergic Populations in Adulthood as a Consequence of Brief Perinatal Glucocorticoid Exposure. *J Neuroendocrinol* 17: 465-482, 2005.

McArthur S, McHale E & Gillies GE. The Size and Distribution of Midbrain Dopaminergic Populations are Permanently Altered by Perinatal Glucocorticoids Exposure in a Sex- Region- and Time-Specific Manner. *Neuropsychopharmacology* 32: 1462-1476, 2007.

McBride WJ, Kimpel MW, Schultz JA, McClintick JN, Edenberg HJ & Bell RL. Changes in gene expression in regions of the extended amygdala of alcohol-preferring rats after binge-like alcohol drinking. *Alcohol* 44(2): 171-183, 2010.

McDonald AJ. Projection neurons of the basolateral amygdala: a correlative Golgi and retrograde tract tracing study. *Brain Res Bull* 28: 179-185, 1992.

McEwen BS & Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm Behav* 43(1): 2-15, 2003.

McLearn GE & Rodgers DA. Differences in alcohol preference among inbred strains of mice. *Q J Study Alcoholi* 20: 691-695, 1959.

Meera P, Wallner M & Otis TS. Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic $GABA_A$ receptors. *J Neurophysiol* 106: 2057-2064, 2011.

Mehta AK & Ticku MK. Ethanol Potentiation of GABAergic Transmission in Cultured Spinal Core Neurons Involves γ-Aminobutyric $Acid_A$-Gated Chloride Channels. *J Pharmacol Exp Ther* 246(2): 558-564, 1988.

Melendez RI, Rodd-Henricks ZA, Engleman EA, Li TK, McBride WJ & Murphy JM. Microdialysis of Dopamine in the Nucleus Accumbens of Alcohol-Preferring (P) Rats During Anticipation and Operant Self-Administration of Ethanol. *Alcohol Clin Exp Res* 26(3): 318-325, 2002.

Melendez RI. Intermittent (Every-Other-Day) Drinking Induces Rapid Escalation of Ethanol Intake and Preference in Adolescent and Adult C57Bl/6J Mice. *Alcohol Clin Exp Res* 35(4): 652-658, 2011.

Mellon SH, Griffin LD & Compagnone NA. Biosynthesis and action of neurosteroids. *Brain Res Rev* 37: 3-12, 2001.

Melo JA, Shendure J, Pociask K & Silver LM. Identification of sex-specific trait loci controlling alcohol preference in C57Bl/6 mice. *Nature* 13: 147-153, 1996.

Melón LC & Boehm II SL. $GABA_A$ receptors in the posterior, but not anterior, ventral tegmental area mediate Ro15-4513-induced attenuation of binge-like ethanol consumption in C57Bl/6J mice. *Behav Brain Res* 220: 230-237, 2011.

Melón LC & Boehm II SL. Role of Genotype in the Development of Locomotor Sensitization to Alcohol in Adult and Adolescent Mice: Comparison of the DBA/2J and C57Bl/6J Inbred Mouse Strains. *Alcohol Clin Exp Res* 35(7): 1351-1360, 2011.

Melón LC, Wray KN, Moore EM & Boehm II SL. Sex and age differences in heavy binge drinking and its effects on alcohol responsivity following abstinence. *Pharmcol Biochem Behav* 104: 177-187, 2013.

Melón LC, Nolan ZT, Colar D, Moore EM & Boehm III SL. Activation of extrasynaptic δ-$GABA_A$ receptors globally or within the posterior-VTA has estrous-dependent effects on consumption of alcohol and estrous-independent effects on locomotion. *Horm Behav* 95: 65-75, 2017.

Merlo Pich E, Lorang M, Yeganeh M, Rodriguez de Fonseca F, Raber J, Koob GF & Weiss F. Increase of Extracellular Corticotropin-Releasing Factor-Like Immunoreactivity Levels in the Amygdala of Awake Rats during Restraint Stress and Ethanol Withdrawal as Measured by Microdialysis. *J Neurosci* 15(8): 5439-5447, 1995.

Middaugh LD, Kelley BM, Bandy AL & McGroarty KK. Ethanol consumption by C57BL/6 mice: influence of gender and procedural variables. *Alcohol* 17: 175-183, 1999.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi Z-P, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW & Homanics GE. Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad* 96(22): 12905-12910, 1999.

Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL & Homanics GE. $GABA_A$-Receptor δ Subunit Knockout Mice Have Multiple Defects in Behavioral Responses to Ethanol. *Alcohol Clin Exp Res* 25(12): 1708-1718, 2001.

Milenkovic I, Vasiljevic M, Maurer D, Höger H, Klausberger T & Sieghart W. The Parvalbumin-Positive Interneurons in the Mouse Dentate Gyrus Express GABAA Receptor Subunits Alpha1, Beta2, and Delta along their Extrasynaptic Cell Membrane. *Neuroscience* 254: 80-96, 2013.

Mody I & Maguire J. The reciprocal regulation of stress hormones and $GABA_A$ receptors. *Front Cell Neurosci* 6(4): 1-6, 2012.

Moore EM, Serio KM, Goldfarb KJ, Stepanovska S, Linsenbardt DN & Boehm II SL. GABAergic modulation of binge-like ethanol intake in C57Bl/6J mice. *Pharmcol Biochem Behav* 88: 105-113, 2007.

Moore EM & Boehm II SL. Site-specific microinjection of baclofen into the anterior ventral tegmental area reduces binge-like ethanol intake in male C57Bl/6J mice. *Behav Neurosci* 123(3): 555-563, 2009.

Morales M & Margolis EB. Ventral tegmental area: cellular heterogeneity, connectivity, and behavior. *Nature Rev Neurosci* 18: 73-85, 2017.

Morris JA, Jordan CL & Breedlove SM. Sexual Dimorphism in Neuronal Number of the Posterodorsal Medial Amygdala is Independent of Circulating Androgens and Regional Volume in Adult Rats. *J Comp Neurol* 506: 851-859, 2008.

Murphy JM, Gatto GJ, Waller MB, McBride WJ, Lumeng L & Li TK. Effects of scheduled access on ethanol intake by the alcohol-preferring (P) line of rats. *Alcohol* 3: 331-336, 1986.

Nachman M, Larue C, & Le Magnen J. The role of olfactory and orosensory factors in the alcohol preference of inbred strains of mice. *Physiol Behav* 6:53-95, 1971.

Naimi TS, Brewer RD, Mokdad A, Denny C, Serdula MK & Marks JS. Binge Drinking Among US Adults. *JAMA* 289(1): 70-75, 2003.

National Institute of Alcohol Abuse and Alcoholism. NIAAA council approves definition of binge drinking. *NIAAA Newsletter*. 2004; 3:3.

Newman EL, Gunner G, Huynh P, Gachette D, Moss SJ, Smart TG, Rudolph U, DeBold J & Miczek KA. Effects of Gabra2 Point Mutations on Alcohol Intake: Increased Binge-Like and Blunted Chronic Drinking in Mice. *Alcohol Clin Exp Res* 40(11): 2445-2455, 2016.

NIAAA. NIAAA council approves definition of binge drinking. *NIAAA Newsletter, No. 3*. Washington, DC: NIAAA, Winter; 2004.

Nie H, Rewal M, Gill TM, Ron D & Janak PH. Extrasynaptic δ-containing $GABA_A$ receptors in the nucleus accumbens dorsomedial shell contribute to alcohol intake. *Proc Natl Acad Sci* 108(11): 4459-4464, 2011.

Nishikuza M & Arai Y. Sexual Dimorphism in Synaptic Organization in the Amygdala and its Dependence on Neonatal Hormone Environment. *Brain Res* 212: 31-38, 1981.

Nixon K & Crews FT. Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J Neurochem* 83(5): 1087-1093, 2002.

Norman KJ, Seiden JA, Klickstein JA, Han X, Hwa LS, DeBold JF & Miczek KA. Social stress and escalated drug self-administration in mice, I. Alcohol and corticosterone. *Psychopharmacology* 232(6): 991-1001, 2015.

Nusser Z, Sieghart W & Somogyi P. Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* 18: 1693-1703, 1998.

Ogilvie K, Lee S & Rivier C. Effect of Three Different Modes of Alcohol Administration on the Activity of the Rat Hypothalamic-Pituitary-Adrenal Axis. *Alcohol Clin Exp Res* 21(3): 467-476, 1997.

O'Malley PM, Bachman JG & Johnston LD. Period, age, and cohort effects on substance use among American youth, 1976-82. *Amer J Publ Hlth* 74: 682-688, 1984.

Obernier JA, White AM, Swartzwelder HS & Crews FT. Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacol Biochem Behav* 72: 521-532, 2002.

Olive MF, Koenig HN, Nannini MA & Hodge CW. Elevated extracellular CRF levels in the bed nucleus of the stria terminalis during ethanol withdrawal and reduction by subsequent ethanol intake. *Pharmcol Biochem Behav* 72: 213-220, 2002.

Olson VG, Zabetian CP, Bolanos CA, Edwards S, Barrot M, Eisch AJ, Hughes T, Self DW, Neve RL & Nestler EJ. Regulation of Drug Reward by cAMP Response-Element

Binding Protein: Evidence for Two Functionally Distinct Subregions of the Ventral Tegmental Area. *J Neurosci* 23(23): 5553-5562, 2005.

Olson VG & Nestler EJ. Topographic Organization of GABAergic Neurons Within the Ventral Tegmental Area of the Rat. *Synapse* 61: 87-95, 2007.

Omelchenko N, Bell R & Sesack SR. Lateral habenula projections to dopamine and GABA neurons in the rat ventral tegmental area. *Eur J Neurosci* 30: 1239-1250, 2009.

Orford J & Keddie A. Gender differences in the functions and effects of moderate and excessive drinking. *Br J Clin Psychol* 24(4): 265-279, 1985.

Otmakhova N, Duzel E, Deutch AY & Lisman J. The Hippocampal-VTA Loop: The Role of Novelty and Motivation in Controlling the Entry of Information into Long-Term Memory. In: Baldassarre G., Mirolli M. (eds) Intrinsically Motivated Learning in Natural and Artificial Systems. Springer, Berlin, Heidelberg, 2013.

Overstreet DH, Knapp DJ & Breese GR. Similar anxiety-like responses in male and female rats exposed to repeated withdrawals from ethanol. *Pharmacol Biochem Behav* 78: 459-464, 2004.

Overstreet LS, Westbrook GL & Jones MV. *Transmembrane Transporters* (ed. Quick MW). Wiley-Liss Inc., Hoboken, NJ, 259-275, 2002.

Paladini CA, Robinson S, Morikawa H, Williams JT & Palmiter RD. Dopamine controls the firing pattern of dopamine neurons via a network feedback mechanism. *Proc Natl Acad Sci* 100(5): 2866-2871, 2003.

Pasqualini C, Olivier V, Guibert B, Frain O & Leviel V. Acute Stimulatory Effect of Estradiol on Striatal Dopamine Synthesis. *J Neurochem* 65: 1651-1657, 1995.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW & Houser CR. $GABA_A$ receptor changes in δ subunit-deficient mice: Altered expression of α4 and γ2 subunits in the forebrain. *J Comp Neurol* 446(2): 179-197, 2002.

Pfaffman C. Taste: a model of incentive motivation. In: Pfaff, D, editor. The psychobiological mechanisms of motivation. New York: Springer-Verlag, 59-97, 1982.

Phillipson OT & Pycock CJ. Dopamine neurons of the ventral tegmentum project to both medial and lateral habenula. *Exp Brain Res* 45(1-2): 89-94, 1982.

Pierce HI, McGuffin RG & Hillman RS. Clinical Studies in Alcoholic Sideroblastosis. *Arch Intern Med* 136(3): 283-289, 1976.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W & Sperk G. $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101(4): 815-850, 2000.

Pruett SB, Collier SD & Wu W-J. Ethanol-induced Activation of the Hypothalamic-Pituitary-Adrenal Axis in a Mouse Model for Binge Drinking: Role of Ro15-4513-Sensitive Gamma Aminobutyric Acid Receptors, Tolerance, and Relevance to Humans. *Life Sci* 63(13): 1137-1146, 1998.

Pupe S & Wallén-Mackenzie A. Cre-driven optogenetics in the heterogenous genetic panorama of the VTA. *Trends Neurosci* 38(6): 375-386, 2015.

Sanchez-Catalan MJ, Kaufling J, Georges F, Veinante P & Barrot M. The antero-posterior heterogeneity of the ventral tegmental area. *Neuroscience* 282:198-216, 2014.

Rabinowicz T, Dean DE, McDonald-Comber Petetot J, & de Courten-Myers GM. Gender Differences in the Human Cerebral Cortex: More Neurons in Males; More Processes in Females. *J Child Neurol* 14(2): 98-107, 1999.

Randall CL, Roberts JS, Del Boca FK, Carroll KM, Connors GJ & Mattson ME. Telescoping of landmark events associated with drinking: a gender comparison. *J Stud Alcohol Drugs* 60(2): 252-260, 1999.

Rassnick S, Pulvirenti L & Koob GF. Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. *Psychopharmacology* 109: 92-98, 1992.

Rehm J, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y & Patra J. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* 373: 2223-2233, 2009.

Rewal M, Jurd R, Gill MT, He D-Y, Ron D & Janak PH. α4-Containing $GABA_A$ Receptors in the Nucleus Accumbens Mediate Moderate Intake of Alcohol. *J Neurosci* 29(2): 543-549, 2009.

Reynolds DS. The value of genetic and pharmacological approaches to understanding the complexities of $GABA_A$ receptor subtype functions: The anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav*, 90(1): 37-42, 2008.

Rhodes JS, Best K, Belknap JK, Finn DA &Crabbe JC. Evaluation of a simple model of ethanol drinking to intoxication in C57Bl/6J mice. *Physiol Behav* 84:53-63, 2005.

Rhodes JS, Ford MM, Yu C-H, Brown LL, Finn DA, Garland Jr T & Crabbe JC. Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain Behav* 6:1-18, 2007.

Richardson HN, Lee SY, O'Dell LE, Koob GF & Rivier CL. Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. *Eur J Neurosci*, 28(8): 1641-1653, 2008.

Rinker JA, Marshall SA, Mazzone CM, Lowery-Gionta EG, Gulati V, Pleil KE, Kash TK, Navarro M & Thiele TE. Extended Amygdala to Ventral Tegmental Area Corticotropin-Releasing Factor Circuit Controls Binge Ethanol Intake. *Biol Psychiatry* 81(11): 930-940, 2017.

Rodaros D, Caruana DA, Amir S & Stewart J. Corticotropin-Releasing Factor Projections from Limbic Forebrain and Paraventricular Nucleus of the Hypothalamus to the Region of the Ventral Tegmental Area. *Neuroscience* 150: 8-13, 2007.

Rodd ZA, Bell RL, Kuc KA, Zhang Y, Murphy JM & McBride WJ. Intracranal Self-Administration of Cocaine within the Posterior Ventral Tegmental Area of Wistar Rats: Evidence for Involvement of Serotonin-3 Receptors and Dopamine Neurons. *J Pharmacol Exp Ther* 313: 134-145, 2005.

Rodgers DA. Factors Underlying Differences in Alcohol Preference Among Inbred Strains of Mice. *Psychosom Med* 28(4): 498-513, 1966.

Roberts AJ, Heyser CJ & Koob GF. Operant Self-Administration of Sweetened Versus Unsweetened Ethanol: Effects on Blood Alcohol Levels. *Alcohol Clin Exp Res* 23(7): 1151-1157, 1999.

Robinson DL, Brunner LJ & Gonazales RA. Effect of Gender and Estrous Cycle on the Pharmokinetics of Ethanol in the Rat Brain. *Alcohol Clin Exp Res* 26(2): 165-172, 2002.

Rodd ZA, Bell RL, Melendez RI, Kuc KA, Lumeng L, Li TK, Murphy JM & McBride. Posterior Ventral Tegmental Area Between Alcohol-Preferring and Wister Rats. *Alcohol Clin Exp Res* 28(8): 1212-1219, 2004.

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

Rodd-Henricks ZA, McKinzie DL, Li T-K, Murphy JM & McBride WJ. Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. *J Pharmacol Exp Ther* 303: 1216-1226, 2002.

Rogan SC & Roth BL. Remote Control of Neuronal Signaling. *Pharmacol Rev* 63: 291-315, 2011.

Rossi DJ & Hamann M. Spillover-mediated transmission at inhibitory synapses promoted by high affinity α6 subunit $GABA_A$ receptors and glomerular geometry. *Neuron* 20: 783-795, 1998.

Rudebeck PH, Mitz AR, Chacko RV & Murray EA. Effects of Amygdala Lesions on Reward-Value Coding in Orbital and Medial Prefrontal Cortex. *Neuron* 80(6): 1519-1531, 2013.

Rudolph, U, Crestani, F, Benke, D, Brunig, I, Benson, JA, Fritschy, JM, Martin JR, Bluethmann H & Möhler H. Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature* 401, 796–800, 1999.

Russell KE & Stern MH. Sex and Strain as Factors in Voluntary Alcohol Intake. *Physiol Behav* 10(3): 641-642, 1973.

Ryabinin AE, Criado JR, Henriksen SJ, Bloom FE & Wilson MC. Differential sensitivity of c-Fos expression in hippocampus and other brain regions to moderate and low dos Möhler es of alcohol. *Mol Psychiatry* 2: 32-43, 1997.

Ryabinin AE, Galvan-Rosas A, Bachtell RK & Risinger FO. High alcohol/sucrose consumption during dark circadian phase in C57Bl/6J mice: involvement of hippocampus, lateral septum and urocortin-positive cells of the Edinger-Westphal nucleus. *Psychopharmacology* 165: 296-305, 2003.

Sakmann B, Hamill OP & Bormann J. Patch-clamp measurements of elementary chloride currents activated by the putative inhibitory transmitters GABA and glycine in mammalian spinal neurons. *J Neural Transmission Suppl* 18: 83-95, 1983.

Salin PA & Prince DA. Spontaneous GABAA receptor-mediated inhibitory currents in adult rat somatosensory cortex. *J Neurophysiol* 75(4): 1573-1588, 1996.

Samson HH. Initiation of ethanol reinforcement using a sucrose-substitution procedure in food- and water-sated rats. *Alcohol Clin Exp Res* 10: 436-442, 1986.

Sander T, Ball D, Murray R, Patel J, Samochowiec J, Winterer G, Rommelspacher H, Schmidt LG & Loh E-W. Association Analysis of Sequence Variants of the $GABA_A$α6, β2, and γ2 Gene Cluster and Alcohol Dependence. *Alcohol Clin Exp Res* 23(3): 427-431, 1999.

Saxena NC & Macdonald RL. Assembly of GABAA Receptor Subunits: Role of the δ Subunit. *J Neurosci* 14(11): 7077-7086, 1994.

Schuckit MA, Anthenelli RM, Bucholz KK, Hesselbrock VM & Tipp J. The Time Course of Development of Alcohol-Related Problems in Men and Women. *J Stud Alcohol* 56(2): 218-225, 1995.

Schuckit MA, Gold E & Risch C. Plasma cortisol levels following ethanol in sons of alcoholics and controls. *Arch Gen Psychiatry* 44: 942-945, 1987.

Schumacher M, Weill-Engerer S, Liere P, Robert F, Franklin RJM, Garcia-Segura LM, Lambert JJ, Mayo W, Melcangi RC, Parducz A, Suter U, Carelli C, Baulieu EE & Akwa Y. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog Neurobiol* 71: 3-29, 2003.

Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy JM, Möhler H & Lüscher B. The γ2 subunit of $GABA_A$ receptors is required for maintenance of receptors at mature synapses. *Mol Cell Neurosci* 24(2): 442-450, 2003.

Sharpe AL, Tsivkovskaia NO & Ryabinin AE. Ataxis and c-Fos expression in mice drinking ethanol in a limited access session. *Alcohol Clin Exp Res* 29: 1419-1426, 2005.

Shughrue PJ, Lane MV & Merchenthaler I. Comparative Distribution of Estrogen Receptor-α and -β mRNA in the Rat Central Nervous System. *J Comp Neurol* 388: 507-525, 1997.

Sieghart W. Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol Rev* 47, 181-234, 1995.

Sieghart W & Sperk W. Subunit composition, distribution, and function of GABA(A) receptor subtypes. *Curr Top Med Chem* 2: 795-816, 2002.

Sillaber I & Henniger MSH. Stress and alcohol drinking. *Ann Med* 36(8): 596-605, 2004.

Simerly RB, Chang C, Muramatsu M & Swanson LW. Distribution of Androgen and Estrogen Receptor mRNA-Containing Cells in the Rat Brain: An In Situ Hybridization Study. *J Comp Neurol* 294: 76-95, 1990.

Smith KS, Bucci DJ, Luikart BW & Mahler SV. DREADDs: Use and Application in Behavioral Neuroscience. *Behav Neurosci* 130(2): 137-155, 2016.

Sommer WH, Rimondini R, Hansson AC, Hipskind PH, Gehlert DR, Barr CS & Heilig MA. Upregulation of Voluntary Alcohol Intake, Behavioral Sensitivity to Stress, and Amygdala *Crhr1* Expression Following a History of Dependence. *Biol Psychiatry* 63: 139-145, 2008.

Somogyi P, Fritschy JM, Benke D, Roberts JD & Sieghart W. The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α2 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacology* 35: 1425-1444, 1996.

Sparrow AM, Lowery-Gionta EG, Pleil KE, Li C, Sprow GM, Cox BR, Rinker JA, Jijon AM, Peña J, Navarro M, Kash TL & Thiele TE. Central Neuropeptide Y Modulates Binge-Like Ethanol Drinking in C57Bl/6J Mice via Y1 and Y2 Receptors. *Neuropsychopharmacology* 37(6): 1409-1421, 2012.

Sparta DR, Hopf FW, Gibb SL, Cho SL, Stuber GD, Messing RO Ron D & Bonci A. Binge Ethanol-Drinking Potentiates Corticotropin Releasing Factor R1 Receptor Activity in the Ventral Tegmental Area. *Alcohol Clin Exp Res* 37(10): 1680-1687, 2013.

Spigelman I, Zhiwei L, Liang J, Cagetti E, Samzadeh S, Mihalek RM, Homanics GE & Olsen RW. Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the $GABA_A$ receptor δ subunit. *J Neurophysiol* 90(2): 903-910, 2003.

Sprow GM & Thiele TE. The neurobiology of binge-like ethanol drinking: Evidence from rodent models. *Physiol Behav* 106: 325-331, 2012.

Stamatakis AM & Stuber GD. Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nat Neurosci* 15(8): 1105-1109, 2012.

Stamatakis AM, Jennings JH, Ung RL, Blair GA, Weinberg RJ, Neve RL, Boyce F, Mattis J, Ramakrishnan C, Deisseroth K & Stuber GD. A Unique Population of Ventral Tegmental Area Neurons Inhibits the Lateral Habenula to Promote Reward. *Neuron* 80: 1039-1053, 2013.

Stell BM, Brickley SG, Tang CY, Farrant M & Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subuit-containing $GABA_A$ receptors. *Proc Natl Acad Sci* 100(24): 14439-14444, 2003.

Stephens MAC & Wand G. Stress and the HPA Axis. *Alcohol Res* 34(4): 468-483, 2012.

Stuber GD, Hopf FW, Hahn J, Cho SL, Guillory A & Bonci A. Voluntary ethanol intake enhances excitatory synaptic strength in the ventral tegmental area. *Alcohol Clin Exp Res* 32(10): 1714-1720, 2008.

Substance Abuse and Mental Health Services Administration (SAMHSA). 2005 National Household Survey on Drug Abuse. Available at: http://www.drugabuse.statistics.samhsa.gov

Substance Abuse and Mental Health Services Administration (SAMHSA). 2015 National Survey on Drug Use and Health (NSDUH). Table 6.84B—Tobacco Product and Alcohol Use in Past Month among Persons Aged 18 to 22, by College Enrollment Status: Percentages, 2014 and 2015. Available at: https://www.samhsa.gov/data/sites/default/files/NSDUH-DetTabs-2015/NSDUH-DetTabs-2015/NSDUH-DetTabs-2015.htm#tab6-84b.

Sun N & Cassell MD. Intrinsic GABAergic Neurons in the Rat Central Extended Amygdala. *J Comp Neurol* 330: 381-404, 1993.Sb

Surmeier DJ, Ding J, Day M, Z Wang & Shen W. D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends Neurosci* 30(5): 228-235, 2007.

Szumlinski KK, Diab ME, Friedman R, Henze LM, Lominac KD & Bowers MS. Accumbens neurochemical adaptations produced by binge-like alcohol consumption. *Psychopharmacology* 190: 415-431, 2007.

Taber MT, Das S & Fibiger HC. Cortical regulation of subcortical dopamine release: mediation via the ventral tegmental area. *J Neurochem* 65: 1407-1410, 1995.

Tang A, George MA, Randall JA & Gonzales RA. Ethanol Increases Extracellular Dopamine Concentration in the Ventral Striatum in C57BL/6 Mice. *Alcohol Clin Exp Res* 27(7): 1083-1089, 2003.

Taniguchi H, He M, Wu P, Kim S, Paik R, Sugino K, Kvitsiani D, Fu Y, Lu J, Lin Y, Miyoshi G, Shima Y, Fishell G, Nelson SB & Huang ZJ. A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron* 71(6): 995-1013, 2011.

Taverna S, van Dongen YC, Groenewegen HJ & Pennartz CMA. Direct Physiological Evidence for Synaptic Connectivity Between Medium-Spiny Neurons in Rat Nucleus Accumbens In Situ. *J Neurophysiol* 91: 1111-1121, 2003.

Thielen RJ, Engleman EA, Rodd ZA, Murphy JM, Lumeng L, Li T-K & McBride WJ. Ethanol Drinking and Deprivation Alter Dopaminergic and Serotonergic Function

in the Nucleus Accumbens of Alcohol-Preferring Rats. *J Pharmacol Exp Ther* 309(1): 216-225.

Thyagarajan B, Guimarães MJ, Groth AC & Calos MP. Mammalian geomes contain active recombinase recognition sites. *Gene* 244(1-2): 47-54, 2000.

Tidey JW & Miczek KA. Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. *Brain Res* 721: 140-149, 1996.

Tsigos C & Chrousos GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res* 53(4): 865-871, 2002.

Tritsch NX, Ding JB & Sabatini BL. Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. *Nature* 490: 262-266, 2012.

Tzschentke TM. Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog Neurobiol* 63: 241-320, 2001.

Ungless MA, Singh V, Crowder TL, Yaka R, Ron D & Bonci A. Corticotropin-Releasing Factor Requires CRF Binding Protein to Potentiate NMDA Receptors via CRF Receptor 2 in Dopamine Neurons. *Neuron* 39(3): 401-407, 2003.

Urban DJ & Roth BL. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogeneic Tools with Therapeutic Utility. *Annu Rev Pharmacol Toxicol* 55: 399-417, 2015.

Vashchinkina E, Manner AK, Vekovischeva O, den Hollander B, Uusi-Oukari M, Aitta-aho T & Korpi ER. Neurosteroid Agonist at GABAA Receptor Induces Persistent Neuroplasticity in VTA Dopamine Neurons. *Neuropsychopharmacology* 39: 727-737, 2014.

Valdez GR, Roberts AJ, Chan K, Davis H, Brennan M, Zorrilla EP & Koob GF. Increased Ethanol Self-Administration and Anxiety-Like Behavior During Acute Ethanol Withdrawal and Protracted Abstinence: Regulation by Corticotropin-Releasing Factor. *Alcohol Clin Exp Res* 26(10): 1494-1501, 2002.

Valentino RJ, Rudoy C, Saunders A, Liu X-B & Bockstaele EJV. Cortocotropin-releasing factor is preferentially colocalized with excitatory rather than inhibitory amino acids in axon terminals in the peri-locus coeruleus region. *Neuroscience* 106(2): 375-384, 2001.

van Eden CG, Hoorneman EMD, Buijs RM, Matthijssen MAH, Geffard M & Uylings HBM. Immunocytochemical localization of dopamine in the prefrontal cortex of the rat at light and electron microscopy level. *Neuroscience* 22: 849-862, 1987.

van Pett K, Viau V, Bittencourt JC, Chan RKW, Li H-Y, Arias C, Prins GS, Perrin M, Value W & Sawchenko PE. Distribution of mRNAs Encoding CRF Receptors in Brain and Pituitary of Rat and Mouse. *J Comp Neurol* 428: 191-212, 2000.

van Zessen R, Phillips JL, Budygin EA & Stuber GD. Activation of VTA GABA Neurons Disrupts Reward Consumption. *Neuron* 6(22): 1184-1194, 2012.

Verdoorn TA, Draguhn A, Ymer S, Seeburg PH & Sakmann B. Functional Properties of Recombinant Rat $GABA_A$ Receptors Depend upon Subunit Composition. *Neuron* 4: 919-928, 1990.

Vertes RP. Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse* 51: 32-58, 2004.

Vithlani M, Terunuma M & Moss SJ. The Dynamic Modulation of GABAA Receptor Trafficking and Its Role in Regulating the Plasticity of Inhibitory Synapses. *Physiol Rev* 91(3): 1009-1022, 2010.

Vollenweider I, Smith KS, Keist R & Rudolph U. Antidepressant-like properties of α2-containing GABA(A) receptors. *Behav Brain Res* 217(1): 77-80, 2011.

Vollstädt-Klein S, Loeber S, Kirsch M, Bach P, Richter A, Bühler M, von der Goltz C, Hermann D, Mann K & Kiefer F. Effects on Cue-Exposure Treatment on Neural Cue Reactivity in Alcohol Dependence: A Randomized Trial. *Biol Psychiatry* 69(11): 1060-1066, 2011.

Wafford KA & Whiting PJ. Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the gamma 2 subunit. *FEBS Lee* 313(2): 113-117, 1992.

Wagner CK, Silverman A-J & Morrell JI. Evidence for Estrogen Receptor in Cell Nuclei and Axon Terminals within the Lateral Habenula of the Rat: Regulation during Pregnancy. *J Comp Neurol* 392: 330-342, 1998.

Walker DL, Toufexis D & Davis M. Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur J Pharmacol* 463(1-3): 199-216 2003.

Walker QD, Rooney MB, Wightman RM & Kuhn CM. Dopamine Release and Uptake are Greater in Female than Male Rat Striatum as Measured by Fast Cyclic Voltammetry. *Neuroscience* 95(4): 1061-1070, 2000.

Wallace M, Luine V, Arellanos A & Frankfurt M. Ovariectomized rats show decreased recognition memory and spine density in the hippocampus and prefrontal cortex. *Brain Res* 1126(1): 176-182, 2006.

Wallner M, Hanchar HJ & Olsen RW. Ethanol enhances $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ γ-aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci* 100(25): 15218-15223, 2003.

Wallner M, Hanchar HJ & Olsen RW. Low dose acute alcohol effects on $GABA_A$ receptor subtypes. *Pharmacol Ther* 112: 513-528, 2006.

Wanberg KW. Prevalent of Symptoms Found Among Excessive Drinkers. *Int Addict* 4(2): 169-185, 1969.

Wand GS. Alcohol, the hypothalamic-pituitary-adrenal axis and the hormonal tolerance. In: Zakhari S, editor. Alcohol and the Endocrine System. Bethesda, MD: National Institute on Alcohol Abuse and Alcoholism; 1993. pp. 251–270. NIAAA Research Monograph No. 23.

Wang B, Shaham Y, Zitzman D, Azari S, Wise RA & You Z-B. Cocaine Experience Establishes Control of Midbrain Glutamate and Dopamine by Corticotropin-Releasing Factor: A Role in Stress-Induced Relapse to Drug Seeking. *J Neurosci* 25(22): 5389-5396, 2005.

Ward RJ, Colivicchi MA, Allen R, Schol F, Lallemand F, de Witte P, Ballini C, Della Corte L & Dexter D. Neuro-inflammation induced in the hippocampus of 'binge drinking' rats may be mediated by elevated extracellular glutamate content. *J Neurochem* 111: 1119-1128, 2009.

Wechsler H, Davenport A, Dowdall G, Moeykens B & Castillo S. Health and Behavioral Consequences of Binge Drinking in College. *JAMA* 272(21): 1672-1677, 1994.

Wechsler H, Dowdall G, Davenport A & Rimm EB. A Gender-Specific Measure of Binge Drinking among College Students. *Am J Public Health* 85(7): 982-985, 1995.

Wechsler H, Dowdall GW, Maenner G, Gledhill-Hoyt & Lee H. Changes in Binge Drinking and Related Problems Among American College Students Between 1993

and 1997: Results of the Harvard School of Public Health College Alcohol Study. *J Am Coll Health* 47: 57-68, 1998.

Wechsler H. Binge Drinking: The Five/Four Measure. Correspondence to the Editor, *J Stud Alcohol* 122-123, January 1998.

Wei W, Zhang N, Peng Z, Houser CR, & Mody I. Perisynaptic Localization of δ Subunit-Containing $GABA_A$ Receptors and their Activation by GABA Spillover in the Mouse Dentate Gyrus. *J Neurosci* 23(33): 10650-10661, 2003.

Wei W, Faria LC & Mody I. Low Ethanol Concentrations Selectively Augment the Tonic Inhibition Mediated by δ Subunit-Containing $GABA_A$ Receptors in Hippocampal Neurons. *J Neurosci* 24(38): 8379-8382, 2004.

Weiss D, Lorang MT, Bloom FE & Koob GF. Oral Alcohol Self-Administration Stimulates Dopamine Release in the Rat Nucleus Accumbens: Genetic and Motivational Determinants. *J Pharmacol Exp Ther* 267(1): 250-258, 1993.

Werner DF, Blednov YA, Ariwodola OJ, Silberman Y, Logan E, Berry RB, Borghese CM, Matthews DB, Weiner JL, Harrison NL, Harris RA & Homanics GE. Knockin mice with ethanol-insensitive alpha1-containing gamma-aminobutyric acid type A receptors display selective alterations in behavioral responses to ethanol. *J Pharmacol Exp Ther* 319: 219-227, 2006.

White A, Castle IJP, Chen CM, Shirley M, Roach D & Hingson R. Converging Patterns of Alcohol Use and Related Outcomes Among Females and Males in the United States, 2002 to 2012. *Alcohol Clin Exp Res* 39(9): 1712-1726, 2015.

Whitten, WK. Modification of the oestrous cycle of the mouse by external stimuli associated with the male. *J Endocrinol* 13:399-404, 1956.

Wholfarth KM, Binachi MT & Macdonald RL. Enhanced neurosteroid potentiation of ternary $GABA_A$ receptors containing the δ subunit. *J Neurosci* 22: 1541-1549, 2002.

Williams CL & Meck WH. The Organizational Effects of Gonadal Steroids on Sexually Dimorphic Spatial Ability. *Psychoneuroendocrino* 16(1-3): 155-176, 1991.

Wisden W, Laurie DJ, Monyer H, Seeburg PH. The Distribution of 13 $GABA_A$ Receptor Subunit mRNAs in the Rat Brain. I. Telencephalon, Diencephalon, Mesencephalon. *J Neurosci* 12(3): 1040-1062, 1992.

Wise RA. Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia* 29: 203-210, 1973.

Wissman AM, McCollum AF, Huang GZ, Nikrodhanond AA & Woolley CS. Sex differences and effects of cocaine on excitatory synapses in the nucleus accumbens. *Neuropharmacology* 61: 217-227, 2011.

Woolley CS & McEwen BS. Roles of estradiol and progesterone in regulation of hippocampal spine density during the estrus cycle in rat. *J Comp Neurol* 336: 293-306, 1993.

Woolley CS, Venczel HJ & Schwartzkroin PA. Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. *J Comp Neurol* 373: 108-117, 1996.

Wu W-J, Wolcott RM & Priett SB. Ethanol Decreases the Number and Activity of Splenic Natural Killer Cells in a Mouse Model for Binge Drinking. *J Pharmacol Exp Ther* 271(2): 722-729, 1994.

Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL & Hjelmstad GO. Nucleus accumbens medium spiny neurons target non-dopaminergic neurons. *J Neurosci* 31: 7811-7816, 2011.

Yang RJ, Mozhui K, Karlsson R-M, Cameron HA, Williams RW & Holmes A. Variation in Mouse Basolateral Amygdala Volume is Associated with Differences in Stress Reactivity and Fear Learning. *Neuropsychopharmacology* 33: 2595-2604, 2008.

Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA & Lusis AJ. Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Res* 16: 995-1004, 2006.

Yetnikoff L, Lavezzi HN, Reichard RA & Zahm DS. An Update on the Connections of the Ventral Mesencephalic Dopaminergic Complex. *Neuroscience* 282: 23-48, 2014.

Yoneyama N, Crabbe JC, Ford MM, Murillo A & Finn DA. Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol* 42(3): 149-160, 2008.

Yim HJ, Robinson DL, White ML, Jaworski JN, Randall PK, Lancaster FE & Gonzales RA. Dissociation Between the Time Course of Ethanol and Extracellular Dopamine Concentrations in the Nucleus Accumbens After a Single Intraperitoneal Injection. *Alcohol Clin Exp Res* 24(6): 781-788, 2000.

Yorgason JT, Ferris MJ, Steffenson SC & Jones SR. Frequency-Dependent Effects of Ethanol on Dopamine Release in the Nucleus Accumbens. *Alcohol Clin Exp Res* 38(2): 438-447, 2014.

Young PT. Motivation and Emotion. Wiley, New York. 1961.

Zahm DS & Brog JS. On the significance of subterritories in the "accumbens" part of the rat ventral striatum. *Neuroscience* 50: 751-767, 1992.

Zeisel A, Muñoz-Manchado AB, Codeluppi S, Lönnerberg P, La Manno G, Juréus A, Marques S, Munguba H, He L, Betsholtz C, Rolny C, Castelo-Branco G, Hjerling-Leffler J & Linnarsson S. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* 347(6226): 1138-1142, 2015.

Zilberman M, Taveres H & El-Guebaly N. Gender Similarities and Differences. *J Addict Dis* 22: 61-74, 2003.

Zuo W, Wang L, Chen L, Krnjević K, Fu R, Feng X, He W, Kang S, Shah A, Bekker A & Ye J-H. Ethanol potentiates both GABAergic and glutamatergic signaling in the lateral habenula. *Neuropharmacology* 113: 178-187, 2017.