Regulation of Ethanol Seeking by the Bed Nucleus of the Stria Terminalis and the Ventral Tegmental Area: Evidence for a Direct Neural Circuit

By

Melanie M. Pina

A DISSERTATION

Presented to the Department of Behavioral Neuroscience

and Oregon Health & Science University School of Medicine

In partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

March 2016

School of Medicine

Oregon Health & Science University

Certificate of Approval

This is to certify that the PhD Dissertation of

Melanie M. Pina

Regulation of Ethanol Seeking by the Bed Nucleus of the Stria Terminalis and the Ventral Tegmental Area: Evidence for a Direct Neural Circuit

Has been approved

[redacted]
Dissertation Advisor

[redacted]
Committee Member

[redacted]
Committee Member

[redacted]
Committee Member

[redacted]
Committee Member

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAV	adeno-associated virus
ACC	anterior cingulate cortex
aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
ArchT	archaerhodopsin
AUD	alcohol use disorder
BAP	basal posterior amygdala
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
CAV-2	canine adenovirus type 2
CeA	central nucleus of the amygdala
ChR2	channelrhodopsin 2
CIE	chronic intermittent ethanol exposure
CNO	clozapine-N-oxide
CNS	central nervous system
CPA	conditioned place aversion
CPP	conditioned place preference
CS	conditioned stimulus
CS-	negative conditioned stimulus
CS+	positive conditioned stimulus
D1	dopamine receptor D1 subtype
D2	dopamine receptor D2 subtype
DA	dopamine
dBNST	dorsal BNST
DNQX	6,7-Dinitroquinoxaline-2,3-dione disodium salt
DREADD	designer receptors exclusively activated by designer drugs
EGFP	enhanced green fluorescent protein
FLEX	flip excision
GABA	Ɣ-aminobutyric acid
GABAA	GABA type A receptor
GABAB	GABA type B receptor
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
HA	human influenza hemagglutinin epitope tag
hEf1α	human elongation factor 1 alpha

List of Abbreviations (cont.)

hM3Dq	M3 muscarinic receptor Gq-coupled DREADD
hM4Di	M4 muscarinic receptor Gi-coupled DREADD
HSV	herpes simplex virus
hSyn	human synapsin promoter
icv	intracerebroventricular
IF	immunofluorescence
iGluR	ionotropic glutamate receptor
IHC	immunohistochemistry
IP	intraperitoneal
IR	immunoreactivity
IRES	internal ribosome entry site
KOR	kappa opioid receptor
KORD	kappa opioid receptor-based DREADD
LDT	laterodorsal tegmental nucleus
LiCL	lithium chloride
LS	lateral septum
mGluR	metabotropic glutamate receptor
NAc	nucleus accumbens
NDS	normal donkey serum
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NpHR	halorhodopsin
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PFC	prefrontal cortex
RASSL	receptor activated solely by synthetic ligands
RVP	rostral ventral pallidum
SN	substantia nigra
TBS	tris-buffered saline
TH	tyrosine hydroxylase
US	unconditioned stimulus
vBNST	ventral BNST
vGluT	vesicular glutamate transporter
VP	ventral pallidum
VTA	ventral tegmental area

Acknowledgments

Frist, I would like to thank Chris for his mentorship over the last 5 years. He has helped shaped me as a scientist and a professional. I would especially like to thank him for allowing me to undertake a considerably risky project and supporting me throughout its completion. Chris - I have learned more over the last 5 years than I could have ever imagined and it has been a pleasure and an honor to learn from you.

I would also like to acknowledge the members of my committee (Drs. Tamara Phillips, Andrey Ryabinin, Matt Lattal, and Susie Ingram) who provided me with their time, support, and thoughtful feedback. A special thanks to Dr. Ryabinin who opened up his lab to me and allowed me to attend his lab meetings. I genuinely appreciate all of the training I received and knowledge I gained as a result.

Many thanks to Megan McClintick, Leah Hitchcock, and Morgan Wirthlin for their friendship and support (both intellectually and personally) throughout the years – it has meant the world to me and has gotten me through some of the toughest of times.

Most important of all – many, many thanks to my family (Margie Crandall, Jon Pina, Natalie Pina, and David Crandall). You have believed in me and I am forever grateful for your unwavering love and support. To my wonderful fiancé, Dr. Jonathan Fay – your love, support, and encouragement have helped me get through graduate school. I have learned so much from you, personally and

scientifically. You are always there to lend me an ear, a brain, or an extra set of hands. Thank you.

Finally, I would like to acknowledge the funding that contributed to the experiments presented in this dissertation. Over the past several years, I have been very fortunate to receive support from the Portland Chapter of the ARCS Foundation, OHSU Brain Institute, OHSU-Vertex Partnership, Tartar Trust, Psi Chi, American Psychological Association, NIH-NIAAA (R01AA007702, T32AA007468), and NIH-NIDA (T32DA007262).

Abstract

Alcohol use disorder is a chronic relapsing and remitting condition, where relapse to drinking is often triggered by an intense desire for alcohol (i.e., ethanol; craving) and the consequent motivation to obtain ethanol (seeking). Environmental stimuli (cues) associated with past ethanol use are believed to strongly contribute to relapse, as exposure to these cues can trigger intense feelings of craving and drive ethanol seeking. Thus, the broad goal of this dissertation was to identify the neurocircuitry underlying cue-induced ethanol-seeking behavior, as indexed through an ethanol-induced conditioned place preference (CPP) procedure. Focus was placed on examining two distinct brain regions – the ventral tegmental area (VTA) and the bed nucleus of the stria terminalis (BNST) – and their adjoining (BNST→VTA) neural circuit. These regions have been previously shown to underlie appetitive behaviors on an individual and circuit level.

In Chapter 1, the involvement of VTA glutamatergic input in ethanol-induced CPP expression was examined. Ionotropic glutamate receptor (iGluR) antagonists selective for NMDA and AMPA/kainate receptors were co-infused into the VTA before the CPP expression (seeking) test. Intra-VTA antagonism of iGluRs blocked ethanol CPP expression, suggesting that glutamatergic input to the VTA is necessary for ethanol-seeking behavior.

In Chapter 2, the involvement of the BNST in ethanol-induced CPP expression was examined. The BNST was inhibited during the expression test using a blend of classical and contemporary techniques that included electrolytic

lesions, pharmacological inactivation, and chemogenetics (aka designer receptors exclusively activated by designer drugs, DREADD). Whereas BNST lesions and DREADDs reduced CPP expression, pharmacological inactivation blocked it altogether. These findings indicate that the BNST is involved in ethanol-seeking behavior.

Based on the findings that the VTA and BNST are individually involved in ethanol-induced CPP expression, the role of a direct projection from the BNST to VTA was assessed. A retrograde intersectional strategy was used to express DREADDs in VTA-projecting BNST cells only. Inhibition of BNST-VTA cells during the expression test reduced ethanol-induced CPP, suggesting that ethanol-seeking behavior is expressed directly through a BNST to VTA neural circuit.

Altogether, these findings demonstrate that VTA glutamate signaling (Chapter 1), BNST activation (Chapter 2), and BNST input to the VTA (Chapter 3) underlie an ethanol-seeking behavior that is relevant to relapse. Future studies are needed to determine the neurochemical nature of BNST inputs to the VTA, as these may be prime targets for treatments aimed at reducing craving and preventing relapse. In summary, this dissertation presents several novel findings that implicate a distinct neural signal, neural structures, and a direct neural circuit in cue-induced ethanol-seeking behavior.

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Introduction

General Introduction

Alcohol use disorders (AUDs) constitute a major global health concern. In 2013 alone, 5.9% of all deaths worldwide were attributed to alcohol (i.e., ethanol) intake (WHO, 2015). This statistic combined with the social, emotional, and other physical consequences of excessive ethanol use, makes it difficult to deny the ongoing need for preclinical research. Of greatest interest is identifying treatments to promote and maintain abstinence in individuals diagnosed with an AUD. Remission however is often compromised by a chronic vulnerability to relapse that is poorly understood. In fact, estimates of long-term relapse rates following remission are as high as 60%, depending on the treatment sought (R. H. Moos & Moos, 2006). Lack of information on the neurobiological antecedents

and psychological determinates of relapse makes AUDs all the more problematic to address.

Further complicating our understanding of the persistent risk of relapse are the complex interactions between internal processes and the external environment. Most noted are the relationships that develop between environmental stimuli (cues), both contextual and discrete, and the internal states produced by ethanol. Over the course of ethanol use, these cues become associated with the effects of ethanol through a Pavlovian learning process, whereby an associative (ethanol-cue) relationship is formed. Once the relationship has been acquired, these associative cues are able to autonomously produce psychological and physiological states that are powerful enough to elicit behavior responses. These responses have been suggested to play an important role in the development of AUDs and relapse.

Even after lengthy periods of abstinence, exposure to drug-associated cues can trigger intense feelings of craving and drive drug seeking (Ciccocioppo, Martin-Fardon, & Weiss, 2004; Ciccocioppo, Sanna, & Weiss, 2001b; Weiss et al., 2001) leading to relapse to drug use. When considering ethanol in particular, this lingering sensitivity to related cues is especially problematic given the omnipresent nature of ethanol and ethanol-related cues in society. Therefore, it is important that the neurobiology of this phenomenon is understood so that more effective and durable treatments for alcoholism can be designed.

In the following sections, I describe common methodologies used to probe the neurobiology underlying primary and conditioned ethanol reward. Specifically,

these methodological sections detail several commonly used animal models and tools to manipulate the brain. Sections that follow will then discuss the neural substrates that have been identified in ethanol-seeking behavior. Finally, a brief overview of the main experiments in this dissertation and a rationale for each are provided.

Animal Models

To gain understanding of the neurobiological mechanisms underlying AUD, several animal models have been developed. These models are designed to reflect various aspects of AUD. The most widely used procedures assess ethanol reward and reinforcement[1] and include drinking, self-administration, and conditioned place preference (CPP). It should be noted that no animal model can fully emulate all aspects of human alcoholism. However, animal models allow for unparalleled access to the brain and thus provide a means to evaluate neural mechanisms involved in aspects of ethanol reward and dependence. These models therefore represent invaluable preclinical tools for identifying potential biological correlates of and treatments for AUD.

Drinking. For nearly a century it has been known that rodents, like

[1] In this dissertation, the terms reward and reinforcement will be distinguished from one another. Reward will be used to refer to the appetitive nature of a stimulus as indicated by the ability of environmental stimuli to elicit approach behavior, whereas reinforcement will refer to experimental contingencies that increase the likelihood of behavior(s) occurring (Bardo & Bevins, 2000; White, 1989).

humans, will voluntarily consume ethanol (Richter, 1926; Richter & Campbell, 1940). For this reason, rodents have long been used in drinking procedures that involve home cage access to ethanol. This represents the simplest way to gauge ethanol reward, through consumption, which is done simply by providing rodents with a bottle and measuring the amount they drink. While ethanol is occasionally the only solution provided in drinking studies, 2-bottle choice procedures are more commonly used in rodents and yield an additional measure of preference for ethanol. In a 2-bottle choice drinking procedure, home-cage access to ethanol and another ethanol-free fluid (typically water) is provided continuously or at temporally controlled intervals. Evidence of ethanol reward is then indicated by the amount consumed and preference for the ethanol-containing solution over the other available fluid. Manipulations that affect ethanol consumption and/or preference but not water or total fluid intake are believed to have interfered with ethanol's rewarding or aversive effects (Gill & Amit, 1989).

Although these studies have high face validity, they are often limited by the fact that like humans, rodents are sensitive to the aversive taste of ethanol. At higher concentrations, ethanol's aversive taste makes it difficult for rodents to drink to a state of intoxication. Therefore, procedures requiring oral intake of ethanol may require water deprivation, slow increases in ethanol concentration, and/or the addition of a sweetener like sucrose to the ethanol-containing solution to help rodents overcome the aversive taste (Meisch, 1977; Samson, Tolliver,

Lumeng, & Li, 1989). For instance, modified sucrose fading[2] techniques (Samson, 1986) are a common strategy that has been used to achieve voluntary consumption of high concentrations of ethanol in rodents. With this technique, sweeteners like sucrose or saccharin are initially added to an ethanol solution then slowly faded out. However, this illustrates a pitfall of these procedures, which is that the underlying motivation for ethanol consumption is not always understood. For example, rodents may freely consume ethanol for the sweetened taste or for its caloric value. Therefore, it is not always evident that ethanol is being consumed in this procedure for its post absorptive pharmacological effects. Additionally, intervention-induced decreases in ethanol intake in this procedure do not always indicate that a manipulation decreased the ethanol reward. It is possible that reduced intake may reflect an enhancement of ethanol's pharmacological effects, resulting in a leftward shift in the dose-response curve, which translates to an increased effect of ethanol at lower amounts. As a result, care must be taken when interpreting results in drinking studies, as the underlying reasons for decreased intake may not always be apparent.

Self-administration. In self-administration procedures, rodents must successfully perform an operant response (e.g., lever press or nose poke) in order to gain access to ethanol that they can orally consume (Samson, Pfeffer, &

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[2] A sucrose-substitution procedure was originally developed to initiate operant ethanol self-administration in rats (Samson, 1986; Samson et al., 1989).

Tolliver, 1988) or, less commonly, intravenously self-administer (e.g., Gass & Olive, 2007). This model is therefore suited to assessing two distinct phases of ethanol consumption: the appetitive phase and the consummatory phase. Whereas the appetitive phase involves ethanol seeking and associated behaviors (e.g., lever pressing), the consummatory phase involves ethanol intake (Samson & Hodge, 1995). One strategy to assess ethanol seeking in the absence of consumption with the self-administration model involves giving animals free access to an ethanol sipper tube after fulfilling a work requirement (Czachowski & Samson, 1999; Samson, Slawecki, Sharpe, & Chappell, 1998). In this way, the animal's willingness to work for ethanol can be assessed in the absence of the ethanol's postabsorptive effects. Thus, the effects of a treatment on ethanol-seeking behavior can be separated from its effects on consummatory drinking. Moreover, additional variations in the procedure can be made to assess the ability of ethanol-associated stimuli (cues) or the self-administration environment (context) to control ethanol-seeking behavior. For example, under second-order schedules, ethanol responding can be maintained not only by ethanol (primary reinforcer) but also by ethanol-associated cues (secondary reinforcer; e.g., Lamb, Pinkston, & Ginsburg, 2015). Additionally, contexts associated with past ethanol reinforcement may serve to maintain or invigorate responding (i.e., seeking) for ethanol (e.g., Sciascia, Reese, Janak, & Chaudhri, 2015).

Self-administration procedures can also be used to model ethanol-seeking behavior that may underlie relapse to ethanol drinking. In these reinstatement

procedures, rodents are first trained to execute a behavior to receive ethanol as described above. After rodents have acquired the task and learned to respond for ethanol (the acquisition phase), they then undergo extinction training, where their responses are no longer reinforced with ethanol. This training continues until their responding for ethanol decreases to low levels, i.e., the behavior is extinguished. Re-emergence (i.e., reinstatement) of extinguished responding for ethanol may then be precipitated or induced by exposure to a stressor (stress-induced), an ethanol-associated stimulus (i.e., cue-induced), or ethanol (drug-induced or primed). Notably, ethanol- and cue-primed reinstatement procedures have been shown to have high criterion validity and are useful for the preclinical evaluation of pharmacotherapies aimed at reducing ethanol relapse (reviewed in Epstein, Preston, Stewart, & Shaham, 2006).

In addition to yielding the rate and pattern of ethanol responding, self-administration procedures also offer an additional measure of amount of ethanol consumed during the consummatory phase. However, similar to drinking studies, the aversive taste of ethanol may be difficult to overcome in self-administration procedures. Therefore, liquid deprivation and sweetener fading strategies have also been used to establish operant responding for and consumption of ethanol. This similarly compromises straightforward interpretations of the underlying purpose for the behavior.

***Place conditioning*.** Another approach to modeling reward in rodents is the conditioned place preference (CPP) procedure. With this Pavlovian (classical) conditioning procedure, a distinct environmental stimulus (conditioned

stimulus, CS) can acquire associative value after being paired with a motivationally significant stimulus (the unconditioned stimulus, US). Ultimately, the previously neutral stimulus (CS) develops the ability to elicit a conditioned motivational response similar to the response elicited by the US. As such, the CPP procedure is widely used to study the rewarding properties of many abused drugs and is considered one of the most popular models of drug reward (Tzschentke, 1998; 2007).

In a standard ethanol-induced CPP procedure, a discrete cue (e.g., visual or tactile stimulus; referred to as the positive conditioned stimulus, CS+) presented in one spatial location[3] is repeatedly paired with ethanol, usually administered by the investigator (i.e., non-contingently). On alternating sessions, a different stimulus not paired with ethanol (the negative conditioned stimulus, CS-) is presented in a location adjacent[4] to where the CS+ was presented. During this acquisition phase, an association develops between the CS+ and the subjective effects of ethanol (US). In the subsequent expression phase, animals are given access to the entire conditioning apparatus and thus exposed to both cues (CS+ and CS-). When given the choice between the CS+ and CS-, animals

[3] While the above description is of a two-compartment configuration, which utilizes spatial cues as well as discrete cues, a one-compartment configuration is also commonly used, where each discrete cue is presented across both spatial locations during CS+ and CS- conditioning trials (Hitchcock, Cunningham, & Lattal, 2014). This gives animals free access to the entire apparatus during conditioning (acquisition).

[4] The location of CS- compartment may be directly next to the CS+ compartment in a two-chamber apparatus or separated by a neutral compartment in a three-chamber apparatus.

will generally approach and maintain contact with (i.e., prefer) the CS+, when a US is rewarding. In other words, if an animal spends a greater amount of time with the ethanol-paired stimulus (CS+) in relation to the nondrug (typically saline)-paired stimulus (CS-) this is taken as an indication of ethanol's positive rewarding effects. Conversely, a greater amount of time spent with the saline-paired stimulus compared to the ethanol-paired stimulus would be considered conditioned place aversion (CPA) and taken to indicate a negative or aversive effect of ethanol. Ethanol's ability to produce CPP or CPA depends on many factors such as past history of ethanol exposure, route of administration, CS+ exposure duration, injection timing, and dose (e.g., Bormann & Cunningham, 1998; Cunningham & Henderson, 2000; Cunningham, Clemans, & Fidler, 2002; Cunningham, Ferree, & Howard, 2003; Cunningham, Okorn, & Howard, 1997). A result of conditioned reward or aversion can vary by species and strain. Although ethanol-induced CPP has been reported in some strains of rat (e.g., Chester, Rodd-Henricks, Li, Lumeng, & Grahame, 2001; Ciccocioppo, Panocka, Froldi, Quitadamo, & Massi, 1999; Morales, Varlinskaya, & Spear, 2012), studies overall have shown conflicting results ranging from lack of CPP (Asin, Wirtshafter, & Tabakoff, 1985) to CPA (Cunningham, 1979) (van der Kooy, O'Shaughnessy, Mucha, & Kalant, 1983). However, ethanol-induced CPP has been found in a several mouse strains (Cunningham, 2014) and thus has much utility as a model of ethanol reward in this species.

Unlike self-administration, CPP does not require a lengthy training phase. In fact, in an inbred strain of mouse, DBA/2J, commonly used in ethanol-induced

CPP, a significant place preference can be conditioned after only two ethanol-cue pairings (Pina & Cunningham, 2014a; 2014b; Pina, Young, Ryabinin, & Cunningham, 2015). Another advantage to this procedure is that it does not involve oral intake of ethanol, which is required in drinking and with most ethanol self-administration procedures. This is highly beneficial in cases where manipulations, such as the use of pharmacological agents, reduce general consummatory behavior in addition to ethanol reward or reinforcement. For example, erroneous conclusions may be made when a drug with anorectic liability reduces oral intake of ethanol. However, since CPP involves noncontingent ethanol administration, the effect of anorectic drugs on ethanol reward can be assessed more accurately (Pina & Cunningham, 2014b). The place preference paradigm also permits for the evaluation of manipulations on the direct (acquisition) versus conditioned (expression) effects of ethanol, which are often difficult to isolate in other procedures. Specifically, when a manipulation disrupts the expression of ethanol-induced CPP (without affecting motor behavior or expression of CPP/CPA induced by other drugs) this is generally taken to indicate that it interfered with the conditioned rewarding effect of ethanol or ethanol seeking. Indeed, since CPP can be used to gauge the conditioned rewarding or motivational value of stimuli, it also serves as an effective method to measure cue-induced ethanol-seeking behavior. However, additional explanations for disrupted CPP expression may include impaired memory recall or reduced CS+ valence. Manipulations that disrupt the development (acquisition) of CPP are thought to impact either associative learning or the

primary rewarding effect of ethanol. To distinguish between these two possibilities, this procedure can be used to assess whether the manipulation also disrupts acquisition of other associations such as CPA induced by ethanol or other drugs (e.g., Pina & Cunningham, 2014a).

One disadvantage of the CPP procedure is that the drug is administered by the investigator and therefore delivered noncontingently. Although this may be considered an advantage given the control over dose it provides, it reduces the face validity of this model. Unlike humans, in this procedure, rodents do not consume ethanol of their own volition. Similarly, humans do not take ethanol via intraperitoneal injections, as is used in this animal model. Moreover, this procedure does not typically involve an escalation in intake as is usually observed in humans. As may occur in to self-administration procedures, manipulations that affect locomotor activity may nonspecifically impact CPP expression. It has been previously demonstrated that increases in activity may disrupt ethanol-induced CPP expression, thereby obscuring its detection (Gremel & Cunningham, 2007). Hence, results obtained by manipulations that increase or decrease preference test activity must be cautiously interpreted. Despite these drawbacks, this model presents a rapid and efficient method to evaluate the primary and conditioned motivational effects of ethanol in rodents.

Summary. In summary, animal models have been used extensively in ethanol research, with several models designed to study various aspects of ethanol-mediated behavior. The most commonly used procedures to model ethanol seeking independent of intake are self-administration and CPP. In this

dissertation, a CPP procedure well-established by our laboratory was used to model ethanol-seeking behavior in mice (Cunningham, Gremel, & Groblewski, 2006). This model was chosen, in part, due to the fast and relatively easy nature of the training phase in addition to the accessibility of each phase of behavior – acquisition (development) and expression (seeking).

Although it should be noted that no one procedure is able to mimic all features of human ethanol use, these models allow for the investigation of underlying neural mechanisms involved in ethanol-related behaviors. In the following sections, several commonly used techniques to probe the neural structures and circuits involved in rodent behavior are described.

Tools to Manipulate Neural Structures

Many techniques have been developed to evaluate distinct brain structures and circuits. These methods allow for the activity of defined brain areas to be manipulated during behavior. Thus, they require intracranial access, typically gained through stereotaxic surgery. Of these methods, the most widely used are lesions and microinjection. However, more modern tools have been developed that harness the capabilities of viral gene transfer in order to more precisely control cells and circuits. Each of these techniques, classic and contemporary, possess inherent benefits and drawbacks that are discussed in detail below and summarized in Table 1.

Classical Tools

Lesions. A classical method used to study brain function involves the

removal or destruction of neural tissue. With this method, experimental lesions are made to defined brain structures through manual, chemical, or electrical means. Behavior is then examined in the absence of this tissue, thus providing insight into the involvement of the lesioned structure. Although studies using this technique have been highly informative, they are complicated by the permanent nature of this procedure. One issue that arises is the propensity for other brain structures to compensate for the damaged region (Jones et al., 2003). Therefore, the function of the damaged structure is not entirely lost as it is being executed by a surrogate region (e.g., Poulos, Ponnusamy, Dong, & Fanselow, 2010). This may severely compromise the interpretation of results obtained from studies using a lesion procedure. Another issue is the difficulty encountered when using lesions to assess effects at distinct phases of a behavior. This again is a consequence of the irreversible damage produced by lesioning. Thus, with many animal models, it is difficult to determine whether ablation of a structure impacted the development (acquisition) or performance (expression) of behavior. Lesions made after acquisition may help pinpoint the effect of the damage to the expression phase. In addition to low temporal resolution, lesions provide low to moderate spatial resolution, the extent of which depends largely on the lesion method (reviewed in Cain & LeDoux, 2008). For example, electrolytic and mechanical lesions provide the lowest spatial resolution as they damage or remove support cells and fibers of passage in addition to cell bodies. Depending on the neurotoxin used, chemical lesions can be cell-body specific, sparing axonal fibers passing through the target region.

Intracranial microinjections. Local administration of pharmacological agents into discrete brain targets is another strategy to control neural activity. This technique typically requires surgical placement of guide cannula in order to allow for later access to otherwise inaccessible brain structures. Small volumes of drug solutions are then administered directly into the brain by threading a smaller gauge injector through the guide cannula. These solutions typically contain drugs that bind to distinct membrane proteins (receptors) expressed within the target brain region in order to enhance or inhibit local cellular activity. This procedure has several major advantages compared to lesions, most of which relate to its ability to produce more temporally specific effects. Unlike lesions, which are permanent, the effects of most pharmacological antagonist and agonist are temporary and can therefore be more precisely controlled. Microinjections can be administered during distinct phases of behavioral procedures, allowing for a more straightforward interpretation of this manipulation's effects on behavior. Additionally, this technique can provide insight into the neurochemical signals involved, as agents selective for distinct receptor types can be infused. Though to a lesser extent than lesions, microinjections also produce damage resulting in reactive gliosis (Cunningham, Ames, Donalds, & Benes, 2008). This damage results from cannula installation and injector placement, which produce damage that is dorsal to and localized within the target structure. Finally, it is difficult to ascertain the exact extent of diffusion of the administered solution. Infusion of large molecule dyes has been used to approximate the extent of a drug's spread in deep brain structures

(Myers, 1966). However, diffusion may depend not only the volume injected but also on the nature of the solution (polarity, hydrophobicity) and is therefore difficult to approximate or predict with other substances. Thus, it is not always clear that the site of infusion is the region directing the observed behavior. For this reason, it is often necessary to include additional groups that receive drug injections in locations proximal to the target structure.

Intracranial stimulation. Another strategy used to manipulate neural structures involves stimulation of discrete brain regions by chronically implanted electrodes. This technique is generally referred to as intracranial self-stimulation (ICSS) or brain stimulation reward (BSR). There are many variations of this procedure, involving contingent (self-administered) or non-contingent (experimenter-administered) stimulation that can be monopolar or bipolar and delivered under varying frequencies and schedules (Carlezon & Chartoff, 2007; Vlachou & Markou, 2010). However, for the purpose of this introduction, focus is placed on the most common application of ICSS – an operant behavioral paradigm where rodents are trained to respond for short electrical pulses. In a classic study by Olds and Milner (1954), it was first discovered that rats would work to obtain electrical stimulation of specific brain structures. This finding demonstrated that direct simulation of certain brain regions was reinforcing and indicated that brain regions supporting ICSS may also underlie the reinforcing properties of natural and drug reward. Importantly, ICSS became recognized as a tool to examine the interaction of drugs of abuse with brain reward systems (Bozarth, Gerber, & Wise, 1980). In these procedures, the effect of

noncontingent administration of drugs on ICSS thresholds (i.e., the minimal amount of stimulation that an animal is wiling to work for) is examined. Therefore, if a drug is rewarding, it will produce leftward shifts in ICSS frequency-rate curves indicating a decrease ICSS threshold or the amount of stimulation required to support responding (Carlezon & Chartoff, 2007). Conversely, drugs with aversive properties are expected to produce rightward shifts and increase the ICSS threshold. The most apparent advantage to this procedure is the ability to directly electrically stimulate discrete brain regions with superior temporal resolution (millisecond timescale). When used to assess abused drugs, ICSS procedures yield a measure of a drug's ability to facilitate or enhance reward and the neurobiology underlying these reward-related shifts (Vlachou & Markou, 2010). However, this technique is invasive, as it requires an intracranial surgery and the installation of permanently indwelling electrodes into the target brain region. Another limitations to this procedure is the poor spatial resolution it affords. Since current is passed through the implanted electrode, all cells and fibers of passage proximal to the electrode tip will be impacted. Therefore, it can be difficult to definitively identify whether the target region (i.e., site of electrode tip), its afferents, or axon collaterals passing through were responsible for the observed effects (e.g., Bielajew & Shizgal, 1986; Corbett & Wise, 1979).

Table 1. Comparison of tools used for neural manipulation

CRITERIA	Classical Tools			Contemporary Tools	
	Lesion	Microinjection	ICSS	Optogenetic	Chemogenetic
Spatial Resolution	+ *depends on lesion type, some damage fibers of passage*	+ *spread difficult to identify*	- *cells and fibers of passage surrounding electrode tip*	++ *labeled spread; targeted to cells*	++ *labeled spread; targeted to cells*
Temporal Resolution	- *permanent*	+ *min to hr depending on drug*	++ *milliseconds*	++ *milliseconds*	+ *min to hr depending on actuator*
Non-Invasive	+ *1 intracranial entry, but involves intentional tissue damage*	- *permanent hardware, repeated intracranial access*	- *permanent hardware*	- *permanent hardware, repeated intracranial access*	++ *1 intracranial entry, activation by peripheral injection*
Cell Specificity	- *marginally possible with chemical lesions*	- *receptors targeted across cell types*	- *all cells and fibers in region targeted*	++ *achieved using transgenic strains and viral promoters*	++ *achieved using transgenic strains and viral promoters*
Minimal Need for Specialized Equipment	- *current generating device and electrodes for electrolytic*	- *Surgical grade stainless steel cannula and injectors of custom gauge, length*	- *Implantable electrodes, programmable stimulator, commutator*	- *Implantable cannula w/ fiber optics of custom length, multi-channel light source*	++ *Requires microinjectors only*
Circuit Specificity	- *Indirect with disconnection procedure*	- *Indirect with disconnection procedure*	- *none*	++ *achieved by axonal light excitation*	++ *achieved by focal actuator injection or antero/retrograde viruses*
Bidirectional Modulation Ability	- *inactivation only*	++ *inhibition and activation depend on drug*	- activation only	++ *depends on combination of opsins and wavelengths*	++ *depends on combination of receptor and actuator*

- low
+ moderate
++ high

Contemporary Tools

In recent years, there has been a rapid emergence of novel tools engineered to control neuronal activity. Of benefit to these tools have been the advancements in recombinant viruses that are capable of gene transfer in the central nervous system (CNS). For example, viruses with low immunogenicity and cytotoxicity such as adeno-associated virus (AAV) can be delivered directly into the brain in order to safely and efficiently express recombinant genes (Mastakov et al., 2002). This provides a means to site-specifically express proteins in the CNS that can be used to modulate the activity of cells in target brain tissue. Optogenetics and chemogenetics represent the two most widely used contemporary tools in behavioral neuroscience, as they can be applied *in vivo* to modulate neural activity in awake behaving mice and rats (Fig. 1). As with the classical tools described above, these modern methods have inherent advantages and disadvantages (Table 1), which are detailed below.

Optogenetics. In this technique, neurons are genetically modified through intracranial injection of a viral vector to express photosensitive proteins such as channelrhodopsin (ChR; excitatory ion channel), halorhodopsin (NpHR; inhibitory ion pump), and archaerhodopsin (ArchT; inhibitory proton pump) (reviewed in (Han, 2012). These light-gated proteins can then be activated by targeted illumination, causing rapid (millisecond timescale) depolarization or hyperpolarization of neurons (reviewed in Fenno, Yizhar, & Deisseroth, 2011). By evoking or inhibiting spike activity with this light-protein interaction, activity of distinct brain regions and cell types can be experimentally controlled, including

during the performance of behavioral tasks (Han, 2012). Since these engineered opsins can be controlled by different light wavelengths, neural activity and behavior can be modulated bidirectionally (i.e., multiplexed), offering a major advantage to this technique. Moreover, the high temporal resolution and transient nature of activation/inhibition afforded by this tool makes it ideal to examine discrete phases of behavior. However, a major issue posed by optogenetics is the possibility of desensitization of the opsin (i.e., a change in channel conformation consistent with decreased activation), which can occur within seconds of photoactivation (Mattis et al., 2012). This possibility then becomes a significant concern when repeated or prolonged stimulation of the opsin is required. This is especially problematic for studies that require inhibition or activation of longer durations, such as is required in certain behavioral tasks. Moreover, repeated or prolonged inhibition may lead to rebound excitation from changes in chloride balance or increased membrane excitability (Mattis et al., 2012).

The extent of viral diffusion and resulting protein expression is easily measurable with this technique, as most viral constructs contain a fluorescent tag. More precision in determining the confinement of activation/inhibition to the target structure is therefore provided by this technique. However, similar to microinjections, implantable hardware is necessary to allow for intracranial insertion of fiber optic probes. This technique also requires specialized equipment such as fiber optic probes and programmable light sources, which can be costly. Tethering the animal to the external light source is also necessary,

which may restrict the range of apparatuses that can be used and behaviors that can be assessed (although for recent developments in wireless technologies see: Jeong et al., 2015; Kim, 2015; Kim et al., 2013b; McCall et al., 2013; Park et al., 2015). Recently, questions regarding the effect of illumination in brain structures have arisen, specifically with regard to thermal effects on neural tissue. It has been suggested that focal illumination, especially when intense and prolonged, can result in phototoxicity, heat-induced cell damage, and oxidative stress that independently alter cellular activity (Gysbrechts et al., 2015). Even more problematic is evidence indicating that heat alone can increase neuronal firing rates (Reig, Mattia, Compte, Belmonte, & Sanchez-Vives, 2010). In fact, even at commonly used intensities, the thermal effect of illumination is sufficient to increase cell firing rates (Stujenske, Spellman, & Gordon, 2015). Therefore, the thermal effects produced by illumination may cloud results obtained from studies using optogenetics. Overall, optogenetics provides a unique tool to control neuronal activity with high spatiotemporal resolution. However, the required implantable hardware, specialized equipment, tethering, risk of desensitization, and light-induction may render this tool less than ideal depending on the experimental question and design.

Chemogenetics. This relatively new technique involves the engineering of G protein-coupled receptors (GPCRs) to interact exclusively with small molecules that were otherwise unrecognized by the GPCR (Sternson & Roth, 2014). These mutated proteins termed receptors activated solely by synthetic ligand (RASSL) were first presented by Coward and colleagues (Coward et al.,

1998). In this report, a RASSL based on the kappa opioid receptor (KOR) was created (termed Ro1), which was insensitive to endogenous ligands and responsive to the synthetic agonist spiradoline at low (nM) concentrations indicating its potential for *in vivo* use. However, use of chemogenetics in neuroscience studies was greatly hindered by the pharmacologically active nature of RASSL effectors and the constitutively active nature of early RASSLs. For example, as a potent KOR agonist, spiradoline was not suitable for use in behavioral studies, as it would produce effects independently of the RASSL (Wadenberg, 2003). The constitutive activity of the Ro1 RASSL also proved problematic, with reports demonstrating that peripheral and central expression led to cardiomyopathy (Redfern et al., 1999), osteopenia (Peng et al., 2008) and hydrocephaly (Sweger, Casper, Scearce-Levie, Conklin, & McCarthy, 2007). However, improved RASSLs termed designer receptors exclusively activated by designer drugs (DREADDs) better suited for in vivo studies were developed (Armbruster, Li, Pausch, Herlitze, & Roth, 2007). These engineered GPCRs possess no detectible constitutive activity and are robustly activated at nM concentrations of otherwise pharmacologically inert compounds but not their native ligands. The first established DREADDs were based on excitatory G_q-coupled and inhibitory G_i-coupled human muscarinic receptors M3 (hM3Dq) and M4 (hM4Di), respectively (Alexander et al., 2009; Armbruster et al., 2007; Nichols & Roth, 2009). Receptors hM3Dq and hM4Di possess no affinity for the

endogenous ligand acetylcholine and are robustly activated by the drug clozapine-N-oxide (CNO)[5], a pharmacologically inert metabolite of clozapine, which is highly bioavailable and produces no pharmacological effect in rodents (Armbruster et al., 2007; Bender, Holschbach, & Stöcklin, 1994). Since their inception, other DREADDs have been engineered which include a G_s-coupled muscarinic-based (rM3Ds) receptor and G_i-coupled KOR-based DREADD (KORD) (Farrell et al., 2013; Guettier et al., 2009; Vardy et al., 2015). Notably, the development of KORD with actuator salvinorin B allows for bidirectional control of behavior when used in combination with hM3Dq receptors and CNO (Vardy et al., 2015).

Comparable to optogenetics, DREADDs can be ectopically expressed in the CNS by focal infusion of a vector encoding for these receptors. A major advantage of chemogenetics over optogenetics, however, is the lack of required specialized equipment and need for permanently implanted hardware. In fact, DREADDs require just one initial intracranial entry to infuse the viral vector carrying the DREADD-encoding gene. The receptors can then be activated by a relatively non-invasive peripheral injection of an actuator. This is highly advantageous when performing sensitive behavioral procedures that are affected by excessive handling (e.g., Bechtholt, Gremel, & Cunningham, 2004). Similar to optogenetics, more precise detection of viral spread and DREADD expression is

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[5] Several new and highly potent DREADD agonists have recently been discovered (Chen et al., 2015).

possible with this technique. This advantage is owed to the use of viral vectors, as they are designed to encode for DREADDs as well as a fluorescent marker, which can even be fused to the DREADD itself.

Unlike optogenetics, DREADDs signal through canonical G-protein pathways. Once activated, the duration of the inhibition or activation produced by the DREADD can be long lasting (e.g., Alexander et al., 2009; Krashes et al., 2011). The duration of effect is also determined by the half-life of actuators, which may remain in central tissue and activate DREADDs for minutes to hours. In some cases, this low temporal resolution may serve as a major shortcoming of this technique. However, a protracted effect is often highly valued in studies where behavioral tasks are of longer duration and long-lasting effects of manipulations are desired. Although it is theoretically possible, no studies have reported DREADD desensitization. However, this presents a reason for including some form of functional confirmation of DREADD effects.

In summary, DREADDs are a useful technique to control neuronal signaling *in vivo*. Considering the sensitivity and duration of many behavioral tasks, the non-invasive nature of DREADD activation (i.e., peripheral drug injection) and longer timecourse of inhibition/activation make chemogenetic strategies highly desirable in behavioral research. Additional background information on DREADDs is provided in Chapters 2 and 3 of this dissertation, where they have also been used experimentally.

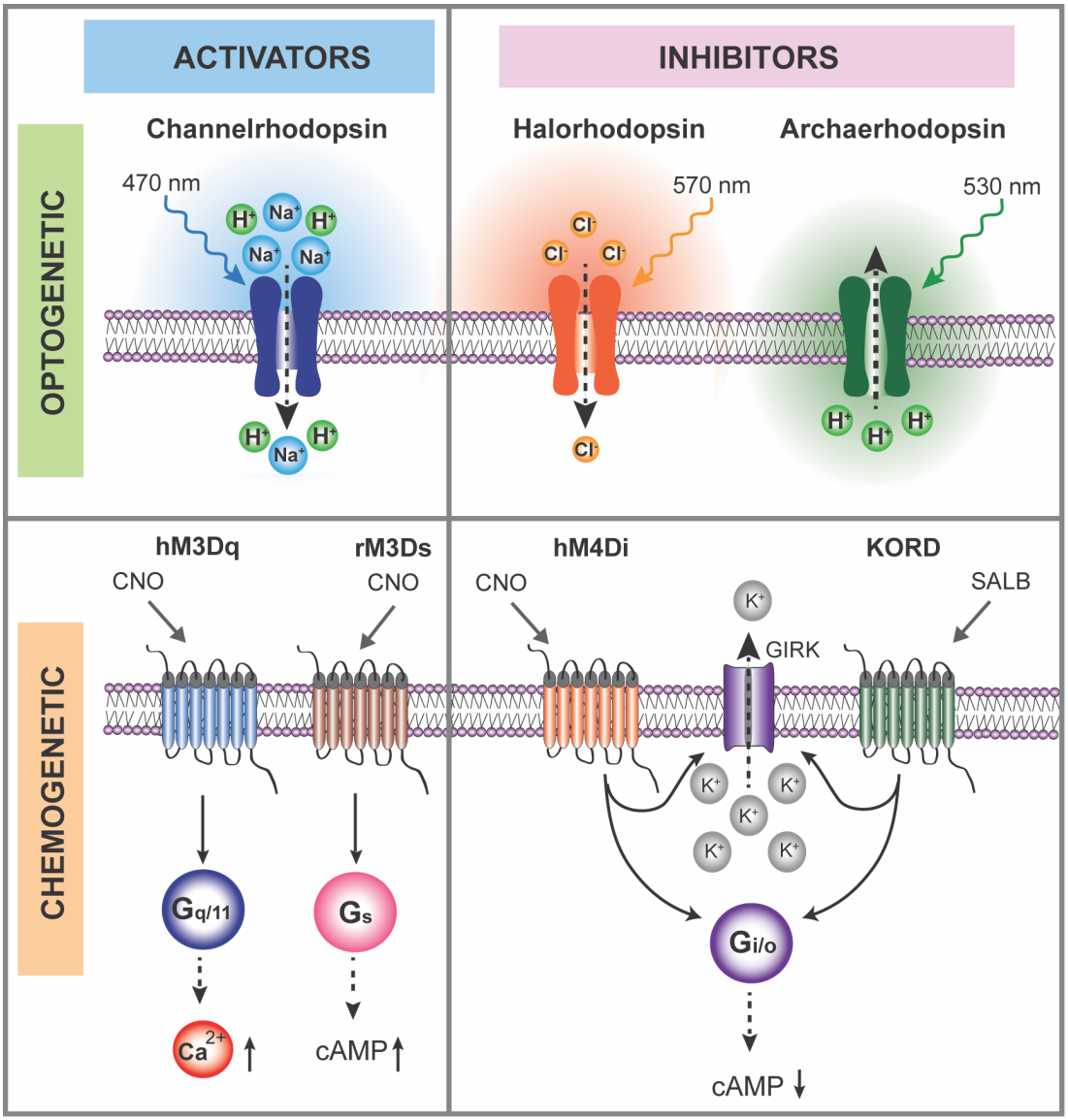

Figure 1. Contemporary tools for neural structure and circuit manipulation. The emergence of optogenetic and chemogenetic tools has provided a new means to selectively manipulate neuronal activity in vivo. These genetically-encoded proteins provide unprecedented access to cell-types and circuits allowing their reversible modulation. Optogenetic photo-opsins are activated by different wavelengths (nM) and can rapidly depolarize or hyperpolarize cells through various mechanisms. Chemogenetic G protein-coupled receptors are engineered to activate or inhibit cell activity through canonical G-protein mechanisms upon binding otherwise inert pharmacological molecule (clozapine-N-oxide, CNO; salvinorin B; SALB). Figure was adapted from Sternson et al. (2015) and permission to reuse the original image was obtained from Elsevier (License #3813990763723).

Summary

The above-described tools provide a means through which to target and manipulate the brain. These tools offer variable degrees of selectivity, with contemporary techniques typically being associated with higher precision in terms of spatial and neuronal targeting. In this dissertation, a combination of classical and contemporary tools was used. Specifically, lesions, microinjections, and DREADDs were used to assess individual neural structures and a specific neural circuit. As the main contemporary tool used in the present work, DREADDs were chosen based on the ease of their activation (i.e., peripheral CNO injection) and longer duration of effect that was more suited for use during a 30-min CPP test. In order to target DREADDs to a defined neural circuit, a modified dual-virus strategy was used. In the proceeding section, this dual-virus strategy, along with others used to target circuitry, is discussed in depth.

Tools to Manipulate Specific Neural Pathways

Historically, direct manipulation of neural circuits has been a challenging task, with much of the difficulty due to limited methodologies. In the past, many of the tools used possessed relatively low selectivity and provided indirect manipulation. Several strategies however have been designed using both classical and contemporary tools to probe the neural circuitry underlying behavior. This section describes several commonly used strategies and includes discussion of their merits and weaknesses.

Classical tools.

Historically, disconnection procedures involving lesions and pharmacological microinjections have been used to evaluate neural circuitry. This strategy involves the disruption of two directly connected brain regions to assess whether their interaction is involved in behavior (e.g., Di Ciano & Everitt, 2004b; Gremel & Cunningham, 2010; Parkinson, Willoughby, Robbins, & Everitt, 2000; Sartor & Aston-Jones, 2012). Typically, a unilateral lesion or inactivating microinjection (e.g., ɣ-aminobutyric acid, GABA, agonists or channel blocker) is made in a source region and another lesion or microinjection (inactivating or receptor-selective antagonist) is made in the contralateral hemisphere of its terminal target. Thus, if a behavior is dependent upon a source-target interchange, then their contralateral disconnection should be more disruptive to behavior than ipsilateral disconnection or unilateral manipulation of each region individually. However, a major weakness of this strategy is the indirect nature of the manipulation on the circuit. Indeed, the imprecision of this method has at times proved problematic, resulting in significant reductions in behavior with ipsilateral and unilateral manipulations alone (e.g., Gremel & Cunningham, 2010). This is likely due to this technique's inability to directly target distinct yet intermixed populations of target-projecting neurons within source regions. Instead, each region is broadly manipulated leading to inhibition of their overall activity and output throughout the brain.

To help visualize and identify the circuit, neuronal tracing has sometimes been used in conjunction with these classical tools. In these studies (reviewed in

Oztas, 2003), tracing agents are injected into the brain to label neurons in a manner that is retrograde (axon terminal back to the soma), anterograde (soma to axon terminal), or transsynaptic (to adjacent neurons retro- or anterogradely; also called transneuronal transfer). In addition to being capable of axoplasmic transport, tracers should be non-neurotoxic if used in long-term studies. In studies of behavior, circuit involvement is inferred by co-labeling of neuronal activity markers like c-Fos with the tracer through immunohistochemistry (IHC) (e.g., Mahler & Aston-Jones, 2012). Cells that are immunopositive for both the tracer and activity marker are then used to identify afferent or efferent projection neurons that were activated during behavior. When used in tandem with disconnection procedures, this provides a means to visually assess the impact of disconnection on circuit activity (e.g., Sartor & Aston-Jones, 2012). Though these procedures help to label neurons within the circuit and gauge their activity, they still fall short of allowing isolated modulation of the circuit. Overall, this remains a major weakness of classical techniques, as results only offer an indirect measure of neural circuit involvement in behavior.

Contemporary tools.

Optogenetic and chemogenetic strategies have provided a refined and more selective means to directly manipulate neural circuits. This is principally due to viral transduction, as proteins (opsins and DREADDs) are trafficked downstream from soma to axon terminals (anterograde) and therefore expressed on presynaptic boutons as well as to cell bodies (Tye & Deisseroth, 2012). In the case of optogenetics, illumination can then be targeted to axons terminals, which

results in depolarization or hyperpolarization of the neuron. This strategy has been successfully implemented in many behavior studies (reviewed in Tye & Deisseroth, 2012). However, a concern that arises when using this method is the possibility of antidromic stimulation of the cell. Stimulation of terminals may result in back-propagation of an action potential that activates the neuron and its collateral inputs to other regions outside the circuit of interest. Therefore, with this strategy there is a potential for activation of multiple circuits, which diminishes the selectivity of the manipulation.

Several chemogenetic-based strategies have been used to modulate neural circuit activity. First, a functional disconnection procedure methodologically similar to that used with lesions or microinjections has been reported (Mahler et al., 2014). In this study, hM4Di receptors were unilaterally expressed in VTA dopamine (DA) cells and contralaterally in rostral ventral pallidum (RVP) cells. In an operant self-administration model, contralateral disconnection of RVP-VTA DA reduced cued reinstatement of cocaine seeking relative to both unilateral RVP inhibition and unilateral VTA DA inhibition. Despite these positive results, issues pertaining to the indirect nature of this manipulation remain. Specifically, it is unclear whether simultaneous inhibition of RVP and VTA DA, regardless of hemisphere, would have been sufficient to produce a similar effect. Thus, with this technique it is important to include ipsilateral as well as unilateral controls. In summary, the indirect nature of disconnection procedures and necessary inclusion of numerous controls renders this a less desirable strategy for targeted modulation of neural circuits.

Another DREADD-based method that has been used involves intracranial injection of CNO. The principal behind this is strategy is similar to that of the optogenetic circuit-selective method outlined above. Here, like illumination, CNO is targeted to the terminal region of DREADD-expressing cells (Mahler et al., 2014; Zhu & Roth, 2014). Focal infusion of CNO therefore serves to activate/inhibit DREADD-expressing cells and/or presynaptic neurotransmitter release from DREADD-expressing nerve terminals (Zhu & Roth, 2014). In this manner, the activity of defined neural circuits can be more precisely controlled. However, given that this strategy requires intracranial microinjections it also carries with it the disadvantage of requiring permanently indwelling hardware (i.e., guide cannula and obturators), repeated intracranial entries, and a resulting increased risk of tissue damage.

An alternative approach to projection-based targeting involves the intersection of multiple viruses that are injected into two distinct yet synaptically connected nuclei. Typically, a retrograde virus encoding for cre recombinase is injected in a target region and a cre-inducible virus encoding for DREADD is injected into the source region. In this manner, the activity of source inputs to the target region (i.e., projection neurons) can be controlled by systemic injection of CNO. This approach has been successfully implemented using canine adenovirus (CAV-2) to retrogradely infect source region cells and selectively express DREADDs (Boender et al., 2014; Carter, Soden, Zweifel, & Palmiter, 2013; Nair, Strand, & Neumaier, 2013). In addition, it is possible to use alternate retrograde viruses such as adeno-associated virus (AAV; with select promoters

and serotypes) or herpes simplex virus (HSV) (e.g., Stamatakis et al., 2013). The use of HSV in this capacity will be described in full detail in Chapter 3 of this dissertation. Notably, this strategy provides an ideal means to control neuronal circuits important for specific behaviors. Not only does this method confer a high degree of selectivity, it does not require implantable hardware and can be robustly activated by a simple peripheral injection of an actuator. Theoretically, it is possible for axon collaterals from source region cells to express DREADDs, which given the systemic nature of DREADD activation may lead to activity in sites outside the circuit. Hence, studies using this method should be careful to address this potential caveat.

Summary

Overall, several techniques involving classical and contemporary tools have been used to manipulate defined neural circuits. On one hand, classical tools provide indirect manipulation and generally require the use of multiple controls to in order to carefully and appropriately interpret results derived using these strategies. Conversely, contemporary tools involving viral-mediated gene transfer confer greater circuit selectivity and have been successfully used to manipulate direct neural projections form source to target region. A dual-virus strategy involving DREADDs was used in Chapter 3 of this dissertation to modulate a neurocircuit and was chosen based on its high degree of selectivity and relatively noninvasive means of activation.

Neural Structures Involved in Ethanol-Seeking Behavior

In the following sections, several neural structures and circuits involved in ethanol-seeking behavior are discussed. Building off of earlier sections, the following studies involved a combination of several different animal models and tools to identify these neural structures and circuits. It should be noted that in the ethanol literature, there is a paucity of evidence for distinct brain regions that drive ethanol seeking. Specifically, studies using site-directed neural manipulations to assess the neurobiology of cue-induced ethanol seeking in the absence of consumption (e.g., using self-administration and CPP procedures) are sparse. This small but relevant literature is discussed below.

Findings from Ethanol Self-Administration Studies

Studies using ethanol self-administration procedures to evaluate the neural mechanisms underlying ethanol seeking have by and large employed intracranial microinfusions into discrete brain regions. Most of the brain regions that have been evaluated in self-administration studies are part of the mesocorticolimbic system (Gardner & Ashby, 2000) and are typically situated downstream of the VTA. As with many drugs of abuse, acutely administered ethanol excites DA neurons within the VTA through direct and indirect mechanisms (Brodie, Pesold, & Appel, 1999b; McDaid, McElvain, & Brodie, 2008; Morikawa & Morrisett, 2010; Mrejeru, Martí-Prats, Avegno, Harrison, & Sulzer, 2015). Downstream, the nucleus accumbens (NAc) and amygdala

receive DAergic input from the VTA (Swanson, 1982) and considerable evidence suggests that this DAergic input to each region underlies associative learning and motivated behavior (Cador, Robbins, & Everitt, 1989; Clark, Collins, Sanford, & Phillips, 2013; Di Chiara et al., 2004; Ikemoto & Panksepp, 1999; Wise, 2004). As such, NAc and amygdala are two of the most well-characterized structures in terms of their involvement in ethanol seeking and several of these studies are described below.

The NAc has been routinely implicated in the reinstatement of drug-seeking behavior (Everitt & Robbins, 2005; McFarland & Kalivas, 2001). Many of these studies have found differential involvement of core and shell subdivisions (Alderson, Parkinson, Robbins, & Everitt, 2000; Fuchs, Evans, Parker, & See, 2004; Fuchs, Ramirez, & Bell, 2008). Similarly, the NAc core and shell have been shown to be differentially involved in cue-induced ethanol seeking. For example, ethanol self-administration studies have shown that transient inactivation of the NAc core but not shell reduces cue-induced reinstatement (Chaudhri, Sahuque, Cone, & Janak, 2008) and context-induced renewal (Chaudhri, Sahuque, Schairer, & Janak, 2010) of ethanol seeking. Other self-administration studies however have demonstrated that NAc shell modulates cue-triggered responding for ethanol (Millan, Reese, Grossman, Chaudhri, & Janak, 2015) and that blockade of DA D1 receptors in the NAc shell but not core reduced spontaneous recovery of ethanol-seeking behavior (Hauser et al., 2015). In addition, DA D1 receptor antagonism in NAc core and shell has been reported to block context-induced renewal of ethanol seeking (Chaudhri, Sahuque, & Janak, 2009).

Although these studies vary in their findings with regard to core versus shell subdivisions, overall they support a role for the NAc in ethanol-seeking behavior and suggest that DA input may underlie its involvement.

The amygdala has also been strongly implicated in drug-seeking behavior, specifically the basolateral (BLA) and central nucleus (CeA) subdivisions as well as the bed nucleus of the stria terminalis (BNST) of the extended amygdala (Crombag, Bossert, Koya, & Shaham, 2008; Erb & Stewart, 1999; Shaham, Erb, & Stewart, 2000). In ethanol self-administration studies, inactivation of the BLA has been shown to reduce context-induced renewal of ethanol seeking (Chaudhri, Woods, Sahuque, Gill, & Janak, 2013) and basal posterior amygdala (BAP) inactivation reduced cue-induced ethanol seeking (Millan et al., 2015). Moreover, involvement of BLA in ethanol seeking may involve a glutamatergic mechanism, as intra-BLA ionotropic glutamate receptors antagonism reduced cue-elicited ethanol seeking (Sciascia et al., 2015). Notably, excitatory transmission from the amygdala to the NAc is believed to underlie reward seeking and suggests an amygdala-NAc circuit may be involved in drug seeking. For example, optical stimulation of BLA glutamate to NAc has been shown to be reinforcing, whereas optical inhibition of BLA-NAc reduced cue-induced sucrose intake (Stuber et al., 2011). Indeed, pharmacological disconnection has revealed that an amygdala-NAc interaction underlies cue-induced cocaine seeking (Di Ciano & Everitt, 2004b).

The CeA and BNST have generally been implicated in stress-induced ethanol-seeking reinstatement but may also play a role in cue-induced ethanol

reinstatement. For instance, intra-CeA but not intra-BLA infusion of mifepristone, a glucocorticoid receptor antagonist, has been reported to suppress reinstatement of ethanol seeking induced by the pharmacological stressor yohimbine (Simms, Haass-Koffler, Bito-Onon, Li, & Bartlett, 2011). Activation of group II metabotropic glutamate receptors blocks stress- and cue-induced reinstatement of ethanol-seeking presumably through CeA and BNST action (Zhao et al., 2006).

Lastly, additional regions that have been implicated in ethanol seeking using self-administration procedures include the dorsomedial striatum (Corbit, Nie, & Janak, 2012; 2014), medial prefrontal cortex (Dayas, Liu, Simms, & Weiss, 2007), prelimbic cortex and VTA (Brown et al., 2015). Importantly, the VTA, which is the chief source of DA input to NAc, amygdala and cortical regions, has been shown to be explicitly involved in context- and cue-induced ethanol seeking (Hauser et al., 2014; 2011; Löf et al., 2007). Overall, self-administration studies have been important in identifying several key neural substrates involved in ethanol-seeking behavior. Several of these structures have also been implicated in cue-induced ethanol seeking through ethanol-induced CPP procedures and thus are discussed in the next section.

Findings from Ethanol-Induced CPP Studies

Studies on the neural mechanism of ethanol-induced CPP can be grouped into two main categories, acquisition and expression. Acquisition studies are those that assess the development of ethanol-induced place preference. These typically include procedures where manipulations occur during the conditioning or

training phase, where animals learn to associate contextual cues with ethanol reward. Conversely, expression studies involve manipulations that occur after the conditioning phase and before preference testing. Expression studies, in particular, are useful in assessing ethanol-seeking behavior and conditioned reward. Below, findings from each of these types of ethanol-induced CPP studies are discussed. Given the focus of this dissertation, emphasis is placed on results obtained from expression studies conducted previously in our laboratory. Of note, relatively few labs study the primary and conditioned rewarding properties of ethanol using a CPP procedure. This is partly due to the difficult and unreliable nature of ethanol-induced place conditioning in rats (Fidler, Bakner, & Cunningham, 2004; Tzschentke, 2007). In view of this, all of the studies from our lab described below used male DBA/2J mice. This inbred strain will intragastrically and intravenously self-administer high levels of ethanol (e.g., Fidler et al., 2011; Grahame & Cunningham, 1997). Most importantly, this strain will rapidly and reliably develop an ethanol-induced CPP, even with a minimal amount of conditioning sessions (Cunningham, 1995; Pina et al., 2015; Pina & Cunningham, 2014a).

In this section, I provide an overview of studies that have directly examined the neural areas underlying the acquisition and expression of ethanol-induced place preference. From these studies, a proposed circuit diagram illustrating the neural structures implicated in each phase of ethanol-induced CPP has been developed and is included in Fig. 2. This diagram will be revisited and updated in the discussion portion of this dissertation (Fig. 19).

Acquisition. As with self-administration studies, the NAc and amygdala are the two most evaluated regions in terms of their involvement in ethanol induced CPP acquisition. Previously, our lab has investigated NAc[6] involvement in ethanol-induced place preference acquisition. In one study, bilateral electrolytic lesions of the NAc before CPP training disrupted the acquisition of CPP (Gremel & Cunningham, 2008). In a later study, bilateral NAc infusions of the D1-like receptor antagonist SCH-23390 disrupted the development of ethanol-induced CPP (Young, Dreumont, & Cunningham, 2014). This finding is similar to a finding reported in rats showing that nonselective DA antagonism prevented CPP induced by intracerebroventricular (icv) infusions of ethanol (Walker & Ettenberg, 2007). Combined, these studies indicate that the NAc is necessary for establishing associative relationships between ethanol reward and environmental cues, likely through a DA D1-like receptor-dependent mechanism. Other work from our lab has looked at the amygdala's[7] role in ethanol CPP acquisition (Gremel & Cunningham, 2008). Using electrolytic lesions, the amygdala was ablated bilaterally before CPP training. Amygdala lesions disrupted acquisition (and/or expression) of ethanol-induced place preference, suggesting this region may also be involved in ethanol-cue learning.

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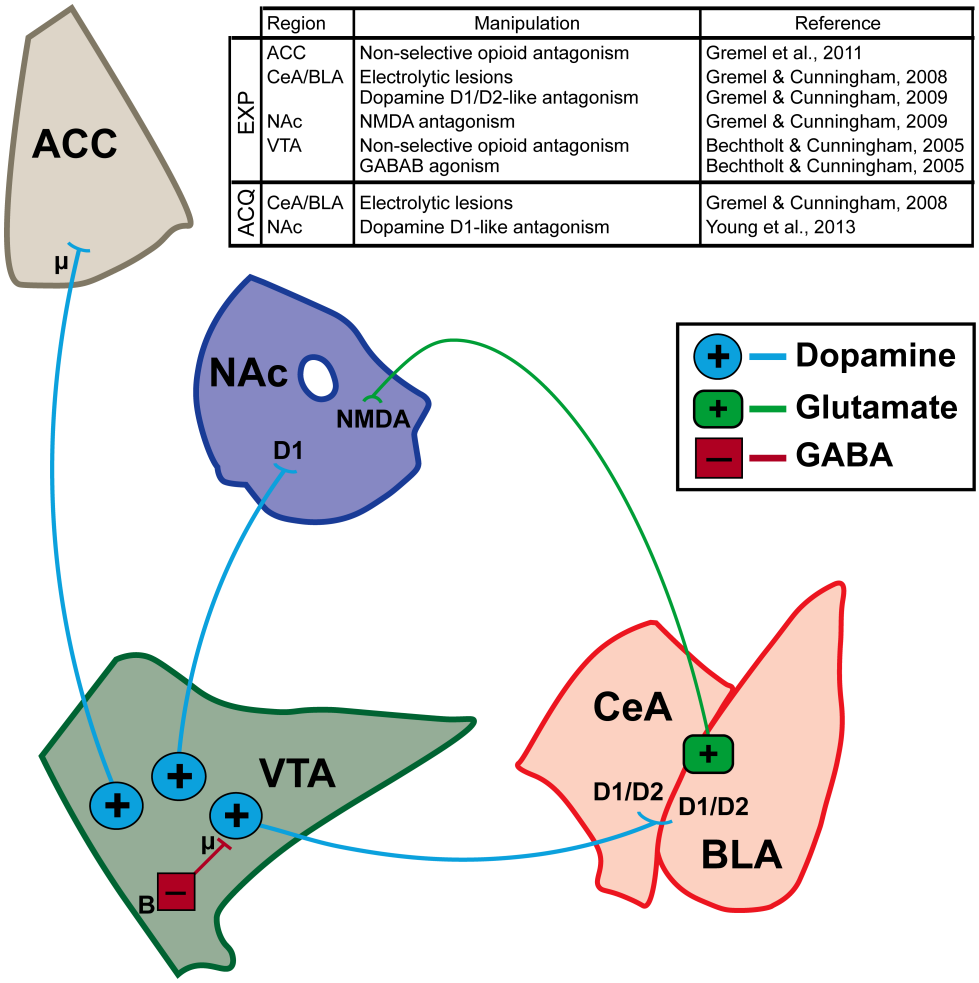
[6] Given the small scale of the mouse brain, divisions within nuclei can be difficult to precisely target without overlap. Therefore, when summarizing previous studies from our lab, no distinction will be made between the NAc core and shell.

[7] As with the NAc, no distinction is made between amygdala subdivisions as manipulations were generally directed at the basolateral (BLA) or central nucleus (CeA) of the amygdala.

Together, these findings demonstrate that these structures downstream from the VTA are necessary for the development of ethanol-induced CPP. Moreover, NAc involvement in ethanol-induced CPP is directly attributed to DAergic innervation, as activity at D1-like receptors in this region is necessary for acquisition. Though the literature here is sparse, it should be noted that prior to the work by Gremel & Cunningham (2008) there were no published studies directly examining the neural structures involved in the acquisition phase of ethanol-induced CPP. Another consideration is that acquisition studies involving microinjections can be problematic given the added handling required to administer the drug. Previous studies have indicated that microinjection-related handling can interfere with the acquisition of ethanol-induced CPP (Young et al., 2014). Though they do not require added handling prior, lesions are also problematic when administered pre-conditioning, as it unclear whether they affected the acquisition or expression phase (refer to Chapter 2 for further discussion).

Expression. Over the last decade, our lab has investigated the involvement of several brain areas in ethanol-induced place preference expression. These include the VTA, NAc, amygdala, and anterior cingulate cortex (ACC). Each of these structures is thought to be involved in reward and motivation partly through DA mechanisms and as such are part of a broader mesocorticolimbic DA system (detailed fully in Chapter 1).



	Region	Manipulation	Reference
EXP	ACC	Non-selective opioid antagonism	Gremel et al., 2011
	CeA/BLA	Electrolytic lesions	Gremel & Cunningham, 2008
		Dopamine D1/D2-like antagonism	Gremel & Cunningham, 2009
	NAc	NMDA antagonism	Gremel & Cunningham, 2009
	VTA	Non-selective opioid antagonism	Bechtholt & Cunningham, 2005
		GABAB agonism	Bechtholt & Cunningham, 2005
ACQ	CeA/BLA	Electrolytic lesions	Gremel & Cunningham, 2008
	NAc	Dopamine D1-like antagonism	Young et al., 2013

Figure 2. Diagram of the neural circuitry involved in ethanol-induced conditioned place preference. Previous studies from our lab have demonstrated the involvement of cortex (ACC), amygdala (BLA/CeA), striatum (NAc) and midbrain (VTA) in ethanol conditioned place preference (CPP). Each of these regions receives dopaminergic input from the VTA (indicated by blue arrows) and is part of a broader mesocorticolimbic dopamine system that underlies reward and motivation. Whereas all of these regions have been evaluated in the expression (EXP) phase of ethanol-induced place preference, fewer have been assessed during acquisition (ACQ). Some of the neurochemical signals underlying the involvement of each region in ethanol-induced CPP have also been identified. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area; +, excitatory; -, inhibitory; blue circles, dopamine cells; green rectangles, glutamate cells; red squares, GABA cells; green arrows, glutamate projections; μ, mu-opioid receptors; B, $GABA_B$ receptor; D1, dopamine D1-like receptor; D2, dopamine D2-like receptor; NMDA, ionotropic glutamate receptor.

While VTA DA cell activity is initially increased by rewarding stimuli, this diminishes over time with repeated reward exposures and subsequent learning (Schultz, 1986). Eventually, DA cell activity is no longer increased by presentation of a reward itself and is instead robustly increased by environmental stimuli that have become associated with the reward and predict its delivery or availability (Ljungberg, Apicella, & Schultz, 1992; Schultz, 1997). Thus, the involvement of this conditioned DA response in ethanol-induced CPP expression can be supported by studies focusing on the VTA or downstream sites, as described below.

Whereas involvement of the NAc in ethanol-induced place preference acquisition is clear, its involvement in expression is more complicated. In early work, the effect of intra-NAc infusion of methylnaloxonium on ethanol-induced CPP expression was tested (Bechtholt & Cunningham, 2005). This manipulation failed to impact preference expression, suggesting a lack of NAc opioid receptor involvement. A later study directed bilateral electrolytic lesions at the NAc after ethanol-induced CPP conditioning and before testing to isolate this structure's involvement in expression (Gremel & Cunningham, 2008). Overall, lesions made at this timepoint did not affect ethanol-induced place preference further suggesting that the NAc may be less involved in ethanol-induced CPP expression than anticipated.

However, additional pharmacological procedures have supported a more specific role for the NAc in CPP expression. In one study, intra-NAc antagonism of DA D1- and D2-like receptors prevented the expression of CPP induced by icv

ethanol (Walker & Ettenberg, 2007). In another study from our lab, NAc DA (D1- and D2-like) and glutamate (N-methyl-D-aspartate, NMDA) receptors were blocked during the CPP expression test using either flupenthixol or AP-5, respectively (Gremel & Cunningham, 2009). Whereas DA receptor antagonism did not affect ethanol-induced place preference, NMDA receptor antagonism reduced it, suggesting that the NAc's involvement in expression is specific to activity at NMDA receptors. In another study, unilateral AP5 administration into the NAc also disrupted ethanol-induced place preference expression (Gremel & Cunningham, 2010). Although this was a disconnection study designed to examine glutamate input to NAc from amygdala[8], its findings further illustrated the importance of NAc NMDA receptor activity in ethanol-induced CPP. Overall, these studies established a role for NAc NMDA but not DA receptors in ethanol-induced place preference expression and suggest that glutamatergic input from the amygdala may also be involved. Although DA input from the VTA to NAc is a hypothesized mechanism underlying drug seeking, these results suggest that it does not underlie ethanol seeking, at least as indexed by CPP. Finally, these studies serve to demonstrate that manipulations more selective than global inactivation or deletion of a structure may be necessary to appropriately gauge that structure's importance in behavior.

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[8] Although findings from Gremel & Cunningham, 2010 appeared to demonstrate that amygdala disconnection from NAc blocked ethanol-induced CPP expression, reduced CPP in mice unilaterally infused with AP5 in the NAc prevented this interpretation.

Accordingly, the amygdala's role in ethanol-induced CPP expression has also been addressed by our lab. Like lesions made prior to conditioning, bilateral electrolytic lesion of the amygdala made before the test phase blocked ethanol-induced place preference expression (Gremel & Cunningham, 2008). In addition, when bilaterally infused into the amygdala, the D1- and D2-like DA receptor agonist flupenthixol blocked ethanol-induced CPP expression (Gremel & Cunningham, 2009). Together, these results illustrate the amygdala's role in ethanol-seeking behavior and indicate the importance of DAergic input to this structure for ethanol-induced place preference expression.

Finally, the ACC has been the only cortical structure evaluated by our lab in the context of ethanol-induced CPP expression. In this study, ACC involvement in expression was assessed by bilaterally infusing the non-specific opioid receptor antagonist methylnaloxonium into the ACC before the ethanol-induced CPP test (Gremel, Young, & Cunningham, 2011). Intra-ACC infusion of methylnaloxonium disrupted ethanol-induced place preference expression, reducing its magnitude at the lowest dose and abolishing it at the highest dose. Hence, the ACC appears to modulate ethanol-induced CPP expression through an opioidergic mechanism.

In summary, findings from expression studies performed in our lab have demonstrated that the VTA, NAc, amygdala, and ACC are all structures involved in ethanol place preference expression. Infusion of a mixed opioid receptor antagonist into the VTA and ACC but not NAc interfered with ethanol-induced CPP expression. Similarly, lesions and mixed DA receptor antagonism disrupted

expression when targeted to the amygdala and not NAc. Involvement of the NAc in ethanol-induced CPP expression appeared to be confined to NMDA receptors only, suggesting a more explicit neurochemical mechanism underlies its involvement in ethanol-induced place preference expression.

Overall, these studies evaluated structures situated downstream from the VTA that have been implicated in drug seeking based on their efferent DAergic input. In addition to these studies demonstrating that downstream sites are involved, findings from Bechtholt & Cunningham (2005) further illustrate the importance of the VTA in ethanol-seeking behavior. As opioid receptors are situated presynaptically on local GABAergic inputs to DA cells, the authors hypothesized that methylnaloxonium blocked the activation of VTA GABAergic interneurons (presumably from endogenous opioid peptide inputs). This likely inhibited VTA DA activity, thereby reducing ethanol-induced place preference expression. Conversely, baclofen presumably reduced CPP by acting directly on VTA DA cells, because they express $GABA_B$ receptors. Although these findings and proposed mechanisms underscore the importance of VTA DA in ethanol-induced CPP expression they provide little information on the external inputs that modulate DA cell activity. Thus, the origins and neurochemical sources of VTA DA cell innervation involved in ethanol-seeking behavior remain unknown. Accordingly, the next section discusses the involvement of VTA input in reward and drug-seeking behavior and identifies several inputs that may be of importance.

Inputs to the Ventral Tegmental Area

Excitatory (glutamatergic) afferents of the VTA arise from virtually all structures to which this region projects with the exception of the NAc and lateral septum (LS), which provide strong GABAergic inputs (Geisler, Derst, Veh, & Zahm, 2007). This suggests that there is a broad network of excitatory reciprocal projections to and from the VTA, with much of the glutamatergic input to VTA neurons arising from subcortical regions that include but are not limited to the amygdala, mesopontine nuclei, lateral habenula and hypothalamus (Omelchenko & Sesack, 2007). Several of the most well supported subcortical sources of VTA input in positive motivational states are included in Table 2. Although this reciprocal flow of neurotransmission is also found between the VTA and cortex, the prefrontal cortex (PFC) serves as the only cortical source of glutamate to the VTA (Carr & Sesack, 2000). Most importantly, these glutamatergic afferents play a critical role in regulating VTA neuron firing. Specifically, glutamatergic input to the VTA appears to be critical for behaviorally relevant burst firing of VTA DA neurons (Overton & Clark, 1997). The resulting phasic release of DA from the VTA is intimately associated with goal-directed behaviors and drug reward (Wanat, Willuhn, Clark, & Phillips, 2009).

In addition to the contemporary methods described earlier, recent advances in immunohistological techniques have facilitated more precise mapping of afferent and efferent projections of the VTA. As a result, an updated view on the role of broad neural circuit activity (including that of the VTA) in relation to behavior has been formed. In this current view, the net result of cell-

specific projections to cell-specific targets are accounted for and the subsequent plotting of these circuits suggest a complex topographical map (Watabe-Uchida, Zhu, Ogawa, Vamanrao, Uchida, 2012). This map indicates the existence of an intricate network of connectivity designed to tightly regulate the activity of neuronal ensembles, which in turn orchestrate complex and divergent behaviors, even from within the same circuits. In the VTA specifically, not all inputs are alike in their behavioral consequences. Here, DA activity is governed by a complex network of cell-type specific neuron-to-neuron connections, the net effect of which can result in vastly different motivational states (Jennings et al., 2013; Lammel et al., 2012).

Several lines of evidence suggest the importance of monosynaptic inputs to VTA DA neurons (one-step inputs) in governing motivational behaviors. For example, investigation of the laterodorsal tegmental nucleus (LDT) has revealed that 80% of its glutamatergic afferents synapse onto VTA DA neurons directly (Omelchenko & Sesack, 2005). The importance of these glutamatergic inputs have been corroborated through electrophysiological analysis, which has shown this region is essential to VTA DA cell burst firing (Lodge & Grace, 2006). Moreover, *in vivo* stimulation of LDT glutamate afferents have been reported to selectively terminate on and stimulate a distinct population of VTA DA neurons, which thereby generate a reward-like state (Lammel et al., 2012).

The bed nucleus of the stria terminalis is another region upstream from the VTA that has been identified as critically involved in regulating the activity of DA cell activity (Georges & Aston-Jones, 2001; 2002; Jalabert, Aston-Jones,

Herzog, Manzoni, & Georges, 2009). Specifically, the BNST positively modulates VTA DA activity putatively through two primary and distinct mechanisms: (1) a direct glutamate projection to VTA DA neurons and (2) a direct GABA projection to VTA GABA neurons (Jalabert et al., 2009; Jennings et al., 2013; Kudo et al., 2014; 2012). Of note, recent evidence derived from studies using more advanced and selective tools suggests a BNST GABA input to VTA GABA neurons is the predominant source (Jennings et al., 2013). Of relevance, behavioral studies in rodents have demonstrated that the BNST underlies cue-elicited drug seeking. For example, transient inactivation of the BNST has been shown to prevent cue-induced reinstatement of cocaine seeking (Buffalari & See, 2011). Direct projections from the BNST to VTA appear to be important for cocaine-related behaviors. For example, disconnection of the BNST-VTA pathway has been shown to reduce expression of cocaine CPP (Sartor & Aston-Jones, 2012), but the role of that projection in ethanol seeking remains unknown. Additional discussion on the BNST and its VTA projections are included elsewhere in this dissertation (refer to Chapter 2, Chapter3, and Discussion sections). These sections further explore the involvement of the BNST and BNST-VTA circuit in ethanol-seeking behavior.

Table 2. Glutamatergic Inputs to VTA

	Input Region		Reference
1	Bed nucleus of the stria terminalis	(BNST)	1, 2, 3, 4, 5
2	Laterodorsal tegmental nucleus	(LDTg)	1, 2, 6, 7, 8
3	Lateral hypothalamic area	(LH)	1, 2, 9, 10
4	Ventral pallidum	(VP)	1, 2, 9
5	Lateral preoptic area	(LPO)	1, 2
6	Parabrachial nucleus	(PB)	1, 2
7	Reticular formation	(RF)	1, 2
8	Medial preoptic area	(MPA)	1, 2
9	Central nucleus of the amygdala	(CeA)	1
10	Subthalamic Nucleus	(Sth)	1

1) Watabe-Uchida et al., 2012; 2) Geisler et al., 2007; 3) Georges & Aston-Jones, 2001; 4) Georges & Aston-Jones, 2002; 5) Kudo et al., 2012; 6) Lodge & Grace, 2006; 7) Lammel et al., 2012; 8) Forster & Blaha, 2000; 9) Geisler & Wise, 2008; 10) You et al., 2001

Rationale for Dissertation

The goal of the work described in this dissertation was to identify the neural mechanisms that underlie the motivational effects of ethanol-associated cues and ethanol-seeking behavior. Specifically, this series of experiments was designed to test the role of intra-VTA ionotropic glutamate receptors (Chapter 1) as well as the involvement of the BNST (Chapter 2) and its input to the VTA (Chapter 3) in ethanol-induced CPP expression using a combination of classical and contemporary tools. This work was based on the hypothesis that ethanol-seeking behavior is driven by a direct input from the BNST to the VTA, putatively glutamatergic in nature.

In Chapter 1, the involvement of VTA ionotropic glutamate receptors (iGluRs) in ethanol-seeking behavior was assessed. A classical pharmacological strategy was used, where antagonists selective for NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors were bilaterally infused into the VTA before the ethanol-induced CPP test. A central hypothesis of this experiment was that glutamatergic input to VTA DA is involved in ethanol-seeking behavior. This idea was based on previous evidence indicating that exposure to drug-predictive cues triggers conditioned glutamate release in the VTA (You, Wang, Zitzman, Azari, & Wise, 2007), which serves to activate DA cells resulting in drug-seeking behavior (reviewed in Kalivas & Volkow, 2011). Although evidence for VTA glutamate input involvement in cue-induced seeking exists for several classes of abused drugs, few studies to date have assessed its role in the context of cue-induced ethanol seeking. As a result,

the contribution of glutamatergic input to the VTA during tasks of ethanol-conditioned reward is unknown. Hence, in this chapter I sought to first determine whether activity at VTA glutamate receptors was important for ethanol-seeking behavior. I hypothesized that blocking iGluRs in VTA would disrupt the expression of ethanol-induced CPP.

In Chapter 2, I evaluated whether a source of input to the VTA, the BNST was itself involved in ethanol-seeking behavior. Considering the previously reported role for the BNST in cue-induced drug seeking, I hypothesized that inhibition of this structure's activity during the expression phase would disrupt ethanol-induced CPP. Over several experiments, a combination of classical and contemporary tools was used to inhibit BNST activity. In the first experiment, bilateral electrolytic lesions of the BNST were made before conditioning or after conditioning but before the preference test. Lesions made at separate phases were designed to disentangle the BNST's involvement in expression (post-conditioning) from acquisition (pre-conditioning). Next, pharmacological microinjections were used to transiently inactivate the BNST during ethanol-induced CPP expression. In this experiment, a mixture of a $GABA_A$ and a $GABA_B$ receptor agonist (muscimol and baclofen, respectively) were bilaterally infused into the BNST just prior to the preference test. The agonists were intended to increase inhibitory GABAergic activity, thereby reversibly inhibiting the BNST during the ethanol-induced CPP test. In the third experiment, the BNST was chemogenetically inactivated during ethanol-induced place preference expression. A viral vector encoding for inhibitory hM4Di DREADDs (AAV5-hSyn-

HA-hM4D(Gi)-IRES-mCitrine) was delivered into the BNST before habituation. This resulted in ectopic expression of hM4Di receptors on BNST cells within 4-6 weeks. To reversibly inhibit BNST activity, hM4Di receptors were activated by peripheral injection of CNO before the ethanol-induced CPP test. A follow-up experiment served to functionally confirm hM4Di receptor-mediated inhibition. In this experiment, the BNST was engaged by ethanol-associated cue (CS+) exposure and its activity was measured by c-Fos immunoreactivity (IR; used here as a general marker of neuronal activity). The effect of CNO versus saline on CS+-induced c-Fos IR in the BNST of hM4Di-expressing mice was then assessed. The central hypothesis of this chapter was that inhibition of the BNST would reduce ethanol CPP expression. For DREADD control studies, I hypothesized that CNO-mediated hM4Di activation would reduce cue-induced BNST c-Fos IR and that CNO alone would not impact ethanol CPP expression.

The final experiments presented in Chapter 3 of this dissertation used a novel contemporary strategy to test the role of a direct BNST to VTA projection in cue-induced ethanol-seeking behavior. Specifically, hM4Di receptors were expressed selectively in VTA-projecting BNST cells using an intersectional strategy similar to those described earlier (Boender et al., 2014; Carter et al., 2013; Nair et al., 2013). Here, a long-term retrograde HSV vector encoding cre recombinase (hEf1α-EYFP-IRES-cre; HSV-cre) was infused into the VTA and a cre-inducible vector encoding for hM4Di receptors (AAV8-hSyn-DIO-hM4D(Gi)-mCherry) was infused into the BNST. Since retrograde transport times vary across regions, the first experiment of this chapter was designed to empirically

determine the optimal time for retrograde transit from the VTA to the BNST. Here, intra-VTA infusion of HSV was used to express a green fluorescent protein (GFP) in VTA-projecting cells. Tissue from the BNST was examined at 2, 3, and 4 weeks post-HSV infusion and the total number of GFP-positive (GFP+) cells was counted and compared between each delay timepoint. This yielded an ideal post-infusion delay for optimal HSV-mediated transgene expression, which was used to determine the total time required to achieve full expression of hM4Di receptors in BNST-VTA cells. Accordingly, both viruses were used in the next experiment to selectively express hM4Di in BNST-VTA cells. On test day, hM4Di receptors were activated by CNO to determine the effect of BNST-VTA circuit inhibition on ethanol-induced CPP expression. In a final experiment, I controlled for the impact of surgery, viral infusion, and CNO administration on ethanol-induced CPP by substituting HSV-GFP (no cre) for HSV-cre. This resulted in GFP but not hM4Di expression in BNST-VTA cells and also confirmed that, as expected, hM4Di expression was confined to BNST-VTA cells only (i.e., no transgene leakage) in the previous experiment. The main hypothesis tested in this chapter was a direct BNST projection to VTA is involved in ethanol-seeking behavior, as measured by CPP. As such, I predicted that chemogenetic inhibition of VTA-projecting BNST cells would disrupt the expression of ethanol CPP and that in the absence of hM4Di (i.e., in GFP control mice), CNO would not disrupt ethanol CPP expression.

In summary, the behavioral experiments described in this dissertation aimed to test the neurocircuitry underlying cue-induced ethanol-seeking

behavior, as indexed by ethanol-induced CPP. Focus was placed on examining the role of VTA glutamate input, the BNST, and BNST input to the VTA in ethanol-induced place preference expression using a combination of techniques. Overall, these experiments present novel findings that implicate distinct neural signals, neural structures, and a direct neural circuit in ethanol-seeking behavior.

Chapter 1

Involvement of Ventral Tegmental Area Ionotropic Glutamate Receptors in the Expression of Ethanol-Induced Conditioned Place Preference

Abstract

The ventral tegmental area (VTA) is a well-established neural substrate of reward-related processes. Activity within this structure is increased by both the primary and conditioned rewarding properties of abused drugs and its engagement is heavily reliant on excitatory input from structures upstream. In the case of drug seeking, it is thought that exposure to drug-associated cues engages glutamatergic afferents of the VTA that signal directly to dopamine cells, thereby triggering this behavior. It is unclear, however, whether glutamate input to the VTA is directly involved in cue-induced ethanol-seeking behavior. In this experiment, the role of glutamatergic input to the VTA in ethanol-cue seeking was evaluated in DBA/2J mice using an ethanol-induced conditioned place preference (CPP) procedure. Intra-VTA ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) were blocked during the ethanol-induced CPP expression (seeking) test by co-infusion of the antagonist drugs 6,7-dinitroquinoxaline-2,3-dione (DNQX; AMPA/kainate) and D-(-)-2-Amino-5-phosphonopentanoic acid (AP5; NMDA). Compared to saline, bilateral infusion of low (1 DNQX + 100 AP5 ng/side) and high (5 DNQX + 500 AP5 ng/side) doses of the AMPA/kainate and NMDA antagonist cocktail into VTA prevented ethanol-induced CPP expression. This effect was site specific, as infusions of DNQX/AP5 proximal to the VTA did not significantly impact preference expression. An increase in test activity was found at the high but not low dose of DNQX/AP5. This indicates that the impairment in ethanol-induced CPP expression was not due solely to a nonspecific effect of the

antagonists on locomotor activity during the preference test. These findings demonstrate that activation of ionotropic glutamate receptors within the VTA is necessary for cue-induced ethanol-seeking behavior, as measured by CPP.

Introduction

Dopaminergic (DA) transmission within the mesocorticolimbic system is thought to play a key role in motivated behavior. The predominant source of central DA, the midbrain (German & Manaye, 1993), has been the focus of considerable research aimed at understanding the neural events that promote reward seeking. Much of this work supports the idea that reward-related signals are predominantly generated by DA cells that originate in the substantia nigra (SN) and ventral tegmental area (VTA) (Wise, 2004).

For example, early work has established that midbrain DA neurons are phasically activated by primary rewards (Ljungberg et al., 1992; Schultz, 1986; Schultz, Apicella, & Ljungberg, 1993). Remarkably, reward-predicting stimuli also appear to elicit similar levels of phasic DA cell firing. In fact, after training and formation of stimulus-reward associations, the activity of midbrain DA neurons is increased almost exclusively by conditioned stimuli and not the primary rewarding stimulus (Ljungberg et al., 1992; Schultz, 1997). The idea that midbrain DA activity mediates reward and cue-induced motivated behavior is also supported by behavioral studies using animal models. For example, conditioned DA release in the nucleus accumbens (NAc) core has been observed following cocaine-associated cue presentation (Ito, Dalley, Howes,

Robbins, & Everitt, 2000). Accordingly, antagonism of DA D1-like receptors within the NAc reduces context-induced reinstatement of ethanol seeking (Chaudhri et al., 2009). Similarly, blockade of amygdala D1- and D2-like receptors inhibits cue-induced ethanol-seeking behavior as suggested by its disruption of ethanol conditioned place preference (CPP) expression (Gremel & Cunningham, 2009).

Additional studies identify the VTA, as a region fundamental to primary reward and cue-induced reward seeking. For instance, VTA inactivation reduced the acquisition and expression of morphine-induced CPP (Moaddab, Haghparast, & Hassanpour-Ezatti, 2009). Activating $GABA_B$ receptors in the VTA, which putatively inhibits DA activation, also reduced morphine-induced CPP acquisition (Tsuji et al., 1996) and ethanol-induced CPP expression (Bechtholt & Cunningham, 2005). Moreover, exposure to an ethanol-associated cue activated the VTA resulting in increased c-Fos immunoreactivity (Hill, Ryabinin, & Cunningham, 2007). These studies illustrate the VTA's importance in the acute rewarding effects of morphine and conditioned rewarding effects of morphine and ethanol.

Although a role for VTA activity in cue-induced seeking behavior has been established, less is known about what neurochemical inputs are responsible for the excitation of VTA DA cells during drug-associated cue exposure. Considering that activity of VTA dopamine cells is regulated in part by several glutamatergic afferents (Sesack, Carr, Omelchenko, & Pinto, 2003), it is highly likely that glutamate may be involved. It has been suggested that glutamate input to the

VTA may serve as a principal source of DA activation that is required for behaviorally relevant burst firing (White, 1996). Some direct evidence does indeed indicate that glutamate input to the VTA plays a critical role in the motivational effects of abused drugs and drug-associated cues. For example, intra-VTA glutamate receptor antagonism blocked the development of place preference for environmental stimuli paired with cocaine and morphine (Harris & Aston-Jones, 2003; Harris, Wimmer, Byrne, & Aston-Jones, 2004). Moreover, conditioned glutamate release in anticipation of drug delivery has been observed in VTA (You et al., 2007). Taken together, this literature suggests that glutamate may serve as an important source of VTA DA innervation and is a likely signal driving cue-induced drug seeking. Despite this, few studies have assessed glutamatergic involvement in conditioned reward using ethanol as a primary reinforcer.

In the present experiment, we assessed whether glutamatergic input to the VTA was involved in ethanol-cue seeking behavior. A well-characterized ethanol-induced CPP procedure (Cunningham et al., 2006) was used to establish an ethanol-cue association (acquisition) in order to evaluate the impact of ionotropic glutamate receptor antagonism on ethanol-cue seeking (expression). N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors were blocked in the VTA during the ethanol-induced CPP expression test. Based on the existing literature, we hypothesized that blocking the action of this excitatory input to the VTA would reduce ethanol-cue seeking.

Materials and Methods

Animals

Male DBA/2J mice (n = 123; The Jackson Laboratory, Sacramento, CA) were 6-7 weeks of age upon arrival. This inbred strain was chosen based on evidence from our laboratory showing DBA/2J mice consistently develop robust ethanol-induced CPP (Cunningham et al., 2006). Males only were included given the abundance of normative data derived from ethanol-induced CPP studies using male mice and the relative paucity of similar studies in female mice. Mice were housed in polycarbonate cages (2-4 per cage) lined with cob bedding in a colony room maintained at 21+/-1°C on a 12:12 h light-dark cycle with lights on at 7:00 am. All procedures were conducted during the light phase (7:00 am – 7:00 pm). Mice were given approximately one week to acclimate to the colony before surgery. During this time, mice were housed in groups of four. After surgeries, mice were housed 2 per cage to reduce headmount damage and cannula loss from allogrooming. Home cage access to lab chow (5L0D PicoLab® Rodent Diet, St. Louis, MO) and water was provided *ad libitum*. Procedures were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and carried out in compliance with the National Institutes of Health Guide For the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011).

Apparatus

Place conditioning was conducted using an unbiased two-compartment apparatus. Conditioning chambers (30 x 15 x 15 cm) composed of acrylic and aluminum were enclosed in ventilated boxes (Coulbourn Instruments Model E10-20) that attenuated light- and sound. Within each apparatus, six infrared phototransistors were used to detect locomotor activity and time spent on each side of the chamber. Infrared light emitting diodes were mounted opposite these detectors at 5-cm intervals, 2.2 cm above the floor on the front and rear sides of each inner chamber. During each session, locomotor activity and chamber position were continuously recorded by computer. Two distinct interchangeable floors placed inside the conditioning chamber served as tactile cues. Floors were characterized by a grid (2.3-mm stainless steel rods mounted 6.4 mm apart in an acrylic frame) or hole (16-ga. stainless steel sheets perforated with 6.4 mm diameter holes on 9.5-mm staggered centers) pattern. These floor cues are equally preferred by experimentally naïve DBA/2J mice (Cunningham et al., 2003), demonstrating the unbiased nature of the apparatus. A removable clear acrylic divider was used to separate floor cues and partition the apparatus into two compartments. To disperse olfactory cues, floors and chambers were wiped clean with a damp sponge between animals. Additional details about the apparatus and procedure can be found elsewhere (Cunningham et al., 2006).

Drugs

Ethanol (95%; Decon Labs, King of Prussia, PA) was prepared in a 20% v/v solution of 0.9% saline (Baxter International, Deerfield, IL) and administered

intraperitoneally (IP) at a dose of 2 g/kg in a 12.5 mL/kg volume. Vehicle injections of saline were also administered IP (12.5 mL/kg). All IP injections were delivered in 1cc Leur-Slip Monoject™ syringes (Medtronic, Minneapolis, MN) with attached 27 gauge, 0.5 inch Monoject™ plastic hub needles (Medtronic).

Stock solutions of the AMPA/kainate antagonist 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX; 1 mg/mL; Tocris, Minneapolis, MN) and NMDA antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (AP5; 10 mg/mL; Tocris) were prepared in artificial cerebrospinal fluid (aCSF; Tocris). Aliquots were stored at -80 ºC and diluted to final concentrations in aCSF then combined the day of use. Drugs were administered as a cocktail in final doses (in ng/100 nL/side) of 1 DNQX + 100 AP5 (DNQX/AP5 1 group) and 5 DNQX + 500 AP5 (DNQX/AP5 5 group).

Surgical Procedure

Anesthesia was induced with 4% isoflurane (Terrell™, Piramal Critical Care Inc., Orchard Park, NY) and maintained with 1-3% in oxygen with a flow rate of 1 L/min. Mice were secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and guide cannulae (10 mm, 25 ga.; Component Supply Co., Fort Meade, FL) were implanted 2.0 mm above the VTA (AP -3.2, ML ± 0.5, DV -4.69 mm, from bregma)[9] and held in place with permanent glass

[9] Coordinates were derived from a standard mouse brain atlas (Paxinos & Franklin, 2001) and selected based on literature suggesting that more medial aspects of the VTA are involved in approach behavior (Ikemoto, 2007).

ionomer luting cement (Ketac-Cem Maxicap; 3M ESPE, St. Paul, MN). Cannula patency was maintained by placing stainless-steel stylets (10 mm, 32 ga.; Small Parts Inc., Miami Lakes, FL) placed inside of each guide shaft. Mice were given 3-6 days of recovery before the start of behavioral procedures.

Histology

To verify the site of injection, tissue was collected within 24 h of the preference test and post-fixed by immersion in 4% (w/v) paraformaldehyde. Using a cryostat (Leica Biosystems Inc., Buffalo Grove, IL), 40-µm thick coronal sections were collected from -2.7 to -4.0 mm posterior to bregma (Paxinos & Franklin, 2001) and mounted on glass slides. Once dry, tissue was stained with 0.5% cresyl violet acetate (Sigma-Aldrich, St. Louis, MO) then coverslipped. Slides from each subject were imaged and recorded on an Olympus BX51 microscope equipped with an Olympus Q-Color 3™ digital camera. Photomicrographs were analyzed and used to determine the putative location of each infusion. Data from one mouse that received a unilateral injection of aCSF was excluded from statistical analyses. Data from mice that received bilateral injections of DNQX/AP5 outside of the VTA were collapsed across dose and analyzed as an additional control group (n = 2, n = 5 in DNQX/AP5 1 and 5 groups, respectively)[10]. A detailed list of total exclusions by group and reason for removal is included in Table 3.

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[10] Breakdown of “Miss” control mice by dose and conditioning subgroup: DNQX/AP5 1, n = 1 G+, n = 1 G-; DNQX/AP5 5: n = 2 G+, n = 3 G-.

Table 3. Subject Removal

			Reason for Removal			
Group	**Initial n**	**Final n**	**Illness/ Death**	**Equip. Error**	**Cannula Issues**	**Infusion Misses**
aCSF	58	48	6	1	3	1*
DNQX/AP5 1	31	25	1	-	3	2
DNQX/AP5 5	30	23	1	-	-	5

Deaths prior to group assignment due to surgery & recovery issues, n = 4

**This mouse received a unilateral infusion and was removed from statistical analyses*

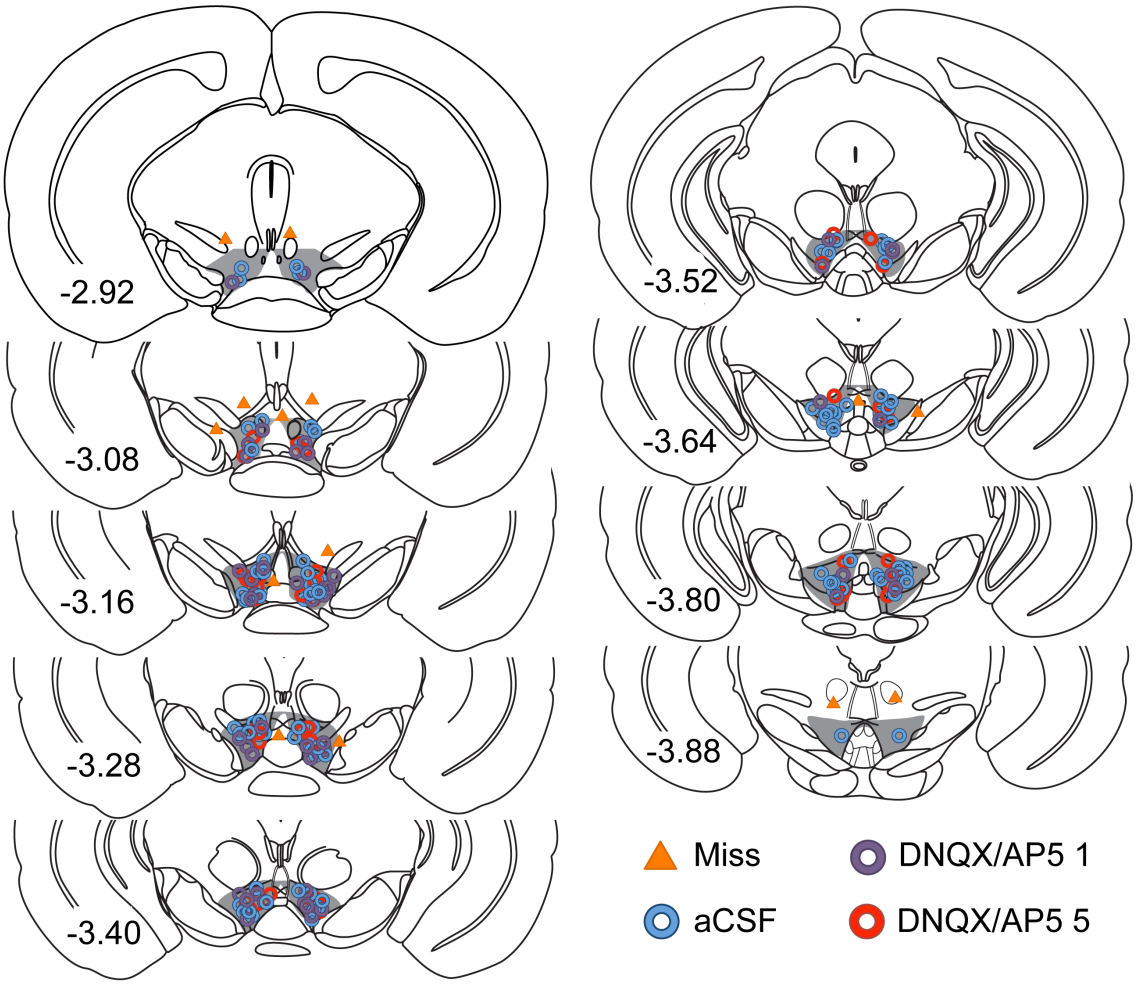

Figure 3. Schematic representation of coronal sections from the VTA showing the sites of infusion. Inclusion area for the VTA is highlighted in gray. Individual circles represent the actual placement of each infusion of aCSF (blue), 1 DNQX + 100 AP5 ng (DNQX/AP5 1, purple), and 5 DNQX + 500 AP5 ng (DNQX/AP5 1, red). The location of misplaced DNQX/AP5 infusions in mice that were included as additional controls (Miss) are presented as triangles. Numbers represent the distance of each section from bregma (in mm) based on a standard atlas of the mouse brain (Paxinos & Franklin, 2001).

CPP Procedure

The CPP procedure was conducted over a period of four days and consisted of the following phases: habituation (one 5-min session), conditioning (four 5-min sessions run twice daily), and preference testing (one 30-min session). Session durations were based on temporal parameters established by our laboratory that have been reliably shown to produce a robust ethanol-induced CPP in DBA/2J mice (Cunningham et al., 1997; 2006).

Habituation. This phase of the procedure was intended to reduce the novelty of the apparatus and stress associated with initial handling and injection. Habituation sessions were run between the hours of 12 pm - 2 pm. Briefly, mice were removed from their home cage, weighed, and given an IP injection of saline (12.5 mL/kg) immediately prior to being placed inside the apparatus on a plain white paper floor. No floor cues were present and the acrylic divider was removed during this phase, allowing the animal to roam the apparatus freely.

Conditioning. Place conditioning sessions were conducted across 2 days, with two sessions (1 CS- and 1 CS+) occurring each day. During conditioning, the acrylic divider was placed in the center of the apparatus to separate grid and hole floors. Mice were randomly assigned to one of three treatment groups based on DNQX/AP5 dose: aCSF (0 ng/side), DNQX/AP5 1 (1 DNQX + 100 AP5 ng/side), and DNQX/AP5 5 (5 DNQX + 500 AP5 ng/side). Each dose group was subdivided into counterbalanced subgroups based on their assigned conditioning floor (Grid+ or Grid-) and left versus right floor cue orientation (GH or HG). On conditioning trials, animals in the Grid+ conditioning

subgroup received ethanol paired with the grid floor and saline paired with the hole floor, whereas animals in the Grid- subgroup received ethanol paired with the hole floor and saline paired with the grid floor. All mice received saline (CS-) trials in the morning (10 - 11 am) and ethanol (CS+) in the afternoon (2- 4 pm).

Preference Testing. Place preference was tested 24 h after the final conditioning session between the hours of 12 pm - 2 pm. Acrylic dividers were removed and both floor cues were presented during the test. Before the start of the test session, mice were gently restrained, stylets removed, and custom-made injectors (32 ga., 12 mm) inserted into the VTA. Polyethylene (PE20; Intramedic™) tubing connected the injectors to 10-µL gastight Hamilton syringes operated by a programmable infusion pump (Model A-74900-10: Cole Palmer, Vernon Hills, IL). Bilateral infusions of 100 nL/side were delivered over 60 s and injectors were left in place for an additional 30 s to minimize the spread of fluid to adjacent brain areas and prevent diffusion up the injection tract. After microinfusions, mice were administered saline (12.5 mL/kg, IP) in place of ethanol and immediately placed in the test chamber.

General Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with the alpha level set at 0.05. Where appropriate, follow-up tests were performed to evaluate the pairwise differences among the means and p-values were Bonferroni corrected for the number of post-hoc comparisons. Analyses specific to preference data and locomotor activity are described in detail below.

Place Preference. Amount of time spent on the grid floor during the test session served as the primary dependent variable. This measure was derived from the recorded time (in sec) spent on the grid side of the apparatus divided by the test duration (30 min). This transformation yielded a dependent variable of time spent on the grid floor in units of sec/min, where 0 sec/min indicated complete aversion to and 60 sec/min indicated complete preference for the grid floor. Preference data were analyzed by two-way ANOVA (dose x conditioning), where dose refers to drug pretreatment groups (aCSF, 1, and 5)[11] and conditioning represents the assigned conditioning subgroup (Grid+ and Grid-). Replication also served as an additional between-groups factor for the common doses used in each replicate. Analyses yielding significant interactions were followed up with post-hoc pairwise comparisons, with p-values Bonferroni corrected for the number of comparisons between group means.

Locomotor Activity. Test activity was analyzed by one-way ANOVA (dose). Conditioning activity data were collapsed[12] across both trials of each type (CS+ and CS-) then analyzed using two-way mixed-factor ANOVA (dose x trial type), where "trial type" corresponds to ethanol (CS+) and saline (CS-) trials.

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[11] An additional control group labeled "Miss" was added after histology was performed. This group was comprised of mice that received bilateral DNQX/AP5 infusions outside of the VTA.

[12] These data were collapsed across trials after analysis of each conditioning trial separately showed that basal (CS-) and ethanol (CS+)-stimulated locomotor activity were generally consistent within each dose group across trials.

Results

A full list of exclusions is provided in Table 3. Mice were removed from analyses due to illness or death (n = 11), missed injections (n = 8), headmount or cannula issues (n = 7), and equipment error (n = 1). Of mice excluded due to missed injections, one received a unilateral infusion of aCSF into the VTA and was permanently removed from analyses. Data from the remaining mice with missed bilateral injections (n = 2, n = 5 in DNQX/AP5 1 and 5 groups, respectively) were combined and used as an additional control group labeled “Miss". This group served to test whether the effects of DNQX/AP5 on preference expression were site-specific. In these mice, infusions were delivered < 500 μm dorsal, medial, or lateral to the VTA (Fig. 3).

Initially, data were analyzed using three-way ANOVA (replication x dose x conditioning) to determine whether there were significant differences in grid times for each dose group (aCSF, 1, and 5)[13] between replications. After confirming there was no main effect of replication and no replication x dose, replication x conditioning, or replication x dose x conditioning interaction (F’s < 1), data from all replicates were combined. Data were then analyzed by two-way ANOVA (dose x conditioning) across all groups (aCSF, 1, 5 and Miss).

[13] The “Miss” control group was not included in these analyses, given the inclusion of both DNQX/AP5 doses and the insufficient number of “Miss” mice per replication (n = 1-4 per replicate).

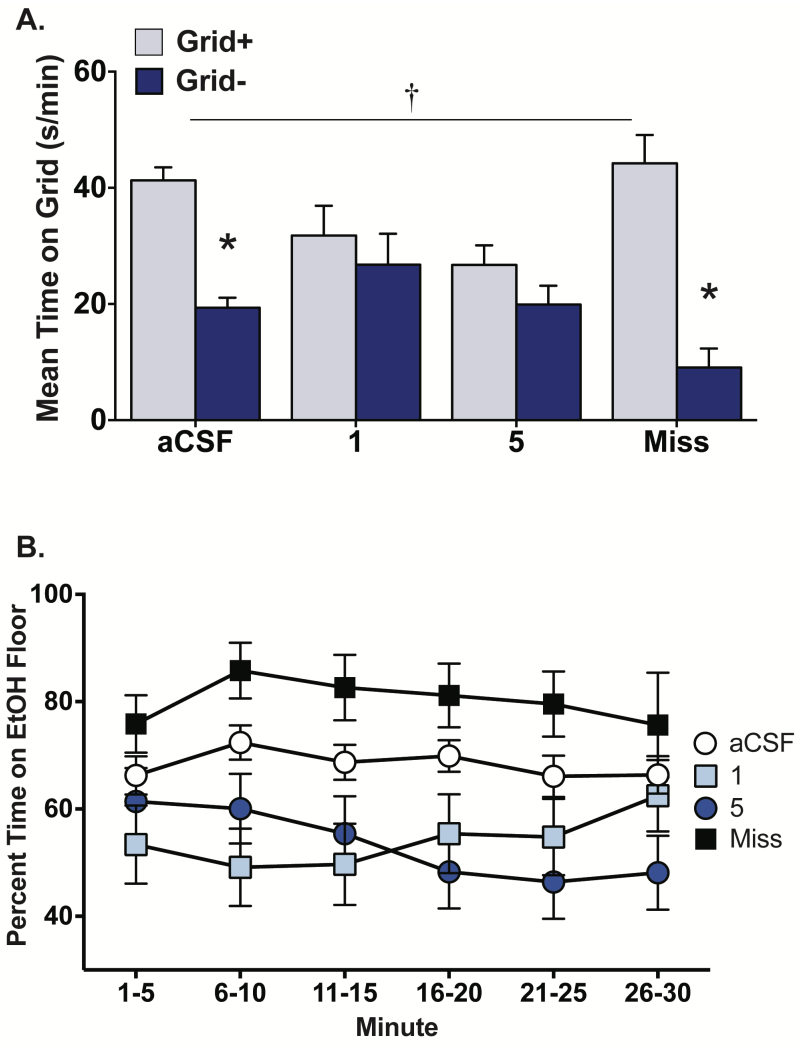

Figure 4. Antagonism of NMDA and AMPA receptors in the VTA blocks ethanol-induced CPP expression. (A) Data are expressed as mean time on the grid floor (s/min + SEM) for groups aCSF, 1 (1 DNQX + 100 AP5 ng/side), 5 (5 DNQX + 500 AP5 ng/side), and Miss. Ethanol-induced CPP expression was blocked by infusion of both doses of DNQX/AP5 into the VTA. Conversely, ethanol-induced CPP was not affected by aCSF infusion into the VTA or DNQX/AP5 infusion outside the VTA. This was supported by a dose (aCSF, 1, 5, Miss) by conditioning (Grid+ vs. Grid-) interaction and a significant difference between Grid+ and Grid- in the aCSF and Miss groups only; † $p = 0.004$ dose x conditioning; *$p \leq 0.001$ between Grid+ and Grid-. (B) Preference expression in 5-min intervals across the CPP test. Data are expressed as Mean (±SEM) percent time spent on the ethanol-paired floor. Across the test, mice that received intra-VTA infusion of DNQX/AP5 (groups 1 and 5) spent significantly less time on the ethanol-paired floor compared to mice in aCSF and Miss groups ($p = 0.002$ main effect of dose). n = 23-48 for aCSF, DNQX/AP5 1, DNQX/AP5 5, n = 7 for Miss.

Table 4. Chapter 1 Activity

Mean Activity Counts per Minute (±SEM) during conditioning and preference test.

Group	n	CS+ trials	CS- trials	Preference Test
aCSF	48	97.2 ± 4.8	46.3 ± 1.9	28.2 ± 1.5
DNQX/AP5 1	25	117.5 ± 5.6[a]	50.4 ± 2.1	23.3 ± 1.8[b]
DNQX/AP5 5	23	96.7 ± 5.2	45.2 ± 5.3	43.7 ± 8.7
Miss	7	84.4 ± 15.0	49.0 ± 1.8	49.4 ± 12.1

[a] Differs from aCSF, $p < 0.02$
[b] Differs from DNQX/AP5 5, $p < 0.05$

Place Preference. As shown in Figure 4A, expression of ethanol-induced CPP was blocked by intra-VTA infusion of DNQX/AP5. This effect was supported by a significant dose x conditioning interaction [$F(3,95) = 4.7$, $p = 0.004$], main effect of conditioning [$F(1,95) = 29.2$, $p < 0.001$], and no main effect of dose. Bonferroni-corrected pairwise comparisons showed that only control mice administered aCSF or DNQX/AP5 outside of the VTA expressed CPP. This was confirmed by significant differences in grid time between Grid+ and Grid- conditioning subgroups (p's ≤ 0.001) in groups aCSF and Miss only.

Time course analyses showed that preference for the ethanol-paired floor was immediately and consistently reduced in mice that received intra-VTA DNQX/AP5 compared to controls (Fig. 4B). Findings yielded a significant main effect of dose [$F(3,99) = 5.4$, $p = 0.002$] but no significant effect of interval or dose x interval interaction.

Test Activity. Test and conditioning activity means (±SEM) for each group are included Table 4. Locomotor activity during the preference test was significantly higher in mice administered the higher dose combination of DNQX/AP5 compared to those administered the lower dose combination. Analyses yielded a significant main effect of dose [$F(3,99) = 5.0$, $p = 0.003$] and post hoc comparisons showed that test activity significantly differed only between DNQX/AP5 5 and 1 dose groups ($p = 0.015$).

Conditioning Activity. Given that intra-VTA infusions did not occur until the test day, conditioning activity was influenced only by the presence of ethanol during this phase of the study (Table 4). Activity was higher on ethanol (CS+)

trials than on saline (CS-) trials, as is typically seen in DBA/2J mice (Cunningham et al., 1992). Significant main effects of trial type [$F(3,99) = 3.9$, $p = 0.012$] and dose [$F(1,99) = 338.9$, $p < 0.001$], but no dose x trial type interaction, were found. When trial type data were analyzed separately, a significant main effect of dose [$F(3,99) = 3.1$, $p = 0.032$] was found for ethanol (CS+) trials only. This was driven by a significant difference between DNQX/AP5 1 and aCSF groups (Bonferroni-corrected $p = 0.046$), as ethanol-induced locomotor activity was significantly higher in the DNQX/AP5 1 group compared to the aCSF group.

Discussion

In the present experiment, intra-VTA blockade of AMPA and NMDA receptors by antagonist drugs DNQX and AP5 prevented the expression of a place preference induced by ethanol. The effect of DNQX/AP5 on ethanol-induced CPP was irrespective of dose, as expression was blocked in DNQX/AP5 groups 1 (1 DNQX + 100 AP5 ng/side) and 5 (5 DNQX + 500 AP5 ng/side). In contrast, neither administration of aCSF in the VTA nor DNQX/AP5 outside the VTA significantly affected ethanol-induced CPP expression. The absence of effect in these controls suggests that reduced expression of ethanol-induced CPP in DNQX/AP5 groups was not due to a nonspecific effect of VTA manipulation (aCSF) or drug action at sites proximal to VTA (Miss), where drug may have spread. Moreover, the impact of DNQX/AP5 on preference expression cannot be solely attributed to its effects on activity. Only the higher dose combination of DNQX/AP5 (5 DNQX + 500 AP5) produced a significant increase

in locomotor activity compared to the lower dose combination (1 DNQX + 100 AP5), which prevented expression without significantly impacting activity. Overall, results demonstrate the importance of VTA glutamate input in ethanol-cue seeking, as indexed by CPP.

This experiment was designed to evaluate the involvement of VTA glutamate input in ethanol-seeking behavior induced by exposure to an ethanol-associated cue. The hypothesis that glutamate input to VTA is involved in this behavior was based on several key findings in the existing literature. First, systemic activity of glutamate systems has been shown to be important for ethanol-seeking behavior. For instance, it has been reported that NMDA/glycine and AMPA receptor antagonism blocks cue-induced reinstatement of ethanol seeking (Bäckström & Hyytiä, 2004). Previous studies have demonstrated a role for metabotropic glutamate receptors (mGluRs) in ethanol seeking. In one study, stress- and cue-induced ethanol seeking were reduced by activation of group II metabotropic glutamate receptors (mGluR) (Zhao et al., 2006), while in another, mGluR subtype 5 antagonism reduced cue-induced reinstatement of ethanol seeking (Schroeder, Spanos, Stevenson, & Besheer, 2008). Cue-induced reinstatement of ethanol seeking was increased by positive allosteric modulation of AMPA receptors (Cannady, Fisher, Durant, Besheer, & Hodge, 2012) and decreased by genetic deletion of the GluR-C AMPA subunit (Sanchis-Segura et al., 2006), further supporting a role for ionotropic glutamate receptors (iGluRs). In addition, recent work has indicated that glutamate inputs to VTA DA neurons influence ethanol relapse through combined action at NMDA and AMPA

receptors (Eisenhardt, Leixner, Luján, Spanagel, & Bilbao, 2015).

Next, the involvement of mesocorticolimbic DA in the conditioned motivational effects of reward-associated stimuli has been well established. Numerous studies have demonstrated increased release and/or activity of DA in terminal fields following exposure to cues associated with previous reward experience (Bassareo, De Luca, & Di Chiara, 2007; Blackburn, Phillips, Jakubovic, & Fibiger, 1989; Duvauchelle, Ikegami, & Castaneda, 2000a; Duvauchelle et al., 2000b). This increase terminal field release appears to promote reward seeking (Phillips, Stuber, Heien, Wightman, & Carelli, 2003) and is likely derived from the VTA, a principal source of DA in this pathway (Cachope & Cheer, 2014; Yun, Wakabayashi, Fields, & Nicola, 2004; Zhang, Doyon, Clark, Phillips, & Dani, 2009).

Beyond this, additional evidence implicates the VTA in reward-seeking behavior. Studies have shown that activation of appetitive behaviors, such as approach is associated with DA activity in the VTA, specifically (reviewed in Ikemoto, 2007; Wise, 2004). Not only is VTA engaged during seeking, but evidence suggests this region must be “online” and active to produce reward-related behavior. For example, transient inactivation of the VTA with lidocaine blocked expression of morphine CPP (Moaddab et al., 2009), whereas bupivacaine blocked pup- but not cocaine-induced CPP expression (Seip & Morrell, 2009). Combined intra-VTA administration of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen has been demonstrated to block drug-primed reinstatement of cocaine seeking (McFarland & Kalivas, 2001) and cue-

induced increases in responding for food (i.e., Pavlovian-instrumental transfer) (Murschall & Hauber, 2006). Notably, our lab has shown that baclofen administered into the VTA blocked the expression of an ethanol-induced CPP (Bechtholt & Cunningham, 2005), thus highlighting the necessity of the VTA and its activation in driving ethanol-induced CPP expression within our procedure.

Finally, there is some evidence to suggest that glutamatergic afferents of the VTA are essential for reward-seeking behavior. Glutamate input to VTA has been shown to be essential for the induction of phasic DA cell firing (for reviews see (Geisler & Wise, 2008; Kalivas, 1993; Overton & Clark, 1997; White, 1996). There is also evidence of drug-cue induced conditioned glutamate release in VTA (You et al., 2007), which indicates the functional relevance of this excitatory input. Moreover, blocking VTA glutamate receptors has been shown to reduce cue-induced reinstatement of morphine seeking (Bossert, Liu, Lu, & Shaham, 2004) and cocaine-primed reinstatement of drug-seeking (Sun, Akins, Mattingly, & Rebec, 2005).

Together, the literature indicates that glutamatergic innervation of the VTA may serve an essential role in triggering drug seeking induced by conditioned cue exposure. Our results support this idea and demonstrate that AMPA and/or NMDA receptor activation within the VTA are necessary for the expression of ethanol-induced place preference. Previously, our lab has shown that the VTA is activated by an ethanol-associated cue and inhibition of the VTA by baclofen blocks ethanol-induced CPP expression (Bechtholt & Cunningham, 2005). Here, we show that ionotropic glutamate receptor activation within the VTA is also

necessary for the expression of ethanol-induced place preference. This work extends our earlier findings and demonstrates that not only is ethanol-induced place preference expressed through the VTA, it is done so through a glutamatergic signaling mechanism.

Since AMPA and NMDA receptor antagonists were co-administered in this study, it is not clear from these data what individual contributions AMPA and NMDA receptors provide. Importantly, the present experiment was designed to more broadly assess the involvement of glutamate input to VTA in ethanol-induced CPP expression. Hence we chose to combine antagonist drugs targeting both AMPA and NMDA to comprehensively block glutamate innervation of VTA. These receptors were targeted based on evidence that VTA DA cells express both AMPA and NMDA ionotropic glutamate receptors (Albin et al., 1992). However, it is possible that one of these receptors may play a more important role in preference expression. Future studies will be needed to determine whether there are distinct roles for AMPA or NMDA receptors in ethanol-induced CPP expression.

As discussed earlier in this document, the hypothesis that VTA glutamate input is involved in reward seeking is largely driven by the idea that it directly excites DA neurons, a known substrate of reward-related behavior. However, given the limitations of intracranial pharmacological manipulations, we cannot be certain that VTA DA cells were targeted directly. Thus, it is possible that ethanol-induced CPP expression is driven by something beyond a direct projection of outside glutamate onto VTA DA cells. Of note, the VTA consists of several cell

types that include dopamine, glutamate, and GABA (Dobi, Margolis, Wang, Harvey, & Morales, 2010; Nair-Roberts et al., 2008; Swanson, 1982). Overall, DA is the most predominant cell type of the VTA and comprises approximately 65% of the region's cells (Nair-Roberts et al., 2008; Swanson, 1982). These stereological estimates suggest that the remaining population of non-dopaminergic cells in the VTA ranges from 30-35% GABAergic (Dobi et al., 2010; Nair-Roberts et al., 2008; Swanson, 1982) and 2-3% glutamatergic (Nair-Roberts et al., 2008). Therefore, it is likely that AMPA and NMDA receptors were blocked across a heterogeneous population of cells and this may have produced the reduction in ethanol-induced CPP. This seems like an unlikely explanation when considering the VTA's population of GABA neurons, however, given their inhibitory function. Indeed, it would be expected that blocking excitatory input onto inhibitory GABAergic interneurons would serve to disinhibit VTA DA activity and produce effects opposite from those observed.

It seems equally unlikely that the present findings were due to iGluR blockade on glutamate cells, given their low prevalence in the VTA. However, it is known that VTA glutamate neurons do make contact with local DA cells, and these synapses have been shown to be functional (Dobi et al., 2010). Therefore, we must consider the possibility that our manipulation blocked ionotropic glutamate receptors on VTA glutamate neurons that serve to locally innervate DA cells. This also suggests that some glutamate input to VTA DA may arise from within the VTA itself.

In addition, several questions still remain about the critical source(s) of

VTA input involved in ethanol-cue seeking, such as 1) what is the neurochemical nature of the input and 2) from where does it arise. Some evidence has implicated opioid signaling to VTA as a potential substrate of ethanol-seeking behavior measured by CPP (Bechtholt & Cunningham, 2005). Other studies detailed earlier have identified glutamate as a key source of VTA input involved in motivated behavior. However, more recent work has indicated GABAergic input may similarly important. Indeed, it is possible that GABAergic input to VTA GABA cells drives seeking behavior by disinhibiting glutamate and DA cells in VTA. This seems plausible given that VTA GABAergic interneurons modulate DA cell activity in a bi-directional manner, indicating their potential importance in reward processes (reviewed in Creed, Ntamati, & Tan, 2014). Notably, a recent study has illustrated the importance of inhibitory input to the VTA, as GABAergic input from the BNST was found to preferentially innervate VTA GABA cells to produce reward-related behaviors (Jennings et al., 2013). These findings strongly suggest that GABAergic afferents to the VTA may be equally important for triggering seeking behavior depending on the type of VTA cells they target. Given the complex interactions between VTA afferents and cellular activity within the VTA, we include a simplified diagram (Fig. 5) to illustrate the possible effects of our manipulation.

During the test, locomotor activity in the higher dose combination (5+500 ng/side) DNQX/AP5 group was significantly increased compared to the lower dose combination (1+100 ng/side) group. Although a trend toward increased activity was found in high dose DNQX/AP5-treated mice compared to the aCSF-

treated controls, neither a low dose of DNQX/AP5 nor infusion of DNQX/AP5 outside of the VTA (Miss) significantly affected activity compared to aCSF. Notably, activity in mice that received misplaced infusion of DNQX/AP5 was elevated compared to the lower dose and aCSF groups, an effect that would likely have been significant if the group size was increased and error decreased. This fits with the finding of increased activity in high dose DNQX/AP5-treated mice, as the majority of mice included in the Miss group received the high dose of antagonist drugs (n = 5 of 7 total). In general, these findings are in agreement with previous studies, as increased activity following intra-VTA antagonism of NMDA receptors with AP5 has been reported (Cornish, Nakamura, & Kalivas, 2001; Harris et al., 2004; Harris & Aston-Jones, 2003; Kretschmer, 1999). Moreover, these studies point to NMDA receptor antagonism by AP5 as the driving force behind the observed increase in basal locomotor activity.

Previously, a significant negative correlation between test activity and preference expression has been reported (Gremel & Cunningham, 2007). Therefore, results from the CPP test must be carefully interpreted when an activity effect is noted, as increased test activity can compete and interfere with preference expression. Although it is possible that increased activity in the higher dose DNQX/AP5 group disrupted ethanol-induced place preference expression, results from the lower dose DNQX/AP5 group would argue against this explanation alone, as preference was disrupted while activity was unaffected. Moreover, test activity levels were similarly elevated in the group (Miss) that received DNQX/AP5 outside of the VTA. Notably, this control group showed

significant place preference suggesting that DNQX/AP5-induced activity increases alone did not impact place preference expression. Thus, it is unlikely that reduced preference in the DNQX/AP5 5 group was simply an artifact of increased activity.

The present findings demonstrate that expression of ethanol-induced place preference requires the activation of ionotropic glutamate receptors within the VTA. This further identifies the VTA as an important neural substrate underlying expression of the conditioned rewarding effects of ethanol and indicates that excitatory input to VTA may drive ethanol-cue seeking behavior. Additional studies are needed to identify the key sources of input to VTA that are responsible for its innervation, specifically related to cue-induced seeking behavior. In the next chapter, I explore one known afferent of the VTA, the bed nucleus of the stria terminalis (BNST), in the expression of ethanol-induced place preference. Notably, the BNST sends a dense projection to the VTA (Dong & Swanson, 2004), is involved in cocaine-induced CPP (Sartor & Aston-Jones, 2012), and is activated by an ethanol-associated cue (Hill et al., 2007). Therefore, Chapter 2 evaluates the role of the BNST in ethanol-induced CPP expression.

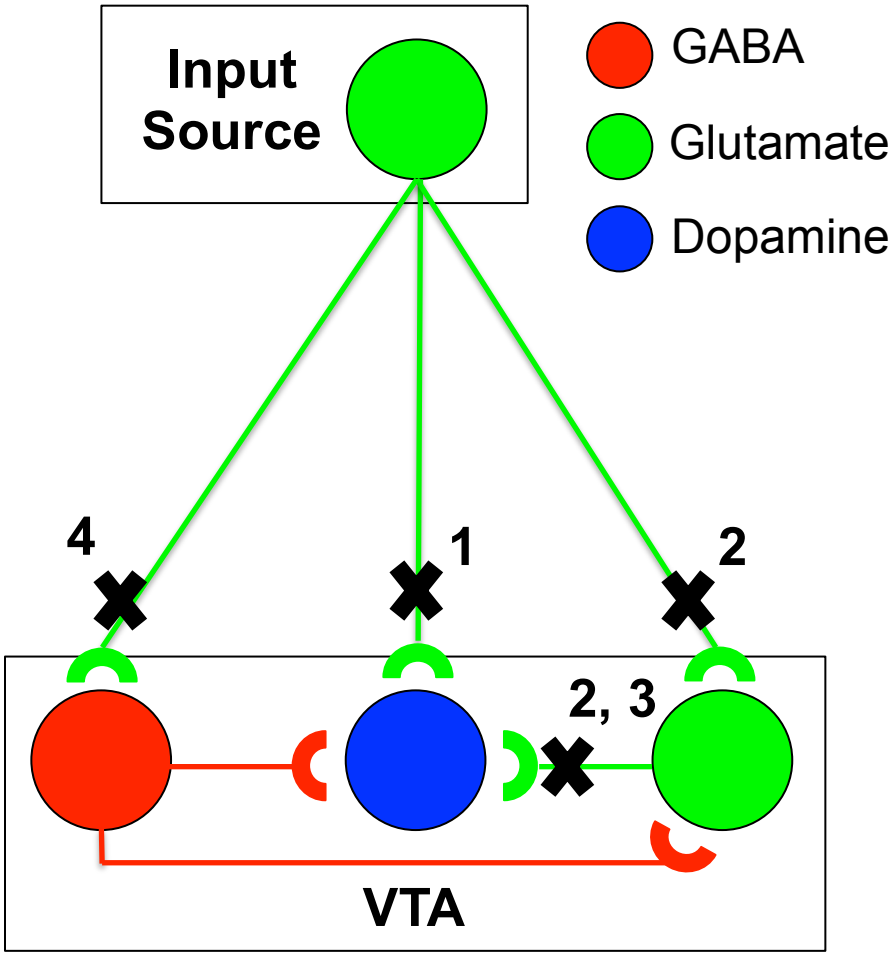

Figure 5. Proposed circuit of afferent inputs to VTA and possible target(s) of ionotropic glutamate receptor (iGluR) antagonism.
1) Direct innervation of VTA DA cells from an outside glutamate source was blocked by iGluR antagonism. 2) Direct innervation of VTA glutamate cells from an outside glutamate source was blocked by iGluR antagonism. This may have additionally blocked local glutamate input to VTA DA. 3) Local innervation of VTA DA cells by VTA glutamate was blocked by iGluR antagonism. 4) Direct innervation of VTA GABA interneurons was blocked by iGluR antagonism. However iGluR blockade on VTA GABA would disinhibit local VTA DA and glutamate cells leading to a net excitatory effect in VTA. X's represent the possible source of glutamate input being antagonized on the target post-synaptic cells.

Chapter 2

The Bed Nucleus of the Stria Terminalis Regulates Ethanol-Seeking Behavior*

*This chapter is based on the following paper: M.M. Pina, E.A. Young, A.E. Ryabinin, C.L. Cunningham, 2015. The Bed Nucleus of the Stria Terminalis Regulates Ethanol-Seeking Behavior in Mice. Neuropharmacology 99, 627–638. Permission to re-use the published content has been obtained from Elsevier (License #3767531344861).

Specific Acknowledgments: C.L.C., E.A.Y., M.M.P. designed research; E.A.Y. performed research in Experiments 1 and 2; M.M.P. performed research in Experiments 3, 4 and 5; A.E.R. provided training and intellectual support.

Abstract

Drug-associated stimuli are considered important factors in relapse to drug use. In the absence of drug, these cues can trigger drug craving and drive subsequent drug seeking. One structure that has been implicated in this process is the bed nucleus of the stria terminalis (BNST), a chief component of the extended amygdala. Previous studies have established a role for the BNST in cue-induced cocaine seeking. However, it is unclear if the BNST underlies cue-induced seeking of other abused drugs such as ethanol. In the present set of experiments, BNST involvement in ethanol-seeking behavior was assessed in male DBA/2J mice using the conditioned place preference procedure (CPP). The BNST was inhibited during CPP expression using electrolytic lesions (Experiment 1), co-infusion of $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen (M+B; Experiment 2), and activation of inhibitory designer receptors exclusively activated by designer drugs (hM4Di-DREADD) with clozapine-N-oxide (CNO; Experiment 3). The magnitude of ethanol-induced CPP was reduced significantly by each of these techniques. Notably, infusion of M+B (Experiment 2) abolished CPP altogether. Follow-up studies to Experiment 3 showed that ethanol cue-induced c-Fos immunoreactivity in the BNST was reduced by hM4Di activation (Experiment 4) and in the absence of hM4Di, CNO did not affect ethanol-induced CPP (Experiment 5). Combined, these findings demonstrate that the BNST is involved in the modulation of cue-induced ethanol-seeking behavior.

Introduction

Drug addiction is a chronic disorder characterized by periods of abstinence and relapse, where relapse to use is often preceded by intense desire for the drug (craving) and the subsequent motivation to obtain the drug (seeking). It is known that environmental contexts and discrete cues therein contribute to relapse by triggering craving (Ehrman, Robbins, Childress, & O'Brien, 1992; Grant et al., 1996; Sinha & Li, 2007) and driving drug seeking even after sustained periods of abstinence or extinction (Ciccocioppo et al., 2001b; Ciccocioppo, Angeletti, & Weiss, 2001a; Crombag & Shaham, 2002; Weiss et al., 2001; Zironi, Burattini, Aicardi, & Janak, 2006). These cues become associated with the rewarding and aversive properties of drugs through a Pavlovian learning process. It is the result of this learning, in addition to drug exposure, that leads to alterations in neural structures associated with motivation and reward.

Over the past several decades progress has been made in identifying the neurobiological substrates underlying drug craving and seeking. One neural structure routinely implicated in relapse and drug-seeking processes is the bed nucleus of the stria terminalis (BNST), a chief component of the extended amygdala (Alheid, 2003). Anatomically, the BNST is a complex cluster of nuclei and there is some disagreement regarding the total number of subdivisions and their boundaries (Ju & Han, 1989). However, it is clear that the dorsal and ventral subdivisions of the BNST (dBNST and vBNST) send dense projections to the ventral tegmental area (VTA) (Dong & Swanson, 2004; 2006b; 2006a; Kudo et al., 2012; Mahler et al., 2014), a region critical for reward seeking (Adamantidis

et al., 2011; Bechtholt & Cunningham, 2005; Di Ciano & Everitt, 2004b). Moreover, BNST inputs to the VTA appear to potently innervate dopamine (DA) neurons (Georges & Aston-Jones, 2001; 2002) leading to their phasic excitation, which is a neural process fundamental to motivated behavior (Adamantidis et al., 2011; Schultz, 1986; Wanat et al., 2009).

Presentation of drug-associated stimuli leads to pronounced activation in dBNST and vBNST, as indicated by increased c-Fos immunoreactivity (Hill et al., 2007; Mahler & Aston-Jones, 2012). In addition, pharmacological inactivation of several BNST subdivisions has been shown to reduce drug-seeking behavior induced by conditioned cue exposure. For example, inactivating the vBNST blocked the expression of cocaine-induced conditioned place preference (CPP) (Sartor & Aston-Jones, 2012). Likewise, inactivation across dBNST and vBNST has been shown to block cue-induced reinstatement of cocaine seeking (Buffalari & See, 2011). In other studies, it appears that vBNST inactivation blocks heroin-primed reinstatement while medial posterior BNST inactivation blocks heroin and cue-primed reinstatement (Rogers, Ghee, & See, 2008). These findings support a role for the BNST in cue-induced drug seeking and suggest that the involvement of distinct subdivisions may vary by drug of abuse.

As illustrated by the above studies, a broad range of work has identified the BNST as an important candidate neural structure involved in relapse. However, the majority of this work has examined cue-induced seeking of cocaine and heroin. Therefore, it is not known whether these findings extend to other drugs such as ethanol. Previously, our lab identified the BNST as one of several

areas activated by presentation of an ethanol-associated cue (Hill et al., 2007). Beyond this, little evidence exists to indicate that the BNST is involved in cue-induced ethanol-seeking behavior. Therefore, our goal was to directly examine this region in the context of cue-induced ethanol seeking using an ethanol-induced CPP procedure that has been well-established by our laboratory (Cunningham et al., 2006).

To evaluate the BNST in ethanol seeking, we used electrolytic lesions, pharmacological inactivation, and chemogenetic inhibition. Given the limitations inherent to each of these intracranial manipulations, we sought to increase the generality of our conclusions by incorporating all three techniques. These manipulations were intended to inhibit BNST activity during ethanol-induced CPP expression. Based on the existing literature, we reasoned that inhibiting the BNST by each of these techniques would disrupt ethanol-induced place preference expression.

Materials and Methods[14]

Animals

Adult male DBA/2J mice (n = 214) were purchased from Jackson Laboratory (Sacramento, CA) at 6-7 weeks of age. Mice were housed 2-4 per

[14] Additional details on the general materials and methods can be found in Chapter 1.

cage in a colony room maintained at 21+/-1°C on a 12:12 light-dark cycle with lights on at 07:00 am. Food and water were available *ad libitum* in home cages throughout the experiment. Surgeries were performed on mice 6-11 weeks of age. All procedures were carried out in accordance with the National Institutes of Health Guide For the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011) and were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Drugs

Ethanol (95%) was prepared 20% v/v in a solution of 0.9% saline and administered intraperitoneally (IP) at a dose of 2 g/kg in a 12.5 mL/kg volume.

In Exp. 2, the BNST was transiently inactivated using a cocktail of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen (M+B; Sigma-Aldrich, St. Louis, MO). Muscimol (0.1 mM) and baclofen (1.0 mM) were dissolved in 0.9% saline and administered bilaterally (100 nL/side) into the BNST. Inactivation of the BNST with these concentrations has previously been shown to reduce cue-induced cocaine and heroin seeking in operant self-administration procedures (Buffalari & See, 2011; Rogers et al., 2008). Infusions were delivered over 60 s and injectors were left in place for an additional 30 s to allow for complete diffusion of drug from the injectors.

In Exps. 3, 4, and 5, clozapine-N-oxide (CNO; Tocris Bioscience, Ellisville, MO) was dissolved in 0.9% saline and administered at 10 or 20 mg/kg (10 mL/kg, IP) 30 min before the CPP test. These doses were selected based on the following considerations. First, compared to Gq-coupled (hM3Dq) DREADDs,

which are very effective at eliciting neuronal firing, Gi-coupled (hM4Di) DREADDs are reportedly less effective at inhibiting activity and may therefore require higher CNO doses (Farrell & Roth, 2013). Indeed, functional assays have demonstrated that CNO is less potent at hM4Di (EC50 = 153 nM) as compared to hM3Dq (EC50 = 29.6 nM) (Armbruster et al., 2007)[15]. Moreover, these doses were based on previously published studies showing that CNO alone produced no physiological or behavioral response in rodents at doses of 10 mg/kg and above (Li et al., 2013; Mahler et al., 2014; Ray et al., 2011; 2013; Vazey & Aston-Jones, 2014). Finally, a maximum dose of 20 mg/kg was specifically chosen for a control experiment as it was, to our knowledge, the highest reported in the literature (Mahler et al., 2014).

Stereotaxic surgery

General procedure. In Exps. 1-4, mice were anesthetized with isoflurane (1-4% in O_2) and placed in a stereotaxic apparatus (Model No. 1900; Kopf Instruments, Tujunga, CA). Non-steroidal anti-inflammatory drugs, meloxicam (0.2 mg/kg) or carprofen (0.1 mg/kg) were injected subcutaneously (in 10 mL/kg) immediately before and 24 h after surgery to minimize post-operative discomfort. Coordinates targeting the BNST (from bregma: AP +0.14 L ±0.8 DV -4.25) were used in Exps. 1a, 1b and 2 based on a standard mouse brain atlas (Paxinos &

[15] The EC50 values reported here were derived from an assay of CNO-induced Ca2+ release from intracellular stores in immortalized human pulmonary artery smooth muscle cells (hPASMCs) expressing hM4Di and hM3Dq receptors (Armbruster et al., 2007).

Franklin, 2001). In Exps. 3 and 4, the lateral ventricles were avoided during virus infusions by approaching the BNST at a 20˚ angle. Burr holes were drilled laterally ±2.3 mm from bregma (AP +0.26) and injectors were lowered 4.33 mm from the top of the skull. These values were derived from the atlas-based coordinates: AP +0.26, ML ±0.8, DV -4.07.

Electrolytic lesions. In Exps. 1a and 1b, electrodes (Rhodes Medical Instruments, Woodland Hills, CA) were lowered bilaterally into the BNST to administer electrolytic (0.5 mA for 10 s) or sham (no current) lesions (Model 3500; Ugo Basile, Varese, Italy). Due to reduced body weights in the BNST lesioned group, mice were given 8-13 days of recovery before the start of conditioning (Exp. 1a) or the CPP test (Exp. 1b). This recovery period ensured weights between lesioned and sham mice were comparable.

Cannulations. In Exp. 2, bilateral cannulae (10 mm, 25 ga) were implanted 2.0 mm above the BNST and held in place with carboxylate cement (Durelon™, 3M, St. Paul, MN) anchored to the skull with stainless steel screws. To maintain patency, stainless-steel obturators (10 mm, 32 ga) were placed inside cannula. Mice were given 3-7 days of recovery before habituation.

AAV vector infusions. To silence BNST neurons, we also used a chemogenetic technique involving Designer Receptors Exclusively Activated by Designer Drugs (DREADDs; Armbruster et al., 2007). AAV5-hSyn-HA-hM4D(Gi)-IRES-mCitrine (3.9×10^{12} virus molecules per mL) was purchased from the University of North Carolina Vector Core (Chapel Hill, NC). Virus was stereotaxically infused using injectors made of 32-ga stainless steel tubing

encased by 26-ga stainless steel. Injectors were attached via polyethylene tubing (PE-20) to 1 µl Hamilton syringes. Infusions of 150 nL/side were delivered by syringe pump (Model PHD 22/2000; Harvard Apparatus, Plymouth Meeting, PA) at a rate of 15 nL/min. To ensure complete diffusion of virus and minimize tracking upon removal, injectors were left in place for 5 min after infusions. Approximately 4-6 weeks were allowed for transgene expression and animal recovery.

Histology

Placement verification. Lesion (Exps. 1a and 1b) and injection (Exp. 2) sites were determined through histological analysis (Fig. 6). Brain tissue was collected within 24 h of the CPP test and immersed in 2% paraformaldehyde (PFA) for 24 h then cryoprotected in 20% sucrose in phosphate-buffered saline (PBS), followed by 30% sucrose/PBS. Using a cryostat, coronal sections (40 µm) were taken from the rostral to caudal end of the BNST (from +0.62 to -0.46 mm from bregma) then stained with 0.1% thionin.

Immunohistochemistry. In Exps. 3 and 4, brains were immersed in 4% PFA/PBS overnight following extraction then cryoprotected as described above. Coronal sections (30 µm) were sliced on a cryostat and stored in a solution of 0.1% NaN_3/PBS at 4ºC until immunohistochemical analysis.

In Exp. 3, mice were deeply anesthetized with CO_2 and transcardially perfused with ice cold 4% PFA/PBS, 24-48 h after the CPP test. Free-floating sections were processed for immunofluorescence to detect the HA-tagged hM4Di protein. Briefly, sections were washed in PBS and permeabilized with 0.4%

Triton X-100/PBS for 1 h, then blocked with 1% bovine serum albumin (BSA)/5% normal goat serum (NGS)/0.4% Triton X-100/PBS for 1 h. Sections were incubated overnight with gentle agitation at 4ºC in 1% BSA/5% NGS/0.4% Triton X-100/PBS containing a rabbit monoclonal antibody against HA (1:500; C29F4, Cell Signaling). Next, sections were rinsed in PBS and incubated for 2 h in Alexa Fluor 555 labeled goat anti-rabbit IgG (1:300; A-21428, Invitrogen). After final washes, sections were rinsed in PBS, mounted on gelatinized slides, and coverslipped using ProLong Gold Antifade mountant with DAPI (Life Technologies). Images were captured with a Leica DM4000 B microscope and cropped and contrast adjusted using Fiji software (NIH).

In Exp. 4, mice were sacrificed via CO_2 and brains were collected 90 min after CS+ re-exposure. Tissue was later processed for c-Fos immunoreactivity (IR) as an indicator of neuronal activity. Sections were washed in tris-buffered saline (TBS) then immersed in freshly prepared sodium borohydride ($NaBH_4$, 1% w/v in TBS) for 30 min to reduce fixative-induced autofluorescence (Beisker, Dolbeare, & Gray, 1987; Tagliaferro, Tandler, Ramos, Pecci Saavedra, & Brusco, 1997). Several washes fully removed $NaBH_4$ before the tissue was blocked with 1% BSA/5% NGS/0.3% Triton X-100/TBS for 45 min. Next, sections were incubated overnight in block containing a rabbit polyclonal primary antibody directed against c-Fos (1:2000; sc-52, Santa Cruz Biotechnology). Sections were then rinsed, blocked as above, and incubated for 2 h in a biotinylated goat anti rabbit secondary antibody (1:1000; BA-1000, Vector Laboratories) followed by 2 h incubation in Alexa Fluor 594-conjugated streptavidin (0.5 µg/mL; 016-580-084,

Jackson ImmunoResearch Laboratories) for immunofluorescent detection. Tissue was mounted and images captured as in Exp. 3. Total c-Fos positive nuclei were manually counted across dorsal and ventral BNST in 2-4 serial sections from mice selected at random from each treatment group (CNO, n = 3; Vehicle, n = 3). Counts were then averaged across subdivisions in each animal and treatment group means were compared using an unpaired two-tailed t-test.

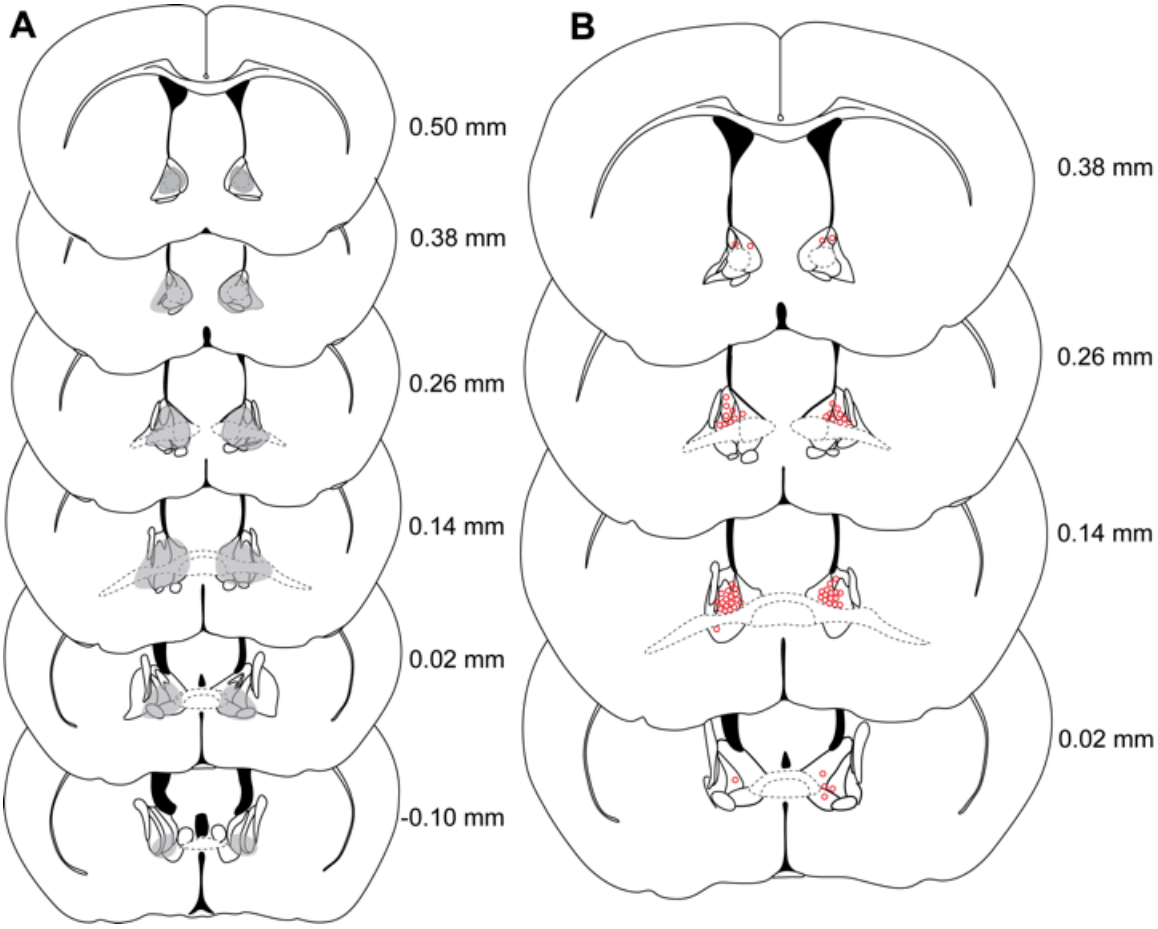

Figure 6. Placement maps indicating the location of lesions and infusions determined after histological analysis. Solid lined structures in section centers indicate the BNST subdivisions and inclusion area. The anterior commissure is indicated by dashed lines. Numbers indicate distance from bregma (in mm) for each coronal section. (A) Maximal extent of pre- and post-conditioning lesions in Exp. 1 is shown in grey. (B) Reconstruction of injector placements for all mice included in Exp. 2. Red circles indicate the location of drug infusion.

Apparatus

Twelve identical acrylic and aluminum chambers (30 x 15 x 15 cm) each enclosed in light- and sound-attenuating ventilated boxes (Coulbourn Instruments Model E10-20) were used to record locomotor activity and amount of time spent on each side of the chamber. Activity and grid time were detected by six sets of infrared photodetectors mounted at 5 cm intervals, 2.2 cm above the floor along the front and rear sides of each inner chamber and recorded by computer (detailed fully in Cunningham et al., 2006).

Chamber floors were composed of grid (2.3-mm stainless steel rods mounted 6.4 mm apart in an acrylic frame) or hole (16-ga stainless steel sheets perforated with 6.4 mm diameter holes on 9.5 mm staggered centers) interchangeable halves that are equally preferred by experimentally naïve DBA/2J mice (Cunningham et al., 2003). Although these stimuli are preferred equally prior to conditioning, they may vary in relative salience or conditionability (Cunningham et al., 2003). Thus, experimentally induced changes in preference for these stimuli by conditioning and other manipulations may not always be symmetrical.

In the one-compartment configuration (Exps. 1a, 1b, and 2), the same floor cue was placed on both sides of the conditioning box (i.e., grid vs. grid, hole vs. hole) during conditioning trials, giving the mouse free access to both sides of the box. In the two-compartment configuration (Exps. 3, 4, and 5), grid and hole floors were separated during conditioning by a clear acrylic divider placed in the center of the apparatus. Previous research in our lab shows no difference in the

magnitude of ethanol-induced CPP produced by these configurations in DBA/2J mice trained in the dark (Cunningham & Zerizef, 2014). Moreover, given the variation in CPP procedures across laboratories, our inclusion of two common configurations allows for greater generality of our conclusions.

Experimental Design

General Procedure. In each experiment, mice were randomly assigned to the following treatment groups: lesion or sham (Exps. 1a and 1b); M+B or vehicle (Exp. 2); CNO or vehicle (Exps. 3, 4, and 5)[16]. We used an unbiased place preference procedure that involved three distinct phases: habituation/pre-test (one session), conditioning (2-4 sessions) and preference test (1-2 sessions). Illustration of procedures and timelines for each experiment are included in Figures 2A, 3A, 5A, 6A, 8A.

Habituation. In Exps. 2-4, animals underwent a 5-min habituation session where they were given an injection of saline and placed in the apparatus on a white paper floor. This phase was intended to reduce the novelty and potential stress associated with the CPP procedure.

Pretest. A 30-min pretest was included in Exp. 1a to examine whether BNST lesions or surgical procedures affected initial bias for the tactile cues. We also included a pretest in Exp. 1b to equate mice in both lesion studies for overall

[16] Contrary to Chapter 1, a “Miss” group was not included in these experiments due to the insufficient number of animals with lesions, infusions, or DREADD expression outside of the BNST.

cue exposure. The pretest was procedurally identical to the place preference test (described below). In neither experiment were there significant group differences in initial preference, floor bias, or activity (data not shown). The overall mean percentages (± SEM) of the pretest session spent on the grid floor were 52.8 ± 2.0 and 50.2 ± 2.0 in Exps. 1a and 1b, respectively.

Conditioning. Within each treatment group, mice were randomly assigned to one of two conditioning subgroups (Grid+ or Grid-). Mice in the Grid+ subgroup received ethanol paired with the grid floor (CS+) and saline paired with the hole floor (CS-), whereas mice in the Grid- subgroup received ethanol paired with the hole floor (CS+) and saline paired with the grid floor (CS-). Each mouse received two or four 5-min conditioning trials before the preference test.

Place preference test. A choice preference test was performed after the last conditioning session. Mice in all experiments were simultaneously exposed to the grid and hole floors for 30 min immediately after a saline injection. The position of each floor type was counterbalanced (i.e., left vs. right) within conditioning subgroups.

Exps. 1a and 1b – Effect of electrolytic lesions of the BNST on ethanol-induced CPP. Exps. 1a (n = 32) and 1b (n = 35) used electrolytic lesions to determine involvement of the BNST in ethanol-induced CPP expression (Fig. 7A). In Exp. 1a, lesions were made prior to the start of conditioning procedures. Since lesions made before conditioning cannot indicate whether disruptions in CPP occurred during the acquisition or expression phase, we conducted a follow up experiment to test effects of BNST lesions induced

after conditioning (Exp. 1b). Thus, Exp. 1a tested whether the BNST was involved in either CPP acquisition or expression, whereas Exp. 1b tested only whether the BNST was involved in ethanol-induced CPP expression. Animals received one conditioning trial per day (CS+ or CS-) over 8 days and the order of exposure was counterbalanced between animals within each conditioning subgroup (Grid+ vs. Grid-).

Exp. 2 – Effect of pharmacological inactivation of the BNST on ethanol-induced CPP. Experiment 2 (n = 30) examined the effect of temporary inactivation of the BNST on ethanol-induced CPP using focal infusions of M+B. As in Exps. 1a and 1b, a one-trial per day procedure was used (Fig. 8A).

Exp. 3 – Effect of chemogenetic inactivation of the BNST on ethanol-induced CPP. In Experiment 3 (n = 45), the effect of chemogenetic inhibition of the BNST on ethanol-induced CPP expression was evaluated (Fig. 10A). During conditioning, mice received two trials per day, with CS- (saline) trials in the morning (10 am – 12 pm) and CS+ (ethanol) trials in the afternoon (2 – 4 pm). Unpublished research in our lab has shown development of similar ethanol-induced CPP using either one- or two-trial per day procedures. Conditioning occurred over a 2-day period for a total of 2 CS- and 2 CS+ trials. Mice expressing hM4Di were injected with CNO (10 mg/kg) or vehicle 30 min before place preference testing. Several studies have shown that vehicle-treated rodents expressing DREADDs do not differ from CNO-treated controls expressing only a GFP transgene (Ferguson et al., 2011; Ferguson, Phillips, Roth, Wess, & Neumaier, 2013). Therefore, in Exp. 3 vehicle-treated mice

expressing the hM4Di construct served as our control.

Exp. 4 – Effect of hM4Di activation on c-Fos IR in the BNST. In Exp. 4 (n = 24), we determined whether CNO would attenuate BNST activity in mice expressing the hM4Di construct (Fig. 11A) as indexed by c-Fos IR after brief exposure to the CS+. Procedures were identical to those described in Exp. 3, except that mice received: 1) a total of 4 CS- and 4 CS+ trials, 2) an abbreviated 15 min CPP test, and 3) a 5-min re-exposure to the ethanol-associated floor cue (CS+) 24 h after the CPP test. A shorter CPP test duration was used to reduce any potential impact on CS-US associative strength since each CS+ presentation without the US serves as an extinction trial (Bouton & Moody, 2004). Additional conditioning trials were given in this experiment to offset any extinction effects and to elicit more robust activity and c-Fos IR in the BNST upon CS+ re-exposure. As in Exp. 3, CNO was administered 30 min before the CPP test and CS+ re-exposure.

Exp. 5 – Effect of CNO alone on ethanol-induced CPP. Experiment 5 (n = 48) was performed to determine whether CNO itself would affect ethanol-induced CPP expression in the absence of hM4Di (Fig. 13A). In Exp. 3, we controlled for possible nonspecific effects of transgene expression (i.e., hM4Di) on behavior by including vehicle-treated hM4Di mice. Here we tested CNO using wild-type mice as controls, since we did not expect them to differ in behavior from mice expressing a control reporter (e.g., GFP). Hence, procedures were identical to those described in Exp. 3, with the following exceptions: 1) mice were not infused with virus and therefore did not express hM4Di, 2) after the first test, mice

received 2 additional conditioning sessions for a total of 4 CS- and 4 CS+ trials, and 3) mice were given a final test after injection of a higher CNO dose (20 mg/kg).

General Statistical Analysis

Preference tests. Preference was defined as a significant difference in time spent on the grid floor between Grid+ and Grid- conditioning subgroups (Cunningham et al., 2003; 2006). Preference data were analyzed by 2-way ANOVA (treatment x conditioning), where treatment refers to the main treatment groups (e.g., sham vs. lesion in Exps. 1a and 1b) and conditioning refers to the assigned conditioning subgroup (Grid+ vs. Grid-). Additional analyses were used to assess the impact of experimental manipulations on CPP expression across test time. To simplify presentation and interpretation of the time course data, preference was expressed as percent time spent on the ethanol-paired floor (CS+) by collapsing across conditioning subgroups (Grid+ and Grid-). Preference data were then averaged across 5-min intervals and analyzed by two-way mixed-factorial ANOVA (treatment x interval). Activity during the preference test was analyzed by one-way ANOVA (treatment).

Conditioning activity. Activity data from conditioning sessions were collapsed across trials of each type (CS+ and CS−) and analyzed by two-way mixed-factorial ANOVA (treatment x trial type), where trial type refers to CS+ vs. CS− trials.

Results

Place preference tests

Exps. 1a and 1b – Effect of electrolytic lesions of the BNST on ethanol-induced CPP. Figure 6A shows the maximum extent of lesion damage to the BNST in Exps. 1a and 1b. In Exp. 1a, four mice were excluded from analyses because lesions were made outside the BNST inclusion area. One mouse was excluded from analysis in Exp. 1b due to a procedural error.

As shown in Fig. 7B, BNST lesions interfered with ethanol-induced CPP. This effect was observed whether lesions occurred before (Exp. 1a) or after conditioning (Exp. 1b) and was supported by significant treatment (lesion vs. sham) x conditioning (Grid+ vs. Grid-) interactions (Exp. 1a, [$F(1,28) = 50.8$, $p < 0.001$]; Exp. 1b, [$F(1,31) = 18.0$, $p < 0.001$]. ANOVA also revealed significant main effects of conditioning in both studies ([Exp. 1a: [$F(1,28) = 155.5$, $p < 0.001$]; Exp. 1b: [$F(1,31) = 84.0$, $p < 0.001$]) and a significant main effect of treatment in Exp. 1a [$F(1,28) = 19.5$, $p < 0.001$], but not in Exp. 1b. Pairwise comparisons between conditioning subgroups within each treatment condition showed that all groups expressed a significant CPP (Bonferroni corrected p's $\leq$ 0.003) in both experiments. These findings indicate that BNST lesions before or after conditioning reduced, but did not completely block ethanol-induced CPP expression.

Analysis of preference across time during the test showed that CPP was immediately reduced in lesioned mice compared to shams in both experiments (Fig. 7C). In Exp. 1a, a significant treatment x time interaction [$F(5,145) = 3.2$, $p =$

0.009] and main effect of treatment [$F(1,29) = 23.3$, $p < 0.001$] were found. Follow-up pairwise comparisons revealed significant differences between groups at all 5-min test intervals (p's ≤ 0.044). In Exp. 1b, analyses yielded significant main effects of treatment [$F(1,33) = 19.0$, $p < 0.001$] and time [$F(5,165) = 3.4$, $p < 0.001$], but no treatment x time interaction. These results indicate that pre- and post-conditioning lesions disrupt preference expression in a relatively consistent manner across the CPP test.

Figure 7. BNST lesions disrupt ethanol-induced CPP

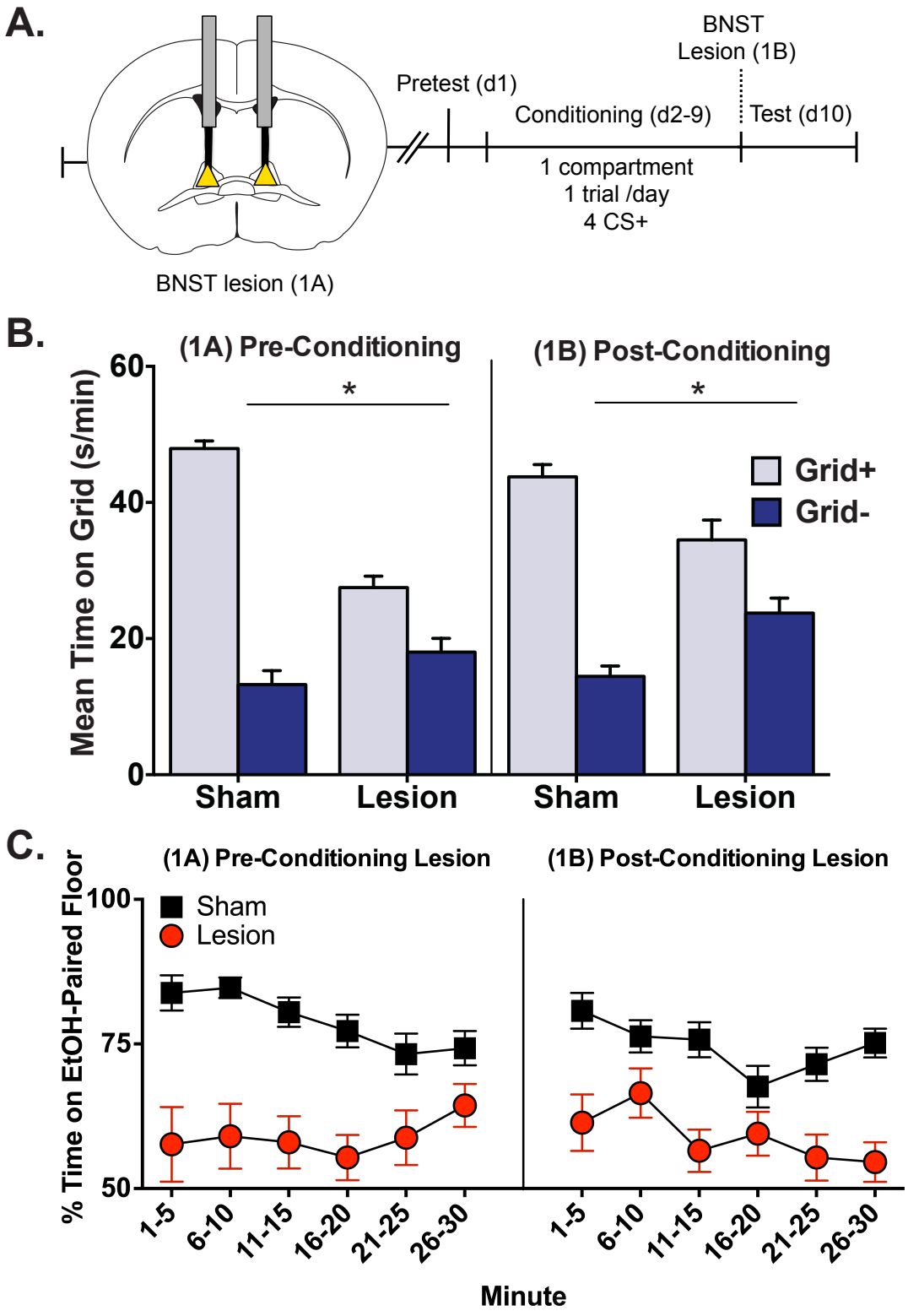

Figure 7. Electrolytic BNST lesions disrupt ethanol-induced CPP expression. (A) Procedural timeline illustrating electrolytic BNST lesions made before (Exp. 1a) or after (Exp. 1b) mice underwent a CPP procedure involving a pretest and 1-compartment, 1 trial/day conditioning procedure where ethanol was paired with a distinct tactile cue (grid or hole floor) every other day for a total of 4 CS+ trials. Preference was measured during a test where mice were allowed equal access to both floor cues. (B) Data are mean time on the grid floor (s/min + SEM) for sham and lesion groups. BNST lesions made before (1a, left panel) and after (1b, right panel) conditioning disrupted ethanol-induced CPP expression. Compared to shams, lesions reduced CPP magnitude as indicated by treatment (sham vs. lesion) by conditioning (Grid+ vs. Grid-) interactions and significant differences between Grid+ and Grid- in sham and lesion treatment groups; † $p < 0.001$ treatment x conditioning; *$p = 0.003$, **$p < 0.001$ between Grid+ and Grid- (n = 6-10/subgroup). (C) Place preference expression in 5-min intervals across the CPP test in Exp. 1. Mean (±SEM) percent time spent on the ethanol-paired floor was significantly reduced in mice that received pre-conditioning (Exp. 1a; left panel) and post-conditioning (Exp. 1b; right panel) electrolytic lesions of the BNST compared to shams (p's < 0.001).

Exp. 2 – Effect of pharmacological inactivation of the BNST on ethanol-induced CPP. Figure 6B illustrates microinfusion injector placements within the BNST. In Exp. 2, mice were excluded from analyses for receiving microinfusions outside of the BNST (n = 2), procedural error (n = 1), lost headmount (n = 1), and histological error (n = 1).

Temporary pharmacological inactivation of the BNST (via microinjection of M+B) blocked expression of ethanol-induced CPP (Fig. 8B). This was confirmed by a significant treatment (M+B vs. vehicle) x conditioning (Grid+ vs. Grid-) interaction [$F(1,26) = 16.0$, $p < 0.001$]. A main effect of conditioning [$F(1,26) = 32.8$, $p < 0.001$], but not treatment, was found. Post hoc analysis of the interaction showed that only mice infused with vehicle expressed significant CPP (Bonferroni-corrected $p < 0.001$), suggesting that activation of the BNST was required for expression of CPP. In addition, mice infused with M+B spent consistently less time than vehicle on the ethanol-paired floor across test intervals (Fig. 8C). This was supported by a main effect of treatment [$F(1,28) = 16.5$, $p < 0.001$], but not time and the absence of a treatment x time interaction.

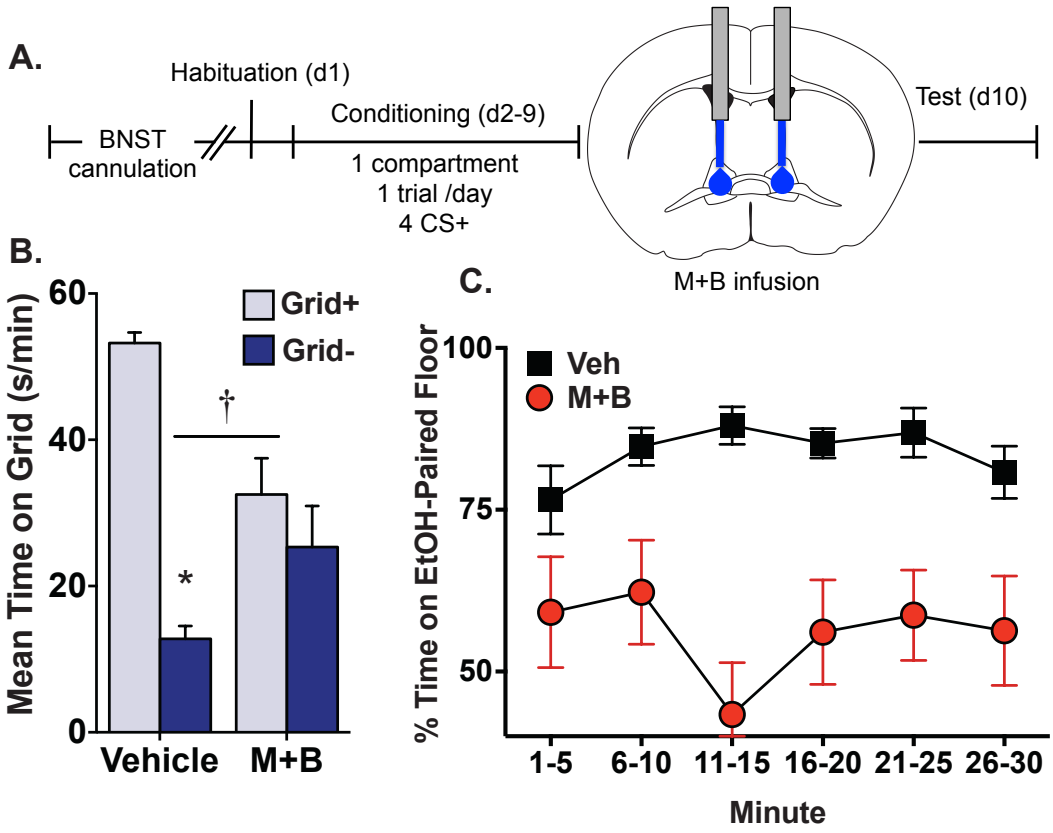

Figure 8. Pharmacological inactivation of the BNST blocks ethanol-induced CPP expression. (A) In Exp. 2, guide cannulae were implanted before the start of the CPP procedure. Mice were habituated to the CPP apparatus then conditioned using a 1-compartment, 1 trial/day procedure where ethanol was paired with a distinct tactile cue (grid or hole floor) every other day for a total of 4 CS+ trials. Immediately before the CPP test, mice received intra-BNST infusions of vehicle or muscimol + baclofen (M+B). (B) Data are mean time on the grid floor (s/min + SEM) for vehicle and M+B groups. Infusion of M+B blocked the expression of ethanol-induced CPP. This was supported by a treatment (vehicle vs. M+B) by conditioning (Grid+ vs. Grid-) interaction and a significant difference between Grid+ and Grid- in the vehicle group only; † $p < 0.001$ treatment x conditioning; *$p < 0.001$ between Grid+ and Grid- (n = 7-8/subgroup). (C) Place preference expression in 5-min intervals across the CPP test in Exp. 2. Data are expressed as Mean (±SEM) percent time spent on the ethanol-paired floor. Mice that received intra-BNST infusion of muscimol + baclofen (M+B) spent significantly less time on the ethanol-paired floor compared to those that received vehicle infusion ($p < 0.001$).

Exp. 3 – Effect of chemogenetic inactivation of the BNST on ethanol-induced CPP. Localized expression of hM4Di in BNST was observed 4-6 weeks after infusion of the viral construct (Fig. 9A), as determined by expression of the hM4Di-fused HA tag (Fig. 9B-C) and mCitrine tag (Fig. 9D). As shown in Figure 10B, activation of hM4Di in the BNST reduced ethanol-induced CPP expression in the CNO-treatment group compared to the vehicle treatment group. When collapsed across conditioning subgroups (Grid+ and Grid-) and analyzed over time (Fig. 10C), preference for the ethanol-paired floor was significantly lower in CNO-treated compared to vehicle-treated animals as indicated by a significant treatment x time interaction [$F(5,220) = 3.0$, $p = 0.012$] and main effect of treatment [$F(1,44) = 7529.4$, $p = 0.004$]. Follow-up analyses revealed significant differences between treatment groups at minutes 11-15, 16-20 and 21-25 (p's $\leq$ 0.024). Overall, these results indicate that hM4Di-induced inactivation of BNST neurons reduced the magnitude of ethanol-induced CPP compared to the control. This effect was delayed, as disruptions in preference expression were not significant before 10 min into the CPP test.

Figure 9. hM4Di expression in BNST

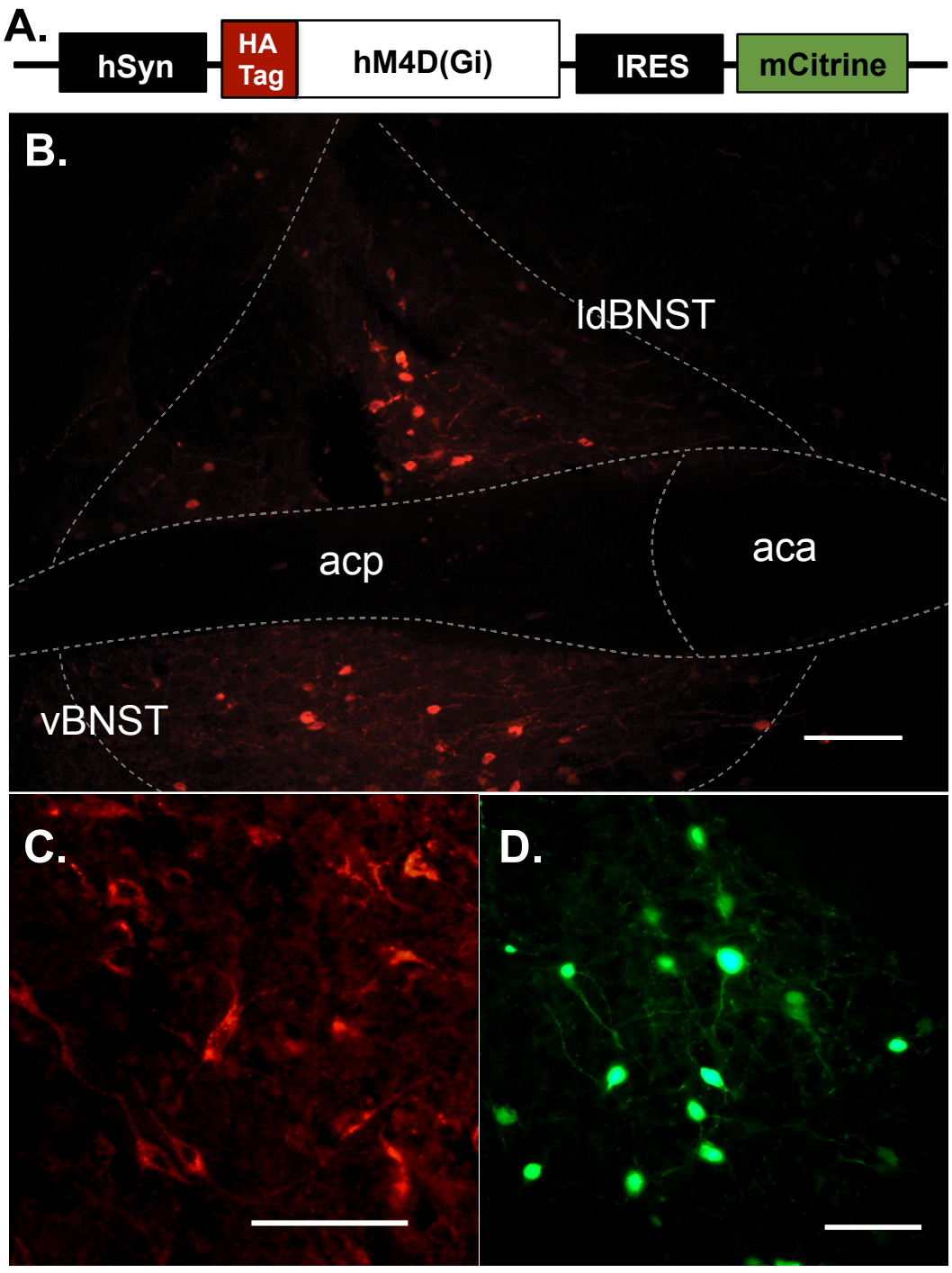

Figure 9. hM4Di expression in BNST. (A) In experiment 3, the hSyn-HA-hM4D(Gi)-IRES-mCitrine adeno-associated virus (AAV; serotype 5) was used to express hM4Di under a neuronal-specific human synapsin promoter (hSyn). HA, influenza hemagglutinin epitope tag; IRES, internal ribosome entry site. (B-D) Localized hM4Di expression in BNST. (B) Representative staining for HA-tagged hM4Di within dorsal and ventral BNST at 10X magnification. (C) Expression of HA-tagged hM4Di on neuronal membranes and processes (20X). (D) Native mCitrine fluorescence showing neurons transduced by AAV vector (20X). Scale bars, 100 μm; aca, anterior commissure, anterior; acp, anterior commissure, posterior; ldBNST, lateral-dorsal BNST; vBNST, ventral BNST.

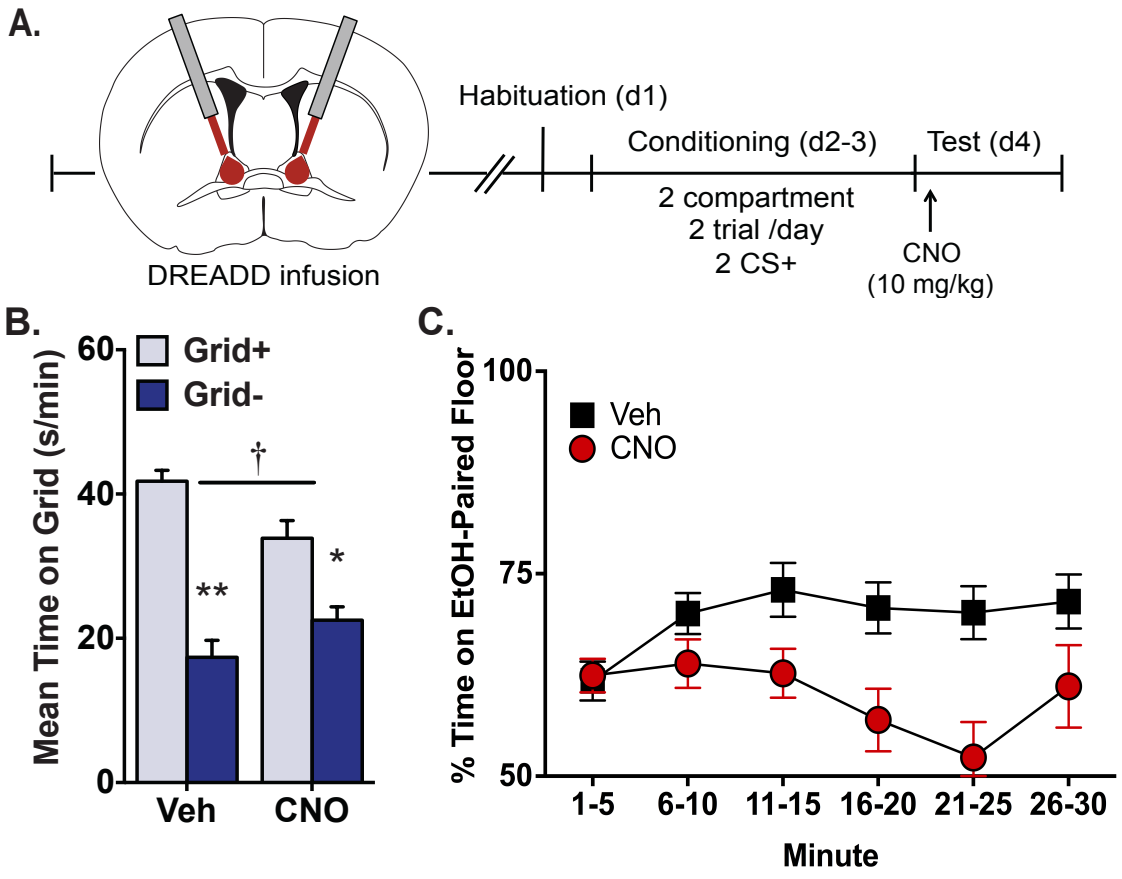

Figure 10. Chemogenetic inhibition of the BNST disrupts ethanol-induced CPP expression. (A) Using an angled approach, an AAV carrying the inhibitory DREADD (hM4Di) was delivered into the BNST 4-6 weeks before habituation. The CPP procedure consisted of a 2-compartment, 2 trial/day procedure where ethanol was paired with a distinct tactile cue (grid or hole floor) each day for a total of 2 CS+ trials before CPP testing. To stimulate hM4Di, CNO (10 mg/kg) was administered before the CPP test. (B) Mean time on the grid floor (s/min + SEM) after 2 trials for vehicle and CNO groups. The magnitude of CPP was reduced in the CNO group as indicated by a treatment (vehicle vs. CNO) by conditioning subgroup (Grid+ vs. Grid-) interaction and significant differences between Grid+ and Grid- in both treatment groups. † $p = 0.003$ treatment x conditioning; *$p < 0.01$, **$p < 0.001$ between Grid+ and Grid- ($n = 12$/subgroup). (C) Place preference expression in 5-min intervals across the CPP test in Exp. 3. Data are expressed as mean (±SEM) percent time spent on the ethanol-paired floor. CNO-mediated activation of hM4Di significantly reduced time on the ethanol-paired floor compared to vehicle ($p < 0.004$). This effect was delayed, as no significant difference between CNO and vehicle was found until interval 11-15 m ($p = 0.027$).

Exp. 4 – Effect of hM4Di activation on c-Fos IR in the BNST. Representative images in Fig. 11B demonstrate the extent of c-Fos IR in the vBNST of hM4Di expressing animals treated with CNO or vehicle. Within the BNST, CS+-induced neural activity was significantly lower in CNO-treated compared to vehicle-treated mice (Fig. 11C) and did not vary by BNST subdivision (Fig. 12). Mean (± SEM) c-Fos positive nuclei were 62.6 ± 2.9 for CNO and 102.1 ± 0.7 for vehicle. Analysis of these data yielded a significant effect of treatment on total c-Fos cells in the BNST [$t(4) = 13.5$, $p < 0.001$], indicating that CNO reduced c-Fos IR.

Perhaps due to the abbreviated length (15 min) of the CPP test in this experiment, we were not able to detect a significant effect of CNO on preference expression (Fig. 11D). This finding is similar to that of Exp. 3, as hM4Di activation had only a marginal effect on preference during the first 15 min the CPP test (Fig. 10C). These findings were supported by analyses of grid time that showed a main effect of conditioning [$F(1,20) = 145.4$, $p <0.001$] but not treatment or a treatment x conditioning interaction ($p = 0.08$). When percent time spent on the ethanol-paired floor was analyzed over 5-min intervals, neither a treatment x time interaction nor main effect of time was found (Fig. 11E). Analyses did reveal a trend toward a main effect of group ($p = 0.070$), similar to that found in first 15 min of Exp. 3 ($p = 0.065$). However, comparisons between these experiments must be made conservatively, as mice received a greater number of CS+ trials in Exp. 4.

Figure 11. BNST activity is reduced by hM4Di activation

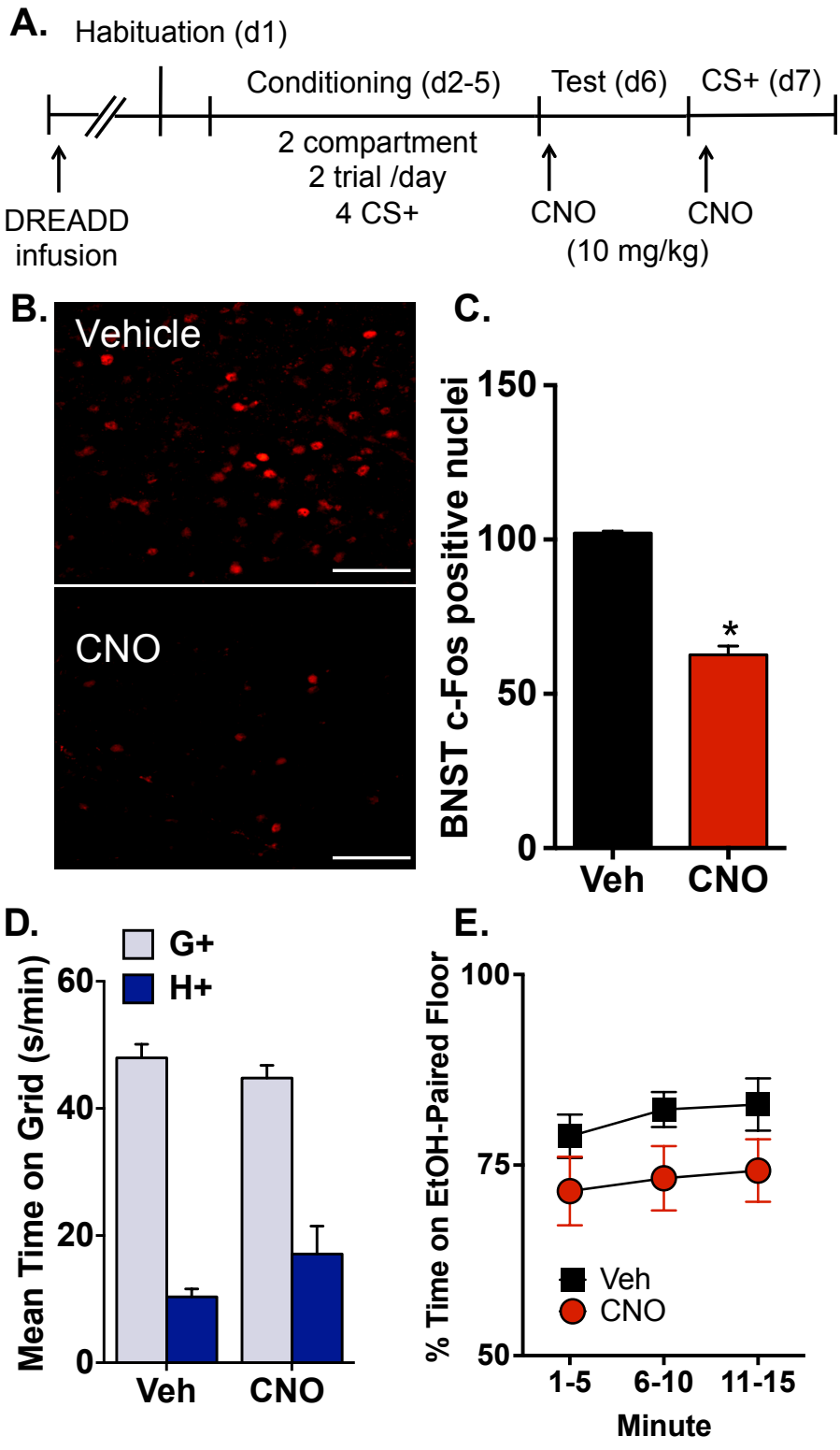

Figure 11. Activation of hM4Di reduces cue-induced activity in BNST.
(A) In Exp. 4, angled bilateral infusions of virus carrying hM4Di was delivered into the BNST before habituation. The CPP procedure consisted of a 2-compartment, 2 trial/day procedure where ethanol was paired with a distinct tactile cue (grid or hole floor) each day for a total of 4 CS+ trials before CPP testing. To stimulate hM4Di, CNO (10 mg/kg) was administered before an abbreviated CPP test. The following day, mice were briefly re-exposed to the ethanol-associated cue (CS+) before tissue was harvested for analysis of c-Fos immunoreactivity. (B) Representative c-Fos immunofluorescence in ventral BNST in hM4Di-expressing mice administered vehicle or CNO before CS+ re-exposure. Scale bars, 100 μm. (C) CNO-mediated stimulation of hM4Di significantly reduced the number of c-Fos positive nuclei in BNST compared to vehicle. n = 3/ treatment group; *$p < 0.001$. (D-E) Place preference expression during the CPP test in Exp. 4. (D) Data are mean (+SEM) time spent on the grid floor in s/min. Compared to vehicle, CNO-mediated activation of hM4Di did not significantly reduce time on the grid floor between conditioning subgroups (Grid+ and Grid-) during an abbreviated (15 min) CPP test. (E) Data are expressed as mean (±SEM) percent time spent on the ethanol-paired floor. When collapsed across conditioning subgroups and analyzed over 5-min intervals, the difference in time spent on the ethanol-paired floor did not significantly differ between CNO and vehicle ($p = 0.70$).

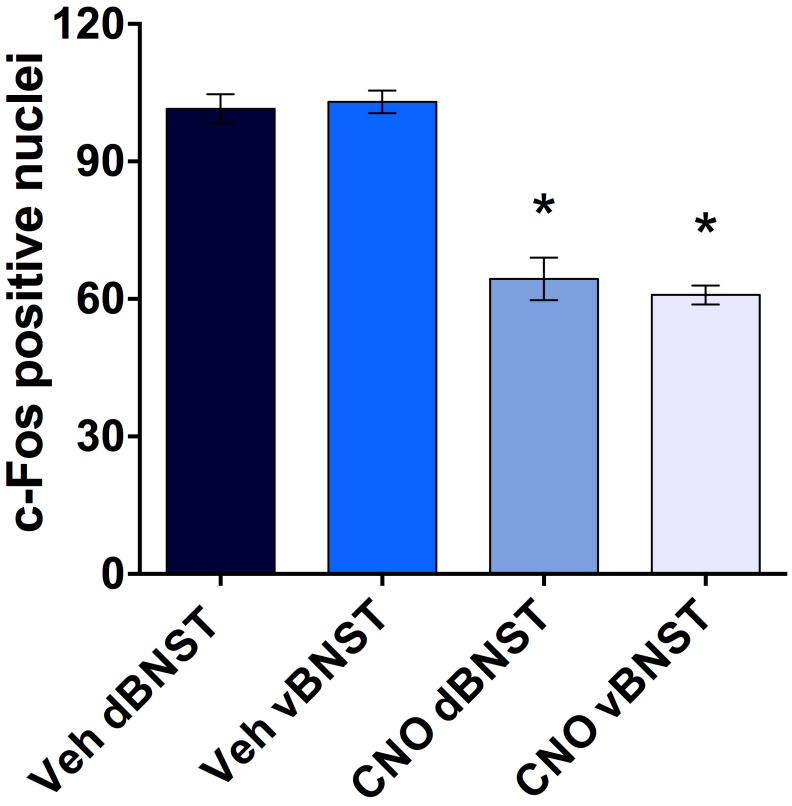

Figure 12. Mean (±SEM) c-Fos positive nuclei in dorsal and ventral BNST (dBNST and vBNST) in mice administered vehicle or CNO. No differences were found in c-Fos expression immunoreactivity between dorsal and ventral subdivisions within each treatment group (vehicle and CNO). Compared to vehicle, CNO-mediated activation of hM4Di significantly reduced c-Fos immunoreactivity in dBNST and vBNST (*p's < 0.001).

Exp. 5 – Effect of CNO alone on ethanol-induced CPP. In the absence of hM4Di expression, CNO at 10- and 20-mg/kg doses did not affect ethanol-induced CPP expression (Fig. 13B). This was indicated for tests 1 and 2 by a main effect of conditioning [p's < 0.001], lack of a significant treatment (CNO vs. vehicle) x conditioning subgroup (Grid+ vs. Grid-) interaction and no main effect of treatment. When analyzed across test intervals (Fig. 13C), percent time spent on the ethanol-paired floor was consistent between treatment groups as indicated by the absence of a significant treatment x time interaction or main effect of treatment on tests 1 and 2 (p's > 0.3). A main effect of time was found on test 2 [$F(5,230) = 2.859$, $p = 0.016$] after mice received a total of 4 CS+ trials but not on test 1 after 2 CS+ trials.

Locomotor activity

Preference tests. In Exps. 1a, 1b, 3, 4, and 5 there were no significant differences in test activity between treatment groups (Table 5). This was confirmed by the absence of a main effect of treatment in each experiment [F's < 1]. In Exp. 2, test session activity was reduced by infusion of M+B as compared to vehicle [$F(1,28) = 10.7$, $p = 0.003$]. However, this reduction in test activity is unlikely to have interfered with preference, as lower test activity is generally associated with enhanced CPP expression (Gremel & Cunningham, 2007).

Conditioning Activity. With the exception of Exp. 1a, CS+ trial activity was influenced only by the presence of ethanol as no treatment manipulation occurred until the test day. As expected with DBA/2J mice (Cunningham et al., 1992), activity during CS+ trials was higher than on CS- trials (Table 5). Analyses

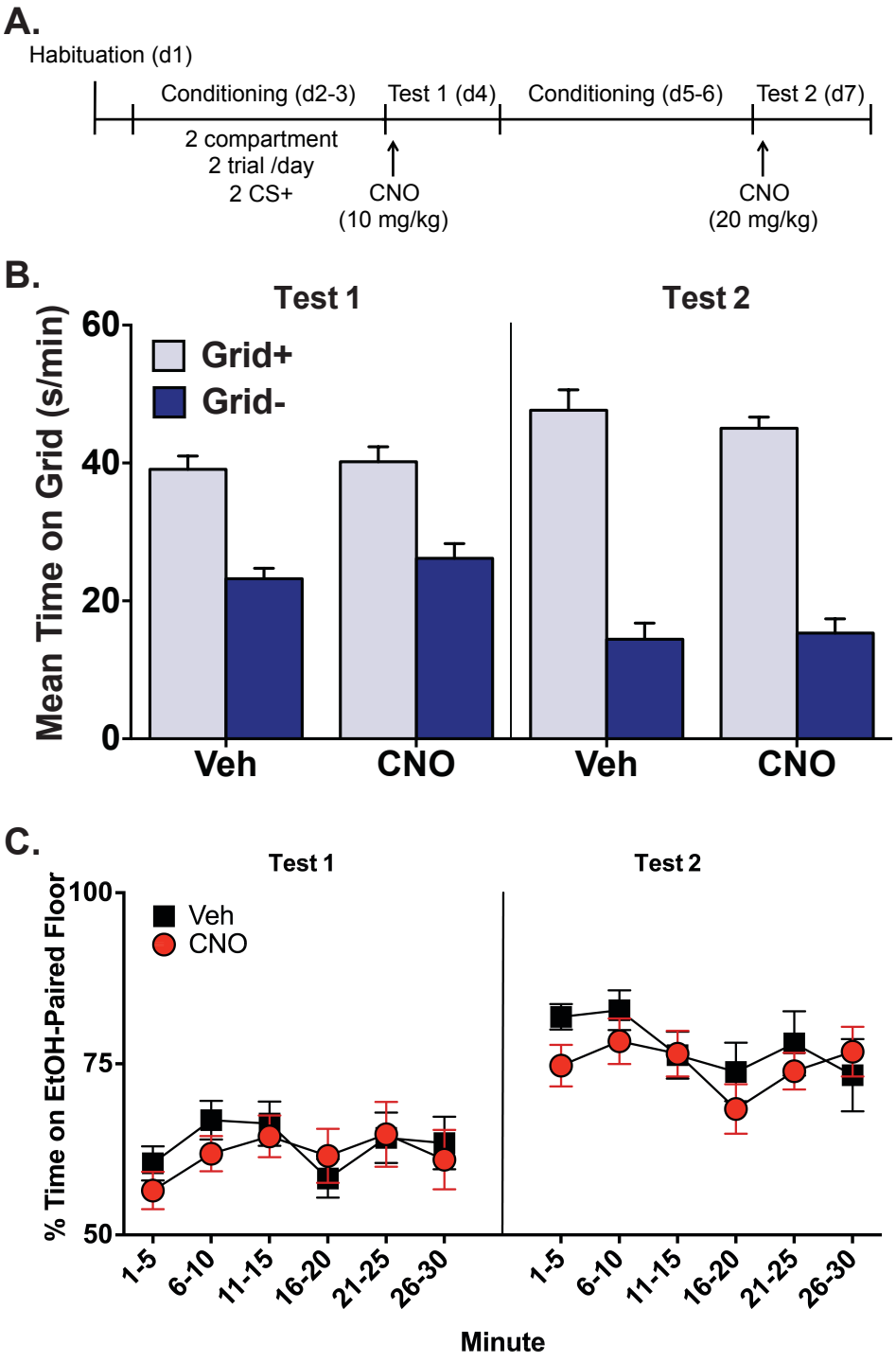
A.
Habituation (d1)
Conditioning (d2-3)
Test 1 (d4)
Conditioning (d5-6)
Test 2 (d7)
2 compartment
2 trial /day
2 CS+
CNO
(10 mg/kg)
CNO
(20 mg/kg)
B.
Test 1
Test 2
Grid+
Grid-
Mean Time on Grid (s/min)
60
40
20
0
Veh
CNO
Veh
CNO
C.
Test 1
Test 2
Veh
CNO
% Time on EtOH-Paired Floor
100
75
50
1-5
6-10
11-15
16-20
21-25
26-30
Minute

Figure 13. In the absence of hM4Di, CNO does not disrupt ethanol-induced CPP expression. (A) In Exp. 5, mice were habituated to the CPP apparatus before conditioning using a 2-compartment, 2 trial/day procedure where ethanol was paired with a distinct tactile cue (grid or hole floor). After 2 CS+ trials, mice were administered vehicle or CNO (10 mg/kg) before a preference test (Test 1). Mice then received an additional 2 CS+ (4 total) trials before a second preference test (Test 2), prior to which vehicle or CNO (20 mg/kg) were administered. (B) In the absence of hM4Di, CNO did not disrupt preference expression. Place preference magnitude did not differ between vehicle and CNO groups on either Test 1 or Test 2. (C) Place preference expression in 5-min intervals across CPP tests 1 and 2 in Exp. 5. No difference in time spent on the ethanol-paired floor was found between vehicle and CNO at 10 mg/kg (Test 1; left panel) and 20 mg/kg (Test 2; right panel).

confirmed that activity was significantly higher on CS+ trials compared to CS- trials in all experiments, as indicated by a significant main effect of trial type [p's < 0.001], but there was no treatment x trial type interaction. In Exp. 1a, BNST lesions reduced locomotor activity during CS+ trials and increased activity during CS- trials. This effect was confirmed by a treatment x trial type interaction [$F(1,30) = 16.3$, $p < 0.001$] and main effects of treatment on CS+ [$F(1,30 = 10.6$, $p = 0.003$] and CS- trials [$F(1,30) = 5.3$, $p = 0.029$].

In Exps. 3-5, locomotor activity was generally lower on conditioning trials than in the other experiments (Table 5). This reflects our use of a 2-compartment configuration in Exps. 3-5, which confines mice to one side of the apparatus during conditioning and therefore provides less space for movement (Hitchcock et al., 2014). Treatment groups in Exps. 3-5 however, showed similar levels of ethanol-induced activation, as confirmed by a lack of a main effect of treatment and a significant main effect of trial type [p's < 0.001].

Table 5. Chapter 2 Activity

Mean Activity Counts per Minute (± SEM) during conditioning and preference test

	Group	CS+ Trials	CS- Trials	Preference Test
Experiment 1a				
Electrolytic Lesion	Sham	176.1 ± 8.8	57.9 ± 3.1	35.5 ± 2.3
Pre-Conditioning	Lesion	128.5 ± 12.0[a]	67.8 ± 2.7[b]	40.1 ± 1.9
Experiment 1b				
Electrolytic Lesion	Sham	203.5 ± 7.9	62.0 ± 3.3	39.5 ± 1.8
Post-Conditioning	Lesion	206.5 ± 6.8	61.4 ± 3.0	44.5 ± 2.3
Experiment 2				
Pharmacological	Vehicle	223.8 ± 12.6	73.7 ± 3.6	36.7 ± 3.8
Inactivation	M+B	220.7 ± 8.4	73.1 ± 3.0	22.5 ± 2.3[a]
Experiment 3				
hM4Di	Vehicle	120.8 ± 4.8	44.2 ± 1.4	40.2 ± 1.3
	CNO	124.1 ± 5.3	46.5 ± 2.0	40.3 ± 1.4
Experiment 4				
c-Fos Control	Vehicle	134.5 ± 4.3	40.1 ± 2.4	45.4 ± 2.4
	CNO	136.1 ± 4.8	40.3 ± 2.0	42.1 ± 2.8
Experiment 5		Trials 1-2		Test 1
CNO Control	Vehicle	120.4 ± 3.5	43.4 ± 1.8	38.7 ± 1.1
	CNO	117.5 ± 4.4	42.4 ± 1.6	38.5 ± 1.3
		Trials 3-4		Test 2
	Vehicle	120.6 ± 4.3	37.2 ± 2.2	31.9 ± 1.5
	CNO	123.2 ± 6.3	35.2 ± 1.7	31.6 ± 1.1

[a] differs from sham or vehicle, $p = 0.003$
[b] differs from sham, $p < 0.03$

Discussion

The present studies used several techniques to directly test the involvement of the BNST in ethanol-seeking behavior using CPP. In Experiment 1, electrolytic ablation of the BNST before (Exp. 1a) and after (Exp. 1b) conditioning reduced the magnitude of ethanol-induced place preference expression. In Experiment 2, CPP expression was blocked and activity reduced by focal infusion of the GABA receptor agonists muscimol and baclofen. In Experiments 3 and 4, activation of hM4Di by CNO reduced CPP magnitude and c-Fos IR, respectively. A control study (Exp. 5) revealed that the reduction in CPP expression was not due to administration of CNO alone, as equal and higher doses of CNO did not impact CPP in the absence of hM4Di.

The time course and extent of CPP disruption observed in these experiments varied across the techniques and procedures used. Preference expression was lower from the beginning of each test after lesions or temporary inactivation (M+B), but this effect was delayed after hM4Di activation. On the basis of our data, we cannot determine why a delayed effect was found in the DREADD experiments. One possible explanation relates to the pharmacokinetic properties of CNO. Given its systemic route of delivery, CNO would not be expected to act as rapidly to inhibit neuronal activity as locally administered drugs like M+B or lesions. *In vivo* experiments in mice have shown that plasma levels of CNO after i.p injection peak within 15 min and clear after 2 h (Wess, Nakajima, & Jain, 2013). However, CNO can produce protracted physiological effects in DREADD-expressing mice, sometimes persisting as long as 8-10 h

(Alexander et al., 2009; Guettier et al., 2009; Krashes et al., 2011). Notably, Alexander and colleagues (2009) demonstrated that neural activity in hM3Dq mice peaked around 45-50 min after CNO injection, an effect that was independent of dose. Assuming a similarly timed peak in suppression of neural activity in hM4Di mice, our findings are in agreement, showing reductions in preference expression 45 min after CNO was administered. Thus, administration of CNO 45-50 min before testing in future studies might show a more immediate reduction in preference, similar to that seen in the lesion and inactivation studies.

Whereas place preference was attenuated by BNST lesions and hM4Di activation (Exps. 1a, 1b, and 3), it was altogether abolished by M+B infusion (Exp. 2). There are several potential explanations for this finding. First, it is possible that the M+B infusion diffused over a broader portion of the BNST, providing greater overall inhibition of this structure. It is also possible that some volume spread to sites adjacent to the BNST and/or throughout the brain as injectors were lowered through the lateral ventricles. Though not a concern for electrolytic lesions, the potential spread into ventricles given the location of the BNST, prompted our use of an angled approach when administering viral infusions in Exps. 3 and 4. This use of differing approaches to target the BNST further expands the breadth of our conclusions. However, it is possible that intraventricular diffusion of M+B may have produced more widespread central inhibition. These explanations are supported by the reduction in locomotor activity that was observed after M+B administration. In addition, this demonstrates a major disadvantage to microinfusion, namely the extent of drug

diffusion. In addition, administering focal microinfusions to awake behaving mice is challenging given the additional handling required. It is plausible that exposure to this handling, which involved light restraint, may have served as a stressor and interfered with preference expression. However, results from our vehicle control group would argue against this explanation. Finally, it is also possible that activating $GABA_A$ and $GABA_B$ receptors using M+B was simply a more effective strategy to inhibit neuronal firing in the BNST.

In interpreting these findings, it is also important to consider the advantages and disadvantages associated with lesions and DREADDs. For example, one major advantage to electrolytic lesions is that they are technically less challenging and can be rapidly administered. Lesions also do not require any additional maintenance or manipulation once they have been administered. This is advantageous from a behavioral perspective, as it removes the possibility of pre-task handling interfering with performance. However, unlike DREADDs and M+B infusion, this also means that the effect of lesions is permanent and not temporally restricted to a discrete experimental time-point. For example, when the BNST was lesioned before conditioning (Exp. 1a), we could not determine if this manipulation impacted associative learning or preference expression. Moreover, this chronic damage may result in compensation by other neural systems, which can be difficult to predict or identify. Given this drawback, we must consider that the full effect of our BNST lesions on CPP expression may have been masked by neural compensation. Another concern is that electrolytic lesions also produce inadvertent damage to nerve fibers passing through the

targeted region. For this reason, when electrolytic lesions alone are employed, there will remain some uncertainty that the effects found are solely due to destruction of cells within the target region. Hence, using additional manipulations may facilitate interpretation of study results. This highlights a major benefit to our combined use of multiple techniques in these studies, which is a strengthened support for our conclusions.

As with focal drug infusions, DREADDs provide a reversible and temporally defined method to manipulate neural activity. A major advantage of DREADDs is that their activation requires only a minimally invasive peripheral injection of an otherwise inert drug (CNO) and therefore necessitates less handling, which may interfere with behavior. Like lesions, viral expression of DREADDs is long lasting and requires only a single intracranial entry, which reduces the risk of infection and damage associated with repeated microinjections. Moreover, DREADD expression is confined to neurons and does not impact fibers of passage within the region being targeted, giving this technique greater selectivity over lesions. Of note, only those neurons expressing the hM4Di receptor will be silenced when activated by CNO administration. As a result of this, the efficacy of this technique depends greatly on the extent of viral expression, which relies on many factors like titer, serotype, and volume administered. Indeed, a noted disadvantage to DREADDs is the degree of difficulty involved in regulating their expression. In our experiments, we achieved highly localized expression in the BNST with AAV serotype 5. This level of expression resulted in viral transduction and hM4Di expression in a subset of

BNST neurons. Therefore, we did not find hM4Di expression throughout all cells contained within this region. Thus it is possible that by using a different AAV serotype or larger vector volume we would have obtained more widespread expression of hM4Di receptors throughout BNST cells. This would likely have resulted in increased CNO-driven inhibition of this region and a greater disruption in CPP expression. Another concern with DREADDs is the potential for perturbation of endogenous receptor activity. Indeed, it is possible that heterologous expression of DREADDs may alter the stoichiometric balance of GPCRs and G proteins in the native system thereby disrupting normal function (Nichols & Roth, 2009). However, the consequences of this possibility on our behavioral outcomes would be difficult to determine. A final concern with DREADDs is the possibility that their activating ligand CNO may have off-target activity or produce effects on its own. This is unlikely given the inability of CNO to back-metabolize to its parent compound clozapine in mice and its subsequent inert nature (Alexander et al., 2009; Armbruster et al., 2007; Guettier et al., 2009). Importantly, our control data illustrate the inert nature of this compound, as doses up to 20 mg/kg had no impact on place preference or locomotor activity. Given the caveats attached to each technique, in combination they provide compelling evidence for tissue-specific regulation of behavior. Here, our finding of BNST involvement in ethanol-induced CPP was consistent across all experimental manipulations and procedural variations. Combined, results from these three techniques provide strong evidence that the BNST modulates ethanol-seeking behavior in mice, as measured by CPP.

In these experiments, we did not distinguish between BNST subdivisions in our manipulations or behavioral analyses because these areas are quite small in the mouse (some extending under 75 μm mediolaterally) and difficult to individually target without overlap using the techniques we employed. Notably, we compared c-Fos IR between dorsal and lateral BNST within each treatment group (CNO and vehicle) and found no significant differences, indicating similar levels of CS+-induced activation and CNO-induced reduction in these subdivisions (Fig. 13). However, there is some evidence to indicate that there exists not only anatomical specificity but also a distinct topology to this region in terms of its regulation of emotion and motivated states. Of note, studies using optogenetics have helped elucidate the specific roles played by intermixed yet distinct neuronal subpopulations within BNST subdivisions. Within the dorsal BNST itself, Kim and colleagues (2013) found that two subregions, the oval nucleus and the anterodorsal BNST, exerted opposite effects on anxiety state. In another study, VTA-projecting glutamate and GABA neurons of the vBNST were shown to differentially regulate anxiety and motivated behavior (Jennings & Sparta et al., 2013). Therefore, it is possible that our results may have varied had we been able to more precisely target discrete subdivisions and/or genetically defined populations of cells within the BNST.

In general, the findings we present here are in agreement with the broader literature indicating that the BNST is involved in reward seeking and appetitive behaviors. Much of this literature has focused on the role of the BNST in stress-induced reinstatement of cocaine seeking (e.g., Erb & Stewart, 1999; McFarland,

2004; McReynolds et al., 2014) and to a lesser extent morphine (Wang, Fang, Liu, & Lu, 2006; Wang, Cen, & Lu, 2001) and ethanol (Funk, Li, & Lê, 2006; Lê, Harding, Juzytsch, Watchus, Shalev, & Shaham, 2000b). However, a growing body of evidence strongly indicates that the BNST is involved in cue-induced cocaine and heroin seeking (Buffalari and See, 2011; Rogers et al., 2008; Sartor and Aston-Jones, 2012). Our findings add to this literature and provide, to our knowledge, the first evidence of BNST involvement in cue-induced ethanol seeking behavior. It is important to note that while studies have supported a role for the BNST in stress-induced drug seeking, cue- and stress-induced seeking are interconnected and likely overlap in their neural circuitry. In fact, in abstinent drug-dependent human subjects, exposure to drug-associated cues can result in concurrent feelings of craving and negative affect (Fox, Bergquist, Hong, & Sinha, 2007). This raises the possibility that the BNST regulates aspects of both stress- and cue-induced relapse. Indeed, it has been suggested that drug-associated cues may themselves serve as psychological stressors by activating the same neural circuits involved in stress (Silberman & Winder, 2013).

Our data further illustrate the broad role of the BNST in ethanol-mediated behavior and support the idea that the BNST mediates several aspects of ethanol abuse from reward to relapse. As previously shown, acute administration of ethanol activates BNST neurons (Chang, Patel, & Romero, 1995; Crankshaw et al., 2003; Demarest, Hitzemann, Mahjubi, McCaughran, & Hitzemann, 1998; Knapp et al., 2001; Leriche, Méndez, Zimmer, & Bérod, 2008) and results in an increase of extracellular dopamine within the BNST (Carboni, Silvagni, Rolando,

& Di Chiara, 2000). Moreover, ethanol self-administration is reduced by intra-BNST antagonism of $GABA_A$ receptors (Hyytiä & Koob, 1995) and D1 dopamine receptors (Eiler, Seyoum, Foster, Mailey, & June, 2003), further demonstrating this structure's role in acute ethanol reward. Withdrawal from chronic ethanol exposure also activates the BNST (Kozell, Hitzemann, & Buck, 2005) as does exposure to an ethanol-associated cue (Hill et al., 2007). The present data now directly show that this BNST activity is important for conditioned preference for an ethanol-associated cue. Collectively, these studies suggest the BNST's role in drug and ethanol addiction is quite broad.

In summary, by inhibiting the BNST through electrolytic lesions (Exps. 1a and 1b), pharmacological agents (Exp. 2), and chemogenetics (Exp. 3), we were able to disrupt expression of ethanol-induced CPP. Taken with a larger literature, our results strongly suggest that the BNST modulates cue-induced ethanol-seeking behavior and indicate that this structure may contribute in multiple ways (i.e., stress and drug-cue sensitivity) to the persistent vulnerability to relapse. Overall, the BNST and its connected circuitry appear to be increasingly promising neural targets for therapies aimed at reducing craving and preventing relapse. Future studies are needed to determine the anatomical and neurochemical nature of the BNST's role in cue-induced ethanol seeking.

In this dissertation, Chapters 1 and 2 demonstrate that the VTA and its afferent, the BNST, are involved in ethanol-induced CPP expression. Combined with the existing anatomic and physiological evidence for BNST regulation of the VTA, our findings may suggest that the BNST is involved in ethanol-induced CPP

expression through a direct neuronal projection to the VTA. Accordingly, the next chapter assesses the role of the BNST-VTA circuit in the expression of ethanol-induced place preference.

Chapter 3

Ethanol-Seeking Behavior is Expressed Directly through an Extended Amygdala to Midbrain Neural Circuit*

*This chapter will be submitted as the following manuscript: M.M. Pina, A.E. Ryabinin, & C.L. Cunningham, (in preparation). Ethanol-seeking behavior is expressed directly through an extended amygdala to midbrain neural circuit. Permission to re-use the published content will be obtained upon acceptance for publication.

Specific Acknowledgments: C.L.C., & M.M.P. designed research; M.M.P. performed research; A.E.R. provided training and intellectual support. Special thanks to Christina Gremel for her original suggestions regarding the intersectional strategy and vectors, Sunila Nair for her advice and support in the implementation of an intersectional approach, and Lindsey Schuette for her help with tissue sectioning.

Abstract

Abstinent ethanol-dependent individuals experience an enduring sensitivity to cue-induced craving and relapse to drinking. There is considerable evidence indicating that structures within the midbrain and extended amygdala are involved in this process. Individually, the ventral tegmental area (VTA) and the bed nucleus of stria terminalis (BNST) have been shown to modulate cue-induced ethanol-seeking behavior. It is hypothesized that cue-induced seeking is communicated through a direct projection from the BNST to the VTA. Previous attempts to discretely manipulate this circuit were limited by existing methodologies, typically pharmacological in nature. In the current experiments, an intersectional viral strategy was used in DBA/2J mice to selectively trace, target, and inhibit BNST projections to the VTA during a test of ethanol conditioned place preference (CPP). In Experiment 1, a herpes simplex virus (HSV)-derived vector was infused into the VTA, resulting in retrograde transport that led to full transgene expression in the BNST within 2 weeks. In Experiment 2, HSV-mediated delivery of cre recombinase (HSV-Cre) in VTA-projecting cells was combined with a cre-inducible adeno-associated virus (AAV) vector to express inhibitory designer receptors exclusively activated by designer drugs (hM4Di, DREADD) in BNST-VTA cells. Before preference expression, clozapine-N-oxide (CNO) was peripherally administered in order to activate hM4Di and inhibit BNST-VTA cells. Ethanol-induced CPP expression was blocked by CNO-mediated inhibition of VTA-projecting BNST cells. A follow-up study revealed this effect was specific to CNO activation of hM4Di as saline- and CNO-treated mice

infused with a control vector (HSV-GFP) showed significant CPP expression (Experiment 3). These findings establish a role for a direct BNST input to VTA in cue-induced ethanol-seeking behavior, as indexed by CPP.

Introduction

It is thought that mesocorticolimbic dopamine (DA) transmission directs reward-related behaviors and is a prime neural signal driving drug seeking and relapse. Originating predominantly from within the ventral tegmental area (VTA), this DA signal has been the focus of much attention. The bulk of this work has centered on the behavioral and functional dissection of neural circuits originating within the VTA and projecting to outside targets such as the nucleus accumbens (reviewed in Ikemoto, 2007). However, growing consideration has been given to VTA afferents and their influence in generating diverse motivational states.

There is emerging evidence that one source of input to VTA, the extended amygdala, is highly involved in regulating VTA-mediated states of reward and aversion. Specifically, the bed nucleus of the stria terminalis (BNST) of the extended amygdala sends strong projections to VTA (Dong & Swanson, 2004; 2006a; 2006b; Kudo et al., 2012; Mahler & Aston-Jones, 2012) that potently innervate DA cells (Georges & Aston-Jones, 2001; 2002) and inhibit gamma-aminobutyric acid (GABA) cells (Jennings et al., 2013; Kudo et al., 2014). Behavioral evidence further implicates the BNST in drug seeking induced by exposure to stress and drug-associated cues. Inactivation of this region has been shown to impair heroin- and cue-primed reinstatement of heroin seeking (Rogers

et al., 2008) as well as stress- and cue-induced reinstatement of cocaine seeking (Buffalari & See, 2011), and cocaine conditioned place preference (CPP) expression (Sartor & Aston-Jones, 2012). The BNST appears to be particularly involved in ethanol seeking. Not only is this structure activated by ethanol-associated cues (Dayas et al., 2007; Hill et al., 2007; Zhao et al., 2006) but its direct inhibition impairs the expression of an ethanol-induced place preference (Pina et al., 2015).

It is unclear however whether the BNST's involvement in cue-induced drug seeking is driven by its connectivity with and input to the VTA. Though studies have attempted to address the role of this circuit in seeking behavior, they were limited by existing methodologies that largely involved a combination of tract-tracing and c-Fos immunohistochemistry or intracranial pharmacological manipulations. Nevertheless, studies using these strategies have been valuable in demonstrating a role for the BNST-VTA circuit in drug seeking. For example, retrogradely labeled BNST projections to the VTA show enhanced c-Fos immunoreactivity following cue-induced cocaine seeking and cocaine place preference expression (Mahler & Aston-Jones, 2012; Sartor & Aston-Jones, 2012). Classic pharmacological disconnection, involving unilateral BNST and contralateral VTA microinjections, has also suggested that a BNST-VTA projection is involved in cocaine CPP expression (Sartor & Aston-Jones, 2012) and stress-induced cocaine seeking (Vranjkovic, Gasser, Gerndt, Baker, & Mantsch, 2014).

Though informative, these techniques do not allow for direct access to and

manipulation of circuits during seeking behavior. Recent advances in viral-mediated gene transfer have provided an unprecedented opportunity for not just cell-type selective targeting but also circuit-selective targeting. Most notably, an intersectional strategy involving infusion of two viruses – a retrograde vector encoding cre recombinase and a cre-dependent adeno-associated virus (AAV) vector – into two distinct yet directly connected nuclei has provided a means for discrete circuit modulation. This approach has been successfully employed in rats using a cre-encoding canine adenovirus-2 (CAV-2) to express designer receptors exclusively activated by designer drugs (DREADD) in projection neurons to and from the VTA (Boender et al., 2014; Nair et al., 2013). This approach enables the direct manipulation of VTA circuit activity during the performance of behavioral tasks. A similar strategy has also been applied in mice using a herpes simplex virus (HSV)-based vector to express channelrhodopsin-2 in distinct populations of VTA efferents (Fenno et al., 2014; Stamatakis et al., 2013). This indicates that HSV can be used in mice for efficient retrograde transduction of projection neurons and long-term transgene expression.

In the present series of experiments, we investigated the role of the BNST-VTA neural circuit in seeking behavior induced by an ethanol-associated cue. We employed a convergent dual-virus strategy to selectively manipulate BNST-VTA projections in mice during ethanol-seeking behavior modeled by CPP. First, BNST projection neurons to VTA were retrogradely traced using an HSV amplicon vector carrying the green fluorescent protein (GFP) gene (Exp.1).

After determining the post-infusion delay necessary for retrograde transport from the VTA to BNST, an HSV encoding cre recombinase was combined with a cre-inducible AAV encoding inhibitory DREADDs (hM4Di). In this manner, hM4Di expression was restricted to BNST-VTA projections neurons, enabling us to inhibit their activity during ethanol seeking modeled in a CPP procedure (Exps. 2-3). We hypothesized that inactivation of BNST-VTA cells during the CPP test would disrupt ethanol-induced place preference expression, thus suggesting that ethanol seeking is conveyed through a direct BNST projection to VTA.

Materials and Methods[17]

Animals

Male DBA/2J mice (n = 125) purchased from Jackson Laboratory (Sacramento, CA) were 7 weeks of age at arrival. Mice were housed in groups of four per cage in a colony room maintained on a 12:12 light-dark cycle (lights on at 07:00 am) at an ambient temperature of 21+/-1°C. Home cage access to food and water was provided *ad libitum* throughout all experiments. All procedures complied with the National Institutes of Health Guide For the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011) and were approved by Oregon Health & Science University's Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

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[17] Additional details on the general materials and methods can be found in Chapter 1.

Drugs

Ethanol (20% v/v) was administered intraperitoneally (IP) at a dose of 2 g/kg in a 12.5 mL/kg volume. Solutions were prepared from a 95% ethanol stock and diluted in 0.9% saline.

To stimulate hM4Di receptors, clozapine-N-oxide (CNO; Tocris Bioscience, Ellisville, MO) was dissolved in 0.9% saline and administered at 10 mg/kg (10 mL/kg, IP) 30 min before the CPP test. This dose of CNO produces no physiological or behavioral response in rodents (Li et al., 2013; Mahler et al., 2014; Ray et al., 2011, 2013; Vazey and Aston-Jones, 2014). In addition, we have shown that CNO does not affect ethanol-induced CPP expression or locomotor activity in DBA/2J mice at 10 or 20 mg/kg (Pina et al., 2015).

Stereotaxic surgery

Mice were anesthetized with isoflurane (4% in O_2 for induction) and secured in a stereotaxic frame (Model No. 1900; Kopf Instruments, Tujunga, CA). Anesthesia was maintained (1-3% isoflurane in O_2) for the duration of the procedure. To minimize post-operative discomfort, the non-steroidal anti-inflammatory drug meloxicam (0.2 mg/kg; 10 mL/kg) was subcutaneously delivered immediately before and 24 h after surgery.

Stereotaxic coordinates for the VTA and BNST were derived from a standard atlas of the mouse brain (Paxinos & Franklin, 2001) and both regions were targeted during the same surgery. For the VTA, injectors were aimed at the more medial aspect of this region (from bregma: posterior (AP) -3.2, lateral (ML) ±0.5, ventral (DV) -4.69). Starting coordinates for the BNST were as follows: AP

+0.26, ML ±0.8, DV -4.07 from bregma. To avoid the lateral ventricles during virus infusion, the BNST was approached at a 20˚ coronal angle. Entry holes were drilled in the skull ±2.3 mm lateral and +0.26 mm rostral to bregma. The head was then tilted 20˚ left or right on a coronal axis and an injector was lowered 4.33 mm from the top of the skull on each side.

Viral-mediated gene transfer

To inhibit the BNST-VTA circuit, inhibitory Designer Receptors Exclusively Activated by Designer Drugs (hM4Di-DREADDs; (Armbruster et al., 2007) were selectively expressed in VTA-projecting BNST cells. To achieve transgene expression in this discrete subset of BNST neurons, a retrograde intersectional approach was used that combines two viral vectors (Fig. 14). First, a retrograde herpes-simplex virus type 1 (HSV)-derived amplicon vector was infused into the VTA to drive long-term expression of cre-recombinase in VTA-projecting cells. Next, a cre-inducible adeno-associated virus (AAV) was delivered into the BNST to selectively express hM4Di in VTA-projecting BNST cells.

Vectors were delivered using injectors made of 32-ga stainless steel tubing encased in 26-ga stainless steel, which was attached via polyethylene tubing (PE-20) to 1 µl Hamilton syringes. Infusions of 200 nL/side were delivered by syringe pump (Model PHD 22/2000; Harvard Apparatus, Plymouth Meeting, PA) at a rate of 20 nL/min. Injectors were left in place for an additional 5 min after infusions to ensure complete diffusion of virus and minimize tracking of the vector up the injector path upon removal. A post-infusion delay of 2-8 weeks, depending on experiment, was given for surgical recovery and transgene expression.

Herpes simplex virus (HSV) vector. All HSV vectors were purchased from Massachusetts Institute of Technology's Viral Gene Transfer Core (Cambridge, MA) and infused bilaterally into the VTA. HSV carrying an enhanced green fluorescence protein-encoding gene (hEF1α-EGFP; > 3 x 10e8 transducing units (TU) per mL) was used in Exps. 1 and 3. This vector (HSV-GFP) served as a (1) retrograde tracer to determine the optimal post-infusion delay required for maximal transgene expression in BNST (Exp. 1); and (2) a control vector to test for nonspecific effects of surgery and transgene expression on behavior (Exp. 3). In Exp. 2, HSV carrying the gene encoding for cre recombinase (hEF1α-EGFP-IRES-Cre; > 3 x 10e8 TU/mL) was used.

Adeno-associated virus (AAV) vector. In all experiments, cre-inducible AAV8-hSyn-DIO-hM4D(Gi)-mCherry (5-7 x 10e12 virus molecules per mL; University of North Carolina Vector Core, Chapel Hill, NC) was infused bilaterally into the BNST.

Immunohistochemistry

Mice were anesthetized with isoflurane and transcardially perfused with ice cold 1X phosphate buffered saline (PBS; pH 7.4), then 4% paraformaldehyde (PFA) in 1X PBS. Brains were removed and immersed overnight in 4% PFA then cryoprotected in 20% sucrose in PBS containing 0.1% NaN_3 (to inhibit bacterial growth) for 24-48 h, followed by 30% sucrose/0.1% NaN_3/PBS for 24-48 h. Using a cryostat, coronal sections (30 µm) were taken from the rostral end of the BNST to the caudal end of the VTA (from +0.62 to -4.0 mm from bregma). In addition, from each replication, mice from CNO and vehicle groups (n = 1, each) were

sagittally sectioned (60 µm) to view the rostral-caudal extent of hM4Di+ BNST projections to VTA. Slices were stored in 24-well plates containing 0.1% NaN_3/PBS until being processed for immunofluorescence (IF). Free-floating sections were processed for IF to detect GFP/YFP (HSV-GFP and HSV-Cre) and mCherry-tagged hM4Di protein. Sections were washed in PBS then immersed in sodium borohydride ($NaBH_4$, 1% w/v in PBS) for 30 min to minimize fixative-induced autofluorescence (Beisker et al., 1987; Kobelt et al., 2004; Tagliaferro et al., 1997). Tissue was rinsed in tris-buffered saline (TBS) to remove $NaBH_4$ before being permeabilized and blocked in 5% normal donkey serum (NDS)/0.3% Triton X-100/TBS for 45 min. Sections were incubated overnight with gentle agitation at 4ºC in 5% NDS/0.3% Triton X-100/TBS containing a goat polyclonal antibody to GFP/YPF (1:2000; ab6673, Abcam, Cambridge, MA) and a rabbit polyclonal antibody to mCherry (1:2000; ab167453, Abcam). Next, sections were rinsed in TBS and incubated for 2 h in a solution of 5% NDS/0.3% Triton X-100/TBS containing Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400; ab150129, Abcam) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:400; ab150076, Abcam). After final washes, sections were rinsed in PBS, mounted on gelatinized slides, coverslipped and sealed in an aqueous mounting medium containing DAPI for nuclear counterstaining (ProLong Gold Antifade, Life Technologies, Eugene, OR).

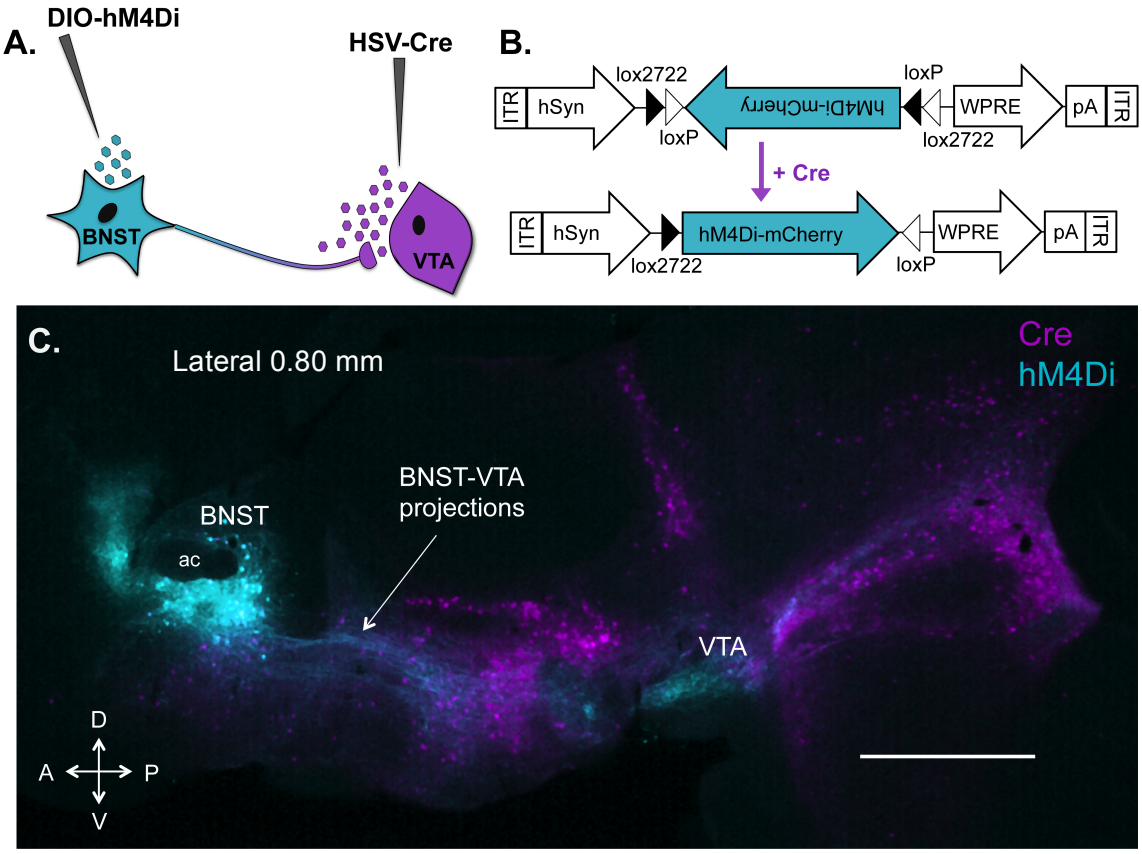

Figure 14. Expression of hM4Di receptors in VTA-projecting BNST neurons using a retrograde intersectional strategy. (A) A dual-viral approach was used to drive expression of inhibitor designer receptors (hM4Di) in a distinct yet intermixed subpopulation of BNST neurons that project to VTA. A long-term retrograde HSV encoding cre recombinase was delivered into the VTA and a cre-dependent AAV-hM4Di was delivered into the BNST. (B hM4Di-mCherry AAV vector design employing the double-floxed inverted open reading frame (DIO) a.k.a. FLEX (flip-excision) strategy. Two pairs of heterotypic, antiparallel loxP-type recombination sites achieve cre-mediated hM4Di inversion and expression under the control of a human synapsin (hSyn) promoter. WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; ITR, inverted terminal repeat, pA, human growth hormone polyadenylation site (C) Sagittal section showing intersection of retrogradely transported HSV-cre (VTA-projecting cre+ cells pseudocolored in magenta were visualized by immunofluorescent (IF) detection of EYFP) and AAV expressing cre-dependent hM4Di (hM4Di+ BNST-VTA cells pseudocolored in cyan were visualized by IF detection of mCherry) 8 weeks after vector infusions. Robust expression of hM4Di is visible in soma (in BNST) and fibers (to VTA) of BNST-VTA neurons. HSV-cre transfected neurons projecting to VTA are visible throughout the brain, with the exception of VTA as retrograde HSV does not infect cell bodies at the site of injection. Note the absence of hM4Di in all HSV-Cre transfected cells outside the BNST. This illustrates that hM4Di is localized to VTA projecting neurons within the BNST only. D, dorsal; V, ventral; A, anterior; P, posterior; scale bar, 1 mm.

Tissue was visualized and recorded on a Leica DM4000 B microscope. Slices from the BNST to VTA of each mouse were analyzed to determine the location and extent of transgene expression. Procedures for IF staining of sagittal sections were similar to those above, except that a 0.4% Triton X-100 was used and tissue was incubated in primary antibodies for 48 h and secondary antibodies for 24 h to allow for better permeabilization and penetration of 60 µm thick tissue. For presentation, channels were merged and images cropped and contrast adjusted using Fiji software (NIH).

Apparatus

Detailed descriptions of the CPP apparatus have been published (Cunningham et al., 2006). In brief, animals were conditioned in 12 identical acrylic and aluminum chambers, each housed in a light- and sound-attenuating enclosure. Locomotor activity and position within the apparatus were recorded by infrared photodetectors that line each chamber. Interchangeable tactile floor halves (grid or hole) that are equally preferred by drug-naïve DBA/2J mice (Cunningham et al., 2003) were used as conditioned stimuli (CS). Floors were separated during conditioning by a clear acrylic divider placed in the center of the apparatus.

CPP Procedure

General methods of our ethanol-induced CPP procedure have been published in detail (Cunningham et al., 2006; Pina et al., 2015). In experiments 2-3, mice were randomly assigned to one of two drug treatment groups, CNO or

vehicle. An unbiased place preference procedure was used, which involved three distinct phases: habituation (one 5-min session), conditioning (four 5-min sessions) and preference test (one 30-min session).

Habituation. Habituation was conducted between the hours of 12 pm – 2 pm and consisted of one 5-min session, where mice were injected with saline and immediately placed inside the apparatus on a smooth white paper floor. This session was designed to reduce the stress of initial handling and injection as well as the novelty of the apparatus.

Conditioning. After initial assignment to drug treatment groups (CNO or vehicle), mice were subdivided into conditioning subgroups (Grid+ or Grid-). In the Grid+ subgroup, ethanol was paired with the grid floor (CS+) and saline was paired with the hole floor (CS-), whereas in the Grid- subgroup, ethanol was paired with the hole floor (CS+) and saline was paired with the grid floor (CS-). During conditioning, a clear acrylic divider separated the floor cues and mice were confined to one half of the apparatus. The position of each floor type was counterbalanced (i.e., left vs. right) within each conditioning subgroup. A two trial per day procedure was used, where saline (CS-) trials were administered in the morning from 10 am to 12 pm and ethanol (CS+) trials were administered in the afternoon from 2 pm to 4 pm. Each mouse received two 5-min conditioning trials of each type (CS+ and CS-) over a 2-day period before the preference test.

Place preference test. A place preference test was performed 24 h after the last CS+ conditioning session. Testing took place between the hours of 12 pm to 2 pm. The acrylic divider was removed before the test and mice were given

free access to the entire chamber consisting of both grid and hole floors for 30 min. No ethanol was administered on the test day; instead, a saline injection was given in place of ethanol. In order to activate hM4Di receptors, an IP injection of CNO (10 mg/kg) was delivered 30 min before the preference test.

Experimental Design

Exp. 1 – determination of retrograde transport time from VTA to BNST. In Exp. 1 (n = 9), we determined the time required for retrograde transport from the VTA to the BNST using an HSV carrying a GFP reporter gene (HSV-GFP). Neural tissue was harvested 2, 3, and 4 weeks after HSV-GFP infusion and mice were randomly assigned to one of three groups (2, 3, 4) based on post-infusion timepoint. Tissue was sectioned then immunostained to enhance the native GFP signal and images were recorded at 20X magnification from the dorsal and ventral BNST (dBNST and vBNST). Within each optical field, the total number of cells expressing GFP (GFP+) were counted manually using the Cell Counter plugin in Fiji. For each mouse, GFP counts were made bilaterally over 2-4 serial sections, each containing dBNST and vBNST, and then averaged. Finally, the mean GFP+ cells per optical field were compared across groups.

Exp. 2 – effect of BNST-VTA inhibition on ethanol-induced CPP expression. In Exp. 2 (n = 70), the involvement of BNST-VTA cells in ethanol-induced CPP expression was assessed. Designer hM4Di receptors were selectively expressed in all mice by delivering HSV-Cre into VTA and a cre-inducible AAV-hM4Di into BNST. After allowing 8 weeks for full transgene

expression, mice underwent CPP conditioning before being tested for preference expression. To activate hM4Di receptors and thereby inhibit BNST-VTA cells, CNO (10 mg/kg, IP) was administered 30 min prior to the CPP test.

Exp. 3 – effect of control transgene expression on ethanol-induced CPP. In Exp. 3 (n = 46), we tested for hM4Di expression in the absence of Cre (i.e., viral leakage) and the effect of CNO and control transgene expression on ethanol-induced CPP. Hence, this control experiment was designed to 1) confirm that expression of hM4Di was dependent on the presence of cre and therefore confined to BNST-VTA cells, and 2) control for the non-specific effects of surgery, transgene expression, and CNO on ethanol-induced CPP expression. All mice received infusion of HSV-GFP (no cre) into VTA and cre-inducible AAV-hM4Di into BNST. As in Exp. 2, mice were conditioned and tested in the CPP procedure 8 wks after viral infusions. On test day, CNO (10 mg/kg, IP) was administered 30 min before placement in the test apparatus.

General Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) with the level of significance set at 0.05, unless otherwise noted. Where appropriate, follow up tests were performed to evaluate the pairwise differences among the means and p-values were Bonferroni corrected for the number of post-hoc comparisons. Analyses specific to preference data and locomotor activity are described in detail below.

Preference tests. The main dependent variable for preference test analyses was time spent on the grid floor. Place preference was denoted when

a significance difference in grid time between Grid+ and Grid- conditioning subgroups was found (Cunningham et al., 2003; 2006). Two-way ANOVA (drug x conditioning) was used to analyze preference data, where drug indicates test pre-treatment (CNO vs. vehicle) and conditioning refers to conditioning subgroup (Grid+ vs. Grid-). To assess the impact of drug BNST-VTA inactivation over the course of the test, data were expressed as percent time on the ethanol-paired floor (CS+) by collapsing across conditioning subgroups (Grid+ and Grid-), then averaged across 5-min intervals and analyzed by two-way mixed-factorial ANOVA (drug x interval). Test activity was analyzed by one-way ANOVA (drug).

Conditioning activity. Conditioning session activity (counts/min) was collapsed over sessions by trial type (CS+ and CS−) and analyzed by two-way mixed-factorial ANOVA (drug x trial type).

Results

Exp. 1 – determination of retrograde transport time from VTA to BNST. In this experiment, the minimal time required for retrograde transport from the VTA to BNST and full transgene expression in BNST was determined. An HSV vector encoding for GFP was infused into the VTA and the total number of BNST neurons positive for GFP (GFP+) were counted in tissue harvested at 2, 3, and 4 weeks after vector infusion. Two weeks following HSV-GFP infusion, VTA-projecting (GFP+) cells were found throughout the anterior and posterior BNST in mediolateral and dorsoventral subdivisions, with exclusion of the oval nucleus (Fig. 15). The total number of BNST-VTA cells expressing GFP did not differ by

time [$F(2,6) = 0.75$, $p > 0.50$], indicating that VTA to BNST retrograde transport and full transgene expression were achieved within 2 weeks of viral delivery (Fig. 16). It has been reported that axonal transport of most AAV serotypes occurs within 4-9 weeks (e.g., Castle, Gershenson, Giles, Holzbaur, & Wolfe, 2014; Salegio, Samaranch, Kells, & Mittermeyer, 2013; Smith, Bucci, Luikart, & Mahler, 2016) and our previous work has demonstrated robust AAV-mediated hM4Di expression in soma and axons of BNST cells within 6 weeks (Pina et al., 2015). Thus, when combining HSV-Cre with an AAV8-DIO-hM4Di, we determined that 8 weeks of incubation would be sufficient to obtain robust hM4Di expression in BNST-VTA cells.

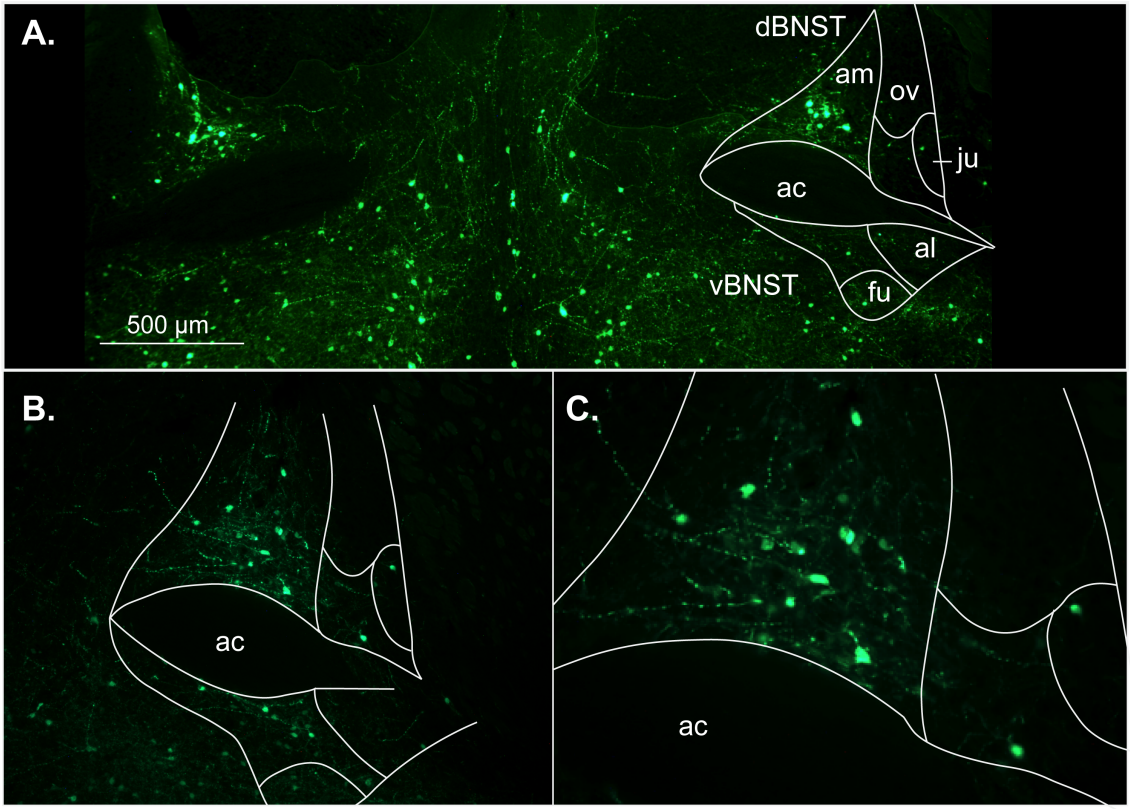

Figure 15. Retrograde HSV vector-mediated GFP expression in BNST-VTA cells 2 weeks after intra-VTA HSV infusion. (A) GFP expression in VTA-projections neurons of dorsal and ventral BNST (dBNST, vBNST) nuclei (outlined in white) and proximal regions. ac, anterior commissure; al, anterolateral BNST. Highest levels of GFP expression were observed in amBNST. GFP expression was absent in ovBNST; fu, fusiform nucleus of BNST; ju, juxtacapsular nucleus of BNST; ov, oval nucleus of BNST. (B-C) GFP expression in dBNST and vBNST subdivisions at 10X and 20X magnification, respectively.

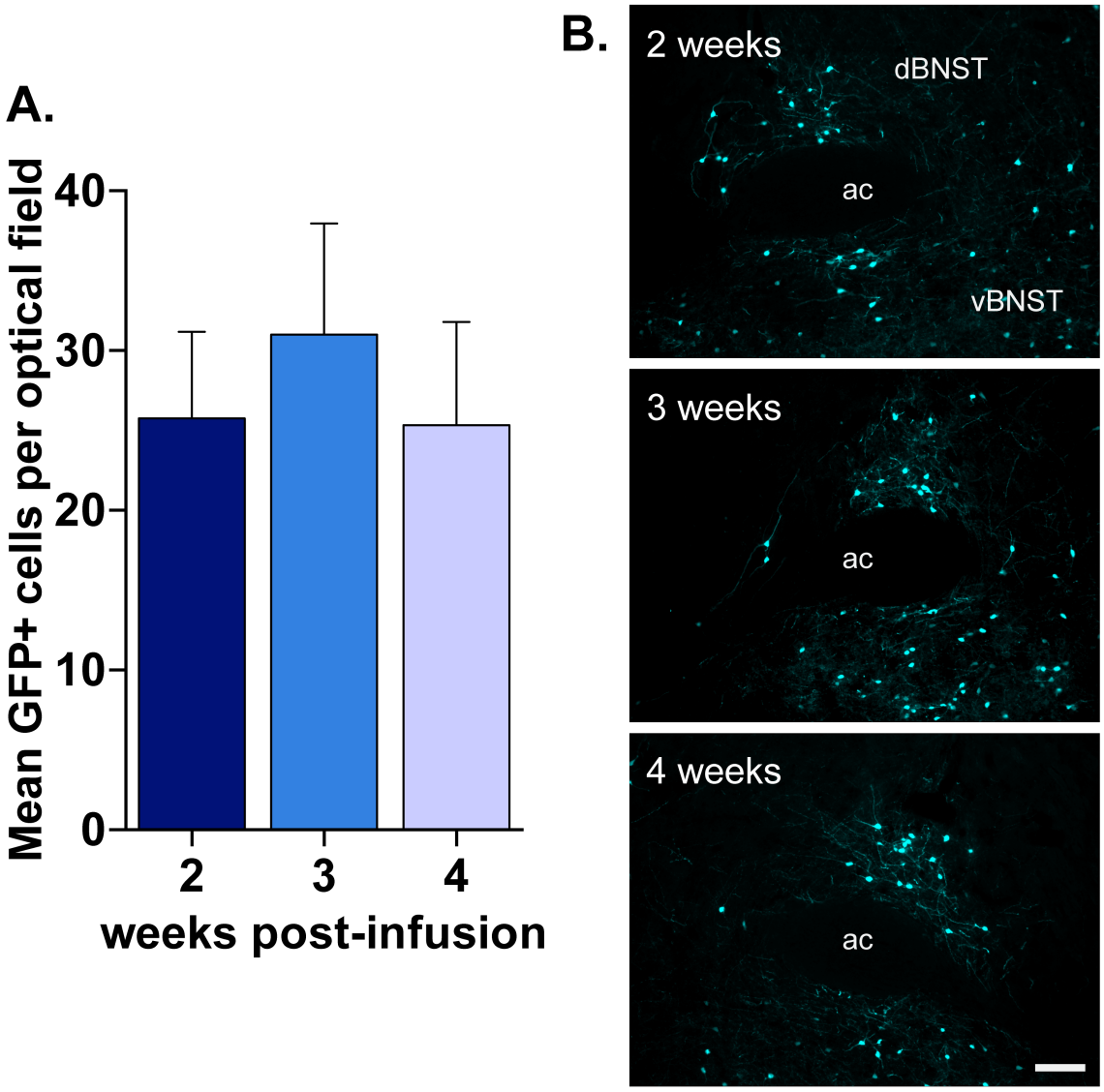

Figure 16. GFP expression in BNST neurons at 2-4 weeks after intra-VTA HSV-GFP infusion. (A) Mean (+SEM) GFP+ cells per optical field. Images were taken from BNST tissue of mice after 2, 3 and 4 weeks of incubation (n = 3/timepoint). Total dBNST & vBNST GFP+ cells were counted bihemispherically in 2-4 serial sections and averaged for each mouse. No difference in the number of GFP+ cells was found across timepoints, suggesting that retrograde transport from VTA to BNST and maximal GFP expression in BNST occurred within 2 weeks of HSV-GFP infusion. (B) Representative photomicrographs taken at 10X magnification illustrating GFP expression (pseudocolored in cyan) in dBNST and vBNST after each post-infusion timepoint. Scale bar, 100 μm; ac, anterior commissure.

Exp. 2 – effect of BNST-VTA inhibition on ethanol-induced CPP expression. The involvement of a BNST projection to VTA in ethanol-induced place preference expression was assessed in Exp. 2. Using an intersectional viral approach, hM4Di receptors were selectively expressed in BNST-VTA cells and activated during the expression test by CNO. Figures 14C and 17A-B illustrate the intersection of AAV-hM4Di (cyan; injected into BNST) and HSV-cre (magenta; injected into VTA). Robust expression of cre recombinase and hM4Di was visible within 8 week of vector infusions in BNST (soma and processes) and VTA (axonal projections). Several mice were excluded from analyses for absent bilateral (n = 1) and unilateral[18] (n = 7) hM4Di expression, hM4Di expression outside the BNST (n = 1), and surgical issues (n = 3), resulting in a final sample size of n = 58 mice. As shown in Fig. 17C, inactivation of the BNST-VTA cells (via CNO-induced hM4Di activation) blocked ethanol-induced CPP expression. This was demonstrated by a significant drug x conditioning interaction [$F(1,54) = 11.48$, $p \leq 0.001$] and main effect of conditioning [$F(1,54) = 32.45$, $p < 0.001$] but not drug ($F < 1$). Bonferroni-corrected post-hoc analyses revealed a significant difference in time spent on the grid floor between conditioning subgroups (Grid+

[18]Drug and conditioning subgroup assignments of all mice with unilateral expression were as follows: CNO Grid+ (n = 1); CNO Grid- (n = 5); Vehicle Grid+ (n = 1). Consequently, we were unable to include mice with unilateral expression as an additional control group in this experiment, as there was an insufficient number of CNO-treated mice in the Grid+ subgroup. Thus, a meaningful comparison could not be made between the mean time spent on the grid floor between CNO-treated Grid+ and Grid- animals.

and Grid-) in the vehicle-treated group only ($p < 0.001$). When assessed over time, CNO reduced the percent time spent on the ethanol-paired floor compared to vehicle across the duration of the test (Fig 17D). Analyses showed a significant main effect of drug [$F(1,52) = 11.44$, $p \leq 0.001$] but not conditioning and no drug x time interaction.

Table 6 includes mean activity counts per min (±SEM) during the preference test and conditioning. CNO-mediated hM4Di activation in BNST-VTA cells did not affect locomotor activity during the preference test, as no main effect of drug was found ($F < 1$). During conditioning (before CNO treatment), animals exhibited robust ethanol-stimulated locomotor activity and there were no group differences on saline or ethanol trials. Analyses revealed a significant main effect of trial type (CS+ vs. CS-) [$F(1,56) = 509.29$, $p < 0.001$], but not group and no group x type interaction (F's < 1).

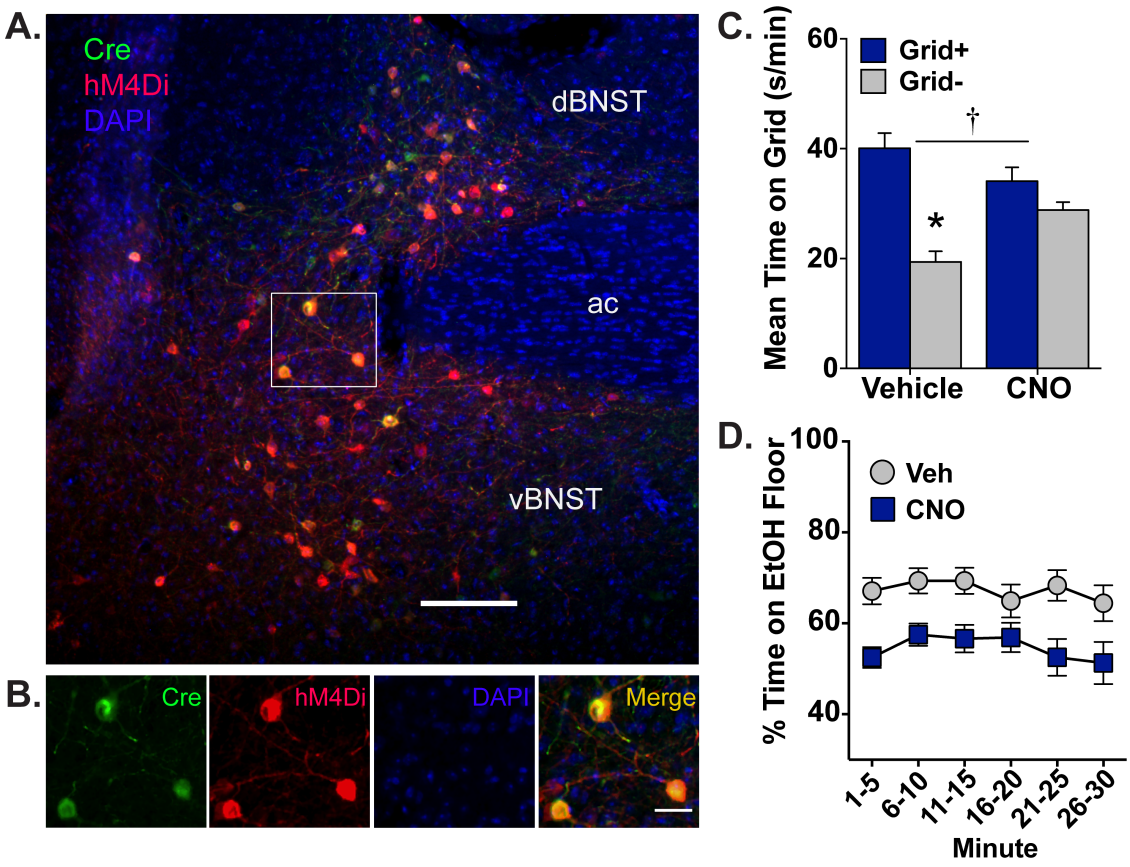

Figure 17. Ethanol-induced CPP expression is blocked by activation of hM4Di receptors selectively expressed in BNST-VTA cells. (A) Heterologous expression of cre-dependent hM4Di (red; visualized by immunofluorescent (IF) detection of mCherry) is observed in VTA-projecting neurons (green; visualized by IF detection of EYFP) of dorsal and ventral BNST. Nuclei are counterstained blue with DAPI; ac, anterior commissure; scale bar, 200 µm. (B) Zoomed in image of region outlined in white box above illustrating overlap HSV-mediated cre expression (green), DIO-hM4Di expression (red), and their overlap (orange-yellow), with nuclei stained blue. Scale bar, 50 µm. (C) Mean (+SEM) time spent on the grid floor (in s/min) during 30-min preference test. VTA-projecting BNST neurons were inhibited via CNO (10 mg/kg)-mediated stimulation of hM4Di. Inhibition of BNST-VTA signaling blocked the expression of ethanol-induced CPP. † $p \leq 0.001$ interaction between drug and conditioning subgroup (Grid+ vs. Grid-); * $p < 0.001$ between conditioning subgroups; n = 13-15/subgroup. (D) Mean percent time (±SEM) spent on the ethanol-paired floor in 5-min intervals across the 30-min

Table 6. Chapter 3 Activity

Mean Activity Counts per Minute (± SEM) during conditioning and preference test

	Group	CS+ Trials	CS- Trials	Preference Test
Experiment 2				
BNST-VTA inactivation	Vehicle	98.0 ± 4.5	37.4 ± 1.2	37.7 ± 1.3
	CNO	100.2 ± 4.3	35.8 ± 1.7	37.0 ± 1.1
Experiment 3				
BNST-VTA control	Vehicle	107.1 ± 5.0	38.9 ± 2.0	39.9 ± 1.2
	CNO	110.9 ± 4.2	40.5 ± 1.4	39.1 ± 2.0

Exp. 3 – effect of GFP expression on ethanol-induced CPP. To control for non-specific effects of surgery, transgene expression, and CNO on ethanol-induced CPP, mice were tested in the absence of hM4Di. A cre-inducible AAV carrying hM4Di was infused into the BNST and a control vector lacking cre (HSV-GFP) was infused into the VTA. Given the absence of cre, no hM4Di expression was observed after 8 weeks of incubation, indicating that no transgene leakage occurred with this strategy (Fig. 18A-B). Moreover, CNO did not affect ethanol-induced CPP in the absence of hM4Di (Fig. 18C-D), as supported by a significant main effect of conditioning [$F(1,42) = 43.13$, $p < 0.001$] but not drug or drug x conditioning interaction (p's > 0.05). No significant differences in percent time spent on the ethanol floor were found between CNO- and vehicle-treated groups across the duration of the test, as indicated by non-significant effects of drug, time, and drug x time. Neither test nor conditioning activity differed between CNO and vehicle groups. As expected, conditioning activity was higher on ethanol trials than on saline trials [main effect of trial type, $F(1,44) = 503.04$, $p < 0.001$].

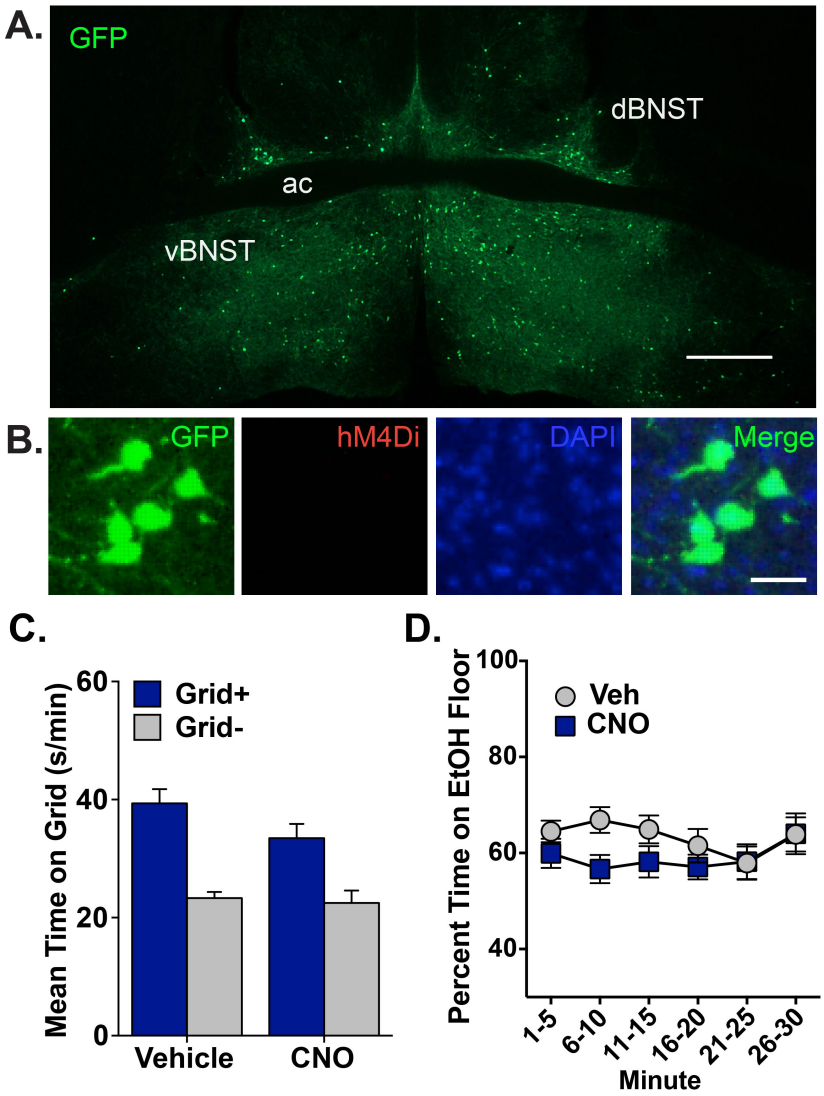

Figure 18. Ethanol CPP expression is not disrupted by CNO in mice expressing GFP in VTA-projecting BNST cells. (A) Expression of GFP in dBNST and vBNST 8 weeks after infusion of HSV-GFP into VTA and DIO-hM4Di into BNST. GFP+ cells (green) indicate VTA-projecting neurons. No hM4Di expression was visible in BNST. ac, anterior commissure; scale bar, 500 µm (B) Zoomed in image from BNST of GFP (green; visualized by immunofluorescent (IF) detection of EGFP), hM4Di (absent; visualized by IF detection of mCherry), nuclei (DAPI) and all channels merged. Note hM4Di is not expressed in the absence of cre; scale bar, 50 µm. (C) Mean (+SEM) time spent on the grid floor (in s/min) during 30-min preference test. CNO did not disrupt ethanol CPP expression in mice expressing GFP but not hM4Di in BNST-VTA cells.; n = 11-12 / conditioning subgroup (Grid+, Grid). (D) There was no significant difference in percent time spent on the ethanol-paired floor between groups when analyzed in 5-min intervals across the 30-min preference test.

Discussion

The present experiments assessed the involvement of a direct neuronal projection from the BNST to the VTA in ethanol-induced CPP expression. A unique intersectional viral strategy was used to selectively target inhibitory DREADDs (hM4Di) to BNST-VTA cells. This involved infusions of a long-term retrograde HSV vector encoding cre recombinase (hEf1α-EYFP-IRES-cre; HSV-cre) into the VTA and a cre-inducible vector encoding for hM4Di receptors (AAV8-hSyn-DIO-hM4D(Gi)-mCherry) into the BNST. This approached allowed for the direct modulation of BNST-VTA cells during ethanol-seeking behavior by CNO-mediated hM4Di activation.

In Exp. 1, we determined that retrograde HSV transport from the VTA to the BNST occurred within 2 weeks of HSV-GFP infusion. This was indicated by robust GFP expression in BNST that did not differ by delay time (2, 3, and 4 weeks post-infusion). In Exp. 2, a retrograde HSV-cre was combined with a cre-inducible AAV-hM4Di to express hM4Di receptors in VTA-projecting BNST cells. Prior to the preference test, hM4Di receptors were activated by injection of CNO, leading to BNST-VTA inhibition. Place preference was blocked in CNO-treated animals, indicating that BNST-VTA circuit activation is necessary for ethanol-induced CPP expression. In Exp. 3, we controlled for nonspecific effects of surgery, transgene expression, and CNO administration on ethanol-induced CPP expression. Procedures were identical to those in Exp. 2 except that mice were administered a control HSV-GFP vector in place of HSV-cre. This resulted in GFP expression in VTA-projecting cells and a marked absence of hM4Di in

BNST. Mice lacking hM4Di receptors exhibited normal levels of ethanol-induced CPP that did not significantly differ in magnitude by CNO or vehicle treatment. These results support Exp. 2 findings and indicate that disruptions in CPP were due to CNO-mediated hM4Di inhibition of BNST-VTA cells. Together, our findings demonstrate that ethanol-induced CPP is expressed through a serial projection from the BNST to VTA.

To our knowledge, this is the first experiment to demonstrate that a BNST projection to the VTA is involved in ethanol-induced CPP. This finding is consistent with other studies that have shown a role for the BNST and BNST-VTA neural circuit in relapse to drug-seeking behavior. For instance, earlier work has indicated that the BNST is activated by cocaine- and ethanol-associated stimuli (Hill et al., 2007; Mahler & Aston-Jones, 2012; Zhao et al., 2006). Furthermore, inactivating the BNST has been shown to reduce cue-induced cocaine-, heroin-, and ethanol-seeking behavior (Buffalari & See, 2011; Pina et al., 2015; Rogers et al., 2008; Sartor & Aston-Jones, 2012). Whereas these studies implicate the BNST in general, additional evidence has suggested this region modulates positive motivational states through a direct projection to the VTA. Of note, dorsal and ventral BNST inputs to VTA are known to potently innervate DA cells (Georges & Aston-Jones, 2001; 2002; Jalabert et al., 2009). This innervation triggers VTA DA cell burst firing, which is a putative mechanism of motivated behavior (Adamantidis et al., 2011; Schultz, 1986; Wanat et al., 2009). More directly, BNST afferents of the VTA are activated by cocaine-associated cue exposure and pharmacological disconnection of this circuit has

been shown to block cocaine place preference (Sartor & Aston-Jones, 2012). Other evidence indicates that a BNST-VTA pathway is explicitly engaged following ethanol exposure and withdrawal. For instance, simultaneous manipulation of $GABA_A$ receptors in BNST and D2 receptors in VTA disrupted ethanol-seeking behavior in preferring (P) rats (Eiler et al., 2003) and chronic ethanol intake and withdrawal enhanced excitatory input onto VTA-projecting BNST cells (Silberman, Matthews, & Winder, 2013). Our findings support this earlier work and further implicate a role of the BNST-VTA circuit in seeking behavior engaged by exposure to an ethanol-associated cue. Combined with work demonstrating ethanol intake- and withdrawal-related enhancements in excitatory input on BNST-VTA cells, our results suggest that cue-exposure may engage an already-upregulated circuit further strengthening ethanol-motivated behaviors.

Much of the work demonstrating BNST modulation of DA cells proposes that excitatory, likely glutamatergic, projections from the BNST directly innervate VTA DA neurons (Jalabert et al., 2009). Indeed, the BNST is known to send monosynaptic inputs to VTA DA cells (Watabe-Uchida et al., 2012), which renders DA activity tractable to modulation by the BNST However, more recent work indicates that BNST efferents preferentially innervate non-DAergic (putatively GABAergic) VTA cells (Jennings et al., 2013). Overall, 70-90% of BNST cells are GABAergic (Le Gal LaSalle, Paxinos, & Ben-Ari, 1978; N. Sun & Cassell, 1993). Moreover, of the three distinct types of VTA-projecting BNST cells (GAD+/VGlut-, VGlut2+/GAD-, VGlut3+/GAD+), 90% are GAD+/VGlut-, i.e.,

GABAergic/non-glutamatergic (Kudo et al., 2012). Thus, it is likely that VTA DA cell activation may result from BNST GABA innervation of VTA GABA cells, the net result of which is DA disinhibition. Notably, Jennings & Sparta et al. (2013) have shown that BNST glutamatergic innervation of VTA generates an aversive state, which is the diametric opposite of the positive motivational state produced by BNST GABA to VTA. In addition, a direct corticotropin releasing factor (CRF) neuronal projection from the BNST to VTA has also been identified (Rodaros, Caruana, Amir, & Stewart, 2007; Vranjkovic et al., 2014) and is likely contained in GABAergic neurons (Dabrowska, Hazra, Guo, DeWitt, & Rainnie, 2013). Notably, a BNST CRF projection to VTA was recently shown to regulate binge-like consumption of ethanol (Rinker et al., 2016).

In considering the above evidence, the possibility remains that the VTA glutamate input involved in ethanol-induced CPP expression (established in Chapter 1) may originate from a source(s) other than the BNST. Thus, while the present work was driven by the hypothesis that BNST glutamate input to VTA DA drives drug seeking, we cannot determine whether this is a mechanism through which ethanol-induced CPP is expressed. Whereas the two-virus strategy employed here enabled circuit-selective manipulation, it did not allow for cell-specific targeting. Although previous studies have established intersectional approaches that target genetically defined cells within neural circuits, they require the use of transgenic animals (Fenno et al., 2014; Stamatakis et al., 2013). Considering the difficult nature of establishing ethanol-induced CPP in numerous mouse strains (Cunningham, 1995; 2014), the use of cre transgenic lines was not

presently feasible. Thus, follow-up studies are needed to identify the exact cell populations within the BNST and VTA that are responsible for ethanol-induced CPP expression.

In addition to cell-specific contributions, another important consideration to be addressed is the involvement of distinct BNST subdivisions in ethanol-induced CPP. While there are discrepancies in the boundaries and total number of nuclei that comprise the BNST (Ju & Swanson, 1989; Moga, Saper, & Gray, 1989), studies have ascribed distinct roles to several divisions. For example, in the dBNST, the oval (ovBNST) and anterodorsal (adBNST) nuclei control varying anxiogenic behaviors (Kim et al., 2013a). The vBNST has been implicated in maternal behavior (Numan & Numan, 1997), cocaine CPP expression (Sartor & Aston-Jones, 2012), and heroin-primed reinstatement (Rogers et al., 2008), whereas the medial posterior (mpBNST) subdivision is involved in both heroin and cue-primed reinstatement (Rogers et al., 2008). Given the scope of these previous studies, the connectivity and terminal output sites of each of these subdivisions were not experimentally addressed. Likewise, the roles of subdivision-specific projections to VTA in behavior and emotional states have not been as well described. The studies that exist have reported involvement of vBNST-VTA CRF in footshock-induced reinstatement of cocaine seeking (Vranjkovic et al., 2014), vBNST-VTA GABA in reward, and vBNST-VTA glutamate in aversion and anxiety (Jennings et al., 2013). Overall, these studies suggest that specific subdivisions, in addition to distinct cell types within each, may differentially contribute to motivation, emotion, and behavior.

Considering the small scale of the mouse brain and spread of hM4Di expression through dorsal and ventral divisions of the BNST, our data provide little evidence of a subdivision-specific mechanism. Nevertheless, some information was provided because of the differential expression obtained in the various BNST subdivisions. The retrograde HSV vector we utilized here produced transgene expression in a broad range of BNST nuclei, spanning across dorsoventral and rostrocaudal gradients. Overall, expression was highest in the medial portions of anterior and posterior vBNST and dBNST. However, expression was notably absent in the ovBNST and weaker in the juxtacapsular (juBNST) nucleus (Fig. 15). Thus, given the weak to absent cre/GFP (and therefore hM4Di) expression in these nuclei, it can be concluded that the ovBNST and likely juBNST were not responsible for the observed disruption in ethanol-induced CPP. Moreover, it is likely that the anteromedial BNST (amBNST) provided the greatest contribution given its high levels of cre/GFP and hM4Di expression. Finally, findings of absent or weak cre/GFP expression should not necessarily be interpreted as evidence of a lack of direct VTA projection. For instance, though we did not find VTA-projecting cells in BNSTov, others have shown this projection exists (Rodaros et al., 2007). Therefore, our results may simply reflect a lack of transgene expression in all neurons[19].

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[19] The human elongation factor 1-alpha (hEf1α) promoter in the HSV-cre/GFP vector used in our experiments is a strong and ubiquitous promoter (Papadakis, Nicklin, Baker, & White, 2004). When combined with HSV (given its natural neurotropism), this should theoretically drive broad neuronal expression (Lachmann, 2004; Neve et al.

Whereas other studies have used a retrograde HSV vector and a DIO or FLEX (flip-excision)-AAV construct to achieve circuit-specific expression of opsins (Fenno et al., 2014; Stamatakis et al., 2013), our experiments are the first to demonstrate circuit-selective DREADD expression using this viral combination. Several previous studies have utilized a similar retrograde intersectional approach to successfully obtain DREADD expression in projection neurons. In these studies, retrograde CAV2-cre was combined with FLEX (a.k.a. DIO) constructs to drive DREADD expression in projection neurons of the rat (Boender et al., 2014; Nair et al., 2013). In these studies, no expression of opsins or DREADDs in the absence of recombinase proteins has been reported. In our experiments, we found no evidence of hM4Di expression outside of cre-positive cells. This null finding is important as it demonstrates the absence of transgene leakage and confirms that hM4Di expression was confined to VTA-projecting BNST cells. Overall, our work further demonstrates the utility of retrograde intersectional approaches and shows that a retrograde HSV can be used in mice for robust long-term transgene expression and combined with AAV-FLEX (a.k.a. DIO) constructs to selectively express DREADDs in serially connected brain nuclei. This approach enables circuit accessibility and highly selective modulation of distinct yet intermixed populations of projection neurons that was previously

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2005). This however does not guarantee all neurons will be transduced as other aspects of the vector such as titer and cell tropism contribute overall to expression levels.

not possible with other techniques.

In summary, we show the successful circuit-selective expression of hM4Di using a unique vector combination. With this approach, VTA-projection neurons in the BNST were inactivated by CNO-mediated hM4Di activation. Inhibition of BNST-VTA cells disrupted the expression of an ethanol-induced place preference. Expression of hM4Di alone did not affect ethanol-induced CPP, as vehicle-treated mice showed significant place preference. As previously shown, in the absence of hM4Di, CNO (10 mg/kg) did not impact ethanol CPP expression (Pina et al., 2015). Here, we further demonstrate that in the presence of a control GFP transgene, CNO produces no effect on ethanol-induced CPP expression. These findings demonstrate that ethanol-induced CPP is expressed through a direct BNST to VTA pathway. In addition to providing support for studies showing BNST and BNST-VTA involvement in cue-induced drug seeking, these experiments demonstrate that the BNST and BNST-VTA circuit are important neural substrates of ethanol-seeking behavior, as indexed by CPP.

Discussion

General Discussion

In the previous chapters, a series of experiments are described that identify a distinct neuronal circuit involved in cue-induced ethanol-seeking behavior, measured using a CPP procedure in mice. First, the role of two individual brain structures, VTA (Chapter 1) and BNST (Chapter 2), in ethanol-induced CPP expression was evaluated. Then, the connection between these structures was assessed during ethanol-induced CPP expression by manipulating a direct neuronal projection originating in the BNST and terminating in the VTA (Chapter 3). Across these experiments, a broad range of techniques was utilized to target these individual brain regions and their connected circuit. Therefore, the discussion of the results will be divided into four separate sections. The first section will describe the relationship between ethanol/drugs, cues and the VTA with specific focus on intra-VTA glutamate regulation of ethanol-induced CPP expression (Chapter 1 findings). The next section will detail ethanol's effects on the BNST and this region's role in seeking behavior, specifically as indexed by ethanol-induced CPP expression (Chapter 2 findings). The third section will discuss evidence of a direct BNST to VTA circuit and its involvement in reward-mediated behaviors, including that of ethanol CPP expression (Chapter 3 findings). Finally, the combined implications of the main findings from each chapter will be summarized and discussed.

Role of VTA glutamate in ethanol-induced CPP expression

Findings from Chapter 1 indicate that the VTA mediates ethanol-induced

CPP expression through ionotropic glutamate receptor (iGluR) activation. In brief, bilateral intra-VTA infusions of an AMPA and NMDA receptor antagonist cocktail (DNQX+AP5) blocked the expression of ethanol-induced place preference. Neither infusion of aCSF into VTA nor DNQX+AP5 into sites proximal to the VTA affected CPP, indicating that expression disruptions were not produced solely by surgical/microinfusion procedures or spread of drug to nearby brain regions. Moreover, locomotor activity was elevated only when DNQX+AP5 was administered at a higher dose or outside of the VTA, demonstrating that DNQX+AP5 effects on activity alone were not responsible for reduced ethanol-induced CPP expression. In summary, these outcomes suggest that glutamatergic input to the VTA is necessary for ethanol-seeking behavior, as measured by CPP. Moreover, findings are in agreement with a sizable body of evidence that demonstrates a vital role for the VTA, DA, and glutamate input to the VTA in the primary and conditioned rewarding effects of ethanol and other abused drugs. This literature is described in detail in the following sections.

Evidence for VTA DA in primary and conditioned drug reward. As a chief source of DA in the mammalian brain, the VTA is tightly linked to the experience of reward and is at the center of many motivational states (Adinoff, 2004). In addition to activation by natural reward presentation (Ljungberg et al., 1992), VTA DA cells are the target of many classes of abused drugs. Whereas activity of VTA DA cells is indirectly increased by drugs like cocaine, heroin, and nicotine (Brodie & Dunwiddie, 1990; Steffensen et al., 2008; Wise, 1996), it is directly increased by ethanol (Brodie, Pesold, & Appel, 1999a; McDaid et al.,

2008; Tateno & Robinson, 2011). Systemically administered ethanol can activate VTA DA cells, even at low doses (Gessa, Melis, Muntoni, & Diana, 1998), and has been shown to enhance somatodendritic DA release (Campbell, Kohl, & McBride, 1996; Kohl, Katner, Chernet, & McBride, 1998) and terminal field release, as indicated by increased DA levels in downstream targets of the VTA such as the amygdala (Yoshimoto et al., 2000) and NAc (Di Chiara & Imperato, 1985). Studies have also shown that rats will self-administer ethanol directly into the VTA (Gatto, McBride, Murphy, Lumeng, & Li, 1994; Rodd et al., 2004). Together, this evidence suggests that the VTA is a focal site of drug and ethanol reward.

Midbrain DAergic systems serve an equally important role in the conditioned rewarding effects of drugs. Evidence of conditioned DA release has been observed in the NAc core following cocaine-associated cue presentation (Ito et al., 2000). Accordingly, in an operant procedure, intra-NAc D1 receptor antagonism has been shown reduce context-induced renewal of punished ethanol seeking (Marchant & Kaganovsky, 2015). Similarly, intra-amygdala D1- and D2-like receptor antagonism has inhibited ethanol-induced CPP expression (Gremel & Cunningham, 2009).

The VTA has been the focus of substantial work, as it is the presumed source of cue-induced DAergic innervation to NAc and amygdala. These studies have identified the VTA as a key neural substrate underlying cue-induced reward seeking. Not only is the VTA robustly activated by ethanol-associated cue exposure (Dayas et al., 2007; Hill et al., 2007), transient inactivation of this region

reduces expression of CPP induced by morphine (Moaddab et al., 2009) and cocaine (Di Ciano & Everitt, 2004a). Likewise, $GABA_B$ agonism and opioid receptor antagonism disrupts ethanol-induced CPP expression (Bechtholt & Cunningham, 2005). Though it is clear that the VTA and DA systems are engaged upon drug-associated cue exposure, the neurochemical input(s) that signal cue-induced VTA DA activation are not well-defined.

Evidence for glutamate-VTA interactions in primary and conditioned drug reward. The neurotransmitter glutamate is known to strongly and directly stimulate DA cells and thus serves as a critical synaptic mechanism regulating VTA DA activity (White, 1996). Through extracellular single unit recordings from rat VTA, previous studies have shown that iontophoretic or pressure ejection of NMDA elicits DA cell bursting (Chergui et al., 1993; Suaud-Chagny, Chergui, Chouvet, & Gonon, 1992) and increases extracellular DA concentrations in terminal fields such as the NAc (Suaud-Chagny et al., 1992). More recent studies employing optogenetics have demonstrated that selective stimulation of glutamate inputs to VTA DA neurons has profound motivational effects, leading to the reinforcement of instrumental behavior and formation of place preference (Lammel et al., 2012; Qi et al., 2014).

As prime targets of ethanol, glutamate receptors have been implicated in ethanol consumption, dependence, tolerance, withdrawal, and craving (e.g., Bernier, Whitaker, & Morikawa, 2011; Blednov & Harris, 2008; Fitzgerald, Liu, & Morzorati, 2012; Griffin, Haun, Hazelbaker, Ramachandra, & Becker, 2014; Kash, Baucum, Conrad, Colbran, & Winder, 2009; McMillen, Crawford, Kulers, &

Williams, 2005; Pignataro, Varodayan, Tannenholz, & Harrison, 2009). Most notably, iGluR activity has been strongly implicated in the development of ethanol-cue associations and the cue-elicited ethanol seeking behavior. For example, previous work has shown that NMDA receptor antagonism blocks the development of ethanol-induced CPP[20] (Boyce-Rustay & Cunningham, 2004) and AMPA/kainate and NMDA receptor antagonism impairs cue-induced reinstatement of ethanol seeking in operant procedures (Bäckström & Hyytiä, 2004; Sanchis-Segura et al., 2006). Additionally, other operant self-administration procedures have shown that AMPA receptor antagonism attenuated cue-induced ethanol seeking in basolateral amygdala (Sciascia et al., 2015) and suppressed habitual ethanol seeking in dorsolateral striatum (Corbit et al., 2014).

Overall, our findings complement previous studies demonstrating iGluR involvement in ethanol-mediated behaviors and reveal that intra-VTA activation of NMDA and AMPA receptors is necessary for the expression of ethanol-induced place preference. The results presented in Chapter 1 strongly suggest that glutamatergic input to VTA is a key neural mechanism driving cue-induced ethanol seeking, as measured by CPP. Although we cannot determine whether AMPA and NMDA receptors were blocked on VTA DA cells directly, we would

[20] In this study, the competitive NMDA receptor antagonist CGP-37849 blocked ethanol-induced CPP acquisition presumably by impairing ethanol-cue associative learning, as this compound also blocked lithium chloride (LiCl)-induced place aversion.

expect that antagonism of these receptors on VTA GABA cells would disinhibit DA and thus enhance CPP. Finally, the present work supports previous research from our lab demonstrating intra-VTA $GABA_B$ and opioid receptor involvement in ethanol-induced CPP expression (Bechtholt & Cunningham, 2005). Here, we confirm VTA involvement in ethanol-induced CPP and report that ethanol seeking is communicated through an intra-VTA iGluR-dependent mechanism. Though these findings underscore the importance of glutamatergic afferents to the VTA in ethanol-induced CPP expression, they fall short of identifying the critical source(s) of these inputs. In the next section, I discuss studies performed in Chapter 2 to address the role of the BNST, a recognized source of VTA glutamate input.

Role of the BNST in ethanol-induced CPP expression

In Chapter 2, the BNST's contribution to ethanol-induced CPP expression was assessed using a combination of classical and contemporary experimental methods. Specifically, the BNST was inactivated during ethanol-induced place preference expression using electrolytic lesions, pharmacological inactivation, and chemogenetic manipulation (aka DREADDs). Inhibition of BNST activity using each of these three techniques reduced ethanol-induced CPP. These results demonstrate a role for the BNST in cue-induced ethanol seeking, as discussed below.

Effects of ethanol on the BNST. The function of the BNST in ethanol-mediated behavior is wide-ranging, spanning from acute reward to relapse. Initial

exposure to ethanol increases extracellular dopamine levels in the BNST (Carboni et al., 2000), presumably through VTA input, and activates BNST cells (Chang et al., 1995; Crankshaw et al., 2003; Demarest et al., 1998; Knapp et al., 2001; Leriche et al., Méndez et al., 2008), leading to increased c-Fos IR (Chang et al., 1995; Knapp et al., 2001; Ryabinin & Wang, 1998; Ryabinin, Criado, & Henriksen, 1997). Ethanol-induced DAergic input to and activation of BNST is putatively reinforcing, as self-administration of ethanol is reduced by intra-BNST antagonism of $GABA_A$ receptors (Hyytiä & Koob, 1995) and D1 receptors (Eiler et al., 2003).

Over time, repeated exposure to ethanol produces neuroadaptations in BNST that drive this structure into a state of enhanced activation. In fact, cycles of chronic intermittent ethanol exposure (CIE) and withdrawal produce upregulation in 5-HT2c receptors and NR2B subunit-containing NMDA receptors in BNST, leading to increased excitability in this region (Kash et al., 2009; Marcinkiewcz, Dorrier, Lopez, & Kash, 2015). Likewise, acute withdrawal from ethanol induces c-Fos IR (Kozell et al., 2005) and increases extracellular CRF levels in BNST (Olive, Koenig, Nannini, & Hodge, 2002). Thus, it has been proposed that patterns of ethanol intake and withdrawal may facilitate the development of ethanol-induced anxiogenic states and stress-induced reinstatement of ethanol seeking (reviewed in Silberman & Winder, 2013).

BNST involvement in stress and anxiety. The BNST is a heterogeneous structure that contains several genetically-defined cell populations (Nguyen, Cruz, Sun, Holmes, & Xu, 2015) and is enriched in CRF

and CRF receptors (Ju & Han, 1989; Van Pett et al., 2000). Thus, the BNST has long been implicated in fear and anxiety, with previous work showing that BNST lesions are accompanied by reduced anxiety and context-associated fear responses (reviewed in Davis, Walker, Miles, & Grillon, 2010). Evidence indicates that BNST CRF systems may underlie aversive and anxiogenic states, as CRF is upregulated in the BNST during chronic stress (Albeck et al., 1997; Stout, Mortas, Owens, Nemeroff, & Moreau, 2000). Within the BNST, CRF neurons have also been shown to modulate conditioned anxiety-like behaviors (Sink et al., 2013) and anxiety induced by ethanol withdrawal (Huang et al., 2010). Notably, CRF signaling in BNST is enhanced by stressors that induce reinstatement of ethanol seeking (Funk et al., 2006; Lê, Harding, Juzytsch, Watchus, Shalev, & Shaham, 2000a). However, no prior work has directly implicated the BNST in stress-induced ethanol seeking. Nevertheless, there is considerable evidence from operant procedures that the BNST is involved in stress-induced cocaine and heroin seeking (Shaham et al., 2000). In summary, a substantial amount of work strongly supports a role for the BNST in reinstatement to drug seeking and relapse. Most of these studies have examined BNST mediation of reinstatement to cocaine seeking induced by exposure to stress. Therefore, it remains unclear whether the BNST is involved in seeking induced by exposure to environmental contexts and cues. The next section presents evidence for the BNST in cue-induced seeking of ethanol and other drugs of abuse.

BNST involvement in cue-induced ethanol seeking. Aside from stress-

induced reinstatement of seeking behavior, the BNST also appears to play a role in cue-induced reinstatement and relapse. Evidence has shown that the BNST is activated by exposure to ethanol-associated cues, as indicated by increased c-Fos IR (Dayas et al., 2007; Hill et al., 2007; Jupp, Krstew, Dezsi, & Lawrence, 2011). However, no studies have directly investigated the role of the BNST in cue-induced ethanol seeking. Thus, the results obtained in Chapter 2 are the first to demonstrate BNST modulation of ethanol-induced CPP expression. In general, these findings agree with studies demonstrating BNST involvement in seeking induced by cues associated with other drugs of abuse.

For instance, previous work has shown that cocaine-induced CPP expression is blocked by inactivation of the vBNST (Sartor & Aston-Jones, 2012), while cue-induced reinstatement is blocked by dBNST and vBNST inactivation (Buffalari & See, 2011). Similarly, inhibition of the mpBNST blocked cue-induced reinstatement of heroin seeking in a self-administration procedure (Rogers et al., 2008). Our results add to this previous work and broaden the role of the BNST from cue-induced heroin and cocaine seeking measured in operant procedures to cue-induced ethanol seeking measured using CPP. thus indicating a more extensive role for the BNST in ethanol- and drug-mediated behavior. Previous studies have identified a direct BNST input to VTA and suggest this pathway may underlie the BNST's involvement in drug-seeking behavior. Thus, in Chapter 3 the involvement of a direct projection from the BNST to VTA was examined and is discussed in detail below.

Role of a BNST to VTA neural circuit in ethanol-induced CPP expression

In the final experiments presented in Chapter 3 of this dissertation, involvement of a BNST to VTA projection in cue-induced ethanol seeking (measured by CPP) was evaluated using a contemporary viral approach. Building on findings from Chapter 2, inhibitory hM4Di DREADDs were selectively expressed in VTA-projecting BNST cells using a retrograde intersectional strategy like those described in previous studies (Boender et al., 2014; Carter et al., 2013; Nair et al., 2013). Briefly, findings from the present work showed that retrograde HSV transport from VTA to BNST occurred within 2 weeks. When HSV-cre was combined with DIO-AAV, hM4Di receptors were confined to VTA-projecting BNST neurons and robustly expressed at 8 weeks post-infusion. Activation of hM4Di receptors inhibited BNST-VTA cells and blocked ethanol-induced CPP expression completely, whereas expression of GFP (control) and CNO (in the absence of hM4Di) did not affect ethanol-induced CPP expression. In total, these results demonstrate that a direct BNST projection to VTA is involved in cue-induced ethanol-seeking behavior, as measured by CPP. In addition, the present findings illustrate the utility of a combined viral approach used in these experiments. Discussions pertaining to the BNST-VTA circuit and this novel vector combination are included in the sections that follow.

Involvement of the BNST-VTA in opposing motivational states. The BNST sends a serial projection to the VTA (Dong & Swanson, 2004; 2006a; 2006b; Kudo et al., 2012; Mahler & Aston-Jones, 2012) that has been shown to innervate DA (Georges & Aston-Jones, 2001; 2002) and inhibit GABA (Jennings

et al., 2013; Kudo et al., 2014) neurons. However, it is not clear whether this BNST-VTA projection motivates ethanol-seeking behavior. Individually, the VTA and BNST contribute to CPP and reinstatement of drug seeking induced by stress and drug-associated cue exposure (discussed in Sections I and II). Therefore, it has been hypothesized that a BNST input to VTA is important for the initiation of drug seeking and relapse (reviewed in Shaham et al., 2000; Silberman et al., 2013). Though prior research has provided some support for this hypothesis, the tools used to test the BNST-VTA circuit have been limited and provided only indirect circuit modulation. Nevertheless, results from these studies have shown that BNST-VTA cells are engaged by cocaine-associated cues (Mahler, Smith, & Aston-Jones, 2013; Sartor & Aston-Jones, 2012) and that disconnection of these regions can disrupt stress-induced cocaine seeking in a self administration procedure (Vranjkovic et al., 2014) and cue-induced cocaine seeking measured by CPP (Sartor & Aston-Jones, 2012). Similar to the BNST literature, few studies have directly examined BNST-VTA input in ethanol seeking. Those that exist have demonstrated that concurrent manipulation of BNST $GABA_A$ and VTA D2 receptors disrupts ethanol-maintained responding (Eiler & June, 2007) and that CIE enhances BNST-VTA glutamate signaling (Silberman et al., 2013). Here, we report for the first time that ethanol-induced CPP is expressed directly through a BNST projection to VTA. This finding supports the hypothesis that a BNST-VTA neural circuit is involved in cue-induced ethanol seeking measured by CPP. These experiments included in Chapter 3 and the resulting novel findings were made possibly through the use of

a contemporary approach involving viral-mediated gene transfer. The implications of this approach and similar strategies are discussed next.

Chemogenetic Targeting of Neural Circuits. In this dissertation, a recombinase-based intersectional approach was used to achieve circuit-selective DREADD expression. This strategy targeted hM4Di expression to VTA-projection BNST neurons using retrograde HSV-Cre and AAV-DIO-hM4Di, where hM4Di expression is controlled by the cre-dependent FLEX switch. Currently, there are no published studies that report using this vector combination. However, several other DREADD-based approaches have been used to manipulate neural pathways and projections to VTA. Below, examples of each strategy and their attached methodological considerations are included.

The most common strategy used for DREADD-based modulation of defined neural circuit involves microinjection of CNO into the terminal fields of DREADD-expressing cells. In one study, for example, ventral pallidum (VP) inputs to the VTA were examined by targeting hM4Di to VP cells and microinjecting CNO into the VTA (Mahler et al., 2014). With this strategy, presynaptic release from VP terminals in the VTA was inhibited, allowing for highly selective inhibition of this input. Despite this strategy's ability to inhibit presynaptic neurotransmitter release, it requires implantation of chronic indwelling cannulae and necessitates additional handling to administer CNO microinjections. Given the experimental design, these requirements may be less than ideal. For example, cannulae and focal injections may lead to excess tissue damage and gliosis within terminal fields (Cunningham et al., 2008) and proximal

regions. Moreover, the additional handing that is necessary to microinject drug can independently impact behavior (e.g., Young et al., 2014). Thus, vehicle-infused controls must be used to assess for nonspecific effects due to tissue damage and handling. Another issue that is inherent to pharmacological microinjections is the difficulty in determining the spread of drug. This is mainly problematic with this approach if DREADD-expressing source neurons also terminate in areas proximal to the target region (i.e., site of infusion). In this case, it would be difficult to determine the precise site of action of CNO and would necessitate the use of additional control animals that receive CNO in sites surrounding the terminal field, where it is plausible that the infusion spread.

Similar to classical disconnection procedures, another approach used involves contralateral manipulation of serially connected source and target regions. For example, by unilaterally expressing hM4Di receptors in each region, Mahler et al. (2014) disconnected rostral VP from VTA DA cells by peripheral CNO injection. Of major benefit to this approach was the lack of invasive intra-VTA CNO microinjection and ability to drive cell type (TH)-specific DREADD expression in terminal field (postsynaptic) cells only. However, as with any disconnection procedure, the manipulation of the circuit is indirect and requires the inclusion of several additional control groups. In fact, since manipulation of each region alone or in combination (regardless of hemisphere) may be sufficient to impact behavior, unilateral and ipsilateral controls are vital to ensure the proper interpretation of results.

Finally, previous two-virus approaches have been used for DREADD-

induced circuit-specific manipulation. In these studies, a retrograde CAV2-cre vector was used to drive DREADD expression in projection neurons to and from the rat VTA (Boender et al., 2014; Nair et al., 2013). As with the present experiments, this technique allows for the direct manipulation of neural pathway activity via a noninvasive systemic CNO injection. Hence, less handling is required, removing the possibility of nonspecific behavioral interference. This strategy is also ideal in that it allows for the selective expression of DREADDs in projection neurons, which are otherwise difficult to target given their intermixed presence in source areas. Further, this technique produces direct inhibition of source neurons in addition to inhibition of presynaptic release in target regions. This may be more or less beneficial depending on whether experimental hypotheses specify pre- and/or post-synaptic mechanisms of action. As such, this technique should not be used when manipulation of presynaptic release only is desired. In this case, focal infusion of CNO into terminal regions should be used. In addition, a recently developed hM4Di-neurexin ($hM4D^{nrxn}$) variant has shown success as presynaptic silencer (Stachniak, Ghosh, & Sternson, 2014). This modified hM4Di receptor is axon-selective and is capable of inhibiting transmitter release across a terminal field by systemic CNO injection. With this strategy, greater spatial resolution (± 500 μm from injection site) is also attainable through intracranial microinjection of CNO (Stachniak et al., 2014).

In Chapter 3, our goal was to attain overall inhibition of the BNST-VTA circuit in a minimally invasive manner. Thus, we settled on using an intersectional strategy given its major benefits over other methods, including circuit-selective

targeting and non-invasive DREADD activation. Although previous studies had used CAV2-Cre in this approach, they were done in rats. Given the reported success of HSV-cre expression in the mouse (Fenno et al., 2014; Stamatakis et al., 2013; Znamenskiy & Zador, 2013), we chose to use this vector in place of CAV2-Cre. However, as discussed fully in Chapter 3, this strategy did not allow for DREADD expression in a genetically defined subpopulation of BNST-VTA cells. Consequently, no specific conclusions can be made regarding the neurochemical nature of BNST-VTA cells involved in ethanol-induced CPP expression.

Considerations and Alternative Interpretations of Results

Broadly, our conclusions are based around the idea that the VTA, BNST, and BNST-VTA circuit regulate seeking behavior induced by an initial drug-free exposure to an ethanol-associated cue. However, several considerations must be made in terms of the impact of our manipulations on neural processes beyond incentive motivation and approach behavior (e.g., memory retrieval, extinction learning). In addition, the influence of nonspecific effects of neural manipulations (e.g., increased test activity and tissue damage) on CPP expression must also be considered. Related concerns and alternative explanations of our findings are discussed at length below.

Impact of manipulations on learning and memory. The most common interpretation of CPP is that of incentive-driven behavior, whereby the animal is seeking out a context or stimulus that has been paired with a rewarding state

(Cunningham, Groblewski, & Voorhees, 2011). The associations that develop as a result of cues and reward state pairings are the result of a Pavlovian learning process. As such, manipulations that impact CPP expression may impact "seeking" by interfering with general memory processes, such as the retrievability of the reward-cue association or inhibitory learning (extinction). Therefore, in the present experiments we must consider the possibility that our findings were due to impairments in retrieval of the ethanol-cue association and/or an enhancement in extinction of the ethanol-cue association.

In the case of the latter, timecourse data from these experiments seems to argue against extinction enhancements. With the exception of hM4Di-induced BNST inhibition (Chapter 2, Exps. 3-4), the manipulations used produced an often initial and consistent reduction in preference expression. For extinction facilitation, we would expect to see initial expression of a place preference followed by a decrement in its magnitude over the course of the test (i.e., nonreinforced trial) as the inhibitory extinction learning occurred (e.g., Bormann & Cunningham, 1997; Cunningham, Dickinson, & Okorn, 1995). Therefore, this alternative explanation cannot be readily applied to our findings, except for those reported in Exps. 3-4 of Chapter 2. Notably, the decrease in preference observed over the test in these experiments could also be attributed to the timecourse of CNO and its peak in central concentration occurring later in the test (described in detail on pp. 110-111). Follow-up studies involving multiple forced (drug-free CS+ exposure) or choice (CPP tests – i.e., drug-free CS+/CS- exposures) extinction sessions would be needed to determine the impact of our manipulations on

extinction.

However, the possibility of our manipulations impairing ethanol-cue associations still remains. Though associative memory formation was likely not impacted given the application of manipulations to the test phase and the normal levels of ethanol-induced CPP expression observed in controls, it is unclear whether associative memory retrieval was disturbed. From the present data alone, we cannot determine whether ethanol-induced CPP expression was disrupted by an impaired ability to retrieve or access the ethanol-cue association from memory. Additional studies would be needed to determine the involvement of VTA iGluR, BNST, and BNST-VTA in memory retrieval. One way to test this alternative would be to apply each of these treatments to a test of CPA induced by ethanol and/or lithium chloride. A finding of disrupted CPA expression would indicate that the manipulation impacted associative memory retrieval. This is because a manipulation affecting seeking would not be expected to affect avoidance unless it impacted retrieval of the cue-drug association.

Impact of test activity on preference expression. It has been previously shown that a competing behavior, such as enhanced locomotor activity, can disrupt the expression of ethanol-induced CPP (Gremel & Cunningham, 2007). Therefore, care must be taken when interpreting results obtained from studies where a manipulation impacted CPP test activity. In this dissertation, there were 2 instances where our manipulation affected test activity. In Chapter 1 test activity was elevated by the highest dose of the AMPA/kainate and NMDA receptor antagonist cocktail used (5 DNQX + 500 AP5 ng/side), including when it

was administered outside of VTA boundaries (Miss group). Although this increase in activity may have impacted expression in the high dose “DNQX+AP5 5” group, it is unlikely that ethanol-induced CPP was blocked by this effect on activity alone. For one, mean activity counts/min in the Miss group were slightly higher than in the DNQX+AP5 5 group, but CPP was not disrupted. Furthermore, activity was not increased in the lower dose combination (1 DNQX + 100 AP5) group, but ethanol-induced CPP was blocked. Thus, when low dose and Miss group outcomes are taken into account, it appears that intra-VTA AMPA and NMDA receptor antagonism blocked ethanol-induced CPP expression, independent of its effects on activity.

In Chapter 2, co-infusion of $GABA_A$ and $GABA_B$ receptor agonists into the BNST blocked ethanol-induced CPP expression and significantly decreased test activity. Given the inverse relationship between test activity and preference expression, it is doubtful that test activity nonspecifically blocked ethanol-induced CPP. In fact, reduced test activity levels have been repeatedly associated with enhanced CPP expression (e.g., Cunningham, 1995; Neisewander, Pierce, & Bardo, 1990; Vezina & Stewart, 1987). Therefore, it can be concluded that pharmacological inactivation of the BNST blocked ethanol-induced CPP expression despite reducing test activity.

Impact of intracranial manipulations on preference expression. The work presented in this dissertation relied almost exclusively on the use of intracranial manipulation. As discussed in the introduction, all tools used to modulate neural activity confer some degree of damage to brain tissue. For

example, lesions involve the intentional destruction of neural tissue, whereas microinjections result in unintentional tissue damage and reactive gliosis (Cunningham et al., 2008). Although we cannot rule out the effects of inflammation and gliosis on our results, in many cases we have attempted to control for this by the inclusion of additional groups. For instance, in Chapter 1, two distinct control groups were included – “aCSF” and “Miss”. Surgical and microinjection procedures in each of these groups were identical to those applied to the drug-treated groups, with the exception of the solution administered (aCSF vs. DNQX+AP5) and site of infusion (proximal vs. intra-VTA). In this case, had excess neural damage impacted preference expression, we would expect to see reduced CPP in all groups tested. However, only intra-VTA drug-treated groups showed blocked ethanol-induced CPP expression, indicating a selective effect of the manipulation. In Chapter 2 Exp. 2, intra-BNST saline-treated mice served a similar function as described above. Given the normal levels of expression in this control groups, it can be concluded intra-BNST pharmacological inactivation selectively impaired ethanol-induced CPP expression.

As lesions involve the intentional destruction of neural tissue and as such this damage directly interfered with preference expression. In Exp. 1 of Chapter 2, lesioned animals showed significantly reduced CPP compared to mice that received a sham procedure, where electrodes were lowered but no current was passed. However, had the surgery itself impacted expression nonspecifically, the sham group would have shown reduced CPP.

In experiments involving viral vector infusion, several control groups were

included to test for effects of surgery/vector infusion, transgene expression, and drug (CNO) treatment on CPP expression. In Chapter 2, all animals were infused with AAV encoding hM4Di and administered saline or CNO (Exps. 3-4). In this way, all animals were treated equally up to the point of drug administration prior to the test. Thus, had surgery/vector infusion or hM4Di expression alone impacted CPP expression, this effect would have been observed in saline-administered mice. However, saline-treated mice showed significant CPP thereby arguing against this alternative explanation. Moreover, in control experiments (Chapter 2, Exps. 4-5), we show that CNO-initiated hM4Di activation reduced c-Fos IR and that in the absence of hM4Di expression, CNO did not impact ethanol-induced CPP. Overall, in this chapter we show that surgery/vector infusion, hM4Di expression, and CNO do not by themselves affect ethanol-induced CPP. Similarly, we controlled for the effects of surgery/viral infusion and transgene expression in Chapter 3 by including vehicle-treated groups that expressed hM4Di and a control GFP transgene as well as a CNO-treated group that expressed GFP only. Notably, GFP-expressing mice received the same set of infusions as hM4Di-expressing mice except that the HSV encoded for GFP in place of cre recombinase. In this manner, we could control for the effects of 4 intracranial infusions, transgene expression (hM4Di and GFP), and CNO administration (i.e., hM4Di activation). As our results showed, only CNO-treated hM4Di-expressing mice showed reduced ethanol-induced place preference expression. This argues against the alternative explanation that nonspecific effects of drug, tissue damage, or cell death alone impacted CPP in these

experiments.

However, it should be noted that we did not directly test for cell death in the DREADD-based experiments. This was, in part, due to the lack of observed effect of transgene expression alone on CPP expression or activity (in addition to non-empirical observations of other overt behavioral effects like abnormal gait). Although the viral vectors used in the present experiments are non-neurotoxic, GFP has been reported to be cytotoxic and can induce apoptosis (Liu, Jan, Chou, Chen, & Ke, 1999). Therefore, if cytotoxicity or excess tissue damage is suspected, additional assays should be performed. These assays may include staining for markers of astrocyte activation (reactive gliosis) such as glial fibrillary acidic protein (GFAP).

Summary and Conclusions

In summary, the present experiments were designed to test the neurocircuitry underlying cue-induced ethanol-seeking behavior, as indexed by ethanol-induced CPP. Focus was placed on examining the role of VTA glutamate input, the BNST, and BNST input to VTA in ethanol-induced place preference expression using a combination of techniques. As such, several major findings are presented in this dissertation that when combined identify a neural circuit involved in ethanol-seeking behavior. These findings show that glutamate input to VTA, BNST activity, and a BNST (putatively glutamatergic) projection to the VTA are involved in the expression of ethanol-induced place preference. Further, our results add to and overlap with the existing circuit of structures, all of which

are part of a larger mesocorticolimbic network, previously shown to be important in ethanol-induced CPP expression and acquisition (Fig. 20).

In this circuit, several key targets of DA input from the VTA are involved in ethanol-induced CPP. These neural targets span from cortex to limbic system and include the ACC, BNST, CeA/BLA, and NAc. Though these structures receive DA input, it is not clear whether DA input underlies their involvement in ethanol-induced CPP. For instance, in the NAc, it appears that DA signaling is involved in acquisition but not expression of ethanol-induced CPP (Gremel & Cunningham, 2009; Young et al., 2013). During expression, intra-NAc glutamate signaling has been shown to underlie ethanol-induced CPP expression and it is hypothesized that the amygdala (CeA/BLA) is the source of this glutamate input (Gremel & Cunningham, 2009). Interestingly, amygdala DA signaling has been found to be important for CPP expression and thus supports the hypothesis that a VTA DA → amygdala glutamate → NAc circuit may underlie ethanol-seeking behavior (Gremel & Cunningham, 2009).

Moreover, the extended amygdala (BNST) and ACC, which also receive DA input from VTA, have been shown to be involved in ethanol-induced CPP expression. Whereas central opioid systems appear to underlie ACC participation in ethanol-induced CPP (Gremel et al., 2011), it is unclear what intra-BNST transmitter system(s) are involved, as global inactivation strategies were used to examine the BNST (Pina et al., 2015). As the chief DA source in the mesocorticolimbic pathway, the VTA has been routinely shown to play a necessary role in ethanol-induced CPP expression. Notably, GABAergic,

opioidergic, and glutamatergic signaling mechanism have been shown to underlie VTA involvement in ethanol-induced CPP. Specifically, ethanol seeking is likely motivated by local inhibition of VTA GABA cells and activation of presynaptic opioid (likely μ-opioid) receptors (Bechtholt & Cunningham, 2005), both of which indirectly impact VTA DA cells. Conversely, activation of iGluRs expressed on VTA DA cells is a more DA-direct mechanism through which the VTA may be involved (described in Chapter 1). In addition, we show here that BNST signaling to the VTA is necessary for ethanol-induced CPP expression (Chapter 3). Given that the BNST potently innervates the VTA (Jalabert et al., 2009), it is possible that a direct BNST glutamate projection to the VTA motivates ethanol-induced CPP expression. However, the exact nature of this BNST-VTA projection is unknown.

Future Directions

Overall, this dissertation presents novel findings that implicate distinct neural signals, neural structures, and a direct neural circuit in ethanol-seeking behavior measured by CPP. Future studies will require further dissection of the BNST-VTA neural circuit in order to determine the neurochemical nature of the BNST inputs to the VTA that regulate ethanol-induced place preference expression. The most informative next step would involve using a three-way intersectional approach that combines cre transgenic mice (Vglut2-ires-Cre or Vgat-ires-Cre) with a similar dual-virus strategy to that described in Chapter 3. A similar strategy has already been successfully implemented (Stamatakis et al.,

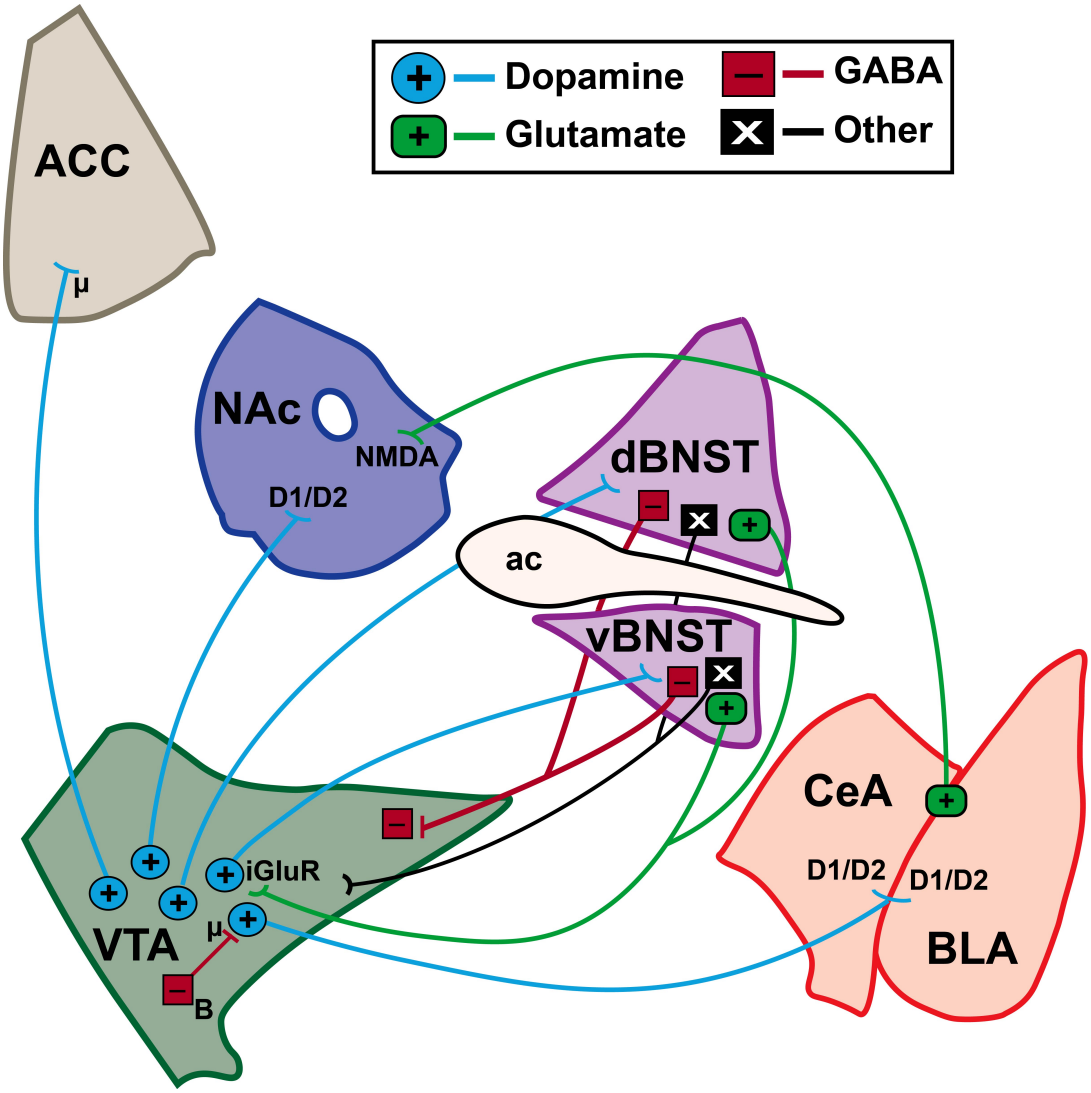
2013), suggesting the feasibility of this technique. Using this approach, hM4Di receptors would be confined to putatively glutamatergic (Vglut2-ires-Cre) or GABAergic (Vgat-ires-Cre) VTA-projecting BNST cells by infusing 1) into the VTA an HSV expressing cre-dependent flippase recombinase (HSV-flpo), and 2) into the BNST a flpo-inducible AAV-DIO-hM4Di. An alternative to this three-way intersectional approach would involve expressing hM4Di receptors (via cre-dependent AAV-DIO-hM4Di) in the BNST of Vglut2-ires-Cre or Vgat-ires-Cre mice and focally infusing CNO into the VTA. With this strategy, presynaptic release from glutamatergic (Vglut2) or GABAergic (Vgat) BNST cells would be inhibited in VTA, as CNO would activate hM4Di receptors expressed on BNST nerve terminals in VTA only. In summary, findings from studies targeting genetically defined populations of BNST cells would help to illuminate the relative contributions of BNST glutamate and GABA projections to VTA in cue-induced ethanol-seeking behavior.

Another issue described earlier in the discussion is the possibility that our findings reflected impairments in general learning and memory processes. As such, follow-up studies could also be performed to test whether our experimental manipulations impacted ethanol CPP expression by disrupting associative (ethanol-cue) memory retrieval. To address the impact of our manipulations on memory retrieval, a similar yet alternative conditioning procedure could be utilized where the aversive compound LiCl is used to induce a CPA (as described in Pina & Cunningham, 2014a). As in the ethanol-induced CPP experiments described in this thesis, each manipulation (VTA iGluR antagonism, BNST &

BNST-VTA inactivation) would be applied to the expression phase of the LiCl-induced CPA. Manipulation affecting place preference expression would not be expected to affect place avoidance unless they more generally impacted memory/retrieval. Thus, if intra-VTA administration of an AMPA and NMDA receptor antagonist cocktail (DNQX+AP5, as described in Chapter 1) disrupted LiCl-induced CPA expression, it could be concluded that iGluR activity in VTA is important for retrieval of a cue-drug associative memory. This finding would suggest that glutamatergic input to VTA is necessary for more general memory processes and not simply incentive-driven behavior, i.e., ethanol seeking. In this manner, the main manipulations presented in each chapter could be applied to a LiCl-induced CPA procedure in order to determine whether the BNST (Chapter 2) and BNST-VTA projection (Chapter 3) may be regulating ethanol seeking or memory/retrieval. Notably, if a manipulation failed to impact LiCl-induced CPA expression, it could be concluded that its disruption of ethanol-induced CPP expression was due to its effect on the incentive motivational properties of the ethanol-paired cue.

In summary, future work should be directed toward characterizing the neurochemical nature of the BNST-VTA signal involved in ethanol seeking as well as the impact of VTA iGluR antagonism, BNST and BNST-VTA inhibition on memory retrieval. Such studies will not only provide novel information about the neural mechanisms involved in ethanol CPP expression, they will help to delineate the nature of each mechanism’s involvement (motivation versus memory).

Figure 19. Updated diagram of the neural circuitry involved in ethanol-induced CPP



	Region	Manipulation	Reference
EXP	ACC	Non-selective opioid antagonism	Gremel et al., 2011
	BNST	Electrolytic lesions	Pina et al., 2015
		GABA agonism	Pina et al., 2015
		Chemogenetic inhibition (hM4Di)	Pina et al., 2015
	CeA/BLA	Electrolytic lesions	Gremel & Cunningham, 2008
		Dopamine D1/D2-like antagonism	Gremel & Cunningham, 2009
	NAc	NMDA antagonism	Gremel & Cunningham, 2009
	VTA	Non-selective opioid antagonism	Bechtholt & Cunningham, 2005
		GABAB agonism	Bechtholt & Cunningham, 2005
		NMDA/AMPA antagonism	Pina & Cunningham, unpublished
	BNST-VTA	Chemogenetic inhibition (hM4Di)	Pina & Cunningham, unpublished
ACQ	CeA/BLA	Electrolytic lesions	Gremel & Cunningham, 2008
	NAc	Dopamine D1-like antagonism	Young et al., 2013

Figure 19. Updated diagram of the neural circuitry involved in ethanol-induced CPP. Previous studies have demonstrated the involvement of cortex (ACC), amygdala (BLA/CeA), striatum (NAc) and midbrain (VTA) in ethanol conditioned place preference (CPP). The present work now demonstrates involvement of the dorsal and ventral bed nucleus of the stria terminalis (dBNST and vBNST) and a direct BNST-VTA circuit. Each of these regions receives dopaminergic input from the VTA (indicated by blue arrows) and is part of a broader mesocorticolimbic dopamine system that underlies reward and motivation. Some of the neurochemical signals underlying the involvement of each region in ethanol-induced CPP have also been identified. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area; +, excitatory; -, inhibitory; blue circles, dopamine cells; green rectangles, glutamate cells; red squares, GABA cells; black boxes, unknown neurochemical signal; green arrows, glutamate projections; μ, mu-opioid receptors; B, $GABA_B$ receptor; D1, dopamine D1-like receptor; D2, dopamine D2-like receptor; iGluR, ionotropic glutamate receptors, NMDA, iGluR subtype; ACQ, acquisition; EXP, expression.

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