Regulation of Basal and Ethanol Abstinence-Induced Affective Behaviors by GluN2B-containin NMDA Receptors in the Bed Nucleus of the Stria Terminalis and Endocannabinoids

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Dedicated to my incredible family:

Mom, Dad, and Sam

and

Joe, Niles, and Daisy

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Katie Holleran

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List of Abbreviations

2-AG	2-arachidonyl glycerol
4E-BP1	eukaryotic initiation factor 4E binding protein 1
AA	arachidonic acid
AAV	adeno-associated virus
ACPC	1-aminocyclopropanecarboxylic acid
ACTH	adrenocorticotropin hormone
AEA	anandamide
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
AUD	alcohol use disorder
AWS	alcohol withdrawal syndrome
BDNF	brain-derived neurotrophic factor
BEC	blood ethanol concentration
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
BNSTGluN2BKD	BNST-specific knockdown of the GluN2B subunit
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CeA	central nucleus of the amygdala
CIE	chronic intermittent ethanol
cort	corticosterone/cortisol

CPP	conditioned place preference
CRF	corticotropin releasing factor
CRFR1	corticotropin releasing factor receptor 1
DA	dopamine
DAG	diacylglycerol
DBS	deep brain stimulation
DCS	d-cycloserine
DG	dentate gyrus
DID	drinking-in-the-dark
dlBNST	dorsal lateral bed nucleus of the stria terminalis
DMSO	dimethyl sulfoxide
DSI	depolarization-induced suppression of inhibition
DSM-5	diagnostic and statistical manual 5th edition
DTs	delirium tremens
eCB	endocannabinoids
ECT	electroconvulsive therapy
eEF2	eukaryotic elongation factor-2
eEF2K	eukaryotic elongation factor-2 kinase
EPM	elevated plus maze
EPSC	excitatory postsynaptic current
EtOH	ethanol
EZM	elevated zero maze
FAAH	fatty acid amide hydrolase

FS	fast spiking
FST	forced swim test
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GSK-3	glycogen synthase kinase-3
HCN	hyperpolarization-activated cyclic nucleotide-gated (channel)
HIC	handling-induced convulsions
HPA	hypothalamic-pituitary-adrenal (axis)
IEG	immediate early gene
IHC	immunohistochemistry
i.p.	intraperitoneally
ISH	*in situ* hybridization
i.v.	intravenously
LTD	long-term depression
LTP	long-term potentiation
LV	lentiviral/lentivirus
LV-Cre	lentiviral Cre recominase
LV-GFP	lentiviral green fluorescent protein
MAG	monoacylglycerol
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder

mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
MSN	medium spiny neuron
mTOR	mammalian target of rapamycin
NaC	nucleus accumbens
NAPE-PLD	*N*-acylphosphatidylethanolamine- hydrolyzing phospholipase D
NAT	*N*-acyltransferase
NET	norepinephrine transporter
NIH	novelty-induced hypophagia
NMDAR	N-methyl D-aspartate receptor
NSFT	novelty-suppressed feeding test
NTS	nucleus of the solitary tract
PAM	positive allosteric modulator
PCP	phencyclidine
PFC	prefrontal cortex
PLC	phospholipase C
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
RDoC	research domain criteria
RS	regular spiking
sEPSC	spontaneous excitatory postsynaptic current

SERT	serotonin transporter
shNaC	nucleus accumbens shell
SSRI	selective serotonin reuptake inhibitor
TRD	treatment-resistant depression
VTA	ventral tegmental area

Chapter 1

The Antidepressant Effects of Ketamine: Involvement of the GluN2B Subunit of the NMDA Receptor in the Bed Nucleus of the Stria Terminalis

1.1 Major Depressive Disorder: From Humans to Rodents

Depression Prevalence, Symptomatology, and Comorbidity

Major depressive disorder (MDD) is one of the most common psychiatric illnesses in humans, with approximately 6.7% of United States adults experiencing an MDD episode every year and 16.6% experiencing at least one episode in their lifetime (Kessler et al., 2005). MDD can be a highly debilitating condition, representing the leading cause of disability worldwide (Lopez and Murray, 1998), and 80% of individuals with depression report some level of difficulty in functioning because of their symptoms (Beck et al., 2011; Pratt and Brody, 2008). Despite this, most people with depression do not receive even minimally sufficient treatment for their condition; only 29% of individuals meeting MDD criteria seek professional help (Pratt and Brody, 2008). This may be due in part to the well-known low efficacy of current pharmacological treatment strategies for managing depression, described below.

MDD is categorized in the Diagnostic and Statistical Manual 5 (DSM-5) as a constellation of symptoms, characterized by at least 5 of the following in a two week period: depressed mood, loss of interest or pleasure (anhedonia), significant weight loss or gain, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue, feelings of

worthlessness and/or guilt, diminished concentration or increased indecisiveness, and recurrent thoughts of death or suicide or suicide attempt (APA, 2013). Importantly an individual must experience either depressed mood or anhedonia in order to meet MDD criteria, and anhedonia will be discussed in greater detail in this chapter.

The DSM-5 is designed for general diagnosis of psychiatric disease based on an individual's symptoms, but these classifications have drawbacks. The broad symptoms experienced by many patients with psychiatric disorders place them into multiple DSM-5 categories at once (for instance, both MDD and generalized anxiety disorder). Conversely, two patients who have been diagnosed via DSM-5 classifications with MDD may have vastly different symptoms and etiologies that could cause disparate treatment outcomes (Barchas and Brody, 2015; Krueger et al., 2014). Indeed, such lumping of patients based on DSM classification may underlie to some extent the low treatment efficacy of current antidepressant treatments. These problems have been inherent to the all iterations of the DSM system of categorizations. While newer editions have attempted to lessen these problems through changes in classification, assigning a strict set of symptoms to each psychiatric illness does not allow for the fluidity between multiple diagnostic criteria that many patients exhibit. Additionally, the lack of biologically-driven delineations of psychiatric disorders (such as biomarkers of disease, genetic contributions, molecular pathways specifically disrupted in a given disease, and alterations in brain connectivity) have lessened the DSM-5's utility, particularly in basic research using rodent models, as described below.

In an attempt to relate biological findings to various disease states, the National Institute of Mental Health (NIMH) began an ambitious project, the Research Domain

Criteria (RDoC) initiative. Instead of strict criteria for each psychiatric illness as in the DSM series, the RDoCs are comprised of (currently) five major domains: negative valence systems, positive valence systems, cognitive systems, systems for social processes, and arousal/regulatory systems, which are clustered based on current neurobiological research relating these domains to similar molecular and neuroanatomical underpinnings (https://www.nimh.nih.gov/research-priorities/rdoc/index.shtml). The RDoCs system represents a crucial step forward in mental health research. It allows for both examination of symptoms shared by a number of different disorders (for example, in the negative valence systems domain, one subcriteria – response to potential harm – could be altered in both anxiety-related disorders and MDD) as well as differing modalities of a certain psychiatric disease that may show differential manifestation between patients. For instance, symptoms described for MDD in the DSM-5 above could be parsed into each of the RDoC domains (negative valence = loss; positive valence = alterations in effort to obtain reward or reward value; cognitive = diminished concentration; social processes = unfounded feelings of lowered self-worth; arousal = insomnia/hypersomnia and feeding disturbances), and assessing contributions of each of these domains to an individual's overall diagnosis could aid in designing more appropriate medication regimens.

While RDoC classifications will be imperative in future research, much of the research discussed in this document predate the RDoC initiative. I will discuss findings with respect to RDoCs classifications, where appropriate (particularly with regards to anhedonia, a dysfunction of the positive valence domain), but for simplicity older studies, particularly in humans, will be discussed with reference to DSM classification of MDD.

While many psychiatric diseases share comorbidity with MDD (Corruble et al., 1996; Kessler et al., 2003), one interaction that is particularly concerning is comorbidity between MDD and Alcohol Use Disorders (AUDs). AUDs impact a vast number of individuals every year with damaging consequences, including incredible financial burden and around 4% total mortality world wide (Rehm et al., 2009). It has long been known that AUDs are strongly comorbid with depression, and, supporting this, a recent study found that both major depressive disorder (MDD) and persistent depression were increased in individuals with DMV-5 AUD criteria (Grant et al., 2015). Conversely, acute alcohol use increases suicidal ideation, even when controlled for concurrent negative life event or individual suicide ideation one hour prior to alcohol use (Bagge et al., 2014). Grant and colleagues highlighted a worrying trend for increased problem drinking in the United States over the past 10-15 years (Grant et al., 2015). This is troublesome, especially given that only approximately one third of individuals with AUDs seek treatment. Though well established, the reason for the relationship between alcohol abuse and depression has been disputed. Because both conditions are relatively common (meaning that a subset of the population will undoubtedly suffer from both conditions, even if the etiologies are unrelated), and because acute alcohol withdrawal can cause affective disturbances (discussed in detail in Chapter 4), it was initially unclear if the two diseases were causally linked. Although it is often generally assumed that alcohol use is a consequence of depression symptoms (i.e. self-medication), research has demonstrated that AUDs often predict MDD development (Fergusson et al., 2011; Hasin and Grant, 2002). Regardless of when manifestation occurs, the presence of either disease hinders the successful treatment of the other (Hasin et al., 1996). Thus, the interaction between AUDs and depression represents a clinically crucial

area of research that will be examined in detail in Chapters 3 and 4. For the remainder of the present chapter, however, I will focus on MDD alone.

Initial MDD Treatment Strategies: the Monoamine Hypothesis of Depression

The primary molecular targets in the treatment of MDD for the past ~60 years have been the monoamines – particularly serotonin and norepinephrine (NE). The so-called monoamine hypothesis of depression came about largely because of serendipitous findings that compounds leading to increased monoamine levels caused excitement or elation and compounds which depleted levels were associated with depressed mood (Robitzek and Selikoff, 1952; Schildkraut, 1965). Specifically, the monoamine oxidase inhibitor (MAOI) iproniazid (Crane, 1956, 1957) and the tricyclic imipramine (Kuhn, 1958) were found to be therapeutically effective in the treatment of depression in humans. However, cardiovascular side effects of these two classes have limited their modern application to only patients who display treatment-resistance to first-line treatments (Pacher and Kecskemeti, 2004). In the 1980's, the development of selective serotonin reuptake inhibitors (SSRIs), in particular fluoxetine (brand names Prozac and Sarafem), represented a major advancement in the field, as these compounds displayed fewer side effects and lower toxicity than MAOI or tricyclic antidepressants.

Despite initial excitement for these monoamine-targeting compounds, major concerns arose. In particular, the lag before therapeutic benefit of weeks to months (Gartlehner et al., 2012; Nierenberg et al., 2000) and low rates of efficacy are enormous pitfalls, particularly in the treatment of a disease in which self-injury and suicidal ideation/ suicide attempts are common side effects. The delayed onset of symptoms relief hints at

downstream molecular mediators of antidepressant effects, as monoamine levels increase after only a day or two of treatment. SSRIs only induce full remission from depressive symptoms in approximately 35% of patients. Although the use of compounds such as venlafaxine that increase both serotonin and norepinephrine (NE) availability can slightly improve remission rates, greater than half of all patients treated with these drugs will not achieve remission (Thase et al., 2001). Failure to respond to multiple antidepressant treatments has been termed Treatment-Resistant Depression (TRD), and represents a clinically crucial area of research into more efficacious treatment strategies (Gaynes, 2009).

Rodent Models of Depression

While significant progress – including the serendipitous discovery of monoamine targeting for depression – has been borne out of human studies, work in rodents is imperative for mechanistic elucidation of these drug actions as well as the analysis of newer compounds potentially lacking the drawbacks described above. Unfortunately, as with many psychiatric disorders, clear disease biomarkers of depression have yet to be definitively described. Thus the study of antidepressant mechanisms and potential new antidepressant compounds in rodent populations has largely relied on pharmacologically validated (through known antidepressants) measurable alterations in behavior of these animals in a handful of tasks (Rupniak, 2003). However, no clear consensus on any one "gold-standard" behavioral task to determine antidepressant efficacy has been reached, and because we cannot truly determine the psychological state of a given rodent, we can only use such measures as a proxy of "depression-like" behavior in these animals.

Many rodent assays of depression rely on a response to an inescapable stressor, often termed "helplessness." Perhaps the most widely-used of these tasks is the forced swim test (FST), in which a mouse or rat is forced to swim in a container of water from which it cannot escape (Porsolt et al., 1977). After a short time (~2 minutes), the animal begins to display immobility – or lack of swimming effort, and the proportion of time spent immobile during the final 4 minutes of the 6 minute swim can be decreased or increased, respectively, through treatment with known antidepressants (Borsini and Meli, 1988) or treatments that worsen mood in humans, such as chronic stress (Petit-Demouliere et al., 2005; Strekalova et al., 2004). Importantly, immobility behavior is resistant to alteration by treatments that target other modalities of negative human affect, such as anxiolytic or antipsychotic drugs. However immobility can be impacted by treatments that alter locomotion, so measurements of locomotor effects of a given compound are vital for proper interpretation of FST data. This general inescapability model is applied in a number of similar tasks, such as the tail suspension and foot shock escape tests (Rupniak, 2003). While FST and related tasks are generally responsive to treatments with antidepressants, some criticisms of this methodology have surfaced. As with any task that is validated by treatment with known compounds, there is a possibility that treatments through novel antidepressant mechanisms may not elicit stereotypical behaviors in such a task, and thus useful treatments for individuals with MDD would go unrecognized. It has also been noted that acute treatments with SSRIs are often capable of reducing FST immobility (Borsini and Meli, 1988; Petit-Demouliere et al., 2005), although chronic treatment can further reduce immobility (Dulawa et al., 2004). The acute efficacy of SSRI treatment in the FST is directly at odds with human literature, requiring weeks to months of administration before relief

becomes apparent (Gartlehner et al., 2012; Nierenberg et al., 2000). Conversely, some studies have shown a lack of efficacy of known antidepressants in reducing immobility time in the widely used C57Bl/6J inbred strain of mice (David et al., 2003; Lucki et al., 2001). Finally, a number of factors, such as age and weight of the animals or time of day, can influence immobility time in the FST (Petit-Demouliere et al., 2005)

Another group of behavioral tasks for observing putative alterations in affect are based on the observation that rodents display reluctance to eat when they are in a novel environment (hyponeophagia). These are conflict-based tasks that pit the rodents' desire to eat (which is elevated either by hunger or appeal of the food) against their novelty-induced decreased desire to consume (Dulawa and Hen, 2005). Although these tasks are generally thought to measure alterations in anxiety-like behavior, they have been used to measure depression-like behavior as well (Li et al., 2011; Santarelli et al., 2003). Interestingly, SSRIs have been demonstrated to reduce hyponeophagia when administered chronically, but not acutely (Bodnoff et al., 1988; Santarelli et al., 2003). This time course is more closely aligned with that of SSRI efficacy for reducing depression related symptoms in humans.

There are two general "flavors" of hyponeophagia-based tasks (both of which will be described extensively in this manuscript). In both instances, the dependent variables are latency to consume and amount consumed (Dulawa and Hen, 2005). First, the Novelty-Suppressed Feeding Test (NSFT) involves food depriving rodents for 24-48 hours followed by exposure to a novel environment containing a pellet of normal rodent chow (Li et al., 2011; Pang et al., 2013). In the Novelty-Induced Hypophagia (NIH) task, a palatable substance (such as liquid Ensure) is utilized, and latency and amount of this substance consumed in a novel environment are measured. The NIH test eliminates the requirement

for food restriction, but training sessions to familiarize the animals with the palatable substance are necessary (Dulawa and Hen, 2005; Louderback et al., 2013). Because this task utilizes a highly appealing substance rather than normal chow, one can easily imagine that this version of the hyponeophagia task would be more likely to engage hedonic circuitry that is often disrupted in MDD and animal models of depression (Strekalova et al., 2004) than the NSFT.

In early work in the lab, I was able to replicate earlier findings demonstrating that the NIH test was bidirectionally sensitive to both decreases (reduced affective behavior) and increases (higher affective behavior) in latency following treatments such as chronic antidepressant and acute amphetamine treatment, respectively (Dulawa and Hen, 2005). I examined latency to consume a palatable substance (Vanilla Shake Ensure™) following either acute restraint stress and diazepam (3mg/kg), respectively, and also demonstrated significant effects on novel cage latency (**Figure 1.1**). Thus, in Chapters 2 and 3, we focus on this behavioral task and NSFT for measuring disturbances in affective behavior.

Although treatments have been available for greater than half a century for individuals suffering with MDD, the low efficacy and long time delay for alleviation to manifest using monoamine-based therapies make the investigation for alternative targets critical. Novel therapeutics are difficult to test in human populations with MDD, but a number of available rodent paradigms examining depression-like behaviors are useful for testing these putative therapies, especially when multiple behavioral assays are utilized in concert.

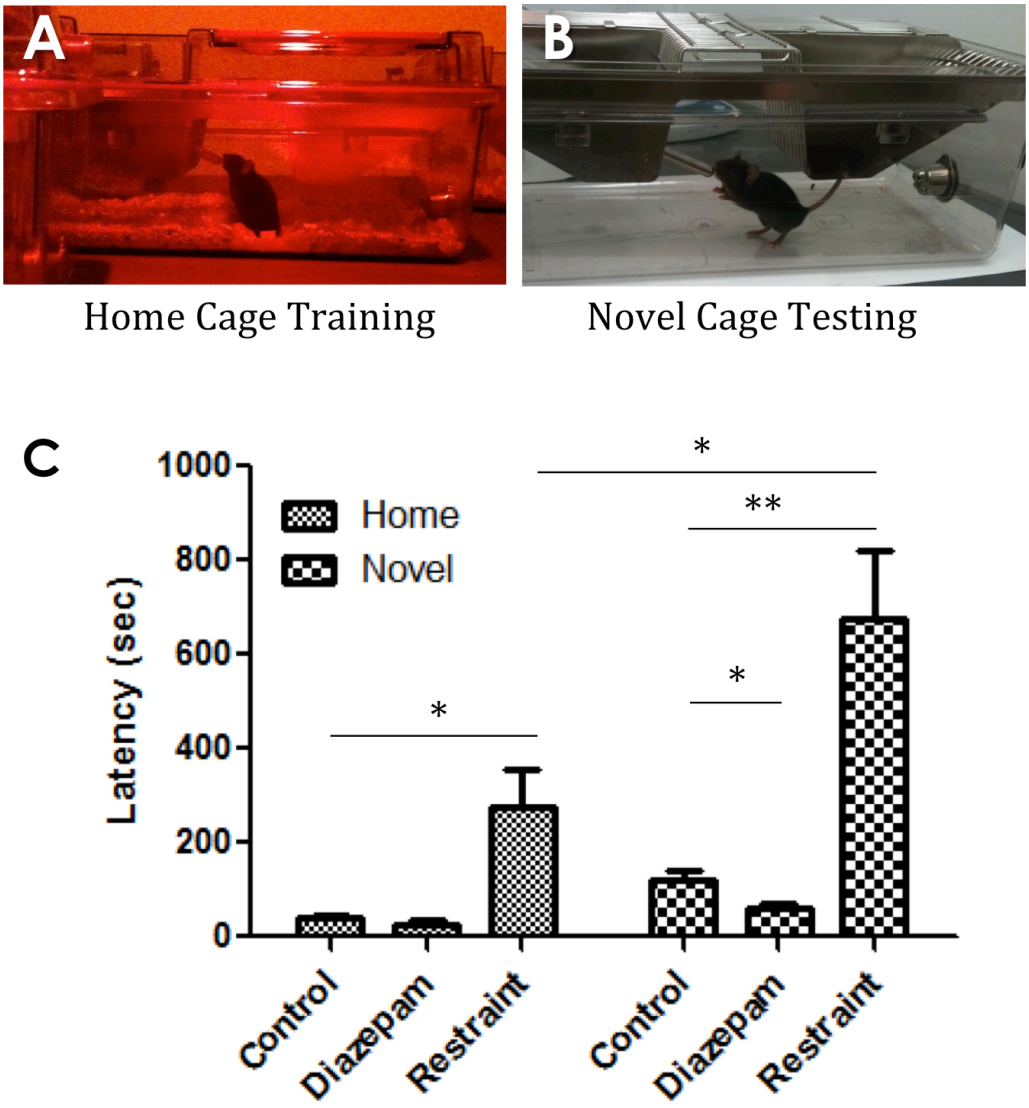

Figure 1.1. The Novelty-Induced Hypophagia (NIH) Task. Mice were trained to drink Ensure in their home cage for 30min/day for 3 days. (A) On day 4, mice were treated with diazepam (3mg/kg; 30 min prior to testing), 60 min restraint stress, or no treatment and tested in their home cage for latency to drink. (B) On day 5, mice were treated again (same treatment groups) and tested in a novel cage devoid of bedding under bright lights for latency to drink. (C) Latency to drink in home vs novel cage for each group (N=10/group). *P<0.05; **P<0.01.

1.2 The Glutamate Hypothesis of Depression

Discoveries Leading to the Use of Ketamine as an Antidepressant

In 1994, Ian Paul of Phil Skolnick's group examined an alternative to the monoamine-centered dogma of antidepressants (Paul et al., 1994). Although increasing monoamine levels had been established as a means to reduce depressive symptoms, no single transmitter system had been causally linked to depression nor demonstrated alterations at clinically relevant time points of administration. In addition, no biomarker (such as serum monoamine levels) had been found that was consistent across all therapeutically beneficial treatments. Contemporary studies revealed that inhibition of the N-methyl D-aspartate glutamate receptor (NMDAR) showed preclinical efficacy in reducing depression-like behavior (Papp and Moryl, 1994; Trullas and Skolnick, 1990). Paul and colleagues examined the ability of 17 antidepressants from numerous different classes that are effective in humans with MDD to alter glycine binding, crucial along with glutamate binding for NMDAR activation, at chronic time points consistent with symptom relief. They found that 16/17 treatments consistently decreased potency of glycine to inhibit [^{3}H]-5,7-dichlorkynurenic acid binding to strychnine-insensitive glycine receptors after chronic antidepressant treatment. In fact, this altered glycine binding was more predictive of antidepressant efficacy than previous putative biomarkers (e.g. β-adrenoceptor down-regulation observed with MAOIs and tricyclics, but not SSRIs). Additionally, no alterations in glycine binding were observed with treatments that occasionally demonstrate false-positives in the FST. Later, the glycine partial agonists d-cycloserine (DCS) and 1-aminocyclopropanecarboxylic acid (ACPC), which inhibit NMDAR functioning at high dosages, were found to reduce FST immobility time in a dose-dependent fashion indicative

of NMDAR antagonism (Lopes et al., 1997). Further findings that NMDAR-inhibiting compounds reduce depression-like behavior and that NMDARs appear to be altered in the brains of suicide victims contributed to the birth of the glutamate hypothesis of depression (Skolnick et al., 1996).

The depression field was forever altered in 2000, when John Krystal's group conducted the first pilot experimentation of NMDAR antagonism in human subjects (Berman et al., 2000). Ketamine hydrochloride, a potent NMDAR antagonist, was infused, i.v., at a low dose (0.5mg/kg over 40 minutes) into a cohort of human subjects with MDD. The results were staggering. Patients not only experienced significant alleviation of symptoms, but the effects were apparent only a few hours after infusion and lasted for a number of days, long after any dissociative side effects of ketamine had worn off. Since this study, ketamine has been demonstrated to be effective not just in MDD, but in TRD with effects lasting days beyond the scope of the original study following a single infusion (Zarate et al., 2006a). In patients with TRD (Zarate et al., 2006a), for whom the average number of failed antidepressant treatments was six (including two individuals given electroconvulsive therapy, ECT), the ketamine infusion seemed like a light at the end of the tunnel. Patients reported: "This is the best I've ever been; I had lost all hope after trying all treatments [failed 16 separate antidepressant trials and ECT]," and "I no longer have depression; it is as if I had never been depressed in my life" (Maeng and Zarate, 2007). Additionally, ketamine was shown to be effective in rodent models of depression (Autry et al., 2011; Li et al., 2011) as well, demonstrating reduction in depression-like symptoms as long as two weeks following a single dosage (Maeng et al., 2008).

In a 4-week study of ketamine's effectiveness of treating patients with TRD, it was found that the average time to relapse following a single infusion was approximately 13 days, although 27% of ketamine responders had still not relapsed when the study was concluded at 4 weeks post-infusion (Ibrahim et al., 2012). Though this represents a fantastic length of improvement following any acute treatment, ketamine's effects are transient, and a single infusion alone is not sufficient to permanently reduce depression symptoms. Thus, an obvious area of investigation would be the utility of repeated dosages of ketamine in a sustained treatment regime for MDD or TRD (Niciu et al., 2014). Initially, two major concerns arose regarding repeated ketamine administration: first, it is entirely possible that response to ketamine treatments could wane over time, eventually rendering symptoms intractable to ketamine. Second, and more concerning, individuals who abuse ketamine recreationally show cognitive deficits (Curran and Monaghan, 2001; Morgan et al., 2004) and reduced prefrontal grey matter (Liao et al., 2011). However, studies examining repeated dosages of ketamine for treatment of MDD have been promising. One study found repeated ketamine infusion (6 infusions over 2 weeks) continued to provide symptom relief, and repeated infusions extended the time to remission to 18 days after the final infusion (Murrough et al., 2013). Additionally, one case study reported continued benefit of ketamine treatment after 40 infusions (Blier et al., 2012). No cognitive impairments have been observed following repeated treatments, likely due to the far lower dosage used in the treatment of MDD compared to recreational ketamine use (Niciu et al., 2014). However, the abuse liability of ketamine remains a concern, and dissociative and cardiovascular effects require medical professionals to be present during infusion. For this

reason, investigations into the mechanisms of ketamine's actions will be crucial in the development of a treatment with lowered abuse potential and fewer side effects.

Ketamine's Actions on the NMDAR

Primarily, ketamine is a noncompetitive antagonist of the NMDAR; however, it also has some actions at non-NMDA glutamate receptors, nicotinic and muscarinic acetylcholine receptors, opioid receptors, and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Chen et al., 2009; Kohrs and Durieux, 1998). Here, I will focus almost exclusively on the NMDAR-related actions of ketamine.

NMDARs are heterotetrameric ionotropic glutamate receptors. In order for channel opening and subsequent permeance to both monovalent and divalent cations, the NMDAR requires membrane depolarization in order to remove a Mg^{2+} blockade from the pore, as well as both glutamate and glycine binding. A number of allosteric sites on the receptor bind a variety of agonists and antagonists, but within the channel, the phencyclidine (PCP) site is where ketamine, a derivative of PCP, binds. The NMDAR is comprised of two obligatory GluN1 subunits and either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Traynelis et al., 2010). While the GluN2 subunit, which binds glutamate, comes in 4 isoforms (GluN2A-D), GluN2A and GluN2B are expressed most commonly in the adult brain. Interestingly, there is a developmental shift in GluN2A vs GluN2B subunit composition in which GluN2A is almost entirely absent a birth and then is increasingly expressed through maturation (Sheng et al., 1994)

The identity of the GluN2 subunit confers a number of properties onto the channel (Traynelis et al., 2010; Yashiro and Philpot, 2008). GluN2A-containing NMDARs have

higher open probability, higher peak currents, and faster decay kinetics than GluN2B-containin NMDARs; however GluN2B gives rise to greater Ca^{2+} conductance primarily due to its longer decay time (Yashiro and Philpot, 2008). This is reflected in the developmental GluN2B→GluN2A shift as faster decay kinetics are observed in the cortex at postnatal day 9 (P9) compared to P4 (Flint et al., 1997). This distinction in kinetics has also contributed to the hypothesis that there are differing subunit-specific synaptic distributions. Recordings of spontaneous NMDAR excitatory postsynaptic currents (sEPSCs) reveal sharper kinetics than evoked NMDAR EPSCs in the hippocampus (Dalby and Mody, 2003). sEPSCs represent smaller glutamate release events, whereby only NMDARs present directly at the synapse would be expected to respond. Conversely, evoked EPSCs following electrical stimulation results in greater glutamate release, and subsequent spillover of glutamate into the extrasynaptic space. Given the kinetic differences observed, it has been hypothesized that GluN2A-containing NMDARs are primarily synaptically localized, whereas GluN2B-containing NMDARs are located perisynaptically. To support this, ifenprodil, an NMDAR antagonist with >400-fold selectivity for GluN2B over GluN2A had no effect on NMDAR sEPSCs, but substantially reduced evoked NMDAR EPSCs (Dalby and Mody, 2003).

Alteration in neuronal plasticity represents a key process by which both beneficial and maladaptive adaptations to stimuli can be achieved. Long term potentiation (LTP) and long term depression (LTD) represents a common modality of experimenter-induced plasticity by which responses to a stimulus (generally, an electrical stimulus) are either augmented or inhibited, respectively. NMDARs have been demonstrated as crucial mediators of both LTP and LTD (Winder and Sweatt, 2001). In hippocampal or cortical preparations, GluN2A-containing NMDARs are critical for the induction of LTP, while

GluN2B-containing NMDARs are not required for LTP. In contrast, cortical and hippocampal LTD requires the GluN2B-containing, but not GluN2A-containing, NMDARs (Liu et al., 2004; Massey et al., 2004). However, this is not true for all brain regions, as described later in this chapter.

Investigations into NMDAR subunit specificity of the antidepressant effects of ketamine have partially implicated the GluN2B subunit, although some studies have also shown GluN2A contributions (Kiselycznyk et al., 2015). Systemic pharmacological targeting of this subunit by the GluN2B selective compound Ro 25-6981 (Fischer et al., 1997) has consistently shown antidepressant-like effects in rodents similar to ketamine (Kiselycznyk et al., 2015; Li et al., 2011; Louderback et al., 2013; Maeng et al., 2008). Although Ro 25-6981 is selective for GluN2B- over GluN2A-containing NMDARs, it has actions at a number of off-target sites, including the serotonin and NE transporters (SERT and NET, respectively) and the dopamine D_4 receptor, that cloud interpretation of changes in depression-like behavior following its administration (Keiser et al., 2009). Another GluN2B-selective antagonist, CP-101,606, has been shown in to reduce depression symptoms in humans with TRD, with less severe dissociative adverse side effects than ketamine (Preskorn et al., 2008). This study was interesting because it required failure of improvement in depression symptoms with SSRI (paroxetine) administered for 6 weeks prior to CP-101,606 treatment. However, though patients did not show improvements in symptoms with SSRIs, paroxetine was administered throughout the study (concurrent with CP-101,606 administration), and further investigations using this compound were halted due to adverse cardiovascular effects.

Recent studies, including our own (described in Chapter 2) have taken advantage of a transgenic mouse line harboring *loxP* sites around *grin2b* (the gene encoding the GluN2B subunit) in order to regionally or cell-specifically target GluN2B-containing NMDARs (Brigman et al., 2010). Deletion of GluN2B from principle cortical neurons by crossing GluN2B-floxed mice with mice expressing Cre-recombinase under the NEX promoter mimics and occludes ketamine's effect on depression-like behavior (Miller et al., 2014). This group also found increased protein synthesis, and in particular increases in brain-derived neurotrophic factor (BDNF), phosphorylated mammalian target of rapamycin (p-mTOR), and GluA1 (a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor), similar to ketamine treatment (described below), which could not be further increased by ketamine injection. The went on to show that ambient tonic glutamate current, measured in the presence of tetrodotoxin, CNQX, and picrotoxin (to block Na^+ channels, the AMPAR, and GABA receptor-mediated currents, respectively), was mediated through GluN2B, as it was blocked by ketamine and the GluN2B antagonist ifenprodil and completely absent in cortical cells lacking GluN2B. Additionally, blockade of this GluN2B-mediated tonic current has been demonstrated to increase the number of functional spines and facilitate AMPAR-EPSCs (Gray et al., 2011) as well as induce the translation of a number of proteins associated with ketamine's antidepressant effects described below (Sutton et al., 2007).

Downstream Effects of Ketamine

Ketamine's rapid and long-lasting effect on depressive behavior hints at a "two-hit" mechanism of action, by which ketamine exerts acute effects contributing to the rapid-

onset phase as well as sustained changes in protein expression or plasticity, which comprise the extended phase (**Figure 1.2**). Though somewhat counter-intuitive, ketamine at subanesthetic, but not higher, dosages has been demonstrated to increase glutamate levels in the prefrontal cortex (PFC) for approximately two hours following administration (Moghaddam et al., 1997). This is believed to be mediated by ketamine-induced inhibition of GABAergic neurons, thus disinhibiting excitatory neurons and driving increased glutamate release. Indeed, this group later showed that, in the PFC of awake rats, ketamine inhibits firing of GABAergic fast spiking (FS) interneurons and augments firing of regular spiking (RS) glutamatergic neurons (Homayoun and Moghaddam, 2007). Additionally, the disinhibition model was temporally supported as FS firing decreased prior to the increased firing of RS cells. Because of simultaneous block of NMDARs by ketamine, this increased glutamatergic tone preferentially activates another ionotropic glutamate receptor, the AMPA receptor. Chronic treatment with antidepressants has been shown to increase AMPAR mRNA expression and RNA editing (Barbon et al., 2006), as well as increase protein expression of GluA1 and GluA2 AMPAR subunits (Du et al., 2007). Interestingly, this potentiation of AMPAR strength required chronic treatment of antidepressants, supporting the hypothesis that monoaminergic antidepressants likely exert their effects through modulation of downstream effectors. Ketamine, alternatively, would immediately enhance AMPAR signaling through increased glutamate release. Supporting the involvement of AMPAR activation in ketamine's antidepressant efficacy, treatment with an AMPAR positive allosteric modulator (PAM), LY392098, rapidly reduced FST immobility time, but this effect was not sustained (Li et al., 2001). Finally, pre-treatment with NBQX, an AMPAR antagonist,

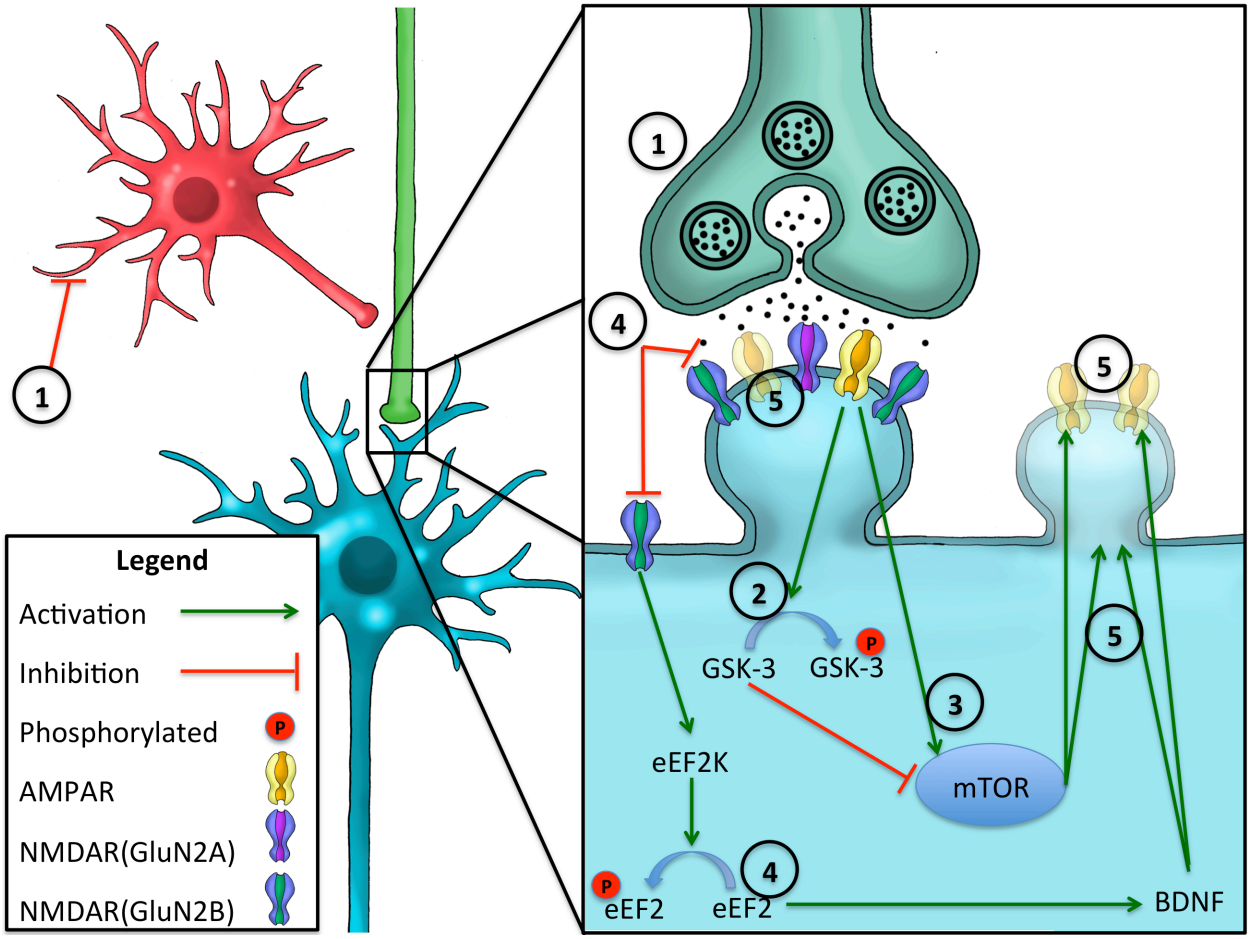

Figure 1.2. Identified Mechanisms of Ketamine. (1) Ketamine increases glutamate release through disinhibition of glutamatergic neurons by inhibition of GABAergic interneurons (red neuron). (2) Increased glutamate preferentially activate AMPARs, which facilitate inhibitory phosphorylation of GSK-3, releasing GSK-3-mediated inhibition of mTOR signaling. (3) AMPAR currents also leads to phosphorylation and activation of mTOR pathway proteins, including p-mTOR, p70S6 kinase, and 4E-BP1. (4) Meanwhile, ketamine inhibits activation of GluN2B-containing NMDARs by tonic glutamate, which then prevents the activity of eEF2K in phosphorylating eEF2. Unphosphorylated eEF2 is then able to induce the translation of a number of proteins, including BDNF. (5) BDNF and mTOR signaling both induce synaptogenesis by increasing functional spine number and translation/insertion of new AMPARs. More details in text.

blocks the acute antidepressant effect of ketamine (Maeng et al., 2008). However, because pharmacological AMPAR activation does not produce sustained reduction in depression-like behavior, this alone does not fully explain long-lasting relief from depressive symptoms following ketamine administration.

In the prefrontal cortex (PFC), ketamine administration has been demonstrated to rapidly and transiently activate the mTOR signaling pathway through AMPAR-dependent increases in phosphorylated, activated forms of p70S6 kinase, eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and mTOR (Li et al., 2010). Activation of this pathway is associated with protein synthesis and new spine formation (Hoeffer and Klann, 2010). Ketamine treatment increased the expression of PSD-95 and GluA1 (both present in new spines), spine density, and EPSCs, and decreased depression behaviors in a sustained and rapamycin-sensitive manner (Li et al., 2010; Li et al., 2011; Liu et al., 2013). Conversely, in postmortem human studies, individuals with MDD had decreased levels of mTOR and associated proteins in PFC tissue compared to non-MDD controls (Jernigan et al., 2011). The inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3), a potent inhibitor of mTOR signaling, is induced by ketamine and required for its antidepressant actions, as ketamine has no effect in mice with constitutively active GSK-3 (Beurel et al., 2011). Furthermore, dosage of ketamine too low to elicit antidepressant-like effects (1mg/kg) is made effective by co-administration of a GSK-3 antagonist (Liu et al., 2013).

However, the involvement of mTOR signaling in the antidepressant effects of ketamine has not been consistently demonstrated. Although rapamycin was determined above to block ketamine's behavioral effect, some investigators found no effect of rapamycin (Autry et al., 2011), and even reductions in depression-like behavior have been

observed following rapamycin administration (Cleary et al., 2008). These differing outcomes of rapamycin administration may be due to differences in timing of behavioral testing following injection, or perhaps through an alternate downstream effector pathway of mTOR (Shimobayashi and Hall, 2014). Additionally, ketamine-induced increase in active phosphorylated mTOR is not consistently observed across brain regions affected by ketamine, such as the hippocampus (Autry et al., 2011), indicating that ketamine may exert its actions through a number of different region-specific mechanisms.

Within the hippocampus, ketamine induces a small but significant augmentation of field potentials, indicative of increased AMPAR tone consistent with observations in the PFC as described above, as well as increased expression of BDNF (Autry et al., 2011). In addition, Dr. Monteggia's group found rapidly decreased levels of phosphorylated eukaryotic elongation factor-2 (eEF2) in the hippocampus, but not the cortex, following treatment with ketamine. Tonic activation of NMDARs maintains eEF2 in a phosphorylated state by activating eEF2 kinase (eEF2K), thereby preventing the translation of a number of proteins (Sutton et al., 2007). In blocking NMDARs, ketamine inhibits eEF2K, decreases eEF2 phosphorylation, and induces protein translation, including that of BDNF within the hippocampus (Autry et al., 2011).

Taken together, these data support an overall role of ketamine in synaptic strengthening and spine formation. Ketamine increases AMPA tone and induces the translation of a number of proteins implicated in synaptogenesis through downstream mechanisms. Finally, observed differences between ketamine's actions may be dependent upon the brain region that is being studied, and these region-specific independent actions may work in concert to elicit antidepressant effects.

Depression Circuitry

Studies investigating the regional substrates of ketamine's antidepressant effects have largely focused on the PFC and hippocampus, as these regions are historically associated with depression and depression-like behavior in humans and rodents (**Figure 1.3**).

Case studies of patients with medial PFC (mPFC) ablations reveal apathetic mood and reduced motivation, implicating the region as a potential player in MDD pathology (Fesenmeier et al., 1990). Immediate early genes (IEGs) in postmortem tissue from the ventral-medial anterior cingulate cortex (a region of the PFC) of patients with MDD who were either untreated or treated but still symptomatic were studied, and IEGs were significantly reduced in MDD patients compared to controls in the mPFC, indicating reduced activity in the region (Covington et al., 2010). While the rodent frontal cortex has significantly reduced complexity compared to that of primates, the mPFC of the rodent, comprised of the prelimbic and ifralimbic cortices, has been used as a general proxy for the human mPFC. Covington and colleagues were able to replicate the reduction of IEGs in the mouse mPFC following chronic social defeat stress, known to cause anhedonia. Finally, they demonstrated that optogenetic stimulation of the mPFC results in decreased depression-like behavior following chronic social defeat stress in sucrose preference (a measure of anhedonia) and social interaction tasks (Covington et al., 2010). Deep brain stimulation (DBS) in the PFC has been used to ameliorate TRD in humans (Mayberg et al., 2005) and similar stimulation protocols in the mPFC also reduce depression-like behavior in rodents

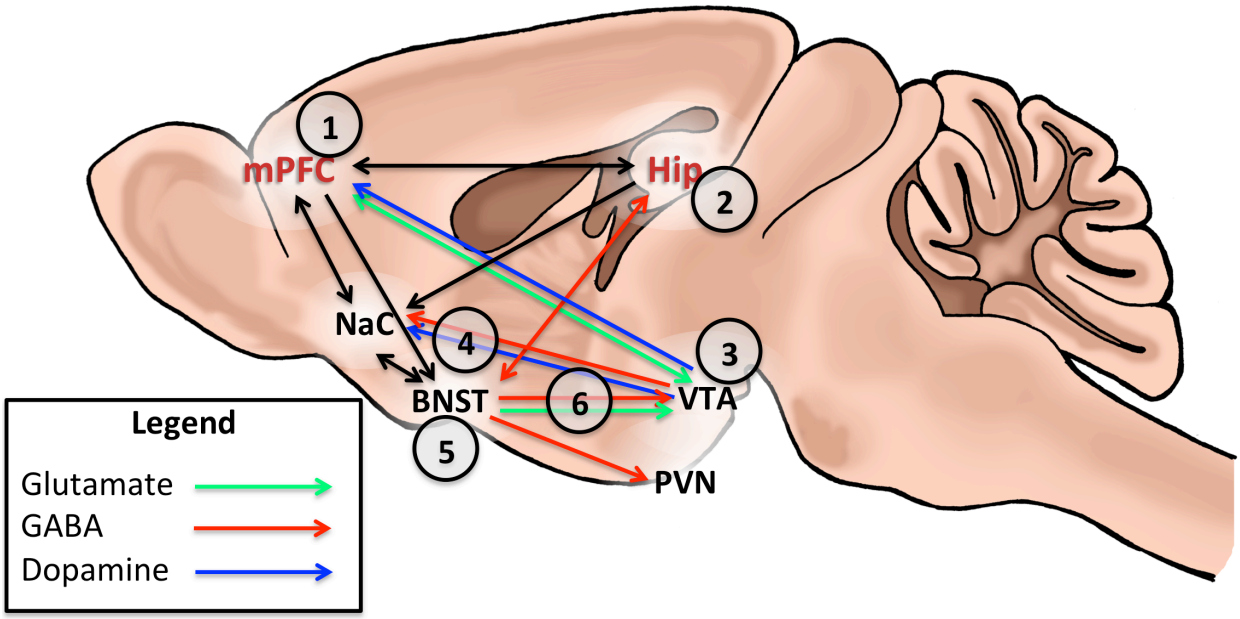

Figure 1.3. Simplified Circuitry of Depression. Ketamine effects have mainly been examined in (1) the mPFC and (2) the hippocampus, due in part to a large body of literature in both humans and rodents implicating these regions in affective disturbances. (3) The VTA is vitally important for reward processing, which is often dysregulated in MDD, however effects of ketamine within this region have not been thoroughly examined. (4) Recent work has highlighted the complex contribution of VTA→NaC circuitry in depression-like behavior, as optogenetic stimulation has been shown to both increase (Chaudhury et al., 2013) and decrease (Tye et al., 2013) these behaviors in rodents. (5) The BNST is another region that, until recently, had not been examined in the role of ketamine's antidepressant effects. It is an extremely heterogeneous region highly connected with stress, reward, and higher order processing circuitry well-suited to govern affective behaviors. (6) Interestingly, glutamatergic or GABAergic BNST projections to the VTA can drive aversive or hedonic behaviors, respectively (Jennings et al., 2013), demonstrating that individual cell types within this region can differentially modulate affective behaviors. mPFC – medial prefrontal cortex; Hip – hippocampus; VTA – ventral tegmental area; NaC – nucleus accumbens; BNST – bed nucleus of the stria terminalis; PVN – paraventricular nucleus of the hypothalamus. More details in text.

(Hamani et al., 2010). This may seem counter-intuitive, as DBS is thought to inhibit firing of neurons whose somas are located near the electrode. The group used radiofrequency lesions, which would destroy all tissue in the area, or ibotenic acid lesions which kill cell bodies but spare axons of passage (Schwarcz et al., 1979) of the mPFC in order to elegantly demonstrate that DBS still produced an antidepressant effect with ibotenic acid lesions, but not following radiofrequency lesions (Hamani et al., 2010). Thus, *activation* of these axons of passage may underlie the antidepressant effects of DBS in the PFC. The PFC has reciprocal connections with the amygdala and the hippocampus and a projection to a subset of ventral tegmental area (VTA) neurons. Dopaminergic projections from the VTA to the PFC comprise the mesocortical DA system, and will be described below. The involvement of the PFC in MDD and depression-like behaviors led to studies examining the effect of ketamine in this region, as previously described (Homayoun and Moghaddam, 2007; Li et al., 2010; Li et al., 2011; Liu et al., 2013).

The hippocampus is another region that has been studied extensively with relation to depression. The hippocampus regulates responses to stress through dense GABAergic projections to a number of regions, including the bed nucleus of the stria terminalis (BNST; described below), the noradrenergic nucleus of the solitary tract (NTS), and the dorsomedial hypothalamus that then relay hippocampal signals to the paraventricular nucleus of the hypothalamus (PVN), a crucial part of the hypothalamic-pituitary-adrenal (HPA) axis (Levinstein and Samuels, 2014). The hippocampus also projects to the nucleus accumbens (NaC) and has reciprocal connections with the PFC. Stress reduces BDNF expression and neurogenesis in the dentate gyrus (DG) of primates (Gould et al., 1998) and rodents (Duman et al., 1997). BDNF infusion in to the DG is sufficient to reduce depression-

like behaviors in the FST and tail suspension test (Shirayama et al., 2002). The effectiveness of chronically administered monoamine-based antidepressant appears to be dependent upon DG neurogenesis (Santarelli et al., 2003). These factors influenced the examination of ketamine's effects within the hippocampus, spearheaded by Dr. Lisa Monteggia's lab. Similar to effective monoamine-targeting antidepressants, ketamine increased BDNF levels and neurogenesis in the hippocampus (Autry et al., 2011)

Anhedonia represents one of two possible core requirements for diagnosis of MDD. Therefore, deficits in reward processing are thought to contribute to symptomatology associated with MDD. The VTA to NaC mesolimbic dopamine system is the primary reward pathway in the brain, and all rewarding substances, including drugs of abuse as well as natural rewards, act in part by increasing dopamine concentration in the NaC. Patients with MDD (as well as those with AUDs) have reduced sensitivity of central dopamine receptors (Schmidt et al., 2001) In rodents repeated restraint stress dampens excitatory synaptic strength on NaC dopamine D1 receptor-expressing medium spiny neurons (D1-MSNs), which was linked to anhedonia-related depression-like behaviors (Lim et al., 2012). Projections from the VTA to the NaC play a role in depression-related behaviors, although thus far results are not entirely clear. One study demonstrated increased susceptibility to social defeat stress-induced anhedonia behaviors when optogenetic phasic stimulation of this pathway was administered during the social defeat task (Chaudhury et al., 2013). However, in the same issue of *Nature*, another group showed that optogenetic phasic stimulation of this pathway after chronic mild stress reduced depression-like behaviors relative to controls while inhibition of this pathway increased these behaviors (Tye et al., 2013). The disparate findings between these two studies could potentially be explained by

a number of methodological differences, including timing of stimulation, type of stressor utilized, and identity of neuronal type contributing to behavior (Lammel et al., 2014). While both groups targeted the VTA to NaC projection that would contain both DAergic and GABAergic neurons, only Tye, et al confirmed that the behaviors they observed were DA-mediated. Further studies will be needed to fully understand the VTA's role in depression behavior. Whether ketamine modulates physiology in the VTA has not been thoroughly examined; however a recent study showed no effect of ketamine treatment on glucose metabolism or tyrosine hydroxylase immunoreactivity in the VTA of rats following chronic unpredictable stress (Baptista et al., 2015). It is possible that the paucity of literature examining ketamine's role in this region could be attributed to the known abuse potential of ketamine.

Given the differences between putative mechanisms of ketamine's antidepressant effects between the PFC and the hippocampus, ketamine's actions are not likely to be identical across all brain regions. Recent work has also implicated the BNST in depression-related behavior, and distinct connectivity and receptor expression patterns within this region, discussed below, suggest that it may be sensitive to ketamine-mediated effects.

1.3 The Bed Nucleus of the Stria Terminalis and Depression

BNST Circuitry

The BNST is a part of an interconnected and related series of nuclei referred to as the extended amygdala, a region in an area previously known as "substantia innominata," or "unnamed substance" (Alheid, 2003; Alheid and Heimer, 1988). This contiguous regional group contains (from rostral to caudal) the shell of the NaC, the BNST, and the central

nucleus of the amygdala (CeA). The BNST contains a highly heterogeneous population of neurons that send and receive myriad projections that cannot be adequately described in full here, though interested readers should be directed to the herculean neuroanatomical mapping work of Hong-Wei Dong and Larry Swanson (Dong et al., 2001b; Dong and Swanson, 2003, 2004a, b, 2006a, b, c). This region is a highly integrative nucleus that participates in stress and anxiety through dense noradrenergic projections from the NTS (Forray and Gysling, 2004), projections (mainly GABAergic) to the paraventricular nucleus of the hypothalamus, and reciprocal connections with amygdalar structures (CeA and basolateral amygdala, BLA) (Dong et al., 2001a). Additionally, the BNST sends dense efferent projections to the VTA. These connections place the BNST at a fundamental junction in mediation between stress and reward circuitry, and, unsurprisingly, the BNST has been identified as a major player in stress-induced relapse to drug seeking and other maladaptive responses to stress (Zorrilla and Koob, 2013). Furthermore, the BNST receives top-down projections from areas such as the PFC and hippocampus involved in decision processing (McDonald, 1998).

In addition to connectivity to stress-related regions, the BNST is also a key player in the corticotropin-releasing factor (CRF) system. CRF is a stress hormone crucial to the HPA axis and stress response. Systemic administration of CRF mimics behavioral characteristics of depression in rodents, including decreased social interaction, feeding, and exploratory behavior (Dunn and Berridge, 1990). Conversely, mice lacking the CRF receptor 1 (CRFR1) display reduced affective behavior (Contarino et al., 1999) and CRFR1 antagonism reduces stress-induced deficits in social interaction (Gehlert et al., 2005). These exciting preclinical results lead to investigation of novel antidepressant therapeutics targeting CRF, but,

unfortunately, clinical trials have not demonstrated efficacy in treating MDD with CRF antagonists (Griebel and Holsboer, 2012). It is possible that this lack of efficacy is due in part to regional differences in CRF's actions. CRF is expressed predominantly in the hypothalamus. However CRF is also expressed in discrete nuclei extra-hypothalamically, most highly in the BNST and CeA (Swanson et al., 1983). A variety of stressors have been repeatedly shown to increase CRF expression in the BNST (Choi et al., 2006; Funk et al., 2006; Kim et al., 2006). Additionally, our lab has shown that BNST CRF neurons are activated by stress, and increase firing in response to NE through β-AR activation (Silberman et al., 2013). This study also found that CRF increases firing rates of VTA-projecting BNST neurons.

The BNST in Anxiety-Related behaviors.

Classically, the BNST has been linked to anxiety-like behavior in rodents. Within the extended amygdala, the CeA is believed to respond to acute stressors, whereas the BNST is engaged following a more chronic stress exposure (Walker and Davis, 2008). Differentiation of these two stressors is vitally important to adaptive responding (Avery et al., 2015). When a threat (for instance, a predator) is in close proximity, escape or evasive behaviors are preferentially enacted. On the other hand, when the possibility of (or chronic exposure to) a threat exists, behaviors aimed to enhance vigilance and decrease food/reward seeking in favor of attention to possible threat are engaged. However, these two behaviors become maladaptive when they are applied during instances of perceived, but not real, threat exposure. Thus, the CeA has been though to underlie maladaptive fear behaviors following acute stress, while the BNST is largely involved in anxiety-related

behaviors induced by chronic exposure to a stressor, representing a neuroanatomical difference between fear and anxiety (Davis et al., 2010; Walker and Davis, 2008). Electrical stimulation of the BNST not only replicated but amplified the behavioral response to restraint stress (Casada and Dafny, 1991). In my early work in the lab, we examined the effect of chronic stressor paradigms, chronic corticosterone (cort) administration and chronic (6-8wk) single housing, compared to control or acute matching stressors (single cort injection or 24hr single housing) in anxiety-related behavior and induction of LTP in the BNST in mice (Conrad et al., 2011). We found that chronic, but not acute, stress decreased open arm time and center time in the elevated zero maze (EZM) and novel open field tests, both proxies for anxiety-like behavior. Chronic stressors also blunt subsequent induction of LTP in the BNST (discussed in further detail below).

Manipulations of the BNST CRF system have also been implicated in anxiety-related behaviors in some studies. Intra-BNST infusion of CRF increase fear-potentiated startle, and co-infusion of a CRF antagonist into the BNST blocks this startle potentiation (Lee and Davis, 1997). A recent study demonstrated that deletion of a $GABA_A$ receptor specifically from CRF cells, thus increasing excitation, increases anxiety-like behaviors, and CRFR1 antagonist infusion into the BNST blocks these behaviors (Gafford et al., 2012).

The BNST in Depression-Related Behaviors

The BNST was initially implicated in anxiety-like behavior induced by chronic stressors by utilizing methodology to broadly either silence/activate the region (such as lesioning and electrical stimulation) or measure its output (such as field potential recording). However, some of these studies demonstrated decreased *depression*-like

behavior following BNST suppression (Crestani et al., 2010; Hammack et al., 2004). Recent studies have investigated the contributions of individual regions and cell types in this heterogeneous region. Alon Chen's group sought to examine the effect of CRF overexpression in the CeA and BNST in affective behaviors (Regev et al., 2011). Lentiviral constructs containing CRF cDNA driven by the CMV promoter were injected into BNST or CeA to drive cell-unspecific overexpression of CRF in either region. Surprisingly, unlike other studies described above, CRF overexpression in the BNST drove *depression*-like, but not anxiety-like, behaviors (Regev et al., 2011). A major caveat of this study was the use of ectopic overexpression of CRF in a number of BNST neurons (not just endogenous CRF-expressing neurons) through the use of a CMV promoter in their lentiviral construct. CRF is not expressed naturally in the majority of BNST neurons, and indeed, we recently found CRF mRNA expression in approximately 10% of dlBNST cells (unpublished data, **Figure E.3**). Elegant optogenetic dissection of BNST projections to the VTA revealed opposing aversive and hedonic actions of glutamatergic versus GABAergic efferents, respectively (Jennings et al., 2013). The proposed involvement of the BNST in depression should come as no large surprise, given the integrative nature of this region in assimilating stress and reward pathways. Anhedonia, or lack of enjoyment in previously pleasurable activities, is a hallmark of MDD in humans and modeled in animal models that induce depression-like behavior (Strekalova et al., 2004). Thus depression can be postulated to represent a functional imbalance between stress (a contributing factor to depression) and reward (diminished in depression), and the BNST sits at a crossroad of these systems.

NMDARs in the BNST

As discussed in a previous section, neuronal plasticity within a region represents a mechanism whereby stimulus exposure can result in altered responses (both beneficial and maladaptive) to subsequent stimulation. LTP is a common artificial proxy of measuring alterations in plasticity in a number of brain regions. Similarly, in the dBNST, NMDAR-dependent LTP can be easily induced and measured following a two train 100Hz tetanus stimulation via field potential recordings (Weitlauf et al., 2004). As previously stated, differences in NMDAR subunit expression, particularly with regards to the GluN2 isoforms, confers differential properties on the channel as a whole, including alterations in plasticity induction. A general trend in the mammalian brain during postnatal development is an increase in expression of GluN2A without concomitant change in GluN2B, contributing to an overall acceleration of NMDAR EPSC decay representing increased GluN2A:GluN2B ratio in adult compared to adolescent tissue (Cull-Candy et al., 2001). It has been hypothesized that the GluN2A:GluN2B ratio in a given brain region contributes to stimulus profiles required to induce either LTP or LTD (Yashiro and Philpot, 2008). In this model, high GluN2A:GluN2B synapses favor LTD induction with lower frequency stimulation, whereas the same stimulus intensity might drive LTP at low GluN2A:GluN2B synapses. Thus low GluN2A:GluN2B synapses would have a lower crossover threshold of stimulus frequency for induction of LTP vs. LTD. It is quite easy to induce LTP in the BNST (Conrad et al., 2011; Weitlauf et al., 2004; Weitlauf et al., 2005; Wills et al., 2012) however electrical induction of LTD in this region has not been successful, indicating a lower GluN2A:GluN2B ratio. Additionally, the BNST demonstrates somewhat unique requirements for plasticity induction. While GluN2A is crucial for LTP induction in the hippocampus and cortex (Liu et

al., 2004; Massey et al., 2004), in the BNST, GluN2A is not required for LTP, as GluN2A knockout mice display no deficits in LTP induction (Weitlauf et al., 2005). On the other hand, GluN2B-containing NMDARs appear to be crucial for BNST LTP. In mice constitutively lacking forebrain GluN2B or in slices following bath application of Ro 25-6981, BNST LTP cannot be induced (Wills et al., 2012). LTP is augmented in the BNST following exposure to chronic intermittent ethanol (CIE), and a coincidental increase in GluN2B expression is also observed following CIE in the region (Wills et al., 2012).

Conclusions

The BNST sits at a nexus of stress and reward circuitry, and thus is uniquely poised for modulation of depression-like behavior. In spite of this, only a handful of studies have investigated this region with regard to depression, perhaps due in part to methodical limitations. However, relatively recently neuroscientists have enjoyed the addition of a number of helpful methods to their toolbox, including cell-specific and regional targeting of viral constructs. The BNST contains high levels of GluN2B-containing NMDARs, necessary for induction of LTP within the region. A growing body of literature supports the hypothesis that ketamine's antidepressant effects are likely mediated – at least in part – by GluN2B-containing NMDARs, and a transgenic floxed-*grin2b* mouse is now available. Chapter 2 examines the possibility that the antidepressant effects of ketamine can be behaviorally mimicked by targeted, non-constitutive, knockdown of GluN2B-containing NMDARs specifically within the BNST.

Chapter 2

Knockdown of BNST GluN2B-containing NMDA receptors mimics the actions of ketamine on novelty-induced hypophagia

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Abstract

Administration of a single low dose of the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine has been demonstrated to elicit long-lasting antidepressant effects in humans with depression, as well as in rodent models of depression. Although pharmacological studies have implicated the GluN2B subunit of the NMDA receptor in these effects, drugs targeting this subunit have off-target actions, and systemic administration of these compounds does not allow for delineation of specific brain regions involved. In this study, we assessed the role of GluN2B in the bed nucleus of the stria terminalis (BNST) in novelty-induced hypophagia (NIH) in mice. First, we verified that ketamine, as well as the GluN2B antagonist Ro25-6981, decreased the latency to consume food in a novel environment in a version of the NIH test. We then hypothesized that GluN2B-containing receptors within the BNST may be a target of systemic ketamine and contribute to behavioral effects. Through the combination of a GluN2B floxed mouse line and stereotaxic delivery of lentiviral Cre recombinase, we found that targeted knockdown of this subunit within the BNST mimicked the reduction in affective behavior observed with systemic ketamine or Ro25-6981 in the NIH test. These data suggest a role for GluN2B-containing NMDARs within the BNST in the affective effects of systemic ketamine.

Adapted from: Louderback KM, et al. Knockdown of BNST GluN2B-containing NMDA receptors mimics the actions of ketamine on novelty-induced hypophagia. Transl Psychiatry. 10.1038/tp.2013.103. (2013)

Introduction

A single, sub-anesthetic dose of the N-methyl D-aspartate receptor (NMDAR) antagonist ketamine exerts a rapid antidepressant action in patients with depression (Berman, et al. 2000), and has been associated with a number of adaptations throughout the brain. However, ketamine acts as a psychotomimetic and has abuse potential, so understanding which specific adaptations are contributing to its positive behavioral effects is crucial for safer treatment options for depression. In this study, we propose that GluN2B subunit-containing NMDARs within the bed nucleus of the stria terminalis (BNST) contribute to the actions of ketamine in a novelty induced hypophagia (NIH) task used to test antidepressant efficacy.

Preclinical rodent studies using ketamine have mirrored the antidepressant actions observed in humans (Li, et al. 2011; Autry, et al. 2011), with studies using the GluN2B-selective antagonist Ro25-6981 specifically implicating GluN2B-containing NMDARs. In rodents, systemic ketamine administration enhances glutamate synaptic function in the medial prefrontal cortex and hippocampus through regulation of homeostatic plasticity (Autry, et al. 2011). Currently however, specific populations of NMDARs have not been linked to antidepressant behavioral effects. We pursued the anatomical underpinning of ketamine's antidepressant actions, focusing on the bed nucleus of the stria terminalis (BNST). The BNST is a component of the extended amygdala that modulates affective behavior, has been implicated in depression and anxiety, and has relatively high adult expression of GluN2B-containing NMDARs (Sink, et al. 2013; Wills, et al. 2012; Regev, et al. 2011). Further, activation of glutamate synapses in the BNST drives negative affect-related behaviors (Kim, et al. 2013). Given the evidence for GluN2B-containing NMDARs in the

antidepressant effects of ketamine, we hypothesized that these receptors within the BNST might govern affective behaviors.

Methods

Animals and Treatment

All animals were housed 2-5/cage and were provided with rodent chow and tap water *ad libitum*. The temperature- and humidity-controlled animal facilities are maintained on a 12:12 h light:dark cycle (lights on 0600-1800 h). All experiments took place during the light phase of the cycle.

For ketamine and Ro25-6981 experiments, male wild-type C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME; 8 wks) were acclimated to the vivarium for a minimum of 1 week prior to experimentation. Following acclimation, mice were handled for 5 days and given habituating saline injections for the last 3 days of handling. Ketamine (3mg/kg body weight, *Ketaved*; *Webster* Veterinary Supply, Sterling, MA), Ro 25-6981 maleate (5 mg/kg; Tocris, Minneapolis, MN), and 0.9% saline were administered intraperitoneally (ip).

Floxed *Grin2b* mice were generated by Eric Delpire as previously described (Brigman, et al. 2010), and floxed *Nr3c1* (GR) mice were generated by Louis Muglia as previously described (Brewer, et al. 2003). Both lines were bred homozygous for the floxed allele in our facility and underwent surgery (described below) at one to four months of age. Prior to behavioral testing, mice were handled for 5 days.

All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Surgical Procedure

Mice were anesthetized with 1.5% isoflurane. 200nL LV-CRE (UNC Vector Core; Titer = $1.3x10^9$) or LV-GFP (UNC Vector Core; Titer = $1.3x10^{10}$) was injected into the dorsal BNST (coordinates: AP: 0.14mm; L: ±0.88mm; D: -4.24mm) at a rate of 50nL/min using an AngleTwo stereotax at a 15.03^o angle to avoid ventricles. Postoperatively, mice were administered ketoprofen (5mg/kg subcutaneously) once/day for 72hrs, then PRN for one week. Mice were given at least two weeks recovery time following surgery before behavioral experimentation.

Novelty-Induced Hypophagia (NIH)

The NIH test consisted of 4 training days followed by a testing day. On all days, mice were given at least 1 hour acclimation to the testing room under low red light (~40 lux), and all mice had access to rodent chow throughout behavioral testing. During training, mice were given 30min/day access to a highly palatable food (liquid Ensure, Home-made Vanilla Shake flavor) in the testing room while group-housed in their home cages under low red light. By the second training day, all mice had consumed Ensure, so no mice were excluded from the study. On test day in the ketamine and Ro25-6981 study, half of the mice were given a one hour restraint stress in 50mL conical tubes while the other half were allowed to remain in their home cages. One half hour following the termination of restraint stress, all mice were given ip injections of ketamine, Ro25-6981 or equal volume saline, yielding 6 groups (no restraint-saline, no restraint-ketamine, no restraint-Ro, restraint-saline, restraint-ketamine, and restraint-Ro). On testing day, each mouse was transferred to an

individual novel cage devoid of bedding under bright lighting (~200 lux) immediately prior to 30 min Ensure access. Cages were cleaned with 30% EtOH before and after each animal.

Elevated Zero Maze (EZM)

An EZM apparatus measuring 34 cm inner diameter, 46 cm outer diameter, placed 40 cm off the ground on 4 braced legs, with two open quadrants and two walled quadrants was used. At the start of the test, mice were placed in the center of one of the open quadrants facing the closed quadrant. The test lasted 5 min. In between subjects, the apparatus was cleaned with 30% ethanol. Mice were considered to be in a quadrant when 75% of their body was in the area. Time spent in the open and closed areas as well as distance traveled were scored using ANY-Maze software (San Diego Instruments, San Diego, CA).

Data and Statistical Analysis

Latency (sec) to the first sip of Ensure and amount (g) consumed was measured in the NIH test. Time spent in open and closed arms and distance traveled was measured in the EZM test. For ketamine and Ro25-6981 studies, statistical significance was calculated via two-way analysis of variance (ANOVA) for treatment x restraint with a *post-hoc* Bonferroni multiple comparison test in the NIH test. For all studies using transgenic animals, statistical significance was calculated via t-test. All data was analyzed using GraphPad Prism 5 (La Jolla, CA). Data is represented as mean±SEM. Significance was set at $P<0.05$.

Results and Discussion

We first validated a version of the NIH test for analysis of the antidepressant efficacy of NMDAR manipulation. In brief, the latency to consume food in a novel environment is quantified as a measure of affective state. Previous studies used chronically stressed rodents and/or food restriction in similar paradigms (Li, et al. 2011; Autry, et al. 2011). We utilized a protocol similar to that described by Dulawa and Hen, in which satiated mice seek a highly palatable food reward (Dulawa and Hen, 2005). This test thus is thought to rely on hedonic drive to consume rather than hunger, which may be more closely aligned with depression-associated anhedonia.

Mice received ketamine (3mg/kg, ip), Ro25-6981 (5mg/kg, ip) or equivalent volume saline one half hour following an hour restraint stressor or no stress. The amount of Ensure consumed and the latency to consume the first sip of Ensure was measured; with increased latency indicating increased negative affective behavior. We found that ketamine and Ro25-6981 each significantly reduced this latency ($F_{(2, 72)} = 12.46$, $P < 0.0001$) in mice, regardless of whether the mice had been previously treated with a one hour restraint stressor or not (**Figure 2.1A**). No difference in consumption was observed with Ketamine or Ro25-6981 administration (**Supplemental Figure 2.S1**). We examined ketamine and Ro25-6981 administration on anxiety-like behavior in the elevated zero maze (EZM) following a forced swim stressor and observed no difference in open arm time or distance traveled (**Supplemental Figure 2.S1**).

While Ro25-6981 is selective for GluN2B-containing NMDARs over non-GluN2B-containing NMDARs, this compound acts on a number of off-target sites also implicated in depression, including serotonin and norepinephrine transporters (Keiser, et al. 2009).

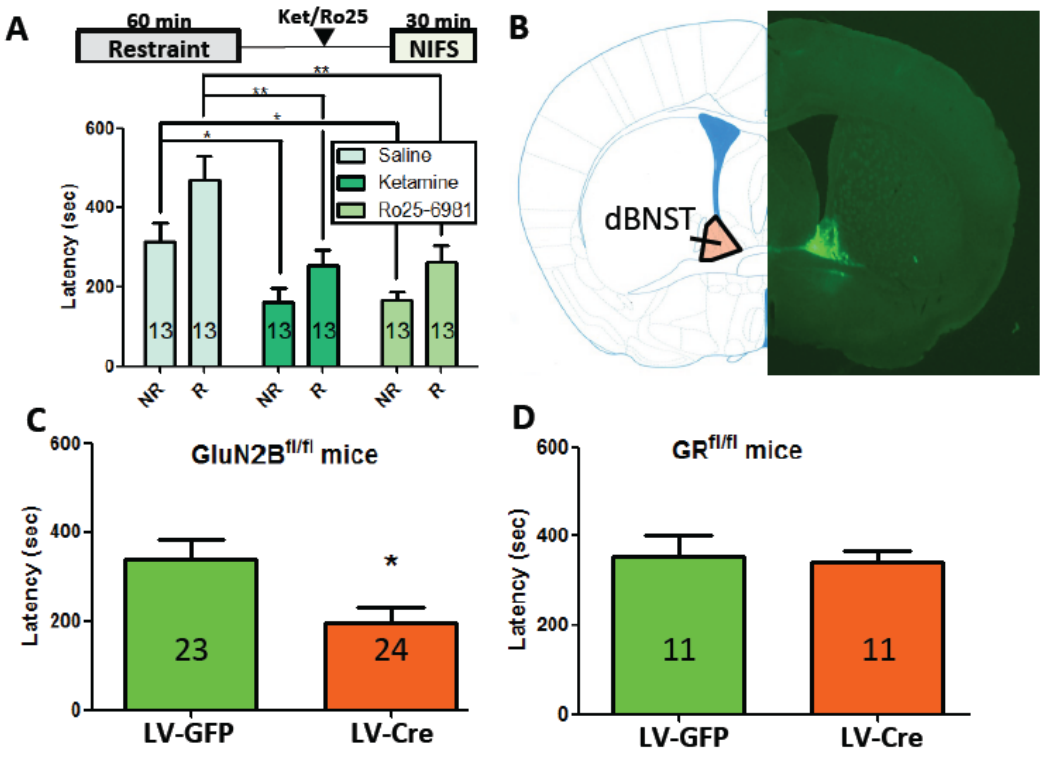


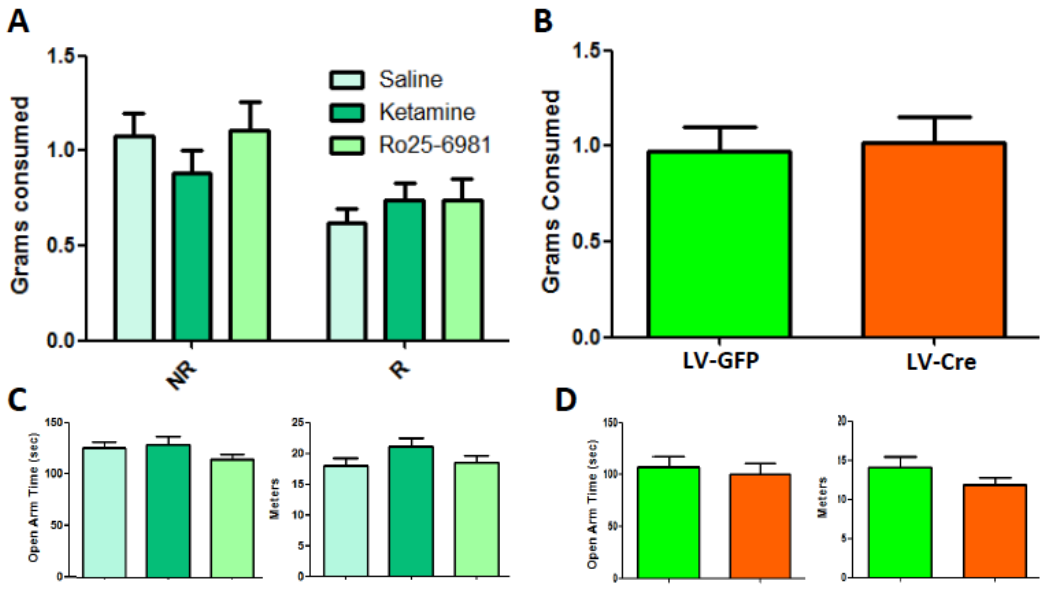
Figure 2.1. Viral deletion of GluN2B from the BNST phenocopies systemic treatment with ketamine or Ro25-6981. (A) Timeline above. Decreased latencies with systemic ketamine (3mg/kg) and Ro25 (5mg/kg) administration in our novelty-induced feeding suppression paradigm were repeated with (R) and without (NR) restraint stress. (B) Coronal atlas image (left) showing dorsal BNST in the inset and LV-GFP injection targeting (right). (C) Viral mediated deletion of GluN2B from BNST (LV-Cre) phenocopies systemic ketamine, (D) however viral mediated deletion of GR from the BNST has no such effect. Data are presented as means with SEM. * indicates P>0.05; ** indicates P>0.01. N's indicated on bars.

Thus, we sought to determine specific neural circuits where GluN2B-NMDARs influence antidepressant action by utilizing a mouse line harboring floxed *Grin2b* alleles (Wills, et al. 2012) in concert with stereotaxic delivery of lentiviral Cre recombinase (LV-Cre) to knock down GluN2B expression within the BNST. This GluN2B floxed line has been used in previous studies by our lab and, when crossed with mice expressing tetO-Cre under the CaMKII promoter, demonstrated 80% reduction in GluN2B levels in the BNST as assessed by Western blot (Wills, et al. 2012). Lentiviral GFP (LV-GFP) was injected as a control as previously described (Fig 1B; Kolber et al. 2008), and functionality of Cre recombinase was confirmed through LV-Cre injection into the BNST of the Ai9tomato reporter mouse line (data not shown). LV-Cre-injected animals displayed a significant decrease in NIH latency compared to LV-GFP controls ($t_{(45)} = 2.44$, $P = 0.0186$; **Figure 2.1C**), mirroring ketamine- or Ro25-6981-treated animals. No difference in total consumption in the NIH test or anxiety-like behavior as measured by the EZM was observed (**Supplementary Figure 2.S1**). It is interesting to note that when GluN2B knockdown in this floxed line is limited to corticohippocampal regions, with no deficit in amygdalar GluN2B expression, no impairment in affective behavior is observed (Brigman, et al. 2010). This indicates that the behavioral phenotype observed in the study outlined here is regionally specific for GluN2B within the BNST.

To control for potential nonspecific actions of the LV-Cre, we performed a parallel study using LV-Cre and LV-GFP injection in a previously described floxed glucocorticoid receptor ($GR^{fl/fl}$) line. In these studies, LV-Cre-injected $GR^{fl/fl}$ mice performed similarly to LV-GFP-injected mice (**Figure 2.1D**). These data indicate that knock down of GluN2B from

the BNST, not nonspecific actions of LV-Cre administration, reduced negative affective behavior.

Taken together, these data indicate that GluN2B-containing NMDARs within the BNST play an important role in regulating depression. Further, our pharmacological data demonstrate that ketamine and Ro25-6981 exert antidepressant-like effects in the NIH paradigm without need of prior stress exposure. Future studies will aim to elucidate the mechanism by which GluN2B blockade in the BNST exerts antidepressant-like effects, and determine if direct pharmacological inhibition of GluN2B within the BNST through cannulated injection of ketamine or Ro25-6981 is able to recapitulate behavioral effects observed with systemic administration.



Supplementary Figure 2.S1. NIH consumption and Elevated Zero Maze behavior. No effect on NIH Ensure consumption or elevated zero maze (EZM) behavior with systemic administration of ketamine or Ro25-6981 or viral deletion of GluN2B from the BNST. Neither (A) systemic ketamine (3mg/kg), systemic Ro25-6981 (5mg/kg), nor (B) BNSTGluN2B knockdown influenced consumption in the NIH paradigm. (C) Ketamine and Ro25-6981 did not alter time spent in the open arm compartment of the EZM 60 minutes following the termination of a forced swim stressor. No alterations in distance traveled within the EZM was observed. (D) Similarly, BNSTGluN2B knockdown did produce alterations in time spent in the open arm compartment or distance traveled in the EZM when assessed 60 minutes following the termination of a forced swim stressor in animals previously tested in the NIH paradigm. R = 60 minute restraint stress; NR = No Restraint

Chapter 3

Ketamine and MAG Lipase Inhibitor-Dependent Reversal of Evolving Depressive Behavior during Forced Abstinence from Alcohol Drinking

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Abstract

While alcoholism and depression are highly comorbid, treatment options that take this into account are lacking, and mouse models of alcohol (ethanol, EtOH) intake-induced depressive behavior have not been well established. Recent studies utilizing contingent EtOH administration through prolonged two-bottle choice access have demonstrated depression-like behavior following EtOH abstinence in singly-housed female C57BL/6J mice. In the present study, we found that depression-like behavior in the forced swim test (FST) is revealed only after a protracted (2 weeks), but not acute (24 hour), abstinence period. No effect on anxiety-like behavior in the EPM was observed. Further, we found that once established, the affective disturbance is long-lasting, as we observed significantly enhanced latencies to approach food even 35 days after ethanol withdrawal in the novelty-suppressed feeding test (NSFT). We were able to reverse affective disturbances measured in the NSFT following EtOH abstinence utilizing the N-methyl D-aspartate receptor (NMDAR) antagonist and antidepressant ketamine, but not memantine, another NMDAR antagonist. Pretreatment with the monoacylglycerol (MAG) lipase inhibitor JZL-184 also reduced affective disturbances in the NSFT in ethanol withdrawn mice, and this effect was prevented by co-administration of the CB1 inverse agonist rimonabant. Endocannabinoid levels were decreased within the BLA during abstinence compared to during drinking. Finally, we demonstrate that the depressive behaviors observed do not require a sucrose fade, and that this drinking paradigm may favor the development of habit-like EtOH consumption. These data could set the stage for developing novel treatment approaches for alcohol-withdrawal-induced mood and anxiety disorders.

Adapted from: Holleran KM, et al. Ketamine and MAG Lipase Inhibitor-Dependent Reversal of Evolving Depressive Behavior during Forced Abstinence from Alcohol Drinking. Neuropsychopharm. (2015, in submission)

Introduction

Alcohol use disorders (AUDs) are strongly comorbid with depression (Grant et al., 2015), and alcohol withdrawal is associated with affective disturbances (Heilig et al., 2010). Developing an understanding of AUD-depression interactions, as well as treatments for these comorbid conditions, represents a clinically crucial research area.

Rodent models of alcohol (ethanol, EtOH) administration, typically non-contingent, have largely focused on anxiety-like behavior during early withdrawal (Kash et al., 2009; Kliethermes, 2005). These and other negative affective behaviors have proven difficult to reliably induce in C57BL/6J mice (Daut et al. 2015) frequently utilized in drinking studies (Kash et al., 2009; Lovinger and Crabbe, 2005). Recent studies in C57BL/6J mice have modeled depression-like behavior following abstinence from contingent EtOH administration (Lee et al., 2015; Pang et al., 2013; Stevenson et al., 2009). Two weeks of abstinence from EtOH induced depression-like behavior in forced swim (FST), novelty-suppressed feeding (NSFT), and saccharin preference tests, but anxiety-like behavior measured via elevated plus maze (EPM) and light-dark box test was unaltered (Pang et al., 2013).

Here, we replicated and extended the findings of Pang and colleagues (2013). We elucidated a timeline by examining behavior 1, 15-18, and 35 days after EtOH removal. Treatments with typical antidepressants (selective serotonin reuptake inhibitors, SSRIs) are notoriously troublesome due to low treatment efficacy and long lag time (weeks to months) before benefits manifest (Gartlehner et al., 2012), and such antidepressants can escalate EtOH consumption in rats (Alen et al., 2013). We examined alternative pharmacological treatments in reversing depression-like behavior in mice. The N-methyl

D-aspartate receptor (NMDAR) antagonist ketamine has been extensively studied for antidepressant efficacy in humans (Berman et al., 2000) and rodents (Autry et al., 2011; Li et al., 2011). Here, we examined the ability of NMDAR antagonists ketamine and memantine to reduce depressive behavior following two weeks of EtOH abstinence. We also examined the role of endocannabinoids (eCBs) in this phenotype, as stress-induced affective behaviors can be ameliorated by increasing eCB levels (Sumislawski et al., 2011). We tested the monoacylglycerol (MAG) lipase inhibitor JZL-184 in reversing disrupted affective behavior following EtOH abstinence, and whether this effect was blocked by the CB1 receptor inverse agonist rimonabant. Further, we examined eCB levels in discrete brain regions during EtOH exposure versus abstinence. Finally, we examined whether motivation for EtOH drinking becomes more habit-like following this paradigm.

Materials and Methods

Animals

232 female C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were used for this study. Females were chosen for two reasons. In humans, females are disproportionately affected by Major Depressive Disorder (MDD) (Grigoriadis and Robinson, 2007). Additionally, female C57BL/6J mice develop higher preference for 10% EtOH over water (Middaugh et al., 1999). Thus, we chose a population that may prove more susceptible to affective disturbances and elevated EtOH preference. Mice were delivered at 7wks of age, then singly-housed and maintained on 12hr light/dark cycle (lights on at 06:00) under controlled temperature (20-25^{0}C) and humidity (30-50%) levels. Treatments were approved by the Vanderbilt Animal Care and Use Committee.

Two Bottle Choice EtOH Drinking

Mice were given access to two sippers (50mL conical tube with stopper and drinking spout) containing water. Mice in control groups (N=69) received water in both sippers. Three days after acclimation to the sipper configuration, mice in EtOH drinking groups (N=143) either went through a sucrose fade (N=85; **Figure 3.1**) or EtOH ramp (N=58; **Figure 3.5**). In the sucrose fade, 10% EtOH and a diminishing concentration of sucrose (10% for 2 days, 5% for 6 days, 1% for 7 days, then 0% for the remainder of the experiment) was used. In the EtOH ramp procedure, no sucrose was given, and an increasing concentration of EtOH (3% for 3 days; 7% for 6 days, then 10% for the remainder of the EtOH exposure period) was used. EtOH was provided during the first 6 weeks, and both sippers were continuously accessible for the length of the experiment. Sipper position was switched weekly to avoid position bias. Mice and sippers were weighed every other day. In order to control for potential drip of the sippers, an empty cage was set up with one water and one EtOH sipper. These sippers were measured every other day, along with mice and experimental sippers, and the average spill value was subtracted from sipper weights. Preference percentage was measured as ((EtOH solution consumed (g) – Drip) / (total consumed (g) – Drip))x100. After this, blood and tissue was collected for blood EtOH concentration (BEC; N=19) and endocannabinoid analysis, respectively, described below. In other mice, following EtOH access, 10% EtOH was replaced with either water for abstinence-related behavioral studies (N=144) or 3% EtOH for the habit-like drinking study (N=10).

Drugs

Ketamine (Patterson Veterinary, Devens, MA; 3mg/kg, 10mL/g bw in saline; i.p.), Memantine HCl (Tocris, Ellisville, MO; 20mg/kg, 10mL/g bw in saline; i.p.), or saline (10mL/g bw; i.p.) were administered 30 minutes prior to NSFT (described below). JZL-184 (Cayman Chemical, Ann Arbor, MI; 8mg/kg, 1mL/g bw in dimethyl sulfoxide (DMSO); i.p.), JZL-184 (as described above) with rimonabant (1mg/kg, 1mL/g bw in DMSO; i.p.), or DMSO (Sigma, St. Louis, MO; 1mL/g bw; i.p.) were administered 2 hours prior to NSFT.

Blood Ethanol Concentration (BEC) Analysis

Trunk blood was collected from mice at 13:00-16:00hrs (7-10hrs into the light cycle) on day 42 of EtOH exposure. Plasma was isolated following a 10 min 10,000 rpm centrifugation of the blood at 4^{O}C. BECs were analyzed in triplicate from 1:100 diluted samples along with EtOH standards using a colorometric horseradish peroxidase- and alcohol oxidase-coupled assay measured at 595 nm as previously described (Prencipe et al., 1987).

Forced Swim Test (FST)

Following 1hr acclimation to the testing room, mice were exposed to a 6-minute forced swim in a cylinder filled with water (23-25^{o}C) such that mice could not touch the bottom. Time immobile (no movement except those required to remain afloat) was scored by blinded observer via video recording during the last 4min of swim.

Elevated Plus Maze (EPM)

As described previously (Kash et al., 2009), mice were tested for time spent on open and closed arms over 5 minutes. Mice were visualized, recorded, and tracked by camera using AnyMaze software (Stoelting Co, Wood Dale, IL). The apparatus is elevated 55cm above the floor and consists of four arms (30.5x6.5cm), two open and two closed (16cm wall height), with a 5x5cm open center zone. Lighting was set to approximately 50 lux

Novelty-Suppressed Feeding Test (NSFT)

As previously described (Pang et al., 2013) mice were food-deprived for 48 hours prior to the NSFT, with a free-feeding window during hours 23-25. Mice were acclimated to the testing room for 1hr prior to testing. Mice were placed into the corner of an arena (32x32cm) covered with 2cm fresh bedding material with a pellet of normal mouse chow (LabDiet 5L0D; West Durham, NC) in the center. Lighting was set to approximately 400 lux. Mice were visualized, recorded, and tracked by AnyMaze software. When a mouse took the first bite of the pellet, the recording was stopped. The latency to take the first bite of chow was measured.

Mass spectrometry to determine endocannabinoid concentration

Tissue punches (0.8mm in diameter in 500mm thick coronal sections) were bilaterally collected from the BLA, CeA, BNST, and ventral striatum after either 42 days of access to EtOH (N=20) or after 15 days of forced abstinence from EtOH (N=20) using a vibratome. Liquid chromatography-tandem mass spectrometry using a Sciex QTRAP 6500 was used to detect endocannabinoids and arachidonic acid in tissue samples (3 mice/sample). Samples

were homogenized in methanol containing deuterated standards (AA-d8, 2-AG-d5, and AEA-d4), bath sonicated, incubated at -20C overnight, and centrifuged; water was added to the supernatant for a final ratio of 75:25 Methanol:Water. Samples (25 µl) were injected onto a C-18 column (50 × 2 mm, 1.7 µm; Acquity) under the following gradient: 40% A and 60% B from 0 to 0.25 min, increased to 5% A and 95% B from 0.25 to 3.75 min held for 2 min, and returned to 40% A and 60% B from 5.75 to 6. Component A was water and B was 2:1 Acetonitrile:Methanol, and each component contained 0.1% formic acid (v/v). Analytes were detected via selective reaction monitoring in the positive (2-AG and AEA) or negative (AA) ion mode using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): AA (m/z 303(311) → 259(267)); 2-AG (m/z 379(384) → 287(287)); and AEA (m/z 348(352) → 62(66)). Quantification was achieved via stable-isotope dilution for AA, 2-AG and AEA.

Statistics

All data are represented as mean±SEM. All statistics were run using Prism 6 (Graphpad, La Jolla, CA). BECs were determined by interpolation of concentrations utilizing absorbance values of known EtOH standards. Differences between groups were assessed using t-tests, one-way ANOVAs and two-way ANOVAs with significance set at a=0.05. When significant main effects were obtained using ANOVA tests, appropriate *post hoc* comparisons between groups were performed.

Results

EtOH Two Bottle Choice Produces Significant EtOH Preference in Singly-Housed Female Mice.

Female C57BL/6J mice were given 24hr access to either 10% EtOH or water for 6 weeks (**Figure 3.1A**). Mice preferred 10% EtOH over water (**Figure 3.1B**; P<0.0001 for each time point; overall preference = 76.39±0.606%), as previously reported (Pang et al., 2013). During the last week, preference (**Figure 3.1C**) and g/kg/day (**Figure 3.1D**) were relatively stable. BECs were sampled on day 42 of EtOH access (31.37±9.47 mg/dl; range 0-172.3mg/dl; **Figures 3.1E and 3.5A**). Animal weights did not differ between groups (P>0.05; results not shown).

Depression-like behavior requires protracted forced abstinence.

The FST and NSFT were utilized to measure depression-like behavior. FST immobility is a well-known proxy for depression-like behavior, and increased NSFT latency indicates depression- or anxiety-like behavior (Bodnoff et al., 1988; Dulawa and Hen, 2005; Li et al., 2011; Pang et al., 2013). Additionally, in the NSFT, chronic treatment with SSRIs is required to reduce depression-like behavior, similar to human treatment (Bodnoff et al., 1988; Dulawa and Hen, 2005). As demonstrated previously (Pang et al., 2013), we observed increased FST immobility in EtOH mice following 18 days of forced abstinence (**Figure 3.2A**; p<0.001, N=10-12/group). However, in another cohort, we found no significant difference in FST immobility between EtOH and control mice 24hrs following EtOH removal (**Figure 3.2B**; p=0.909, N=13-14/group), suggesting that depression-like behavior in this paradigm requires protracted abstinence.

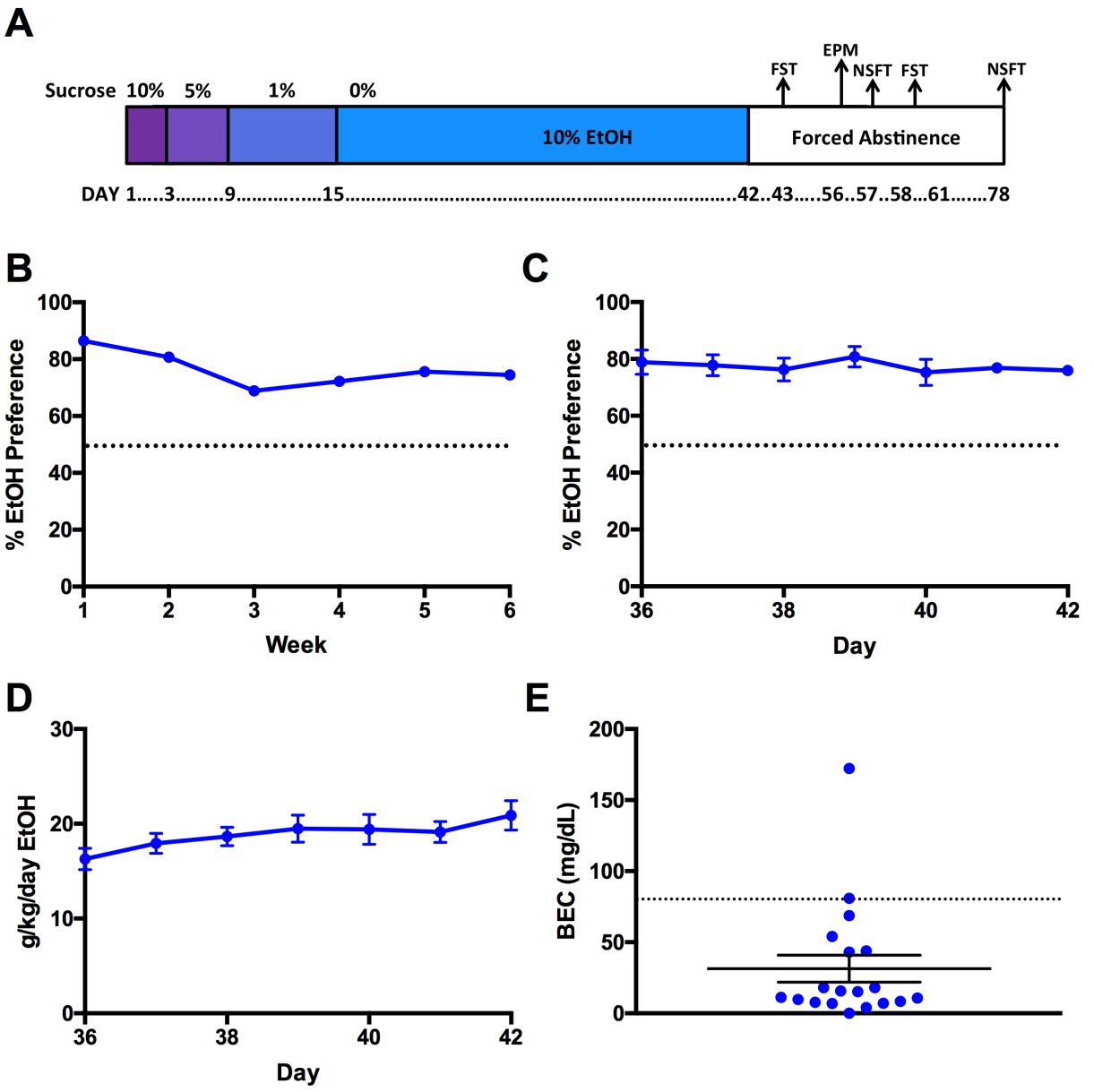


Figure 3.1. EtOH two bottle choice with sucrose fade reliably induces significant EtOH preference. (A) Timeline of EtOH two bottle choice with sucrose fade. 10% EtOH given from day 1-42 with sucrose fade as indicated. FST=Forced Swim Test; EPM=Elevated Plus Maze; NSFT=Novelty-Suppressed Feeding Test. (B) Preference for EtOH-containing sipper during 6 week access to EtOH (N=85). (C) Daily preference for EtOH-containing sipper during the last week of access in the first cohort (N=12). (D) Daily EtOH consumption (g/kg/day) during the last week of access in the first cohort (N=12). (E) Individual BECs for mice after 42 days of drinking. Data represented as mean±SEM. Dashed line indicates 50% preference in (B) and (C); dashed line indicates 80mg/dl in (E).

Forced abstinence from EtOH does not affect EPM behavior.

Anxiety-like behavior occurs in C57BL/6J mice following early withdrawal from intermittent vaporized EtOH (Kash et al., 2009; Kliethermes, 2005), but not at later time points (Daut et al., 2015; Pang et al., 2013). We examined whether forced abstinence from EtOH drinking in this paradigm would alter anxiety-like behavior measured by the EPM, and found no effect on open arm time (**Figure 3.2C**; p=0.738, N=11/group) or distance traveled (**Figure 3.2C'**; p=0.835) after 14 days of EtOH forced abstinence.

Forced abstinence from EtOH increases depression-like behavior in the NSFT that is long-lasting.

We aimed to replicate earlier findings (Pang et al., 2013) as well as examine persistence of this depression-like phenotype. Mice were tested at 15 and 35 days following EtOH removal (**Figure 3.2D**). A two-way repeated-measures ANOVA revealed main effects for time ($F(1,31)=9.769$, $P=0.004$), treatment ($F(1,31)=21.40$, $P<0.0001$), and matching subjects ($F(1,31)=2.037$, $P=0.026$), but no interaction ($F(1,31)=0.2576$, $P=0.6153$). *Post hoc* analysis using Sidak's multiple comparisons tests revealed that EtOH-exposed mice had higher latencies at both 15 ($P<0.001$) and 35 days ($P<0.01$) compared to controls, indicating a persistent depression-like phenotype following EtOH forced abstinence.

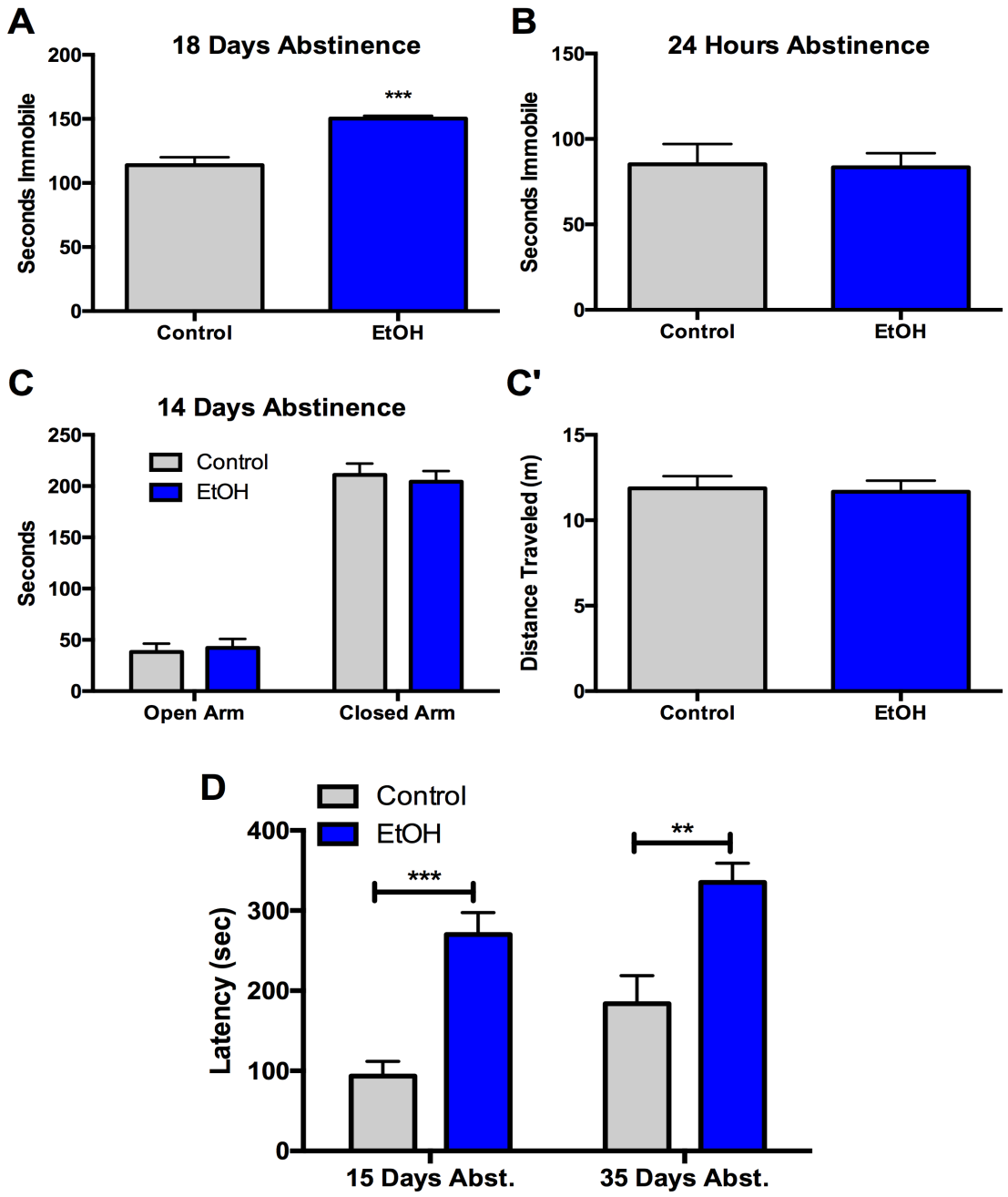


Figure 3.2. Removal of EtOH induces long-lasting depression-like behavior that requires an abstinence period. (A,B) Abstinence from EtOH significantly increases immobility time in the FST (A) 19 days, but not (B) 1 day, after EtOH removal. (C) Anxiety-like behavior in the EPM is unaffected 14 days after EtOH abstinence as measured by time spent in the open or closed arms of the apparatus. (C') Locomotion, measured by distance traveled in the EPM apparatus, is unaltered after 14 days of EtOH abstinence. (D) Depression-like behavior induced by EtOH abstinence is long-lasting. EtOH-exposed mice demonstrate depression-like behavior – increased latency to consume chow in the NSFT – at both 15 and 35 days following removal of EtOH. Data represented as mean±SEM. **P<0.01; ***P<0.001.

Increased latency in NSFT following EtOH forced abstinence is ameliorated by Ketamine and JZL-184, but not Memantine.

Ketamine has rapid and long-lasting antidepressant effects in humans (Berman et al., 2000; Zarate et al., 2006a; Zarate et al., 2006b) and rodents (Louderback, et al. 2013; Li et al, 2011; Autry, et al. 2011). We postulated that ketamine would ameliorate depression-like behaviors induced by EtOH abstinence. EtOH and control mice were treated with saline or ketamine (3mg/kg in saline; N=6-7/group) 30 minutes before the NSFT (**Figure 3.3**). We chose a dose that reduces depression-like behavior in rodents at this time point (Autry et al., 2011; Louderback et al., 2013). Two-way ANOVA revealed main effects of EtOH exposure ($F(1,23)=9.649$, $P=0.005$) and drug ($F(1,23)=10.66$, $P=0.003$), but no interaction ($F(1,23)=3.633$, $P=0.069$). *Post hoc* analysis using Fisher's LSD revealed that only EtOH+Sal mice significantly differed from Contr+Sal ($P=0.001$), and that ketamine+EtOH mice were indistinguishable from Contr+Sal mice ($P>0.05$). Ketamine reversed depression-like behavior in EtOH exposed mice to baseline levels.

Because EtOH and ketamine both inhibit NMDARs we wondered if ketamine was eliciting antidepressant-like effects in this model by mimicking EtOH. To test this, we utilized memantine – another NMDAR antagonist. This drug interested us because it has not been shown to reduce depression in either humans (Zarate et al., 2006b) or rodents (Gideons et al., 2014). We administered memantine (20mg/kg in saline; N=11-12/group) in control and EtOH mice 30 minutes before the NSFT (**Figure 3.4A**) after 15 days of abstinence (main statistics below). We chose this dosage used in a previous study examining memantine for antidepressant efficacy (Gideons et al., 2014). *Post hoc* analysis

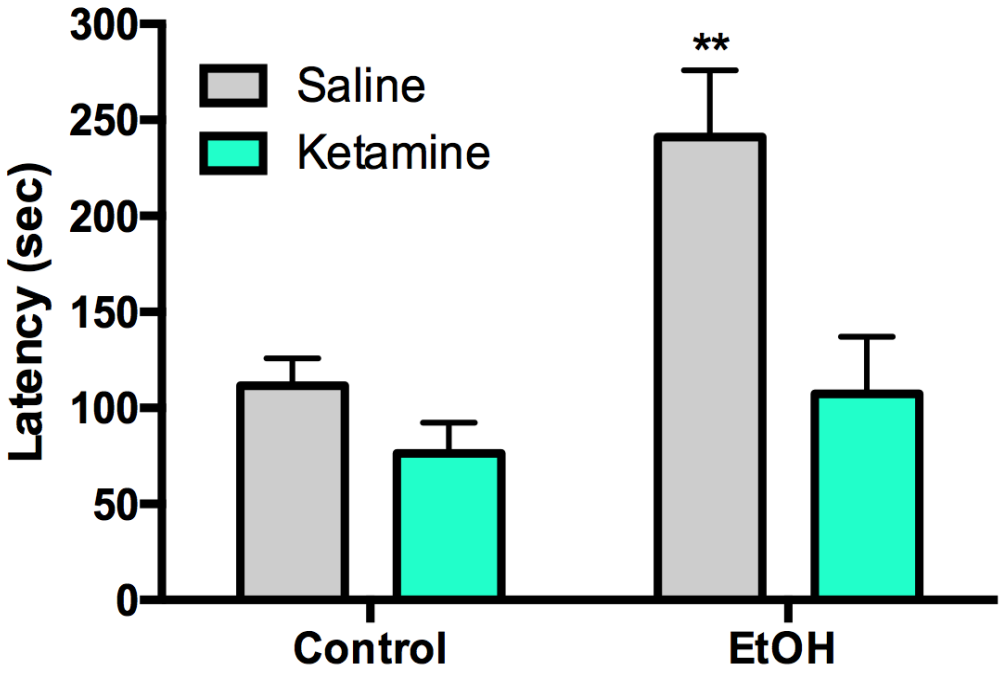
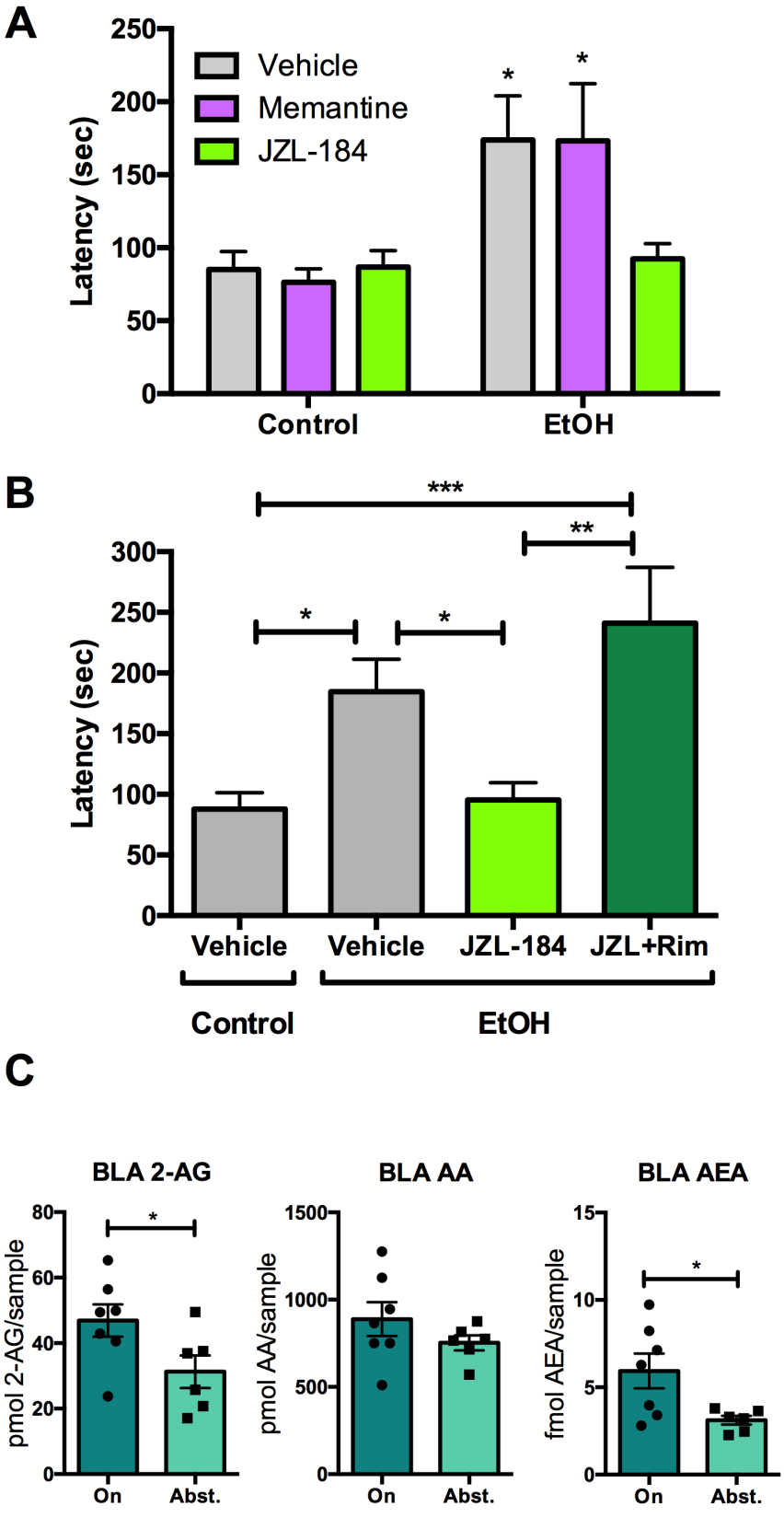


Figure 3.3. Ketamine (3mg/kg; 30 min prior to testing) was able to reduce NSFT latency to control levels in EtOH mice 15 days after EtOH removal. Groups compared to Control-Vehicle for statistics. Data represented as mean±SEM. **P<0.01

with Fishers LSD showed latencies for both EtOH+DMSO and EtOH+Mem groups were significantly higher than the Control+DMSO group ($P<0.05$ for both groups), indicating that memantine did not reduce depression-like behavior.

The eCB system has been implicated in depression- and anxiety-like behavior (Fowler, 2015; Morena et al., 2015). Increasing 2-AG levels through MAG lipase inhibition by JZL-184 reduces stress-induced anxiety-like behavior (Sumislawski et al., 2011). Additionally, ethanol is not able to substitute for cannabinoids in discrimination tasks in rodents (Jarbe et al., 2010; McMahon et al., 2008). We administered JZL-184 (8mg/kg in DMSO; N=7-8/group) or vehicle (DMSO; N=7-8/group) in control and EtOH mice 15 days after EtOH removal (**Figure 3.4A**). Two hours later, mice were tested in the NSFT. Two-way ANOVA revealed a main effect of EtOH ($F(1,45)=10.51$, $P=0.002$) but no main effect of drug ($F(2,45)=1.531$, $P=0.227$) or interaction ($F(2,45)=2.140$, $P=0.130$). *Post hoc* analysis with Fishers LSD showed that EtOH+JZL-184 mice were not significantly different from the Control+DMSO group ($P>0.05$). Like ketamine, JZL-184 reversed depression-like behavior induced by EtOH abstinence.

To demonstrate that the effect of JZL-184 was mediated by the CB1 receptor, we co-administered JZL-184 with the CB1 inverse agonist rimonabant (1mg/kg in DMSO, i.p.) versus JZL-184 alone (8mg/kg in DMSO, i.p.) or vehicle (N=9-10/group) in control and EtOH mice 15 days after EtOH removal (**Figure 3.4B**). Two hours following injection, mice were tested in the NSFT. This rimonabant dosage does not alter affective behavior in a similar task when administered alone (Gamble-George et al., 2013). One-way ANOVA revealed a significant effect of treatment ($F(3,34) = 6.578$; $P=0.0013$). Fisher's LSD *post-hoc*


A
Latency (sec)
250
200
150
100
50
0
Vehicle
Memantine
JZL-184
*
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Control
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Vehicle
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Control
EtOH
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BLA 2-AG
pmol 2-AG/sample
80
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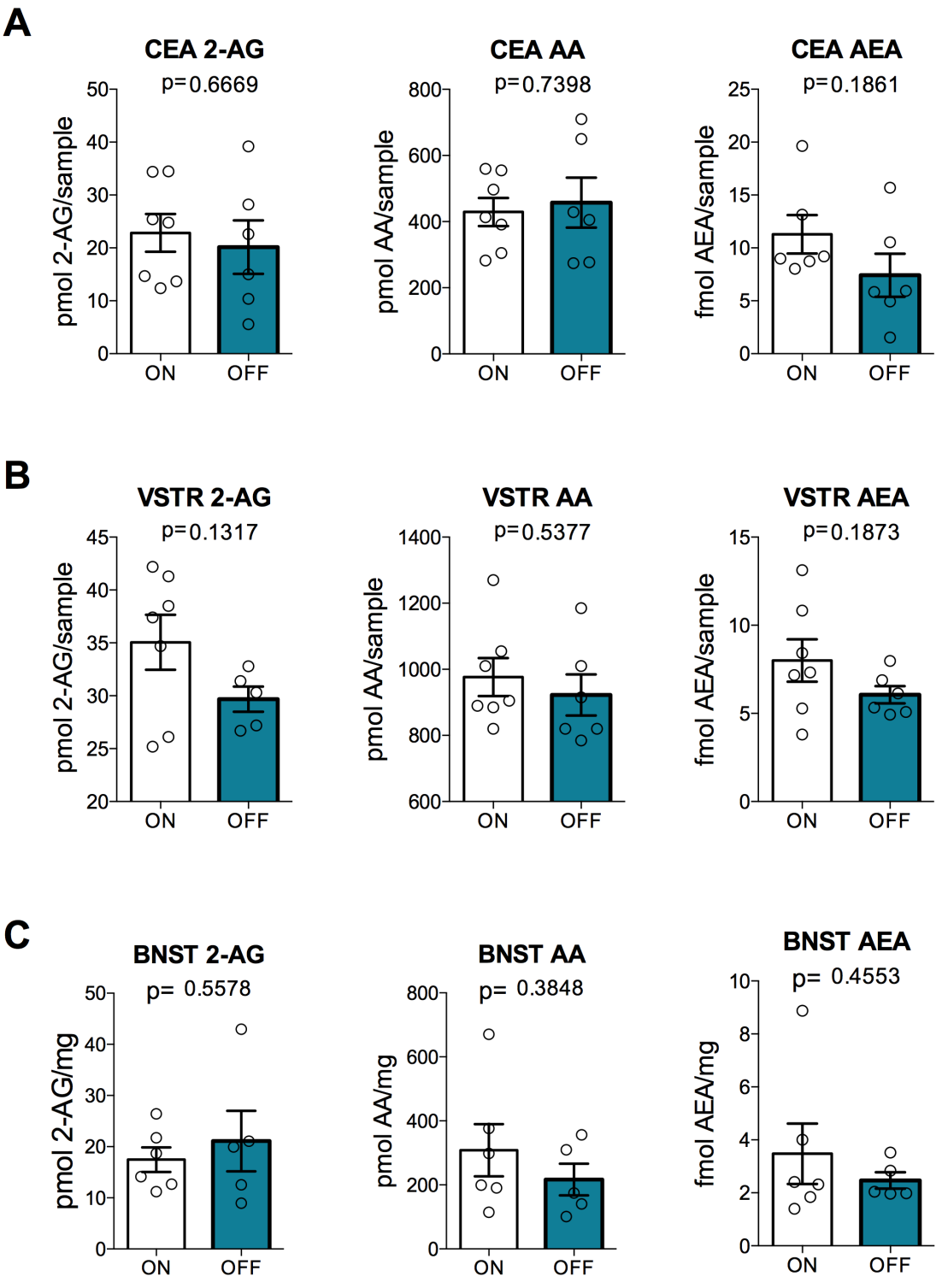
Abst.
BLA AA
pmol AA/sample
1500
1000
500
0
On
Abst.
BLA AEA
fmol AEA/sample
15
10
5
0
*
On
Abst.

Figure 3.4. Endocannabinoid modulation of depression-like behavior and eCB levels during 2 bottle choice paradigm and EtOH abstinence. (A) JZL-184 (8mg/kg; 2 hours prior to testing) completely reduced NSFT latency to baseline levels in EtOH abstinent mice after 15 days of abstinence, but memantine (20mg/kg; 30 min prior to testing) had no effect on depression-like behavior. (B) Rimonabant (1mg/kg; 2 hours prior to testing) blocked the effect of JZL-184 in EtOH mice 15 days after EtOH removal. All drugs administered i.p. In (A), groups compared to Control-Vehicle for statistics. Groups compared to every other group in (B). (C) Levels of 2-AG, AA, and AEA in the BLA after 42 days of drinking (On) or after 15 days of EtOH abstinence (Abst.) Data represented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

test revealed significant differences between control-vehicle mice and EtOH-vehicle ($P<0.05$) and EtOH-JZL+Rim ($P<0.001$) mice, between EtOH-vehicle and EtOH-JZL ($P<0.05$) mice, and between EtOH-JZL and EtOH-JZL+Rim ($P<0.01$) mice. No differences were found between control-vehicle and EtOH-JZL ($P>0.05$) mice or between EtOH-vehicle and EtOH-JZL+Rim ($P>0.05$) mice. Thus, rimonabant prevented the antidepressant-like effect of JZL-184.

Endocannabinoid levels differ between EtOH access and EtOH abstinence.

Given the robust modulation of abstinence-induced depression-like behavior through eCB manipulation, we postulated that discrete brain regions may display altered levels of eCBs during drinking versus abstinence. Mass spectrometry was used to examine levels of 2-AG, anandamide (AEA), and arachidonic acid (AA) in tissue punches from coronal brain sections taken from basolateral amygdala (BLA), central nucleus of the amygdala (CeA), ventral striatum, and bed nucleus of the stria terminalis (BNST). Punches were taken from mice on day 42 of EtOH drinking (N=6-7; 2-3 mice/sample) or after 15 days of EtOH abstinence (N=5-6; 3 mice/sample). In the BLA, both 2-AG ($P=0.049$) and AEA ($P=0.027$) were higher in mice drinking EtOH than mice in EtOH abstinence, while AA levels were unchanged between groups (**Figure 3.4C**). No differences in eCB levels were observed in the CeA, ventral striatum, or BNST (**Supplementary Figure 3.S1**).



Supplemental Figure 3.S1. Levels of 2-AG, AA, and AEA in tissue punches of (A) CeA, (B) ventral striatum, and (C) BNST during EtOH consumption (On) and 15 days after EtOH removal (Abst.). Data represented as Mean±SEM.

Drinking induction is not dependent on sucrose fade, and drinking shifts to habit-based consumption after 6 weeks of EtOH exposure.

Because sucrose engages reward circuitry, some behaviors observed may be confounded as only EtOH-exposed animals were given sucrose. To address this, we utilized an EtOH ramp (N=28) in lieu of sucrose fade (EtOH (Sucr); N=85; **Figure 3.5A**). Repeated-measures two-way ANOVA of preference utilizing EtOH (Sucr) vs EtOH Ramp (**Figure 3.5B**) revealed a significant main effect of time ($F(5,555)=7.894$, $P<0.0001$) and significant interaction ($F(5,555)=35.41$; $P<0.0001$), but no main effect of EtOH administration method ($F(1, 111) = 0.009$;, $P=0.9264$). *Post hoc* analysis revealed significant differences between groups on Week 1 ($P<0.0001$), Week 3 ($P<0.05$), and Week 4 ($P<0.001$).

We postulated that previous exposure to the EtOH paradigm might increase habit-based EtOH consumption. A shift from goal-directed to habit-based consumption is thought to characterize alcohol intake in humans with AUDs (Edwards and Koob, 2013; Everitt and Robbins, 2005). Mice did not show innate preference for 3% EtOH (**Figure 3.5C**). We hypothesized that mice previously exposed to 10% EtOH would have increased preference for 3% EtOH. We replaced 10% EtOH with 3% EtOH (and reversed bottle locations to avoid location preference) in mice after 42 days of EtOH access. Mice with previous EtOH exposure (N=10) had significantly increased preference for 3% EtOH over the naïve mice (N=9; **Figure 3.5C**; $P=0.047$). We also examined both the average amount of EtOH solution consumed (**Figure 3.5D**) and the average g/kg/day EtOH (**Figure 3.5E**) in three groups: mice during the final week of 10% EtOH exposure (10% EtOH; N=10), naïve mice given 3% EtOH (3% (Naïve); N=9), or mice with previous 10% EtOH exposure given 3% EtOH (3%

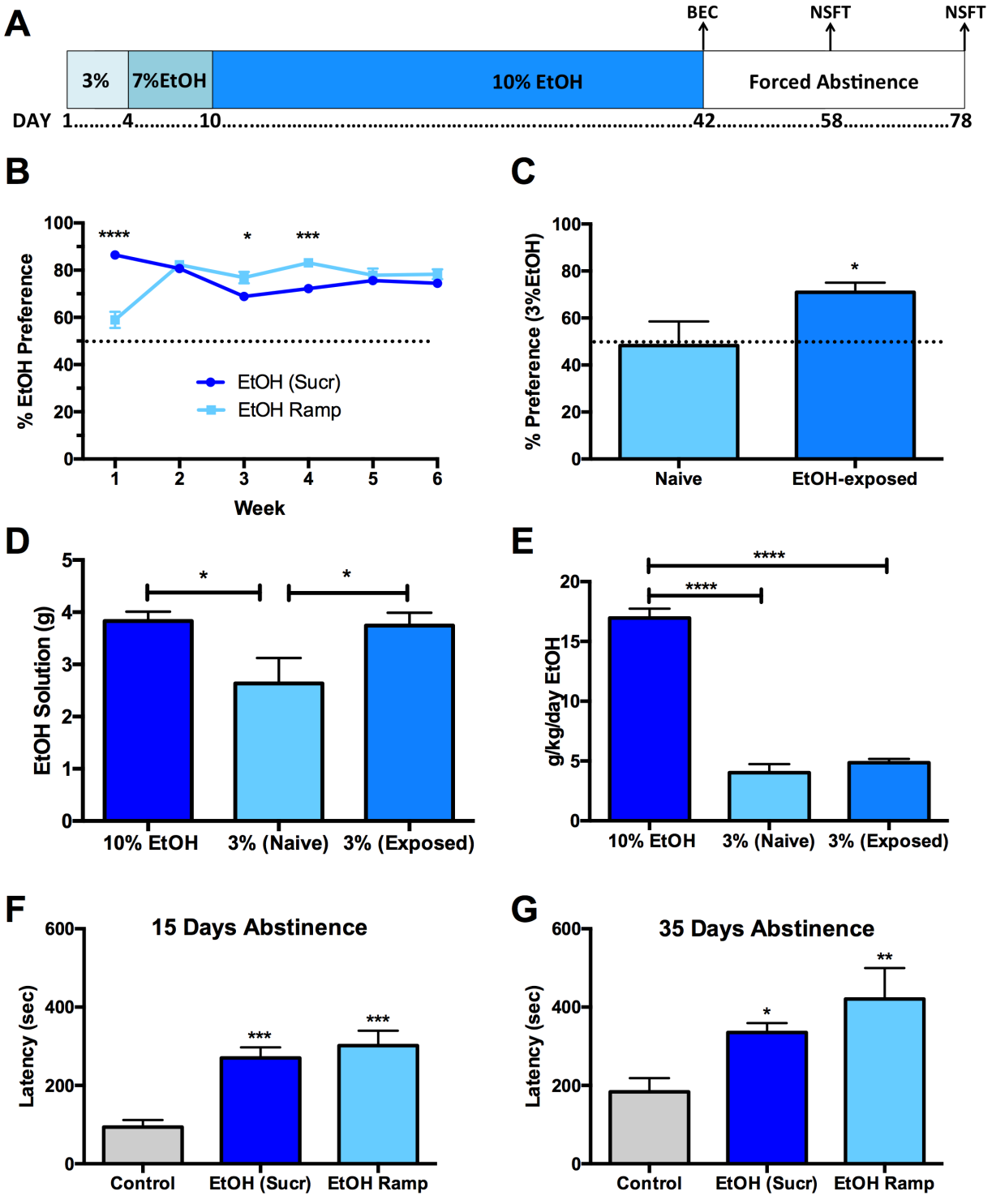


Figure 3.5. Assessment of factors governing EtOH consumption and depressive responses. (A) Timeline of EtOH Ramp drinking paradigm. EtOH is gradually introduced and no sucrose is used. (B) Comparison of sucrose fade EtOH (EtOH (Sucr), N=85) and EtOH Ramp (N=28). (C-E) Preference, consumption, and dosage of a 3% EtOH solution over 2 days in

naïve (3% (Naïve); N=9) or EtOH-exposed (3% (Exposed); N=10) mice, compared to mice drinking 10% EtOH at the end of the two bottle choice paradigm (10% EtOH; N=10). (C) Preference for 3% EtOH is increased in mice with previous exposure to 6 weeks of 10% EtOH. (D,E) Consumption of 3% EtOH is putatively habit-driven. (D) The same average daily volume of EtOH solution is consumed by 10% EtOH mice and 3% (Exposed) mice. Both groups drink a greater volume than 3% (Naïve) mice. (E) Neither 3% (Naïve) or 3% (Exposed) mice drink sufficient quantities to match dosage (g/kg/day) of 10% EtOH mice. (F,G) The EtOH Ramp procedure induces similar depression-like behavior in the NSFT following (F) 15 and (G) 35 days of abstinence. Data represented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

(Exposed); N=10). We hypothesized that habit-driven consumption would manifest in two ways: first, similar volumes of both 10% and 3% EtOH solution would be consumed. Second, we expected 3% (Exposed) mice would not drink sufficient quantities of EtOH to match dosage (g/kg/day) of 10% EtOH mice. One-way ANOVA of consumption (**Figure 3.5D**) revealed a significant main effect of treatment ($F(2, 26)=4.225$; $P=0.026$). 3% (Exposed) mice drank a similar volume of EtOH solution as 10% EtOH mice, and the 3% (Naïve) group drank less than either other group ($P<0.05$) using Fisher's LSD *post hoc* analysis. One-way ANOVA revealed a significant main effect of treatment on g/kg/day EtOH (**Figure 3.5E**; $F(2, 26)=130.8$; $P<0.0001$). g/kg/day EtOH was significantly higher in the 10% EtOH group compared to either 3% EtOH group ($P<0.0001$ for both groups) using Fishers LSD *post hoc* analysis. These data indicate that consumption of 3% EtOH following 6wk exposure to 10% EtOH is possibly habit-driven.

Depression-like behavior following EtOH Forced Abstinence is not dependent on sucrose fade

EtOH ramp procedure produced high levels of EtOH preference similar to the sucrose fade, so we hypothesized that similar behavior may be induced. As in **Figure 3.2D** we tested for increased latency in the NSFT at both 15 (**Figure 3.5F**) and 35 (**Figure 3.5G**) days following EtOH removal in three groups: control (N=10), EtOH with Sucrose fade (EtOH (Sucr); N=23), and EtOH Ramp (N=9). After 15 or 35 days of forced abstinence, one-way ANOVAs revealed a significant difference between groups (15 days: $F(2,39)=10.32$, $P<0.001$; 35 days: $F(2,39)=6.415$, $P=0.004$), and *post hoc* analyses using Dunnett's multiple comparison tests showed both EtOH (Sucr) and EtOH Ramp had significantly higher NSFT

latencies than control mice (15 days: $P<0.001$ for both groups; 35 days: $P<0.05$ for EtOH (Sucr) and $P<0.01$ for EtOH Ramp).

Discussion

In this study, we replicated and extended previous findings that forced abstinence from EtOH drinking produces long-lasting, reversible depressive behavior in mice. A continuous 6 week two-bottle choice EtOH drinking paradigm induced high preference for 10% EtOH over water in singly-housed female mice. BECs after 42 days of 10% EtOH access were relatively low; however, continuous access to EtOH likely limits our ability to observe peak BECs across all animals at any given time. We replicated earlier findings (Pang, et al. 2013) that subsequent forced abstinence resulted in depression-like behavior, and further demonstrated that depression-like behavior requires protracted abstinence and is long-lasting. Both ketamine and JZL-184 reversed depression-like behavior to control levels via two disparate pharmacological strategies. Endocannabinoid levels differed in the BLA of mice currently drinking EtOH versus mice in EtOH abstinence. Finally, we provided evidence that this drinking paradigm induces a shift to habit-based consumption of EtOH.

Increased FST immobility is observed after 18 days – but not 1 day – of forced abstinence from EtOH. This is consistent with previous studies (Stevenson et al., 2009), and may indicate priming of depression-like behaviors during EtOH administration that require an abstinence incubation period to manifest. In contrast, a recent study utilizing the drinking in the dark (DID) paradigm demonstrated depression-like behavior 1-2 days following EtOH withdrawal (Lee et al., 2015). We believe our study may have uncovered an

interesting distinction between our paradigm and DID. Because DID is a limited access paradigm, each daily drinking session is followed by an abstinence period. These repeated short withdrawals – not present in the current paradigm – could induce development of depression-like behavior during the course of the DID regimen. Thus, although these two paradigms model a similar component of alcohol withdrawal (depression), subtle discriminating characteristics of DID and continuous two-bottle choice may lead to a more elegant understanding of the development of depression-like behavior during abstinence.

Depression-like behavior following EtOH forced abstinence is long-lasting, with increased NSFT latencies 35 days following EtOH removal. This timing agrees with substantial previous literature demonstrating incubation of drug craving during abstinence in rodents for a number of drugs of abuse (Pickens et al., 2011), including ethanol (Bienkowski et al., 2004). The protracted depression-like behavior outlined here may underlie some of the negative reinforcement thought to drive relapse to alcohol-seeking in abstinent individuals with a history of AUD (Gilpin and Koob, 2008).

Although depression is highly comorbid with alcohol use, specific investigations of drugs to treat individuals for these disorders in tandem have been woefully lacking. Typical clinical strategy regarding comorbid patients has been to treat one disorder prior to treating the other; however, as each of these disorders tend to worsen symptoms of the other, treatments for both disorders at once are needed (Pettinati et al., 2013). NMDARs represent potential targets for treating depression, as low doses of the NMDAR antagonist ketamine reduce symptoms of MDD (Berman et al., 2000) and treatment resistant depression (Zarate et al., 2006a). Ketamine also reduces depression-like behavior in rodents (Autry et al., 2011; Li et al., 2011; Louderback et al., 2013). Interestingly, family

history of alcohol use predicts greater efficacy (Phelps et al., 2009) and longer-lasting relief of depression symptoms with ketamine (Niciu et al., 2014). We hypothesized that ketamine would be particularly effective in reversing depression-like behavior following EtOH abstinence. Indeed, ketamine reduced NSFT latency to baseline levels.

We examined another NMDAR antagonist, memantine, in reducing depression-like behaviors. Because ketamine produces dissociative symptoms (Berman et al., 2000) and has abuse potential, similar drugs with fewer side effects are needed for the treatment of depression. Memantine has fewer reported side effects than ketamine (Parsons et al., 1999). We found that memantine at a relatively high dosage (20mg/kg) did not reduce depression-like behavior. This is in line with previous data showing that ketamine, but not memantine, is capable of reversing depression-like behavior in mice (Gideons et al., 2014) likely because these two ligands differentially affect the NMDAR (Emnett et al., 2013; Gilling et al., 2009).

One major caveat is that NMDAR antagonists can substitute for ethanol in discriminative stimulus tasks (Kostowski and Bienkowski, 1999; Shelton, 2004). Additionally, ketamine induces alcohol intoxication-like subjective effects in humans (Dickerson et al., 2010; Krystal et al., 1998). Therefore, future studies investigating specific actions of ketamine in reducing affective behavior following EtOH abstinence will be invaluable in identifying therapeutic compounds lacking ketamine's abuse potential. Our lab and others have demonstrated that Ro25-6981, a selective antagonist of the NMDAR GluN2B subunit, mimics the antidepressant effects of ketamine (Li et al., 2011; Louderback et al., 2013). Although we did not examine subunit specificity of depression-like behavior

here, future studies will aim to determine mechanistic actions of NMDAR antagonism in reducing this behavior.

Increasing levels of 2-AG through MAG lipase inhibition by JZL-184 reduces stress-induced affective disturbances (Fowler, 2015; Morena et al., 2015; Sumislawski et al., 2011). Additionally, unlike ketamine, cannabinoids and ethanol do not share discriminative stimulus profiles in rodents (Jarbe et al., 2010; McMahon et al., 2008). We sought to determine if JZL-184 could reduce EtOH abstinence-induced depression-like behavior. Indeed, JZL-184, like ketamine, reversed depression-like behavior in the NSFT. Additionally, co-administration of the CB1 receptor antagonist rimonabant with JZL-184 completely blocked this effect.

Modulation of the eCB system profoundly affected the depressive phenotype of mice following EtOH removal, so we wondered if brain eCB levels differed between mice exposed to EtOH and mice 15 days following EtOH removal. We found that 2-AG and AEA were reduced in the BLA during abstinence compared to current EtOH drinking. Because we only examined eCB levels in these two conditions, a caveat is that we could not determine which of these conditions produces aberrant eCB levels when compared to mice naïve to EtOH. Acute stress reduces amygdalar AEA levels (Hill et al., 2009), and, conversely, enhanced eCB tone reduces the stress response (Patel et al., 2004). However, chronic stress has been demonstrated to increase eCB levels in mice (Patel et al., 2009). Further investigations are crucial to determine specific contributions of eCBs to both EtOH consumption and abstinence-related behaviors using this paradigm. These findings may indicate cannabinoids as potential therapeutic targets in alcoholism and depression comorbidity.

We were initially concerned with the use of sucrose in this paradigm, since hedonic circuitry altered in a depressed-like state would certainly be engaged by sucrose. However, we found that an EtOH Ramp procedure induced both high preference drinking and similar phenotypes in the NSFT.

To assess potential habit-driven consumption following exposure to our paradigm, we further examined preference for 3% EtOH. Mice did not prefer 3% EtOH over water unless they had previous exposure to 10% EtOH. Mice with previous EtOH exposure drank the same volume of 3% EtOH solution as they had 10% EtOH solution, and did not increase consumption to sufficiently match dosage (g/kg/day) of 10% EtOH, lending evidence toward habit-based consumption. Typically, habit versus goal-directed consumption is assessed following devaluation of the rewarding stimulus (Colwill and Rescorla, 1990). Animals will decrease effort to acquire a devalued reward if consumption is goal-directed, but habit-driven consumption proves inflexible to devaluation. Here, the 3% EtOH solution could be thought to represent an innately devalued stimulus, as it does not induce preference in naïve mice. 3% EtOH becomes rewarding only in mice that have acquired preference for 10% EtOH. Although it is tempting to interpret these findings as a shift to habit-driven consumption of EtOH, similar to humans with AUDs (Everitt and Robbins, 2005), future studies will be necessary to fully parse out motivation in EtOH consumption.

Here we have shown that depression-like behavior following a two-bottle choice 10% EtOH paradigm requires protracted abstinence and is long-lasting. Latency in the NSFT is reduced to baseline levels with ketamine, but not memantine. Similarly JZL-184 reduced latency to baseline levels, and this effect was blocked with co-administration of

rimonabant. 2-AG and AEA levels in the BLA were higher in mice currently drinking EtOH compared to mice after 15 days of EtOH abstinence. This paradigm increased putative habit-driven consumption of a 3% EtOH solution. Future studies will aim to uncover alterations in circuit-level plasticity induced by both exposure to and abstinence from EtOH.

Chapter 4

Conclusions and Future Directions

4.1 GluN2B Knockdown in the BNST

In Chapter Two, I recapitulated previous work demonstrating reduction in depression-like behavior following systemic administration of ketamine or Ro25-6981. Others have demonstrated this behavior basally using the FST in mice (Autry et al., 2011) or following chronic stress using the NSFT in rats (Li et al., 2011). We wished to utilize mice in our study design, as current transgenic capabilities limit such studies to mice, and the *grin2b* floxed mouse line was to be used for subsequent deletion studies (Brigman et al., 2010). Though the FST is often the first-line behavioral task used for depression-related experimentation and prospective antidepressant efficacy in rodents, the task is fraught with disadvantages (Petit-Demouliere et al., 2005). In particular, certain factors (including animal sex, age, and weight) influence immobility time, and more importantly inbred strains, including the C57Bl/6J strain used exclusively throughout this manuscript, have not reliably responded to known antidepressants with reductions in immobility time (David et al., 2003; Lucki et al., 2001). Thus, we wanted to utilize a different task in the identification of affective changes with response to treatment.

Due to its high sensitivity and dynamic range (see **Figure 1.1**), we chose to use the NIH test in measuring the ability of ketamine (3mg/kg) or Ro 25-6981 (5mg/kg) to reduce affective behaviors, and indeed were able to replicate previous findings by demonstrating reductions in NIH latency. Interestingly, unlike previous work in rats that relied on chronic

stress for ketamine's effects to manifest (Li et al., 2011), we found significant effects of ketamine and Ro 25-6981 in both naïve and stressed animals. Further, we were able to demonstrate that targeted knockdown of the GluN2B subunit – the target of Ro 25-6981 and putative target of ketamine – specifically within the BNST was able to produce a similar decrease in latency in the NIH test (**Figure 2.1**) with no effect on anxiety-like behavior measured by the EZM (**Figure 2.S1**).

Perhaps an obvious future experiment in these animals would be to demonstrate an occlusion of ketamine's ability to further reduce latency in the NIH test, as was shown with GluN2B deletion specifically from principal cortical neurons (Miller et al., 2014). While this certainly is a valid experiment to perform, interpretation of the potential results obtained is somewhat difficult. For instance, although further reduction in latency in BNSTGluN2BKD mice following ketamine administration would demonstrate a failure of occlusion, one could not be certain if this was due to independent mechanisms of reductions in depression-like behavior, or if it was due to incomplete deletion of GluN2B-containing NMDARs within the BNST, as described in further detail in Appendix D. Similarly, a finding of no further reduction in NIH latency in BNSTGluN2BKD mice following ketamine injection may seem like an occlusion, but could just as easily represent the manifestation of a floor effect in the task itself. However, the demonstration of lower possible latency values in the NIH task (as in **Figure 1.1**) reduces concerns of a floor effect phenomenon.

As discussed in Chapter 1, GluN2B-containing NMDARs are necessary for LTP induction in the BNST, as neither mice with constitutive forebrain-wide knockout of GluN2B nor slices acutely treated with Ro 25-6981 show BNST LTP (Wills et al., 2012). In

future studies, the effects of both ketamine and regional GluN2B deletion on plasticity within this region should be explored. Preliminary data showed ketamine (3mg/kg; 30 minutes prior to making slices) completely blocked LTP induction in BNST (**Figure 4.1**), as we had expected. Curiously, this effect was not mimicked in slices from BNSTGluN2BKD mice compared to control virus-injected mice. In fact, targeted GluN2B knockdown *increased* amplitude of responses during the first 5 minutes following the tetanus, and there was no difference between groups at later time points. Future work will be needed to further parse out the effect of this adult-onset regional knockdown of GluN2B on plasticity within the region. The timing of knockdown in BNSTGluN2BKD animals differs from GluN2B inhibition described in Wills et al., as it is neither acute (such as slice application of Ro 25-6981 or ketamine) nor present throughout the lifetime (such as the forebrain wide GluN2B knockouts), but represents a mid-range time point that may contribute to apparent distinctions between groups. In studies moving forward, as described below and in Appendix C, a viral Cre recombinase that expresses a fluorescent reporter protein will be an invaluable tool.

Finally, as described in the subsequent section, it will be interesting to determine the effect of this regional deletion of GluN2B-containing NMDARs in other assays, such as the two bottle choice EtOH drinking assay described in Chapter 3.

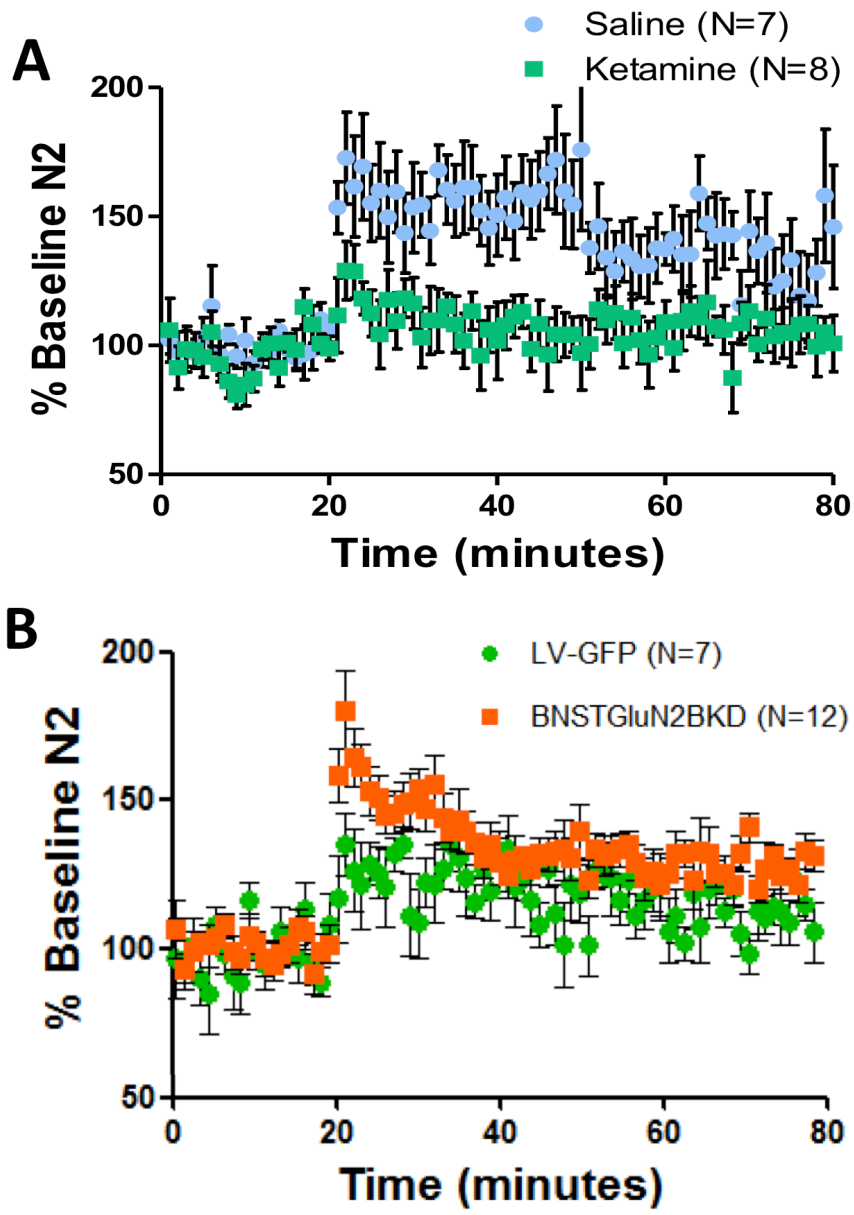


Figure 4.1. LTP induction in the BNST in ketamine-treated and BNSTGluN2BKD mice. Following maintenance of a stable baseline, a tetanus consisting of 2 100Hz 1sec trains was administered in BNST-containing slices from (A) mice given either saline or ketamine (3mg/kg; i.p.) 30 minutes prior to slice preparation or (B) *grin2b* floxed mice injected with LV-GFP or LV-Cre (BNSTGluN2BKD).

4.2 Implications of Depression-like behavior induced by protracted forced abstinence from a two-bottle choice EtOH drinking paradigm

In Chapter Three, I used a previously reported two bottle choice paradigm, in which female singly-housed mice have continuous access to 10% EtOH and water for six weeks, that has been shown to induce depression-like behavior two weeks after removal of EtOH (Pang et al., 2013). We found three things: first, depression-like behavior required a prolonged period of abstinence from EtOH, as no depression-like phenotype was observed 24hrs after EtOH removal, and it was long-lasting with depression-like behavior still observed 35 days after EtOH removal. Second, we were able to ameliorate this behavior with ketamine – but not memantine – and the MAG lipase inhibitor JZL-184. The JZL-184 effect was abolished when it was administered in conjunction with rimonabant, the CB1 receptor inverse agonist. Additionally, we found that endocannabinoid levels in the BLA were higher in mice on day 42 of EtOH drinking when compared to mice that had undergone 15 days of forced abstinence. Finally, we argued that there might be a behavioral switch in drinking motivation from goal-directed to habit-based drinking during the course of the 6-week exposure to EtOH.

These data suggest that chronic, possibly low levels, of contingent EtOH consumption are capable of altering affective and motivational states, as well as circuitry governing these behaviors. We found ketamine and eCB manipulations strongly regulate these affective behaviors, and alterations in eCB levels are induced by this paradigm. The relationship between eCBs and EtOH is complex and will be described in detail below. First, I will highlight an intriguing recent body of work, including our own, which seems to indicate that the induction of depression-like behaviors in mouse models of drinking may

require contingent EtOH administration, and does not seem to require the large doses of alcohol typically administered in non-contingent paradigms.

Unveiling important affective behaviors following contingent EtOH consumption

Human AUDs are characterized by high levels of alcohol drinking and are highly comorbid with affective disorders such as depression and anxiety (Grant et al., 2015). In order to model this human condition with rodents, many researchers have focused on attaining high BEC levels in mice or rats exposed to EtOH. Unfortunately, though methods such as two-bottle choice described above are perhaps the easiest way to administer alcohol, we and other researchers have noted that the continuous nature of administration does not allow for easy determination of maximal BEC levels attained (Becker, 2012). To remedy this, many studies rely on non-contingent methods of EtOH administration, such as EtOH in the (only available) drinking water or liquid diet, EtOH injection or gavage, or exposure to vaporized EtOH (Kliethermes, 2005; Tabakoff and Hoffman, 2000). These administration techniques easily allow regulation of EtOH dosage, and thus subsequent BEC levels are typically above a threshold of intoxication typically set at 80mg/dl to match the human legal limit of blood alcohol concentration. Repeated or long-term exposure to such techniques induces physical EtOH dependence, and so these techniques are often used in an attempt to understand a plethora of withdrawal-related symptoms observed in the human population suffering from AUDs. However, as I will discuss below, these methods may not adequately model all aspects – particularly affective symptoms – of EtOH cessation.

Withdrawal from the aforementioned non-contingent high EtOH paradigms reliably induces handling-induced convulsions (HICs) that peak at approximately 7-10 hours following EtOH cessation, used as a proxy of true physical withdrawal from EtOH (Goldstein, 1973; Kliethermes, 2005). HICs are thought to be similar to seizures and delirium tremens (DTs) associated with complicated alcohol withdrawal syndrome (AWS) in humans with AUDs during acute withdrawal. However, many people with AUDs have a milder phenotype and will not experience such symptoms. In fact, out of all patients admitted to the hospital with a risk of developing complicated AWS, only about 20% will experience these severe symptoms (Maldonado et al., 2015). Thus, these non-contingent rodent models of EtOH administration may overestimate the amount of EtOH necessary for all modalities of withdrawal seen in the human population. Indeed, significant comorbidity of mood disorders is observed in humans with lifetime prevalence of even a mild AUD (Grant et al., 2015), indicating a need to examine the comorbidity of alcohol use and affective disorders in populations that are at significantly reduced probability of experiencing complex AWS-like symptoms.

Despite the induction of severe physical withdrawal symptoms, non-contingent EtOH paradigms are often ineffective in reliably producing affective symptoms. Some groups have observed long lasting depression-related behavior in rats following vaporized EtOH (Slawecki et al., 2004; Walker et al., 2010), but most studies using these techniques have focused on anxiety-related behaviors shortly after withdrawal (Kliethermes, 2005). Though admittedly better in rats, such studies have yielded tepid results in mice of both anxiogenic-like (Kash et al., 2009; Perez and De Biasi, 2015; Watson et al., 1997) and null effects (Daut et al., 2015; Finn et al., 2000; Kiefer et al., 2003) in anxiety-related behavior

typically 4-12 hours after ethanol withdrawal. Additionally, in both species, the presence of locomotor effects clouds interpretation these findings.

Recently, contingent models of EtOH drinking have uncovered previously underappreciated depression-related behaviors in mice during EtOH abstinence (Lee et al., 2015; Pang et al., 2013; Stevenson et al., 2009). First, work from Clyde Hodge's lab showed depression-like behavior measured by the FST in male singly housed C57Bl/6J mice given 4 weeks of two-bottle choice access to 10% EtOH at two weeks, but not one day, following EtOH cessation. Pang et al. and our group went on to show even more robust affective disturbances in female C57Bl/6J mice in a longer, 6-week two-bottle choice paradigm. Females displayed depression-like behavior after 15 days of forced abstinence, but this behavior was not present after 1 day (**Figure 3.2**, and T. Pang, personal communication). In addition, we demonstrated significant EtOH preference not assessed by Hodge's group, and likely not present considering preference for 10% EtOH has been shown in female, but not male, C57Bl/6J mice (Moghaddam et al., 1997). While Stevenson and colleagues found high BECs 2-3hrs into the dark phase (112.8 ± 7.4mg/dl), we found substantially lower BEC levels (likely due to sampling 7-10hrs into the light phase, during which mice are less active), and Pang et al. did not analyze BECs.

The Drinking in the Dark (DID) paradigm has been utilized to induce high levels of contingent drinking and allow more accurate BEC characterization than continuous free-access models. In this model, mice are typically given 2hr access to EtOH solution 3 hours into the dark cycle, and repeated exposure to this paradigm yields increased consumption during access periods and BECs usually >80mg/dl. The intermittent nature of this paradigm is thought to correlate to other discontinuous procedures that produce increased

consumption over time, such as contingent chronic intermittent access every other day (Wise, 1973) or chronic intermittent EtOH (CIE) administered via vaporized EtOH (Lopez and Becker, 2005). It has been found that in DID, the physical changing of sippers when EtOH is being provided acts as a cue for EtOH availability and drives the increased consumption primarily during the first 15 minutes of the procedure (Wilcox et al., 2014).

The DID paradigm was recently shown to induce depression-like behavior in mice during EtOH abstinence, although the time course of this behavioral development was somewhat different than that observed when using continuous model of access, such as in our study. Specifically, DID induced depression-like behavior as soon as 24hrs following EtOH removal (Lee et al., 2015). We were initially perplexed by these findings, as never before had depression-like behavior been observed so soon following EtOH cessation. However, these data, in concert with our own, may reveal a subtle characterization of depression-like behavior induced by EtOH abstinence in mice. Namely, it appears that this behavior may depend on two distinct variables: contingency of administration and presence of prolonged withdrawal. While the former condition is apparent given the nature of both continuous two-bottle choice and DID paradigms, the latter seems at odds with the DID data presented, given that behavior was observed 24hrs after EtOH cessation. However, the very design of the DID paradigm itself includes repeated short-duration withdrawals. The 6 week DID procedure utilized by Dr. Szumlinski's group consisted of 2hr EtOH access/day for 5 days/week (Lee et al., 2015). Therefore, every week of EtOH access also contained four 22hr withdrawal periods and one two-day withdrawal period, which may be crucial for the development of depression-like behaviors.

The recent work by our lab and others has highlighted a potentially critical model of induced depression-like behavior following EtOH abstinence. In particular, we find that the high levels of EtOH typically administered through non-contingent methodology are likely not necessary for disturbances in affective behavior following the cessation of EtOH, as evidenced by the relatively low BECs observed in our study. Rather, it appears that, at least in mice, contingency of EtOH administration may play a role in manifestation of depression-like behaviors. Further, each of the studies in mice outlined in the preceding passage (Lee et al., 2015; Pang et al., 2013; Stevenson et al., 2009) – as well as in the few non-contingent studies finding depression-related behavior in rats (Slawecki et al., 2004; Walker et al., 2010) – require a prolonged period(s) of withdrawal, which can be either continuous or intermittent. We have not fully assessed the requirement of contingency for these behaviors, and it will be important to examine depression-related behaviors in mice following protracted abstinence from non-contingent (e.g. CIE) paradigms. Future studies will also be necessary to determine the minimal length of withdrawal necessary to induce this phenotype, whether anxiety-like behavior is observed during acute withdrawal from the two bottle-choice paradigm, and, if so, when the behavioral shift from anxiety to depression occurs. This transition time point, if it is found to exist, will represent a crucial tool for analysis of neurochemical and neuroanatomical substrates underpinning these behaviors.

These observations may additionally be of unique clinical relevance. While affective disturbances have been relatively difficult to detect in non-contingent pre-clinical models of EtOH administration, they represent a major hurdle for individuals with AUDs. The possibility that exceptionally high BECs are not a requirement for the induction of these

behaviors would be well suited to match the human population, in which even mild AUDs demonstrate comorbidity with mood disorders. In particular, the two-bottle choice paradigm most closely mimics actual human alcohol drinking, in which alcohol is virtually always available and consumed in a contingent manner.

Endocannabinoids and Ethanol Consumption

We found that depression-related behaviors following EtOH withdrawal were powerfully regulated by modulation of the endocannabinoid (eCB) system. Increased availability of 2-AG through the inhibition of MAG lipase by JZL-184 was capable of reducing EtOH abstinence-induced depression-like behaviors to control levels, and JZL-184's antidepressant-like effects were completely blocked by co-administration of the CB1 receptor antagonist, rimonabant. In addition, there were higher levels of both 2-AG and AEA within the BLA of mice on the final day of drinking compared to mice following 15 days of forced abstinence.

The eCB system (**Figure 4.2**) is comprised primarily of two ligands, AEA and 2-AG, and two receptors CB1 and CB2. This system is relatively distinct among the known neurotransmitters, as synthesis of eCBs is activity-driven postsynaptically and retrograde signaling reduces neurotansmision of both glutamate and GABA through presynaptically located receptors (Kano et al., 2009; Pacher et al., 2006; Pava and Woodward, 2012). Both ligands are synthesized from phospholipids in the postsynaptic membrane. 2-AG is produced primarily through combination of phospholipase C β (PLCβ) and diacylglycerol (DAG) lipase activity while AEA synthesis is less clear, but at least one mechanism involves Ca^{2+}-mediated activation of *N*-acyltransferase (NAT) and *N*-acylphosphatidylethanolamine-

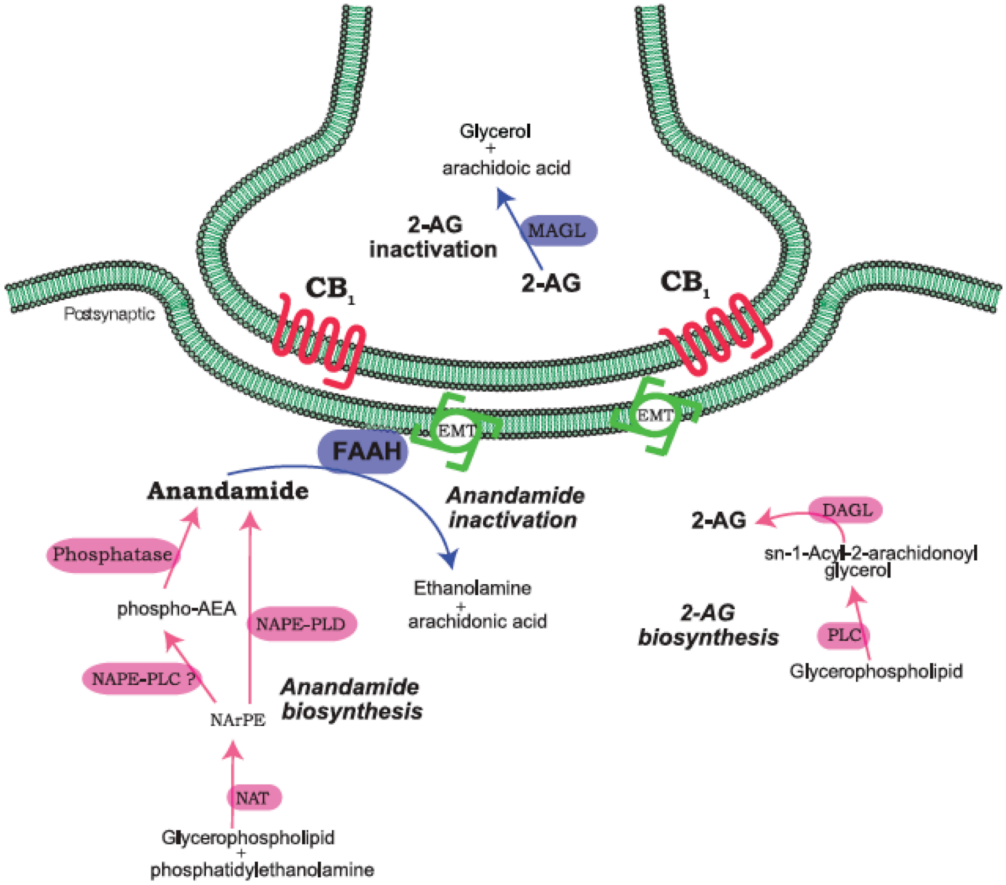


Figure 4.2. The Endocannabinoid System. Schematic representation of the endocannabinoid system in pre- and postsynaptic neurons. The presynaptic terminal is located in the top, whereas the postsynaptic neuron is located in the bottom. EMT, endocannabinoid membrane transporter; MAGL, monoacylglyceride lipase; DAGL, DAG lipase; AEA, anandamide; NArPE, *N*-arachidonyl phosphatidylethanolamine; NAT, *N*-acyltransferase. [Reprinted with permission from (Pacher et al., 2006)]

hydrolyzing phospholipase D (NAPE-PLD). 2-AG and AEA are degraded by MAG lipase presynaptically and fatty acid amide hydrolase (FAAH) postsynaptically, respectively. While there are two receptors for eCBs, CB2 is found chiefly in the periphery (Munro et al., 1993), while CB1 is found throughout the brain, but in particular the cortex, striatum, and cerebellum. Both receptors are $G_{i/o}$-coupled G-protein-coupled receptors (GPCRs) and inhibit adenylate cyclase and cAMP production.

Ethanol and the eCB system are intricately and complexly entwined (for excellent review, see Pava and Woodward 2012). Acute ethanol administration comparable in concentration to intoxication levels drives the synthesis of both 2-AG and AEA in hippocampal cultures, and thus these eCBs contribute to the reduction in glutamate release observed following EtOH treatment (Basavarajappa et al., 2008). Conversely, other groups found decreased AEA levels in the striatum, cerebellum, NaC, hypothalamus, hippocampus, and amygdala (Ferrer et al., 2007; Rubio et al., 2007) and decreased 2-AG levels in the PFC (Rubio et al., 2007) following EtOH administration. Acute EtOH can inhibit depolarization-induced suppression in inhibition (DSI), and CB1 activation can prevent EtOH-mediated increases in GABA transmission in BLA (Talani and Lovinger, 2015), CeA (Roberto et al., 2010), and cerebellum (Kelm et al., 2008), indicating that these systems can function in opposition as well. The role of acute ethanol administration on the eCB system varies widely between regions examined and method of EtOH delivery (Pava and Woodward, 2012).

Alterations in basal levels of eCB tone appear to play a role in inherent preferences for alcohol. For instance, C57Bl/6J mice have lower CB1 expression than the DBA/2 mouse strain, known as near-teetotalers (Hungund and Basavarajappa, 2000), and an alcohol-

preferring rat line has reduced CB1 activity as well as reduced FAAH and, to a lesser degree, MAG lipase expression (Hansson et al., 2007). Conversely, EtOH consumption can be increased through inhibition of FAAH (Hansson et al., 2007) or MAG lipase (Gutierrez-Lopez et al., 2010). Similarly, postmortem studies show reduced CB1 and FAAH in alcohol-dependent humans (Vinod et al., 2010). Decreased CB1 expression in genetic strains of high EtOH consumption are likely compensatory for increased eCB levels, as acute treatment with the CB1 agonist CP-55,940 increases preference and rimonabant reduces preference (Vinod et al., 2008). CB1 activation seems to drive this EtOH preference, as CB1 null mice display both decreased preference and consumption of EtOH, and also display more severe withdrawal symptoms (Naassila et al., 2004). We observed increased levels of 2-AG and AEA within the BLA (but not other regions examined) on the final day of our 6-week EtOH two bottle choice paradigm when compared to animals in withdrawal, perhaps mirroring the above observations. It is important to note, however, that examination of eCB metabolites may have been underpowered (N=5-7/group), preventing observation of significant findings in some regions that showed trending differences in eCB levels (namely, the CeA and the ventral striatum). In addition, we did not examine a control group of animals (6-week two-bottle choice with only water) to determine whether aberrant eCB concentrations occur during drinking or abstinence compared to basal levels. Additional work will be needed to determine whether other regions show alterations in eCB levels during these conditions, compare the eCB levels during drinking and withdrawal to basal eCB concentrations, as well examine CB1, FAAH, and MAG lipase activity during EtOH administration and withdrawal compared to basal activity using our paradigm.

Preclinical data lead to the investigation of rimonabant's utility in curbing EtOH cravings in human subjects. However, it was ineffective in both reducing consumption in heavy drinkers not seeking treatment (George et al., 2010) and preventing relapse in treatment-seeking individuals (Soyka et al., 2008). Interestingly, with regards to our data examining the nexus of EtOH, eCBs, and affective disturbances, rimonabant was discontinued clinically due to significant increases in both anxiety and depression (and consequentially, suicide) in patients using the drug to treat obesity (Topol et al., 2010). Conversely, the eCB uptake inhibitor AM404 showed antidepressant effects in the FST (Hill and Gorzalka, 2005). While CB1 agonists have been shown to increase reinstatement to EtOH in rodents (Lopez-Moreno et al., 2004), no alteration in EtOH reinstatement was observed following FAAH inhibition using URB597 (Cippitelli et al., 2008). The latter study also showed decreased EtOH withdrawal-induced anxiety-like behaviors, similar to our observation that JZL-184 reduced EtOH withdrawal-induced depression-like behaviors. Thus, while overt agonism of CB1 is clearly not well suited for individuals with comorbid AUDs and depression due to relapse potential, it is possible that inhibition of eCB degradation may still have utility in reduction of affective behaviors associated with alcohol withdrawal.

4.3 Future Directions utilizing the two-bottle choice EtOH drinking paradigm

Sucrose two-bottle choice

Our recent unpublished work utilizing this paradigm has yielded interesting findings with regards to another hedonic substance, sucrose. Given the similarities between drugs of abuse and natural palatable food rewards in behavior and physiology, we

wished to assess behavioral consequences of sucrose consumption using the two-bottle choice assay described above. Briefly, in lieu of EtOH, mice were presented with a sucrose solution (10% for 3 days, then 5% for the remainder of the experiment) for 6 weeks and then underwent a forced abstinence period identical to that described above for EtOH. Mice were compared to control (N=9) and EtOH (N=23) groups that were run in tandem. Mice displayed extraordinarily high preference for the sucrose solution, significantly higher than preference for EtOH, and average daily consumption of the sucrose solution was significantly higher than that of 10% EtOH (**Figure 4.3**). Astonishingly, mice consumed a volume of sucrose solution equivalent to approximately 70-75% of their body weight daily. When tested in the NSFT 15 or 35 days following the removal of sucrose, mice displayed significantly increased latency to feed comparable to mice following forced abstinence from EtOH.

Region-specific targeting on drinking and depression-like behavior

Given our findings that regionally targeted deletion of GluN2B-containing NMDARs within the BNST gives rise to reductions in depression-like behavior similar to systemic administration of ketamine, we were eager to assess such a deletion in the two-bottle choice EtOH paradigm. We hypothesized that we would also observe reduced withdrawal-induced depression-like behavior and perhaps alterations in drinking patterns. Due to difficulties in validation using the LV-Cre recombinase described in Chapter 2 (see Appendix C), we wished to utilize a different viral construct that co-expressed a reporter protein along with Cre in order to more easily visualize injection sites for analysis of knockdown. Our lab had previous success with using an adeno-associated viral (AAV)

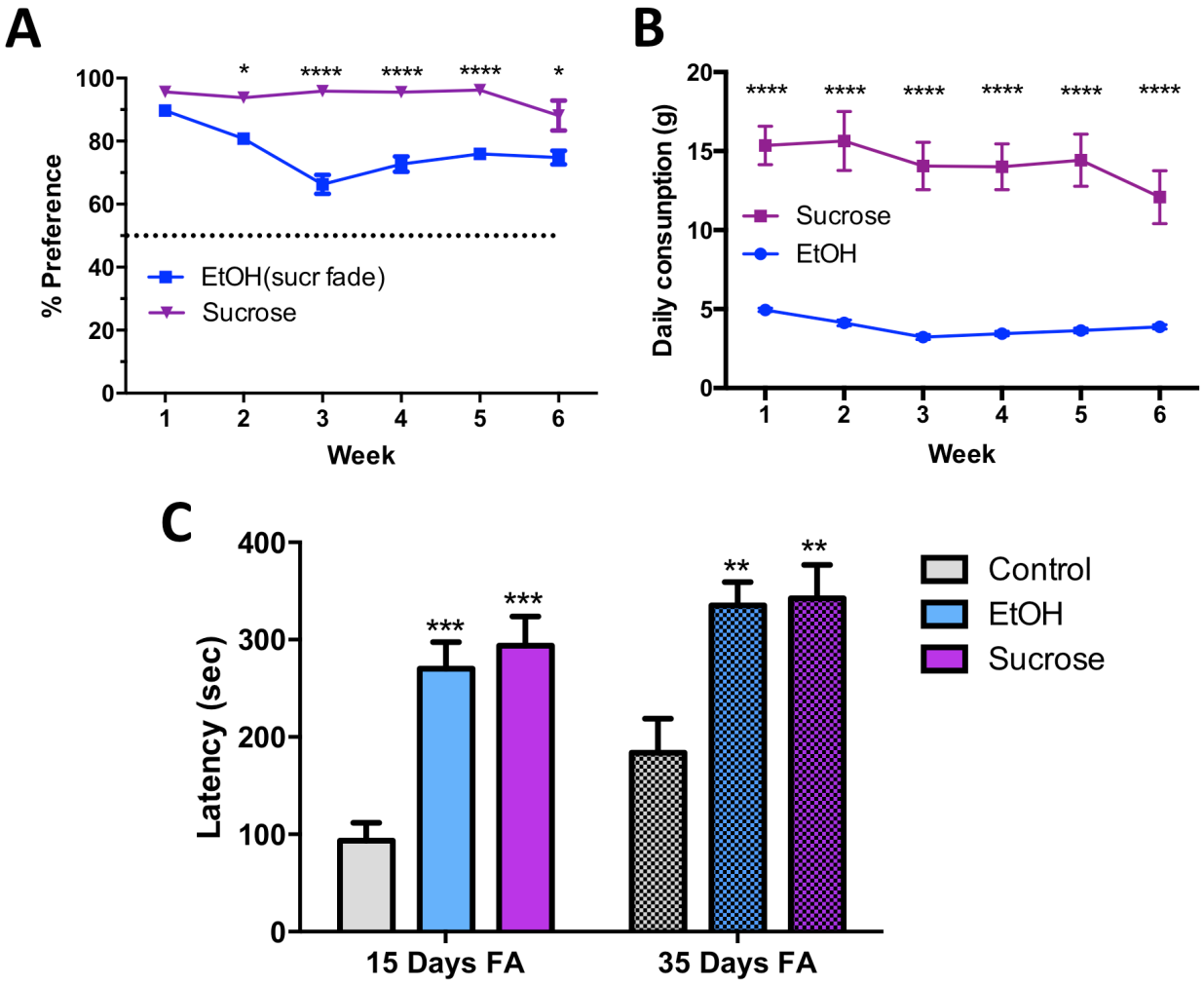


Figure 4.3. Mice have high preference and consumption of 5% sucrose and develop depression-like behaviors during forced abstinence (FA). (A) Average weekly preference for either 5% sucrose (purple; N=12) or 10% EtOH (blue; N=23). (B) Average daily consumption (g) of either solution. (C) Latency to feed in the NSFT after 15 or 35 days of forced abstinence (FA). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

strategy (Flavin et al., 2014), and so we hoped to employ a similar viral construct in this study.

We utilized a virus containing a GFP-Cre fusion protein (AAV9.CMV.HI.GFP-Cre.WPRE.SV40; AAV9-GFP-Cre) or GFP alone (AAV9.CMV.PI.eGFP.WPRE.bGH; AAV9-GFP) from the Penn Vector Core (Perelman School of Medicine; Philadelphia, PA). Mice were bilaterally injected with one of the two viruses into the dlBNST as described in Chapter 2. Two weeks after surgery, mice were transferred to the Vanderbilt Murine Neurobehavior core and underwent the two-bottle choice EtOH paradigm with sucrose fade as described in Chapter 3. Mice were tested for latency in NSFT and immobility time in FST, and no significant differences between groups were observed (**Figure 4.4**).

Unfortunately, after initiating these studies, a report was brought to our attention indicating AAV9-induced inflammation and neuronal loss (Ciesielska et al., 2013). Four mice were examined for histology that had been given unilateral injections of either virus ~8wks prior to examination. We found substantial lesion-like damage to the BNST and surrounding periventricular areas in mice treated with AAV9-GFP-Cre. Such damage was not observed in AAV9-GFP treated animals, but small sample size does not allow us to preclude the possibility that this virus may also induce damage, as the original report cited damage in both construct-containing and reporter lines using the AAV9 serotype (Ciesielska et al., 2013). In addition, although GFP fluorescence can be easily seen in the AAV9-GFP treated mice, very little fluorescence is present in Cre-treated animals.

Although I did not pursue regional deletion further using this paradigm, future studies using such regionally restricted methods will be crucial in elucidating circuitry and systems involved in both the induction of EtOH preference and behaviors related to

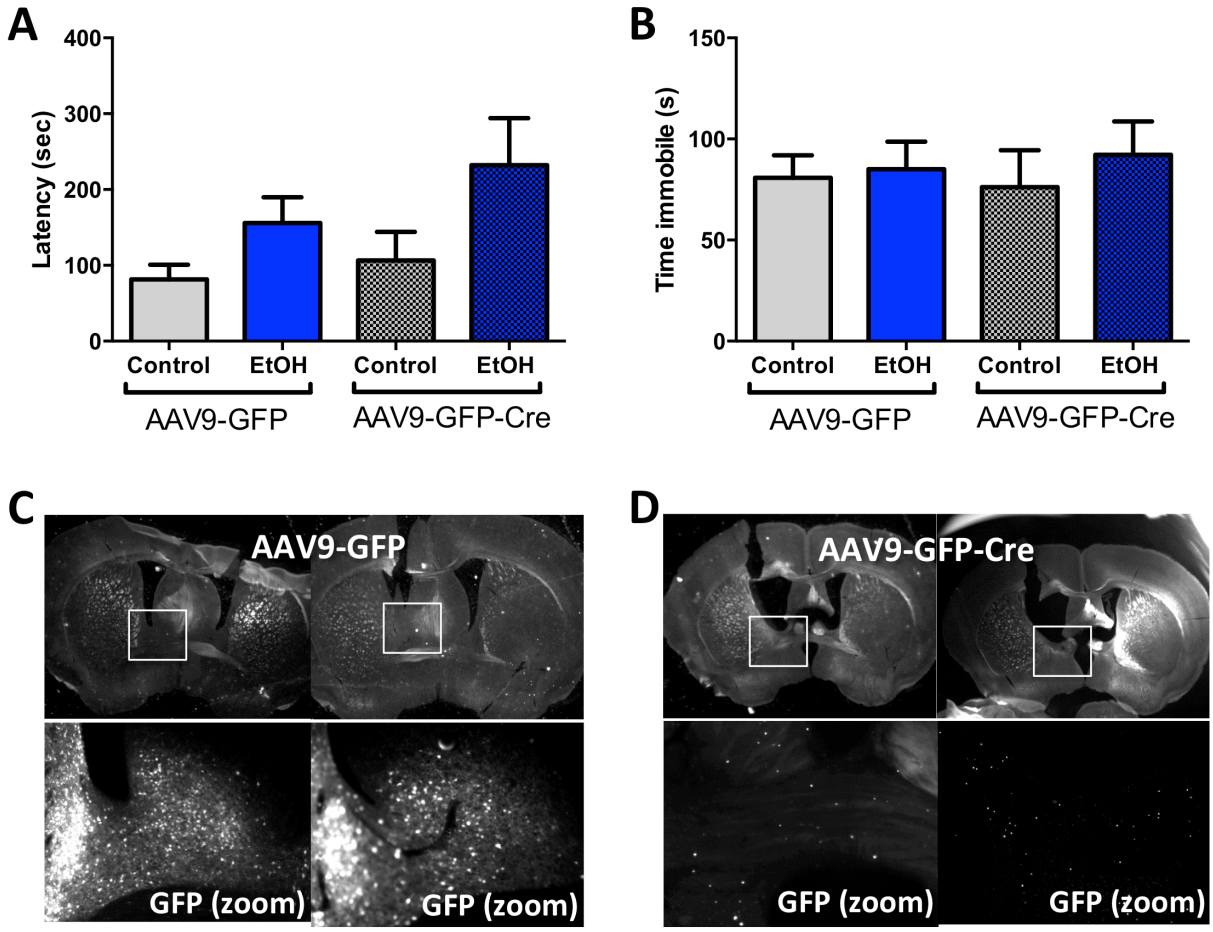


Figure 4.4. Lack of behavioral effect in the NSFT following EtOH withdrawal and AAV9-eGFP-Cre induced neurotoxicity in *grin2b*-floxed mice injected with AAV9-eGFP or AAV9-eGFP-Cre. (A) NSFT latency and (B) FST immobility in mice with bilateral injections of AAV9-GFP or AAV9-GFP-Cre into the BNST 2 weeks prior to initiation of two-bottle choice (N=7-8/group). (C-D) Light microscopy image of coronal brain sections from four animals with unilateral (C) AAV9-GFP or (D) AAV9-GFP-Cre injections. Higher magnification images are of areas delineated by rectangles.

withdrawal. Examination of BNSTGluN2BKD mice in this paradigm still represents an important experiment (though it should be implemented using a different AAV serotype, such as AAV2 or AAV5). Additionally, given the interesting eCB data obtained through this paradigm, an obvious future experiment should involve region-specific manipulation of eCBs. We currently have a DAG lipase floxed mouse line, and one could imagine that deletion of DAG lipase, thus leading to increased AEA, within the BLA may cause alterations (likely increases) in preference and/or reductions in withdrawal-related depression-like behavior.

Alterations in Plasticity following withdrawal from EtOH two-bottle choice

Our lab has demonstrated alterations in intrinsic excitability and plasticity in the BNST during acute withdrawal (~4hrs) following CIE (Kash et al., 2009; Wills et al., 2012). Expression of GluN2B was significantly elevated at this time point, without concomitant changes in GluN1 or GluN2A, and this led to increased decay time observable in NMDAR EPSCs. CIE exposure also inhibited the ability of acute EtOH to reduce NMDAR EPSC amplitude (Kash et al., 2009). CIE augments LTP in the BNST through increased GluN2B expression, as CIE fails to increase LTP in forebrain-wide GluN2B knockout mice (Wills et al., 2012).

In future studies, two comparisons should be made. First, examining a series of time points following CIE, including one that corresponds to behavioral abnormalities in withdrawal from two-bottle choice, will be instructive to determine a time line of alterations within the region, and whether these electrophysiological observations are truly long-lasting responses to EtOH or more representative of acute withdrawal. Second,

the inverse experiment will be extremely useful. That is, the determination of what, if any, alterations in excitability and plasticity are apparent following prolonged withdrawal from two-bottle choice, as well as during early withdrawal.

4.4. Final Remarks

Investigations aimed at improving current treatment strategies for MDD continue to represent a vitally important task in neurobiology, as current strategies require weeks of administration before efficacy is apparent and response rates are woefully inadequate. Additionally, treatments are necessary for individuals suffering from MDD and AUDs in tandem. We have shown that knockdown of GluN2B-containing NMDARs in the BNST recapitulates the effect of systemic ketamine, a rapid-acting and long-lasting antidepressant. Clinical work targeting this subunit has demonstrated reduced dissociative side effects induced by these drugs compared to ketamine, but cardiovascular side effects persist, so more work must be done to find therapeutic treatments targeted to this subunit.

We have also demonstrated long-lasting depression-like behavior induced by withdrawal from chronic contingent EtOH exposure in female mice. This behavior was sensitive to ketamine, but the similarities in subjective state experienced by ketamine and EtOH likely preclude the utility of ketamine in treating MDD in AUD patients. However, we found that increased 2-AG tone through the use of the MAG lipase inhibitor JZL-184 also attenuated affective behavior following EtOH withdrawal. We also found alterations in eCB levels in the BLA during EtOH drinking compared to during withdrawal. Thus, inhibitors of eCB degradation may represent a pharmacologic target in the treatment of MDD, and particularly in MDD experienced by individuals recovering from AUDs.

Taken together, I have aimed here to increase understanding of the neuroanatomical and pharmacological substrates of MDD and MDD/AUD comorbidity, particularly with regard to anhedonia. Future work will be necessary to move preclinical studies, including work described above, into clinical treatments for suffering individuals.

APPENDIX A

Effects of BNST-Restricted Glucocorticoid Receptor Deletion on Affective Behavior.

A.1: Glucocorticoid Receptor-Mediated Effects within the Extended Amygdala

Stress has been associated with a number of adverse effects, including anxiety disorders and addiction. Glucocorticoids are released during stress and bind to glucocorticoid receptors (GRs) present in every cell of the body. Limbic areas, such as the hippocampus, express high levels of GR, and the receptors have been extensively examined within these regions for their ability to alter synaptic plasticity. GRs within one limbic region, the extended amygdala - consisting of the shell of the nucleus accumbens (NAc-Sh), the central nucleus of the amygdala (CeA), and the bed nucleus of the stria terminalis (BNST), have received relatively less attention. Given the prominent role of the extended amygdala in the integration of stress and reward circuitry, and the demonstrated capability of GRs to alter synaptic plasticity, it is somewhat surprising that GRs within the region have not been studied to a greater extent. This review will examine current literature of GR-mediated effects within the extended amygdala. In short, GR activation seems to increase excitability in the extended amygdala. GRs facilitate dopamine release in response to drugs of abuse and stress within the NAc-Sh, facilitate fear conditioning and anxiety within the CeA and appear to decrease anxiety and potentially maintain excitability within the BNST. Activation of GRs within the extended amygdala plays a pivotal role in response to stress and reward, and disregulation of GRs within the region could lead to maladaptive responses, such as anxiety disorders and addiction.

Stress is prevalent in everyday life, and can be defined as "a condition or feeling experienced when a person perceives that demands exceed the personal and social resources the individual is able to mobilize"(Fink, 2010). While surmounting stress experienced is crucial to survival, maladaptive responses to stress or prolonged stress can prove detrimental. Stress has been associated with anxiety disorders such as generalized anxiety disorder, post-traumatic stress disorder (PTSD), and panic disorder, as well as addiction. Indeed, stress is a commonly cited reason for relapse to drug use in addicts, and diagnosis of an anxiety disorder is significantly associated with drug use(Sareen et al., 2006). A deeper understanding of the effects of stress on anxiety and reward circuitry will prove invaluable for treating and preventing anxiety and addiction behaviors such as stress-induced reinstatement of drug seeking.

The HPA Axis and Glucocorticoid Release

Upon exposure to a stressful stimulus, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Corticotropin releasing factor (CRF) is first released from the parvocellular neurons of the hypothalamus to the pituitary through the portal system, triggering the release of adrenocorticotropin (ACTH)(Harris, 1948). ACTH then acts upon the adrenal cortex, leading to the release of glucocorticoids (cortisol in humans and corticosterone in rodents; CORT) into the blood stream. CORT binds to two receptor types, the mineralocorticoid receptor (MR) and the GR, which has about 10-fold lower affinity for CORT than MR(Reul and de Kloet, 1985). While MRs are almost entirely occupied under basal conditions, the lower affinity GR is only activated when high circulating

concentrations of CORT are present – such as during the circadian peak of CORT release and during stress – and acts as negative feedback to inhibit the HPA axis(Reul and de Kloet, 1985). Interestingly, it has also been demonstrated that administration of drugs of abuse and drug withdrawal leads to an increase in plasma CORT levels in rodents (Ellis, 1966; Levy et al., 1991) and humans (Adinoff et al., 2003; Mello and Mendelson, 1997; Mendelson et al., 2005) and subsequent activation of GRs. Thus, GRs may play a role in drug addiction and withdrawal, in addition to its role in the stress response.

Complete knockout of GR is lethal in mice, indicating that this receptor is necessary for survival (Cole et al., 1995). Site-specific genetic or pharmacological alterations in GR function have proved more useful in assessing the receptors' roles. GRs are expressed ubiquitously within the brain (Morimoto et al., 1996; Reul and de Kloet, 1985) and show highest expression within a number of limbic regions, including the hippocampus, CeA, BNST.

Effects of Glucocorticoid Receptor Activation

The effects of GR activation are extensive, and involve two distinct mechanisms: the genomic pathway and the non-genomic pathway (Prager and Johnson, 2009). In the genomic pathway, GRs within the cytosol bind CORT that diffuses freely through the plasma membrane. Unbound GR is maintained in a protein heterocomplex in the cytoplasm (Morishima et al., 2000; Smith and Toft, 1993) The binding of ligand leads to the increased phosphorylation of GR (Wang et al., 2002). This phosphorylation allows GR to form a dimer with other transcription factors (TFs) or another GR (Kassel and Herrlich, 2007; Presman et al., 2010) and translocate to the nucleus (Wang et al., 2002). GRs can function as a

homodimer or with other TFs in order to *trans*-activate or *trans*-repress genes. Within the nucleus, the ligand-bound GR homodimer is able to bind glucocorticoid response elements (GREs) that are present upstream of the promoter of a number of genes (Becker et al., 1986; Chandler et al., 1983) or the homodimer can bind another TF in order to enhance or inhibit its transcription effect (Schoneveld et al., 2004). In fact, transcription can be altered by GRs in an estimated 1-2% of all genes (Joels et al., 2009).

It has been demonstrated that CORT is also able to induce rapid effects within minutes, a time frame not compatible with transcriptional effects of GR, via a putative membrane-bound GR (mGR). For instance, injection of the specific GR agonist dexamethasone to the paraventricular nucleus of the hypothalamus (PVN) is able to inhibit ACTH release in response to restraint stress within minutes (Evanson et al., 2010). Dexamethasone conjugated to BSA, which is membrane impermeable, is able to recapitulate this effect, giving further evidence for a mGR. Some debate does exist over the involvement of the classical GR in these rapid effects of CORT and dexamethasone. A possible yet-undetermined G-protein coupled receptor (GPCR) has been implicated as pituitary cell lines were able bind CORT and dexamethasone at the membrane with no apparent affinity for the GR antagonist RU486 (Maier et al., 2005). The binding of ligand in this study was blocked pertussis toxin, which uncouples G-proteins from their GPCR. However, GR antagonism has been shown to inhibit some rapid effects of CORT or dexamethasone (Karst et al., 2010). In addition, possible mechanisms have been identified that could localize the classical GR to the membrane – such as the presence of a conserved palmitoylation site that has been shown to link the estrogen receptor to the membrane

(Groeneweg et al., 2011; Pedram et al., 2007) and direct binding of GRs to caveolin (Matthews et al., 2008).

GR-mediated Alterations in Synaptic Plasticity

A predominant effect of mGR activation is the recruitment of the endocannabinoid (eCB) system. Through the activation of PLC, GRs induce the production and retrograde release of the eCB 2-arachidonoylglycerol (2-AG) from the postsynaptic bouton (Di et al., 2003). 2-AG binds to the cannabinoid receptor (CB1), leading to a decrease in presynaptic neurotransmitter release (Kano et al., 2009). In this way, mGRs can inhibit excitatory transmission (Evanson et al., 2010; Karst et al., 2010) or inhibit GABAergic projections to glutamatergic neurons, thus disinhibiting excitatory transmission (Hill et al., 2011; Patel et al., 2009).

GRs have been implicated in alterations to synaptic plasticity and excitability. The hippocampus, in particular, has been studied extensively due to long-established electrophysiological recording techniques and high expression of both GRs and MRs. GRs are generally thought to reduce neuronal excitability within the hippocampus. For instance, in the CA1 region, GRs mediate impairments in NMDA-dependent long-term potentiation (LTP) through a slow genomic mechanism (Krugers et al., 2005; Wiegert et al., 2005), as well as facilitate metabotropic glutamate receptor-dependent long term depression (LTD) by lowering the threshold for LTD induction (Chaouloff et al., 2008).

The Extended Amygdala

Although the involvement of GRs in many limbic areas has been examined (Joels et al., 2009; Sousa et al., 2008), one region that has received relatively less attention is the extended amygdala. The extended amygdala consists of the shell of the nucleus accumbens (NAc-Sh), the central nucleus of the amygdala (CeA), and the bed nucleus of the stria terminalis (BNST) (Alheid, 2003; Alheid and Heimer, 1988; Cassell et al., 1999). This region is situated at the crossroads of stress and reward circuitry and has thus been heavily implicated in the negative affect associated with stress disorders and withdrawal from drugs of abuse (Koob and Volkow, 2010). The CeA and BNST have both been implicated in stress-induced reinstatement of drug seeking (Briand et al., 2010; Yamada and Bruijnzeel, 2011). While the involvement of noradrenergic (Smith and Aston-Jones, 2008) and CRF signaling (Koob, 2010) within the extended amygdala in anxiety and addiction behaviors has been studied extensively, the role of GRs within this region is less clear. GRs within the extended amygdala are poised to alter synaptic plasticity and behavioral responses to stress and drugs of abuse. Given the prominent role of this region in stress response and HPA axis modulation, the high expression of GRs, and the proven ability of GR to alter synaptic transmission, the paucity of literature examining GRs within the extended amygdala is somewhat ironic. This review will explore current literature of GR-mediated effects within the extended amygdala, particularly in the context of anxiety and addiction behavior.

Nucleus Accumbens

Within the NAc-Sh, GR activation has been associated with an increase in neuronal excitability and extracellular dopamine (DA) levels. It has long been known that the NAc-Sh is more responsive to glucocorticoids than the NAc core region (Barrot et al., 2000). Recently, Campioni, et al. demonstrated that the activation of GR leads to increased neuronal excitability in the NAc-Sh (Campioni et al., 2009). The AMPA/NMDA ratio was increased in the shell following a cold water forced swim stress, and this effect was postulated to be GR-mediated, as it was mirrored with CORT application and abolished by RU486. Increased AMPAR miniature excitatory postsynaptic current (mEPSC) amplitude and reduced rectification of AMPA currents suggested that the increase in excitability was primarily due to an increase in the number of functional GluR2-containing AMPA receptors (AMPARs) present at the postsynaptic membrane. In accordance, it has been demonstrated in cell cultures that long-term corticosterone application facilitates the lateral diffusion of AMPARs though a GR-mediated mechanism that can be blocked with a GR antagonist (Groc et al., 2008). Because GR-facilitated integration of AMPARs to the post synaptic membrane is delayed and can be blocked by the protein synthesis inhibitor cycloheximide, it has been suggested that this effect is mediated through the genomic pathway (Martin et al., 2009). The observation of increased AMPAR mEPSC amplitude in the NAc is mirrored by earlier work in the CA1 region of the hippocampus in which CORT or a selective GR agonist was able to enhance amplitude – but not frequency – of AMPAR mEPSCs (Karst and Joels, 2005). The enhanced excitability within the NAc in response to GR activation could play a role in the increased drive to obtain a drug of abuse or perform a compulsive behavior in an addicted individual experiencing stress.

Dopaminergic projections from the ventral tegmental area (VTA) to the NAc are crucial in the reward system (Willuhn et al., 2010), and it has been demonstrated that stressors are capable of initiating relapse to drug seeking in humans (Brown et al., 1995; Kosten et al., 1986) and rodents (Erb et al., 1996; Shaham and Stewart, 1995). Thus, the effect of stress on dopamine release within the NAc is worthy area of research. Indeed, footshock stress is capable of increasing extracellular DA levels within the NAc shell in the rat, with no change in the NAc core (Kalivas and Duffy, 1995). This effect appears to be mediated through stress-induced CORT release, as adrenalectomy selectively lowers extracellular DA in the shell but not the core (Barrot et al., 2000). The DA spike observed in the NAc-Sh following stress or the administration of various drugs of abuse may be due in part to the activation of GRs. The hyperlocomotion and increase in extracellular NAc DA following systemic morphine administration can be attenuated by i.c.v. treatment with the GR antagonist, RU486 (Marinelli et al., 1998). In fact, direct infusion of RU486 to the NAc is capable of preventing conditioned place preference to morphine in rodents (Dong et al., 2006). It has also been found that mice lacking GRs in D1 dopamine receptor-containing (dopaminoceptive) neurons showed decreased DA release in the NAc following cocaine administration (Barik et al., 2010). These studies suggest that GRs have a central role in the release of DA within the NAc following drug administration.

The rise in extracellular DA within the NAc following stress or administration of drugs of abuse could be partially due to GRs in the VTA. Acute stress has been demonstrated to increase the AMPA/NMDA ratio in DA neurons within the VTA to a greater extent than acute administration of drugs of abuse (Saal et al., 2003), and this effect is

blocked completely the GR antagonist RU486. Morphine, cocaine, nicotine, and forced swim stress impair the ability of GABAergic synapses to induce LTP onto VTA DA neurons. This leads to a disinhibition of these projections to the NAc, and increased DA release within the NAc (Niehaus et al., 2010). The stress-induced impairment of GABAergic LTP is rescued by RU486, and thus believed to be GR-mediated. Further, direct infusion of CORT to the VTA is sufficient to induce NAc DA release, and this is effectively blocked by coapplication of RU486 (Tye et al., 2009). Because exposure to a stressor is capable of initiating DA release within the NAc through a GR-dependent mechanism, and because drugs of abuse have been demonstrated to induce a similar DA spike within the NAc, it can be postulated that GRs within the NAc and regions projecting to the NAc are crucial for drug-seeking behaviors such as stress-induced reinstatement. Elevation in NAc DA following exposure to a drug of abuse is a key component in the early rewarding stages of drug addiction (Koob and Volkow, 2010). Exposure to a stressor after a long period of drug abstinence would cause a GR-mediated DA spike within the NAc that may be reminiscent of the rewarding effects of such early drug use. This could lead a previously addicted individual to return to their drug of choice in order to mediate the rewarding effects while simultaneously blocking the negative affect caused by the stressor.

Central Nucleus of the Amygdala

The CeA is well situated to contribute to the HPA axis response to a stressor. Electrical stimulation of the CeA leads to an HPA response with increased serum CORT (Weidenfeld et al., 1997). Stimulation of GABAergic projections from the CeA to the BNST

quiets BNST GABAergic projections to the PVN, thus leading to a disinhibition of the HPA response (Herman et al., 2003). The CeA has been particularly implicated in the response to an acute stressor, and has been argued to mediate stimulus-specific fear-like behavior associated with such a stressor (Davis and Shi, 1999; Walker et al., 2009). Indeed, ablation of the CeA completely eliminates cue-induced potentiation of startle response to a footshock(Davis and Shi, 1999).

The CeA contains the densest expression of GR within the amygdala (Morimoto et al., 1996), potentially implicating GRs in the fear response mediated by the CeA. This has recently led some investigators to examine the effects of GRs within the CeA on fear- and anxiety-like responses in rodents. Selective pharmacologic activation of GRs within the CeA elevates GR expression levels, increases anxiety-like behavior in the elevated plus maze (EPM), and increases the plasma CORT in response to the stress of exposure to the EPM (Weiser et al., 2010). Conversely, increased anxiety-like behavior on the EPM following implantation of a CORT pellet into the CeA can be blocked with co-administration of the GR antagonist RU486 into the CeA (Myers and Greenwood-Van Meerveld, 2007).

The development of a transgenic "floxed" mouse harboring loxP sites around exons 1 and 2 of the GR gene, *NR3C1*, has been an invaluable resource for determining region-specific involvement of GRs within the brain as the mouse exhibits a loss of GR expression in regions exposed to Cre-recombinase (Kolber et al., 2008). Lentivirally-mediated delivery of Cre-recombinase has allowed precise site-specific deletion of GR in this mouse line, and was recently utilized in order to examine the effect of GR deletion within the CeA (CeAGRKO) on anxiety- and fear-related behaviors (Arnett et al., 2011; Kolber et al., 2008). The resulting 65% deletion of GRs within CeA neurons did not lead to alterations in

locomotor activity or circulating plasma levels of CORT, and the apparent incongruity with the pharmacological HPA data described above may be attributable to the incomplete GR deletion observed in the present study. In accordance with the CeA's role in fear-like behavior, CeAGRKO mice exhibited impairment in both cue and contextual fear conditioning when compared to mice injected with lentiviral GFP (Kolber et al., 2008). Interestingly, the effect of GRs specifically within the CeA on fear-conditioning was further confirmed by mice with forebrain GR knockout (FBGRKO). These animals lack GRs in the cortex, hippocampus, BLA, and striatum but do not show GR disruption in the CeA or PVN (Boyle et al., 2005). FBGRKO mice do not demonstrate impairments in fear conditioning, indicating that the fear-like CeAGRKO phenotype is region-specific. Further, it was demonstrated that adrenalectomized mice show impairments in contextual fear conditioning, but have intact cue fear conditioning (Pugh et al., 1997). Thus, there may be mechanisms in place within other brain areas to account for global brain reductions in CORT signaling, as specific impairment of CeA GRs leads to a more robust fear conditioning phenotype than adrenalectomy. Thus, GRs within the CeA are capable of inducing HPA axis activation in response to an acutely stressful stimulus, which defies the classical role of GRs in the negative feedback of the HPA axis upon activation. Thus, an overabundance of GR activation within this region could be postulated to cause chronically elevated CORT levels and future anxiety related disorders.

Bed Nucleus of the Stria Terminalis

Despite direct projections to the PVN and heavy GR expression, the roles of GRs within the BNST are very poorly understood. Dunn demonstrated that electrical

stimulation of the lateral aspect of the BNST decreased plasma CORT levels, presumably through activation of GABAergic projections to the PVN (Dunn, 1987). Adrenalectomy decreases expression of CRF mRNA within the dorsolateral aspect of the BNST (dlBNST) and the CeA while increasing extracellular norepinephrine and DA in the dlBNST (Santibanez et al., 2005). Our group has recently shown that chronic stress or systemic CORT administration increases anxiety-like behavior in mice and blunts LTP within the dlBNST (Conrad et al., 2011). It has long been known that chronic treatment with CORT downregulates GR function within the brain (Spencer et al., 1991), leading to impaired negative feedback of the HPA axis. Thus, the inhibitory effect of CORT treatment on BNST plasticity may represent decreased GR function and reflect a role of GRs in the maintenance of excitability within the region. In support of this, specific deletion of BNST GRs using the floxed GR mice described above seems to exacerbate anxiety-like behavior in response to chronic stress as well decreasing locomotion in a stressful situation (EPM), mimicking the effects of chronic CORT administration described above (unpublished data). An important future study will examine alterations in excitability within the BNST of these mice, as well as the consequence of pharmacological manipulation of GRs on BNST excitability. It can be hypothesized that one role of GRs within the BNST is to maintain excitability in order to inhibit the HPA axis in response to a stressor. Thus, chronic stress exposure could downregulate GRs within the region and lead to hyperactivity of the HPA axis and associated conditions, such as anxiety-related disorders and addiction.

Conclusions

While GRs have been postulated to reduce excitability in other limbic regions such as the hippocampus (Chaouloff et al., 2008; Krugers et al., 2005; Wiegert et al., 2005), emerging literature seems to indicate that GRs in the extended amygdala strengthen excitability. GRs in this region likely mediate appropriate response to stressful events in healthy individuals, but disregulation of GRs, through drug addiction or chronic stress for example, may place these receptors in a more sinister role. For instance, GR-mediated enhancement of glutamate response and extracellular dopamine in the NAc might contribute to the salience of natural rewards under normal circumstances, but may lead to stress-induced relapse to addiction in individuals with altered extended amygdala circuitry as a result of previous drug use. Activation of CeA GRs causes anxiety-like responses and CORT release (Myers and Greenwood-Van Meerveld, 2007; Weiser et al., 2010), whereas selective deletion of CeA GRs appears to alleviate fear-like behavior (Kolber et al., 2008). Thus, CeA GRs likely initiate an HPA response to a frightening stimulus by strengthening GABAergic projections to the BNST and disinbibiting the PVN. However, an individual with unusually high GR tone within the CeA would likely have a lowered threshold for fear-like responses, may be susceptible to anxiety disorders and addiction. Finally, GRs within the BNST may maintain excitability in order to inhibit the HPA axis following exposure to a stressor. However, in an individual that has undergone chronic stress, BNST GRs could be downregulated, and the ability of the BNST to inhibit the HPA axis would be impaired. This could result in anxiety disorders or stress-induced relapse to drug seeking, a behavior that is dependent upon the BNST (Briand et al., 2010). GRs within the extended amygdala appear to be important in mediating anxiety- or addiction-like responses to stress.

Extensive further study of the effects of GRs within the region on plasticity and anxiety- and addiction-like behaviors will prove crucial to complete understanding of such maladaptive responses to stress.

A.2: Region-delimited deletion of GRs within the BNST do not alter affective behavior in mice.

The paucity of literature examining GRs within the BNST led us to investigate the behavioral consequence of targeted deletion of these receptors within the region. We utilized a floxed GR mouse line harboring loxP sites around exon 2 of the *Nr3c1* gene (Brewer et al., 2003) and stereotaxic delivery of LV-Cre as described in Chapter 2. Immunohistochemical (IHC) analysis using dual labeling with NeuN (Chemicon, Millipore) and GR (Santa Cruz) antibodies on paraffin embedded sections (8μm) was performed and revealed significant reduction in GR protein in LV-Cre-injected vs. control animals (**Figure A.1**).

We wished to assess a number of behavioral outputs of GR knockdown with in the BNST, and to do so we utilized a battery of behaviors outlined in **Figure A.2**. First, basal anxiety- and/or depression-like alterations were assessed using the NIH task, as described in Chapter 2. We found no alterations in NIH latency between groups (P=0.8032). Because GRs are low affinity receptors for corticosterone, we were not altogether surprised by a lack of phenotype in the marginally stressful environment of the NIH novel test (bright lighting and novel cage). We believed, however, that BNSTGRKD mice might display differences from control virus injected GR-floxed mice during higher levels of stress. Thus,

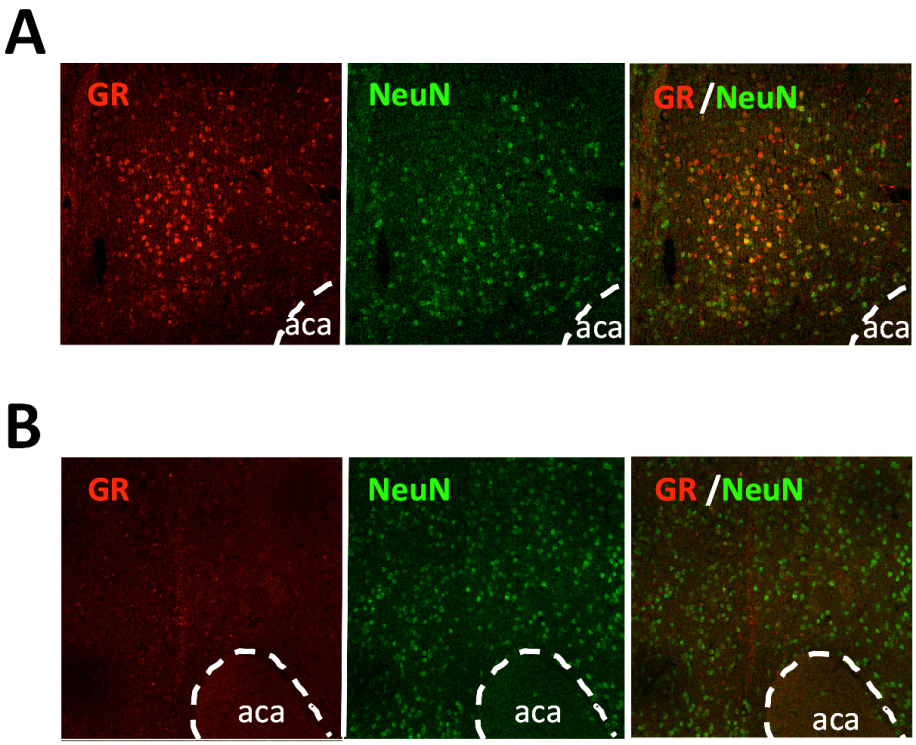


Figure A1. Significant reduction of GR protein expression in BNST of BNSTGRKD mice. Dual IHC labeling of GR (red) and NeuN (Green) reveals plentiful GR expression in the BNST of (A) GR-floxed control mice and (B) significant reduction in GR expression in GR-floxed mice with BNST-targeted stereotaxic injection of LV-Cre.

we utilized the FST as both a test of depression-like behavior as well as an acute stressor, and mice were subsequently tested in the elevated zero maze (EZM; similar in concept to the EPM described in Chapter 3). We saw no alterations in FST immobility time (P=0.6590) or time spent in the open arms of the EZM (P=0.2660). Finally, because the BNST has been implicated in responses to chronic – but not acute – stress, we postulated that a chronic stress paradigm may be required for affective alterations in BNSTGRKD mice. We used a chronic restraint stress paradigm in which mice were restrained for 1hr/day for 10 days in a 50mL conical tube with breathing holes. On the day after the last restraint session, mice were tested in the EPM. Although overall decreases in open arm time were observed following chronic stress, similar to previous assays, we saw no effect of BNSTGRKD on open arm time in the EPM (P=0.1108). These data were not unique, as we tested a total of 3 cohorts of animals following the paradigm outlined in **Figure 4.2** (only cohorts 2 and 3 are represented, as the first cohort utilized a different control, although results were similar).

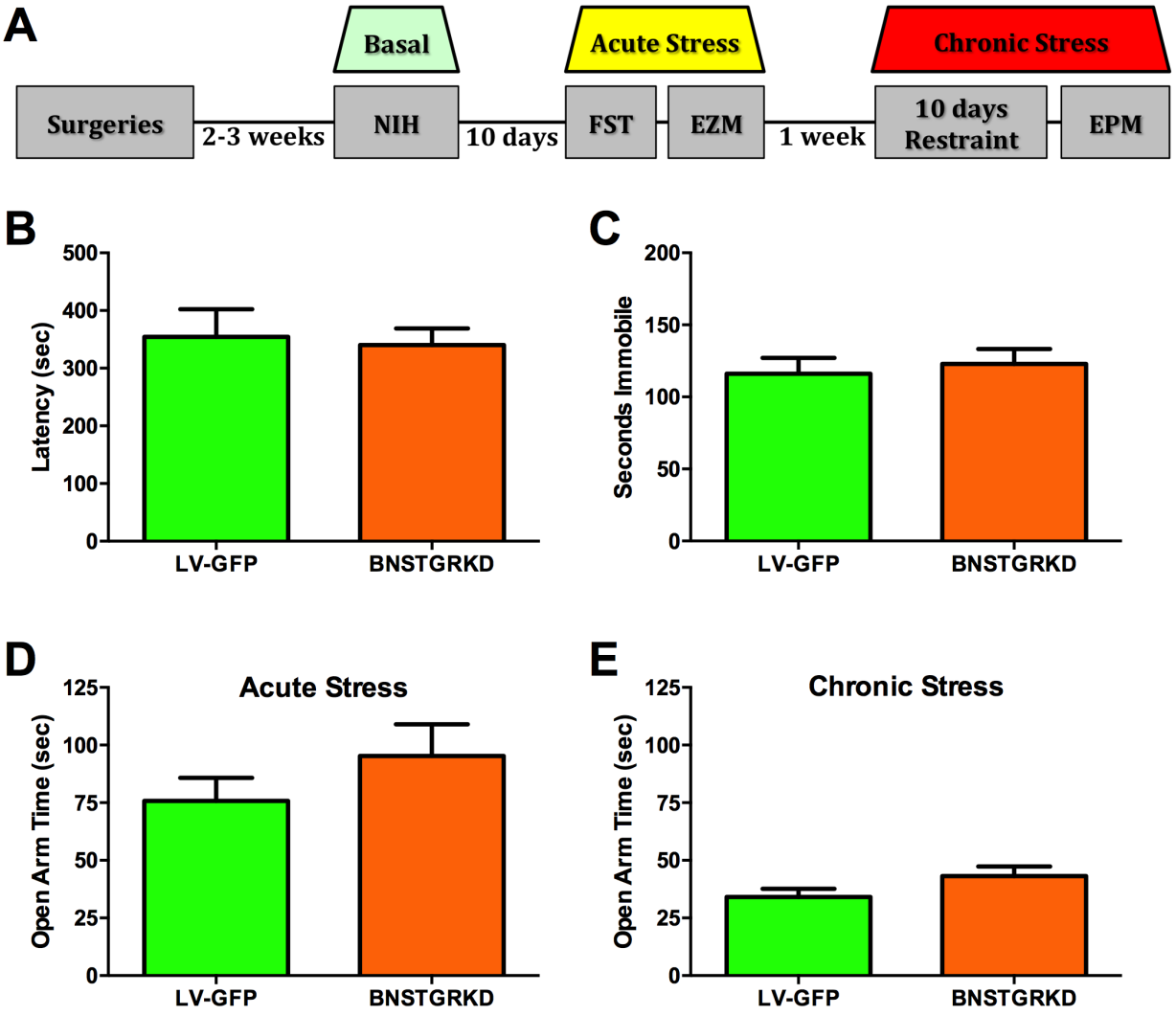


Figure A.2. GR knockdown in the BNST does not influence affective behavior. A) Timeline of experimentation. (B-E) Alterations in affective behavior were observed in a number of behavioral assays. (B) Neither latency in the NIH, nor (C) immobility time in the FST was altered, indicating no changes in depression-like behavior. Open arm time (D) measured in the EZM following acute forced swim stress and (E) in the EPM following 10 days of restraint stress were also unaltered, revealing no effect of BNST GR knockdown on anxiety-like behavior. N=11/group

APPENDIX B

Cocaine-Induced Anxiogenesis

B.1 Assessment of Acute Cocaine-induced Anxiogenesis using EPM

While intake of cocaine reportedly elicits excitation and euphoric mood, cocaine is also associated with anxiety and agitation, particularly after the initial rush of euphoria. Similarly, cocaine was shown to increase anxiety-like behaviors in mice when administered 20 minutes prior to testing (Yang et al., 1992). More recently, this behavior was shown to be absent in dopamine β-hydroxylase knockout mice (Schank et al., 2008). Heterozygous animals, however, showed significant cocaine-induced anxiogenesis that could be blocked by giving the β adrenergic receptor (β-AR) antagonist, propranolol, 10 minutes prior to cocaine injection.

We sought to replicate the findings from the Weinshenker lab in C57Bl/6J mice. In the initial run, we attempted to replicate the procedure exactly (**Figure B.1**). Mice were given an injection of either saline (vehicle and cocaine groups) or propranolol (5mg/kg; i.p.; propranolol group) 10 minutes prior to a second injection of either saline (vehicle, Veh, group) or cocaine (20mg/kg; i.p.; cocaine and propranolol, Coc+Prop, groups). Twenty minutes following this second injection, mice were tested in the EPM for open arm time and locomotion as described in Chapter 3. We found no effect of treatment on open arm time ($P>0.05$). We did, however, observe significantly increased distance traveled in either group receiving cocaine, typical of cocaine treatment.

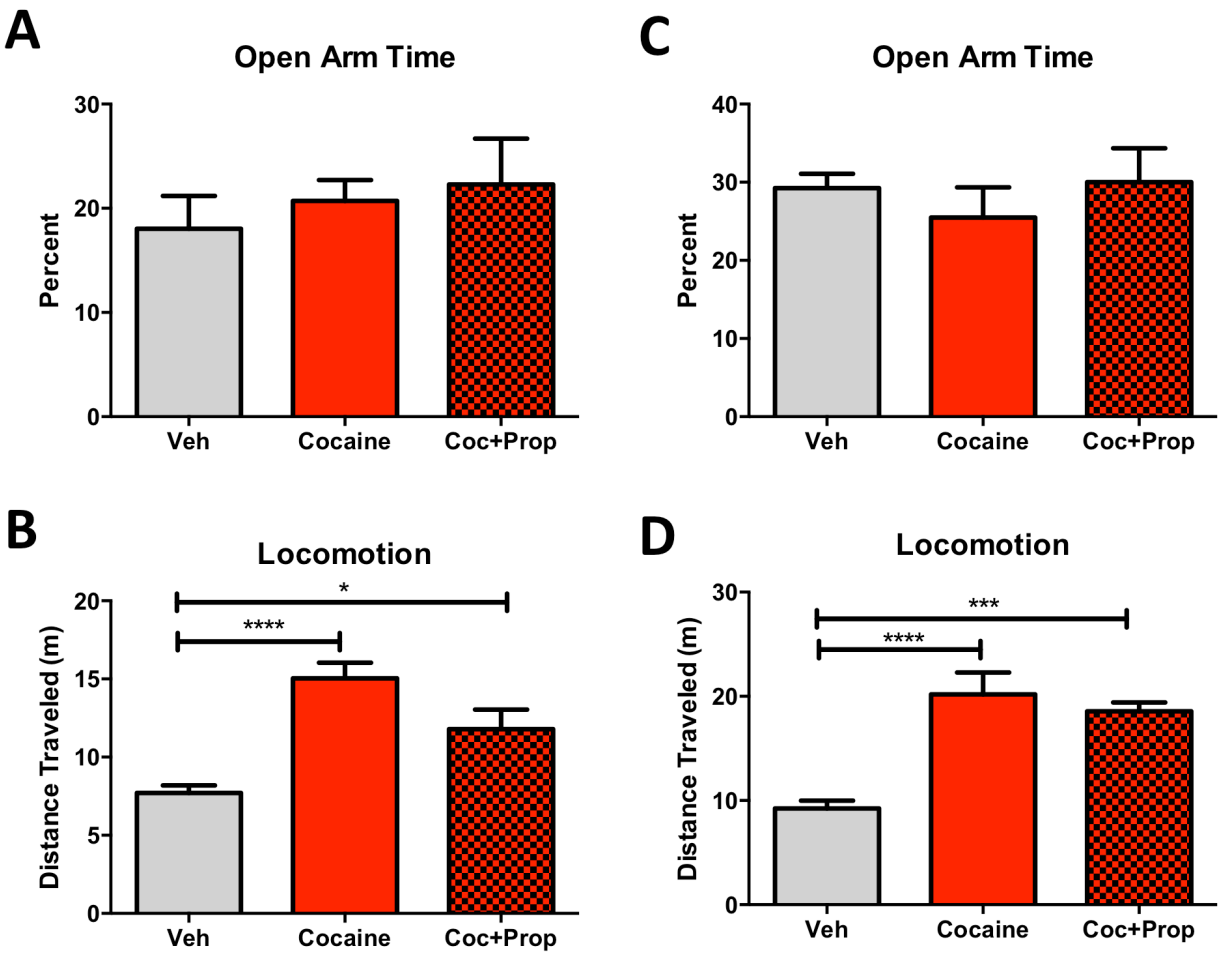


Figure B.1 Acute cocaine administration does not increase anxiety-like behavior in EPM. Mice were tested with (A, B) or without (C, D) handling prior to injection. (A, C) Neither cocaine (Coc; 20mg/kg; 20 min prior to test) nor pretreatment with propranolol (Prop; 5mg/kg; 10 min prior to cocaine) had an effect on open arm time in the EPM. (B, D) However, cocaine treatment was associated with significantly increased distance traveled. N=8/group. *P<0.05; ***P<0.001; ****P<0.0001.

A 5-day handling protocol prior to behavioral experimentation is standard for the majority of studies performed by the Winder lab. However, not all groups incorporate handling into their paradigms, and often – even if handling was performed – it is not included in the methods section in many manuscripts. Schank and colleagues did not explicitly state that they had handled their mice prior to behavioral testing, and we postulated that this could represent a discrepancy between their study and our own attempt. Thus, we repeated the experiment exactly as described above, only in this case handling procedures were omitted. **Figure B.1C** and **B.1D** show that we found identical negative results whether mice were handled or not.

Next, we hoped to elicit a cocaine-induced anxiogenic phenotype by administering chronic cocaine, as had been demonstrated previously (Yang et al., 1992). I administered cocaine (20mg/kg) or saline daily for 10 days (as illustrated in the timeline in **Figure B.2**). On day 10, half of the cocaine mice received propranolol (5mg/kg), and the other cocaine- and saline-treated mice received saline 10 minutes before cocaine/saline. On day 11 mice were tested on the EPM ~20hrs following their last cocaine dosage. No effect on open arm time or distance traveled was observed. These mice were then used in the NIH task. ~20-22hrs prior to testing each day, mice received cocaine or saline with propranolol or saline pre-treatment. No effect on NIH latency or consumption on the test day was observed.

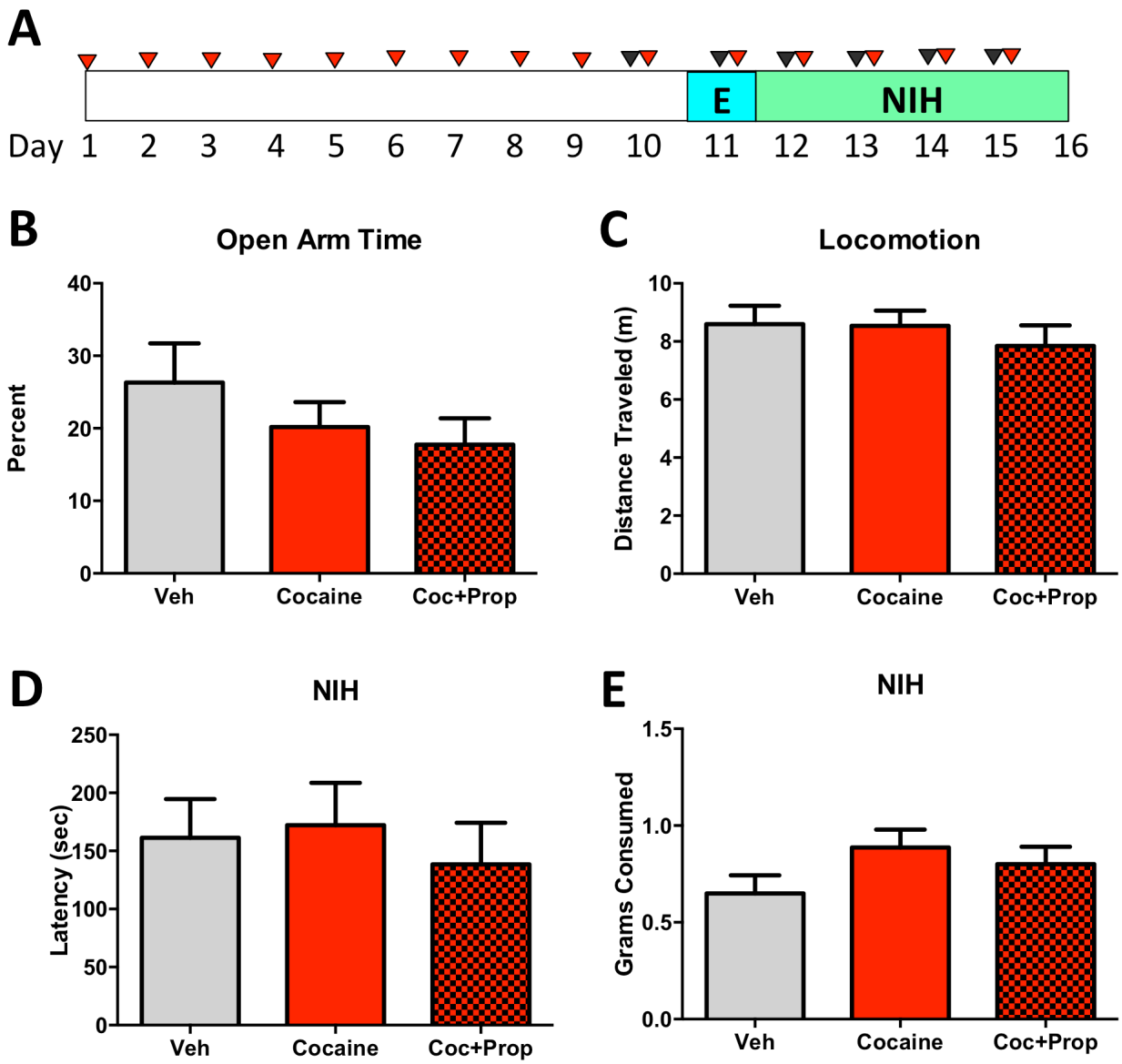


Figure B.2. Chronic cocaine treatment had no effect on anxiety-like behavior in the EPM or NIH tests. (A) Timeline of treatment. Mice were treated with cocaine (20mg/kg) or saline (red arrows) daily for 15 days, and pretreated with propranolol (5mg/kg) or saline (black arrows) on days 10-15. Injections were given 20-22hrs prior to behavioral testing. Mice were tested on the EPM (E) on day 11, and underwent the NIH procedure on days 12-16, with novel testing on day 16. (B) No effects on open arm time or (C) locomotion were observed in the EPM. (D) Similarly, no effect of either treatment was observed on latency or (E) consumption in the NIH novel cage test.

APPENDIX C

Validation of GluN2B Knockdown in BNST following LV-Cre Injection

C.1 Western Blot Analysis of GluN2B knockdown in BNST

IHC is notoriously difficult for membrane-bound proteins, including NMDAR, and so we therefore sought to determine deletion efficiency in our BNSTGluN2BKD mice using a western blot approach to measure protein levels as previously shown (Kash et al., 2009). Similar methodology demonstrated an approximately 80% reduction in GluN2B protein in the BNST in forebrain-wide GluN2B knockout mice using the same *grin2b* floxed line we used for regional targeting (Wills et al., 2012). We obtained tissue punches (0.35mm diameter) of the dlBNST from 300μm thick coronal sections. Tissue was homogenized and protein content was assessed via NanoDrop. 5μg protein was run in each lane, and GluN2B protein levels were normalized to PSD-95 levels. Membranes were blotted for GluN2B and PSD95 simultaneously using two channels on the Odyssey CLx system. Figure B.1 shows results of western blot analysis. No significant differences in GluN2B protein levels were determined (**Figure C.1**), although this does not necessarily indicate that knockdown was not achieved in the BNST. The lentiviral Cre recombinase used does not contain a fluorescent reporter protein. Thus, we could not target BNST punches to areas confirmed to be infected, and we could not confirm successful targeting/viral delivery in LV-Cre treated animals (although, LV-GFP treated animals showed consistetn BNST labeling, demonstrating that our stereotaxic coordinates were correct).

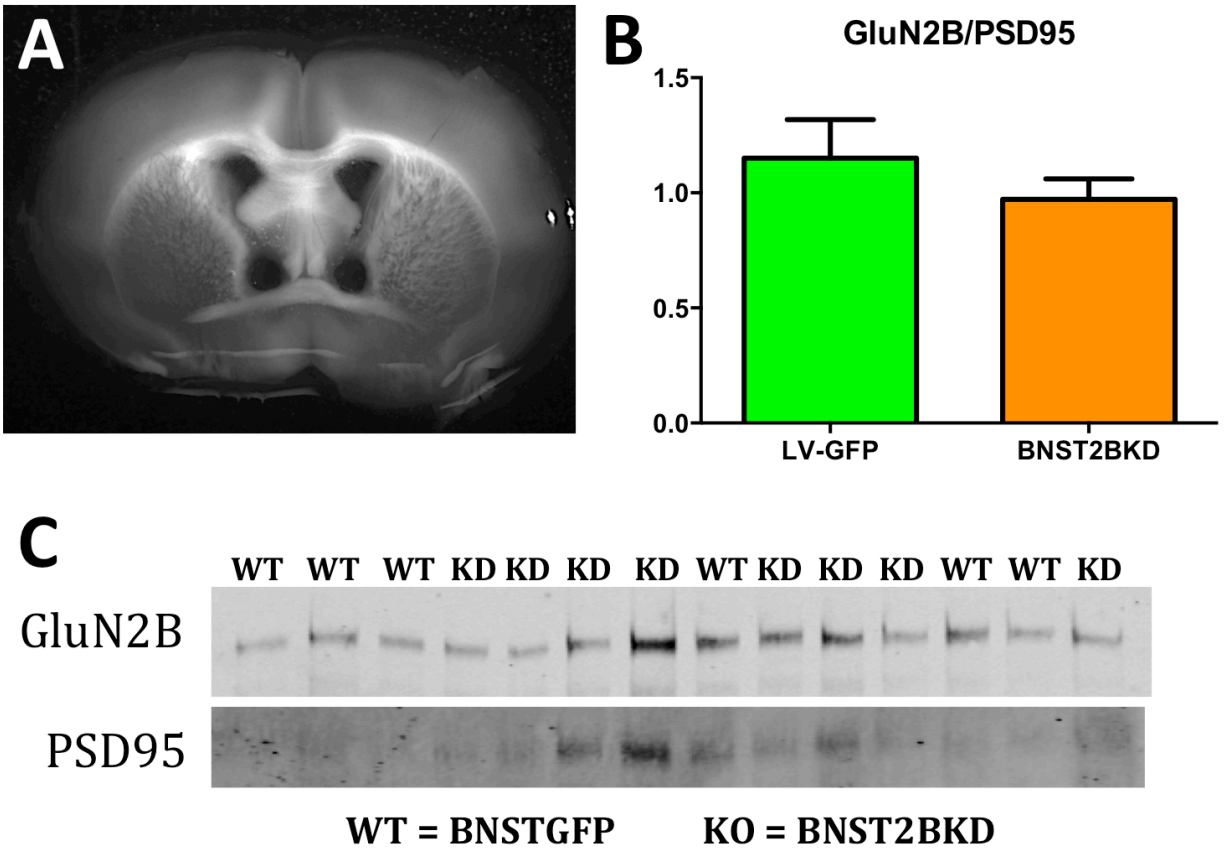


Figure C.1. Assessment of GluN2B protein level within the BNST in BNSTGluN2BKD versus control mice. (A) Representative image showing tissue punches (0.35mm diameter from 0.30mm thick slices) of BNST collected from coronal section from BNSTGluN2BKD (N=8) and control (N=6) mice. (B) GluN2B expression in BNST normalized to PSD95 expression. (C) Image of blot for GluN2B and PSD-95.

C.2 Other Approaches to GluN2B Analysis in BNST

In an attempt to determine anatomical localization of GluN2B expression in BNSTGluN2BKD and control mice, we turned to *in situ* hybridization (ISH) to measure mRNA expression within the BNST. This method is advantageous over immunoblotting because it allows regional examination of expression in slices, rather than expression in a tissue punch that might not accurately reflect location of viral injection. We probed for GluN2B in wild type mice. We also probed for the D_2 dopamine receptor as a positive control. Despite trying two separate GluN2B probes, we were unable to detect GluN2B transcripts using ISH (**Figure C.2**).

We were able to detect GluN2B transcripts in wild type tissue via qRT-PCR (methodology described in greater detail in Appendix D). However, this method inherently has the same caveats as immunoblotting described above. Tissue is acquired by micropunch, and without a fluorescent reporter in the viral construct, we cannot be certain that tissue collected was successfully targeted by the virus.

In order to circumvent these problems in the future, a viral construct containing a fluorescent reporter will be crucial. As alluded to in Chapter 4, we did attempt to use such a virus. We examined both AAV1 and AAV9 serotypes for infection ability and targeting measured by GFP expression and for Cre expression by injecting these viruses into the BNST of a Rosa26^{tomato} reporter line (B6.Cg-Gt(ROSA)26Sor<tm14(CAG-tdTomato)Hze>/J) and examining red tomato fluorescence (**Figure C.3**). One mouse was used per viral construct, and sections were taken for fluorescent expression evaluation ~4 weeks after surgery was performed. Both GFP and GFP-Cre fusion constructs with the AAV1 serotype displayed weak labeling with GFP. Additionally, though it appears that there is strong red

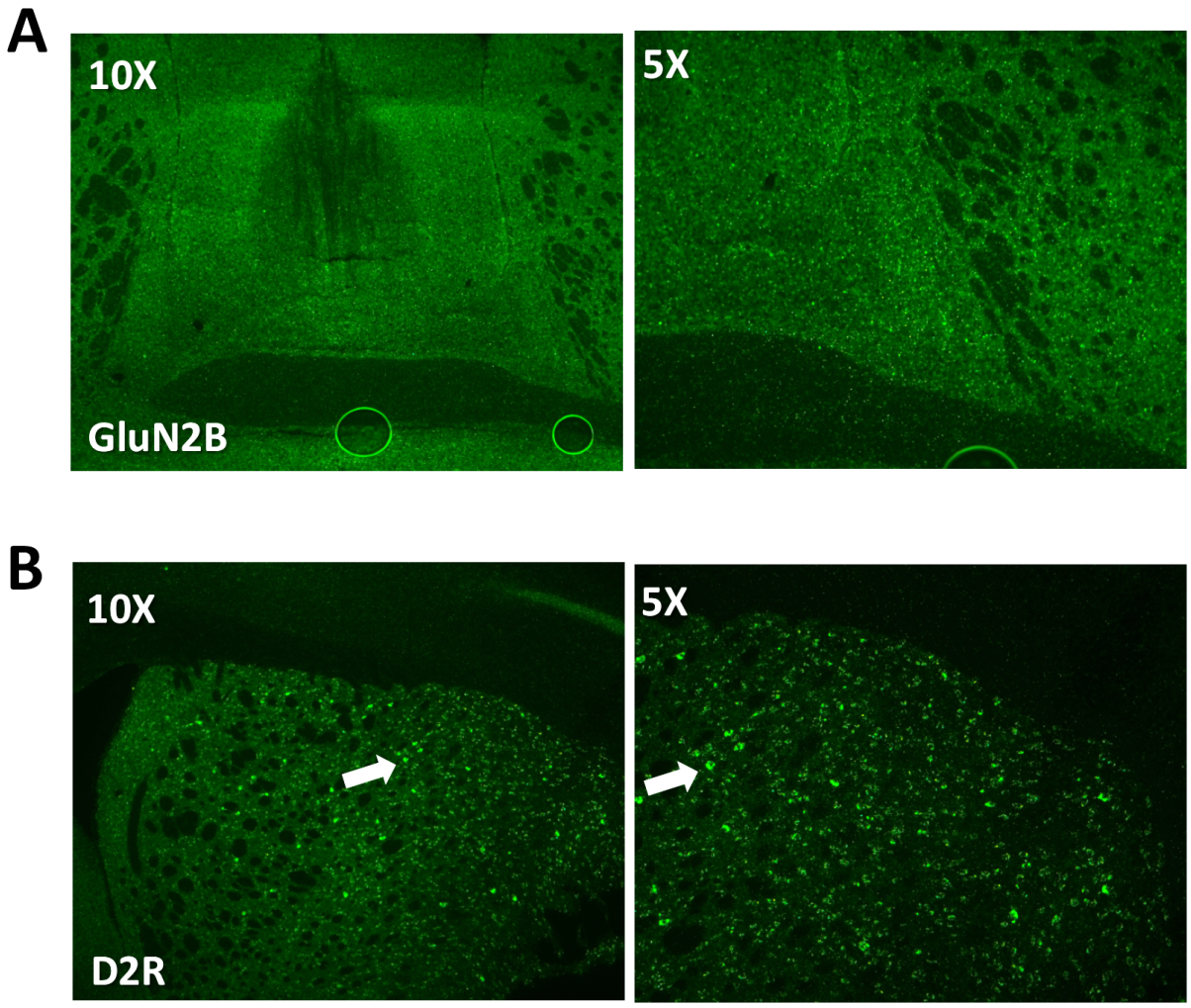


Figure C.2. *In situ* hybridization of GluN2B in BNST and D_2R in striatum. (A) GluN2B antisense probe in the BNST failed to clearly label GluN2B positive neurons. (B) By comparison, D_2R-expressing neurons can clearly be observed in the striatum at both high and low magnification (arrows indicate cell with clear labeling).

tomato fluorescence observable with AAV1-GFP-Cre, this actually appears to be bleedover from the GFP fluorescence (images in **Figure C.3** are false-colored). AAV9 appeared to have both strong GFP and tomato fluorescence, and because of this, we utilized this virus in the two-bottle choice experiment described in Chapter 4. However, anatomical abnormalities are apparent in this image (including enlarged ventricles and tissue degeneration) that were initially overlooked as only a single mouse was examined. These neurotoxic effects seem to be a hallmark of AAV9-GFP-Cre injections into the BNST, and therefore this virus cannot be used in future studies.

We have high expectations for upcoming experiments utilizing a GFP-Cre fusion protein using either AAV2 or AAV5 serotypes, as these have not been shown to cause damage to tissue. Additionally, our lab has been very successful in using a new IHC technique, RNAscope (as described in Appendix E), and we believe that this tool will be imperative in determining alterations in mRNA transcript expression in a variety of potential experiments.

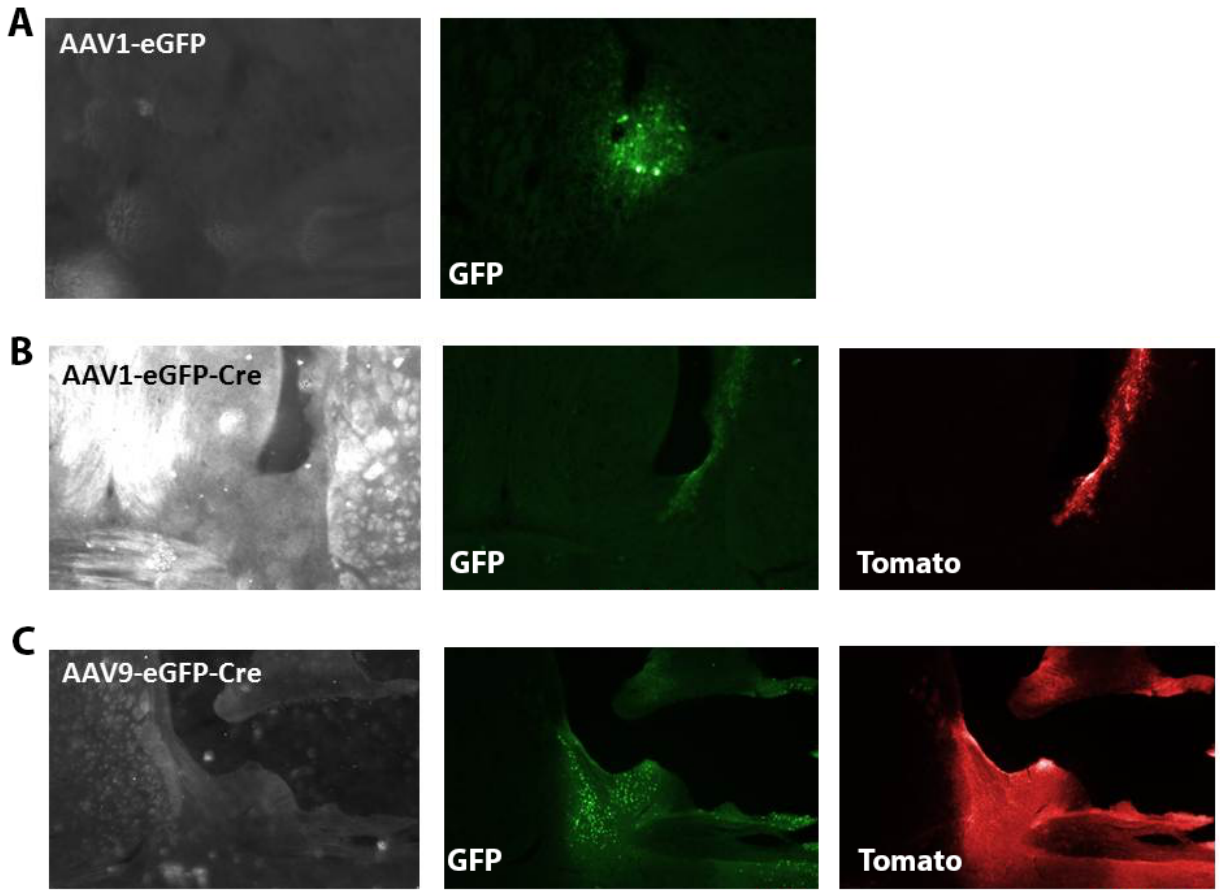


Figure C.3. GFP and red tomato fluorescence in BNST of Rosa26^{tomato} mice following stereotaxic delivery of AAV1-eGFP, AAV1-eGFP-Cre, or AAV9-eGFP-Cre. (A) AAV1-eGFP and (B) AAV1-eGFP-Cre showed rather weak GFP expression. (C) AAV9-eGFP-Cre showed both GFP and red tomato fluorescence, but tissue damage can be observed near Cre-infected sites (red). GFP and Tomato panels are false-colored.

APPENDIX D

D.1: Assessment of the Impact of Pattern of Cocaine Dosing Schedule During Conditioning and Reconditioning on Magnitude of Cocaine CPP, Extinction, and Reinstatement.

Kelly L. Conrad[1,#], Katherine M. Louderback[1,2,#], Elana J. Epstein[1] and Danny G. Winder[1,2,3]
[#]These authors contributed equally to this work.

Abstract

Rationale and Objective: We sought to examine the impact of differing cocaine administration schedules and dosing on the magnitude of cocaine conditioned place preference (CPP), extinction, and stress- and cocaine-induced reinstatement of CPP.
Methods: First, in C57Bl/6J mice, we investigated whether total cocaine administration or pattern of drug exposure could influence the magnitude of cocaine CPP by conditioning mice with a fixed- low dose (F_L; 7.5 mg/kg; 30 mg/kg total), a fixed- high dose (F_H; 16 mg/kg; 64 mg/kg total), or an ascending dosing schedule (Asc; 2, 4, 8, and 16 mg/kg; 30 mg/kg). Next, we investigated if cocaine or saline is more effective at extinguishing preference by reconditioning mice with either a descending dosing schedule (Desc; 8, 4, 2, and 1 mg/kg) or saline. Finally, we examined if prior conditioning and re-conditioning history alters stress (~2-3 minutes forced swim test) or cocaine (3.5 mg/kg)-induced and reinstatement.
Results: We replicated and extended findings by Itzhak and Anderson (2011) demonstrating Asc conditioning produces a greater CPP than either the F_L or F_H conditioning schedules. The magnitude of extinction expressed was similar in the Desc reconditioned and saline groups. Moreover, only the saline, and not the Desc reconditioned mice, showed stress and cocaine-induced reinstatement of CPP.
Conclusions: Our results suggest that the schedule of cocaine administration during conditioning and reconditioning can have a significant influence on the magnitude of CPP and extinction of preference, and the ability of cocaine or a stressor to reinstate CPP.

Adapted from: Conrad KL & Louderback KM, et al. Assessment of the Impact of Pattern of Cocaine Dosing Schedule During Conditioning and Reconditioning on Magnitude of Cocaine CPP, Extinction, and Reinstatement. Psychopharmacology (Berl). 10.1007/s00213-012-2944-1 (2013).

Introduction

Conditioned reward studies routinely involve associating a distinct environment with a reward by using a fixed daily dose of a drug during training. However, a fixed pattern of drug administration does not mimic the progressive human pattern of drug intake frequently found in the clinical literature (Gawin, 1991) and may fail to engage the same neural substrates and learning and memory processes (Ahmed et al., 2002; Ferrario et al., 2005; Koob and Kreek, 2007; Porrino et al., 2002; Smirnov and Kiyatkin, 2010; Vanderschuren and Everitt, 2004; Zhou et al., 2011).

The conditioned place preference (CPP) paradigm has been increasingly used over the past few decades and is now one of the most commonly used behavioral reward models (Tzschentke, 2007). CPP is a robust non-contingent model of drug reward and is frequently used to examine the motivational and conditioned rewarding properties of drugs of abuse. An important recent study by Itzhak and Anderson (2011) demonstrated that conditioning mice with one of two ascending dose schedules of cocaine led to a more robust expression of cocaine CPP than a fixed high dose of cocaine. Interestingly, the total cocaine administered was lower in the ascending groups (45 or 30mg/kg total) than in the fixed-high dose group (64mg/kg total). These findings suggest that the pattern, rather than the total cocaine administered, may be of primary importance in establishing a reliable animal model that replicates the increased intake over time reported in the clinical literature (Angarita et al., 2010). However, it is possible that the 16mg/kg dose used in that study was not optimal and a lower fixed dose of cocaine would have produced a more robust CPP (Mantsch et al., 2010). Moreover, the Itzhak and Anderson (2011) study also demonstrated that the pattern of drug administration – although in a descending dose pattern - may be

similarly important during extinction training in providing resistance to subsequent cocaine-induced reinstatement of cocaine CPP.

Here we have replicated and extended key findings of the Itzhak and Anderson (2011) work. First, we replicated their findings in B6:129S F2 hybrid mice using C57BL/6J mice, demonstrating the ability of this dosage schedule to be relevant for inbred lines. Next, we extended their findings by demonstrating that an ascending schedule of cocaine conditioning produced a greater magnitude of cocaine CPP than a low-fixed dose cocaine administration schedule that resulted in the same overall level of cocaine exposure. Finally, we examined the ability of reconditioning with saline or a descending dose of cocaine to extinguish cocaine CPP. Our results are consistent with the previous report finding that a descending dose of cocaine is as effective as saline at extinguishing preference for the cocaine-paired context. However, reconditioning training with a descending dose of cocaine offered resistance to subsequent cocaine priming and stress-induced reinstatement of cocaine CPP.

Methods

Animals

Experiments were conducted on male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 8–12 weeks and housed 2-5/cage with *ad libitum* access to food and water. Testing commenced at least 1 week after acclimation to the facilities. All procedures were approved by the Vanderbilt University Animal Care and Use Committees and were in accordance with the Animal Welfare Act. The number of mice used is reported in the figure legends.

Place Conditioning Apparatus

The place conditioning apparatus used here was described in detail previously (Davis et al., 2008). Briefly, the two chamber place conditioning apparatus consists of a box insert that slides into the open field chamber (Med Associates; St. Albans, VT). The two compartments of the conditioning box were separated by a single removable guillotine door. One chamber has metal mesh flooring, which was always paired with saline, and the other chamber has bar flooring which was always paired with cocaine (Med Associates inc., product number ENV-3013-2). Each chamber was equipped with horizontal sensors and a 4cm null zone on each side of the guillotine door was used to increase the likelihood of mice being fully within one compartment when their presence was counted. Med Associates Activity Monitor software was used to track and score time spent in each compartment and distance traveled.

General Conditioning Procedures

All conditioning and testing was carried out in a dedicated CPP test room (Vanderbilt Murine Neurobehavior core: http://kc.vanderbilt.edu/mnlcore/) located in the same facility as the mouse housing room. Mice were kept on our regular animal facility light-cycle (lights on at 06:00 and lights off at 18:00). Mice were exposed to the CPP chamber for 20 minutes in all sessions. All conditioning days had 2 sessions/day, Session I was conducted between 10:00-12:00 Session II was conducted between 14:00-16:00. Saline was always given in Session I, unless otherwise noted, and saline-injected mice were always restricted to exploration of the mesh-flooring compartment (guillotine door in

place). Cocaine was always administered in Session II and mice were restricted to exploration of the bar compartment (guillotine door in place). All mice were tested (described below) and allowed to free explore (described below) in a drug-free state, with unrestricted access to both compartments, and the were given between 12:00 and 14:00 to prevent prior experience with a drug given at the same time during the day on previous conditioning sessions from providing a temporal cue and unduly influencing behavior on a particular test. Previous testing by our group on this particular place conditioning apparatus has reliably produced a small but significant innate bias for the mesh flooring, however mice expressing a preference of greater than 33% for one side on the pre-test were removed from the study (1/48 miceBecause the lesser preferred bar flooring was always paired with cocaine,mice that express cocaine CPP were thus overcoming this small innate preference.

The schedule is shown in **Figure D.1**. On day 1, mice habituated to the chamber during a Pre-test in which access to both compartments is unrestricted (no guillotine door in place). Conditioning occurs on days 2-5, and two sessions are held on each of the four conditioning days (eight conditioning sessions total). Saline and cocaine were administered intraperitoneally (i.p.). All injections were given immediately prior to placing the animal in the conditioning chamber. At the end of each session, the chamber was thoroughly cleaned with 70% EtOH and detergent supplied by the mouse neurobehavioral core.

During conditioning Session II on days 2-5, mice were evenly divided based on pre-test preference data and placed into one of 3 groups that differed in cocaine schedules: fixed (low) dose- (F_L; 7.5 mg/kg/day for 4 days), n=16, 30 mg/kg total intake; fixed (high) dose- (F_H; 16 mg/kg/day for 4 days), n=15, 64 mg/kg total intake; or ascending dose (Asc;

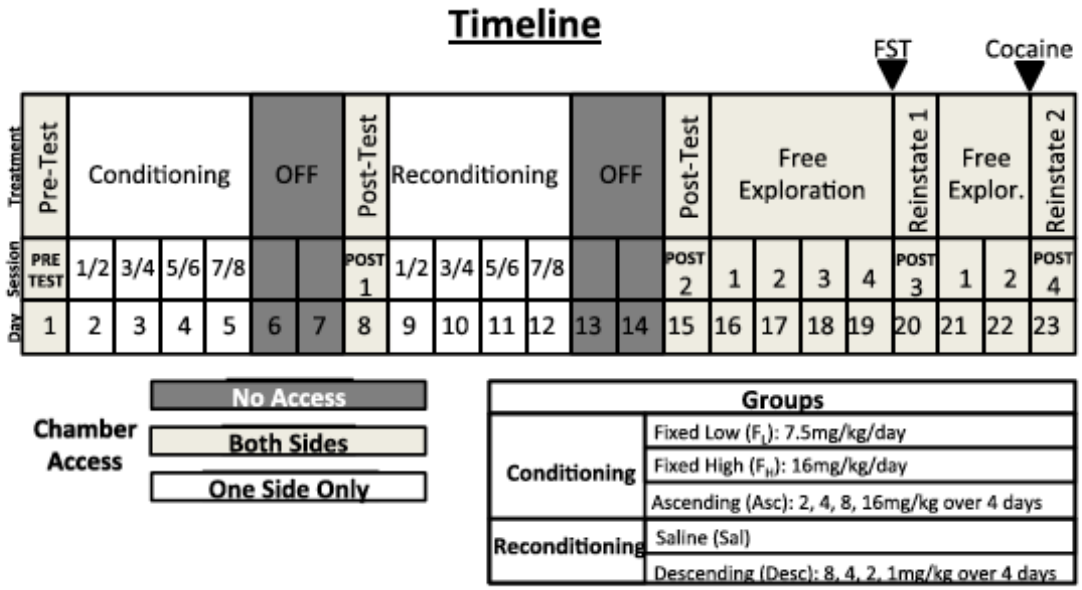


Figure D.1: Timeline of CPP paradigm*:* All testing days (Pre-test and Post-tests) were carried out at 12:00-14:00. 'Conditioning' represents days 2-5, with two sessions per day (morning session, saline, 10:00-12:00; afternoon session, cocaine, 14:00-16:00) during which mice were assigned to one of three groups (F_L, F_H, or Asc; described in the Groups box). 'Off' represents time in which mice are left undisturbed in home cages in housing facility, and are shown in dark grey in the timeline. 'Post 1' represents post-test 1 in which mice were tested for initial preference for the cocaine-paired side.; 'Reconditioning' represents days 9-12 during which two sessions of training was held per day (morning session, saline, 10:00-12:00; afternoon session, cocaine or saline, 14:00-16:00) and mice were assigned to one of two groups (Sal or Desc; described in the Groups box). 'Post 2' represents post-test 2 in which mice were tested for preference for the cocaine-paired side following reconditioning. 'Free-exploration' represents extinction sessions in which no injections were given (12:00-14:00). Stress administration/priming injections are denoted with arrows above treatment line. 'FST' represents administration of the ~2-3 minute forced swim stress, and 'Cocaine' represents the 3.5 mg/kg priming injection of cocaine. 'Reinstate 1' and 'Reinstate 2' represents the stress-induced (FST) reinstatement test (Post 3); and the cocaine-induced reinstatement (Post 4; both administered 12:00-14:00). Restricted access to either the mesh or bar chamber is shown in white. Unrestricted access to both compartments of the chamber, as occurs during testing and free exploration days, is shown in light grey. The 'Groups' box describes the schedule of conditioning and reconditioning.

2 mg/kg on day 2; 4 mg/kg on day 3; 8 mg/kg on day 4; and 16 mg/kg on day 5), n=15 30 mg/kg total intake.

The F_H dose was chosen for our study based on previous work showing that this dose produced significant cocaine CPP (Itzhak and Anderson, 2011) and 16 mg/kg was also the highest dose used on the Asc dose schedule. The F_L dose was chosen based on studies showing that maximal cocaine CPP in C57Bl/6J mice is achieved using a fixed cocaine dose <15 mg/kg cocaine (Mantsch et al., 2010; Miner, 1997; Schindler et al., 2010). Further, this F_L schedule using 7.5 mg/kg dose allowed us to investigate, in addition to schedule of reinforcement, whether total amount of cocaine administered (30 mg/kg in both F_L and Asc groups) is a contributing factor in the magnitude of observed cocaine CPP. Finally, this Asc schedule was based on previous work showing that this schedule and higher Asc dosages of 3, 6, 12, and 24 mg/kg produced cocaine CPP of comparable magnitude (Itzhak and Anderson, 2011).

Expression of Cocaine CPP

On day 8, ~72 hours after the last conditioning session, mice were tested for the expression of cocaine CPP (Post-test 1). During the post-test, mice were treated as they were for the pre-test with unrestricted access to both compartments and no injection given. Mice were placed on mesh side at start of test, and locomotor activity and time spent in the cocaine- and saline- paired chambers were recorded.

Extinction Procedures

Reconditioning

On days 9-12 (**Figure D.1**), all mice were divided into two reconditioning groups, descending (Desc) and saline (Sal), with six subgroups. The first reconditioning group was exposed to a descending schedule of cocaine dosing (Desc; 8 mg/kg on day 9; 4 mg/kg on day 10; 2 mg/kg on day 11; and 1 mg/kg on day 12), resulting in 3 descending subgroups based on prior conditioning history: fixed-low (7.5 mg/kg) dose-descending (F_L-Desc) subgroup, fixed-high (16 mg/kg) dose-descending (F_H-Desc) subgroup, and ascending (2, 4, 8, 16 mg/kg) –descending (Asc-Desc) subgroup. The second group of mice was reconditioned with saline, resulting in 3 additional reconditioning subgroups based on prior conditioning history fixed-low (7.5 mg/kg) dose -saline (F_L-Sal) subgroup, fixed-high (16 mg/kg) dose –saline (F_H-Sal) subgroup, and an ascending (2, 4, 8, 16 mg/kg)-saline (Asc-Sal) subgroup. As with the first round of conditioning, all mice were exposed both to a morning (Session I; saline) and afternoon (Session II; descending cocaine schedule or saline) reconditioning sessions, for a total of 8 sessions over 4 days.

Post-test 2 (Following Reconditioning)

On day 15, ~72 hours after the last reconditioning session, all mice were re-tested for expression of cocaine CPP, in a manner identical to that described in the Expression of Cocaine CPP section (above).

Reinstatement Tests

Free Exploration

To ensure extinction of cocaine CPP, mice were exposed to two rounds of free exploration sessions (extinction sessions) with one session per day on days 16-19 and again on days 21 and 22. During free exploration, mice were given access to both sides of the chamber and no injection was administered. Such sessions provided additional data on the rate of extinction. Similar to 'test' sessions, locomotor activity and time spent in the cocaine- and saline-paired chambers were recorded.

Stress-induced Reinstatement

On day 20, ~24 hours after the final session of the first round of free exploration, mice were exposed to a ~2-3 minutes forced swim (FS) in warm (22-28^{0}C) water. At the end of the FST, mice were towel dried and placed immediately into the mesh-side of the chamber, with access to both compartments (post-test3). Locomotor activity and time spent on the cocaine- and saline-paired sides were recorded.

Cocaine-induced Reinstatement

On day 23, ~24 hours after the final session of the second round of free exploration, mice were administered a 3.5 mg/kg 'priming' dose of cocaine and immediately placed into the mesh side of the chamber, with access to both compartments (post-test 4). Locomotor activity and time spent on the cocaine- and saline-paired sides were recorded.

Statistical Analysis

When determining whether a cocaine-dosing schedule had any significant effect on the expression of CPP, a two-way Analysis of Variance (ANOVA) was used [schedule (F_L, F_H, Asc) x test (pre, post)]. A two-way repeated measures ANOVA was used to determine if reconditioning had an effect on the magnitude of extinction [schedule history (F_L-Sal, F_L-Desc, F_H-Sal, F_H-Desc Asc-Sal, and Asc-Desc) x test [data analyzed as extinction ratio (x test/post-test 1, where x represents: post-test 2, free exploration day 1, and free exploration day 3)]. A three-way ANOVA was used to determine if prior schedule history had an effect on stress-induced reinstatement [conditioning schedule (F_L, –F_H, or Asc) x reconditioning schedule (Sal or Desc)x test (post-test 2, last day of free exploration or stress reinstatement)]. To determine if previous schedule history had an effect on cocaine-induced reinstatement, a three-way ANOVA was used [conditioning schedule (F_L, F_H, or Asc) x reconditioning schedule (Sal or Desc) x test (last day of free exploration or cocaine reinstatement)]. In all experiments, locomotor activity was also analyzed using a one-way ANOVA. A P value < 0.05 was considered significant. When an interaction effect was significant, Bonferroni (for two-way ANOVA) or Student Newman-Keuls (SNK; for three-way ANOVA) *post hoc* tests were performed. All statistical analyses were performed using Prism GraphPad v.6.0.

Results

Experiment 1: Conditioning with an ascending dosing schedule results in a higher magnitude of cocaine CPP than conditioning with fixed dosage schedules.

This experiment was designed to investigate how different schedules of cocaine and total intake influence the magnitude of CPP. The magnitude of CPP was calculated as the total

amount of seconds spent on the cocaine-paired side. No differences were found on locomotor activity between groups (data not shown). The magnitude of CPP (seconds spent on the cocaine-paired side), as the dependent variable, showed significant differences between the groups on pre-test compared to post-test 1 [$F(1,44)$ = 66.24, $P < 0.05$; **Figure D.2**] and an interaction of test and treatment [$F(2,43)$ = 3.02, $P < 0.05$; **Figure D.2**]. A Bonferroni *post hoc* analysis revealed that all three groups showed a significant increase in time-spent on the cocaine-paired side on the preference test (post-test 1), suggesting all three dosing schedules effectively establish cocaine CPP ($P < 0.05$). However, the magnitude of CPP expressed on post-test 1 in the ascending dose (Asc) group (838 ± 21 seconds) was significantly higher than that of the fixed low (F_L) group (746 ± 18 seconds; $P < 0.05$) and the fixed high (F_H) group (731 ± 25 seconds; $P < 0.05$). No significant differences were observed between the F_L and F_H groups.

Our results also suggest that total cocaine dosing was not a factor. By the completion of the 4 conditioning sessions, the Asc group and the F_L groups had both received 30 mg/kg cocaine, yet the magnitude of preference expressed in the Asc group was significantly higher than the F_L group. Likewise, the F_H group received more than twice as much cocaine (total intake = 64 mg/kg) compared to the F_L group (total intake = 30 mg/kg), yet the magnitude of preference expressed was not significantly different between the two fixed dose groups. These findings suggest that the conditioning pattern was more important than the total amount of cocaine administered.

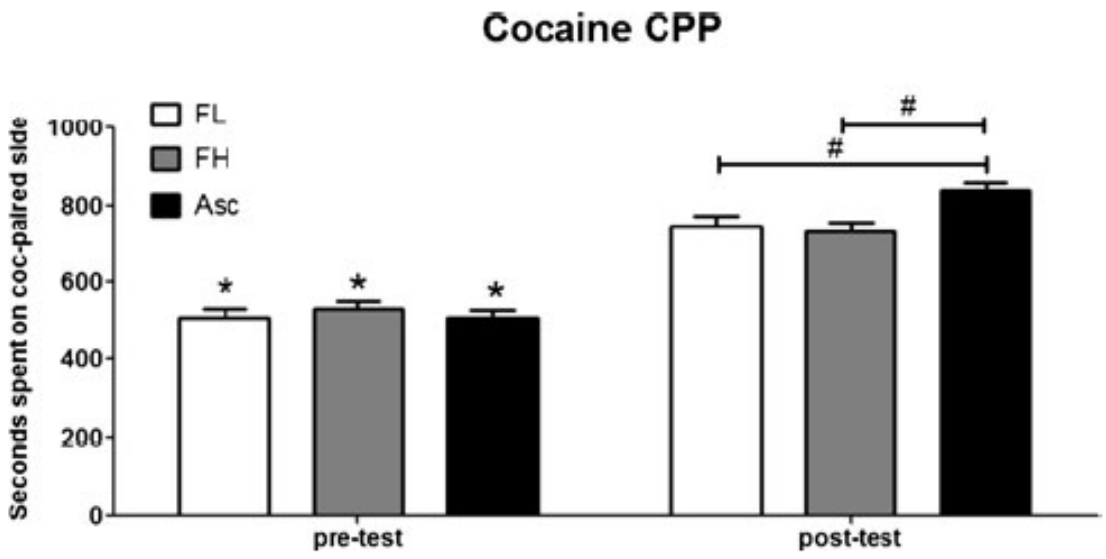


Figure D.2: Mice in the Ascending dosing schedule display a higher magnitude of cocaine conditioned place preference (cocaine CPP) compared to the F_L or F_H groups. All 3 groups showed a significant (*p < 0.05 when difference is within group) increase in time (seconds) spent on the cocaine-paired side on the post-test compared to pre-test. The Ascending (Asc) group showed a significantly higher magnitude of cocaine CPP (#p < 0.05 when difference is between groups) compared to the fixed low (F_L) and fixed high (F_H) groups. Data are represented as mean ± SEM, *p > 0.05, n = 15-16 mice per group.

Experiment 2: Reconditioning with either a descending cocaine dosing schedule or saline results in a similar magnitude of extinction.

This experiment was designed to investigate whether reconditioning mice with a descending schedule of cocaine following CPP induction would be as effective as traditional extinction training in which saline is used to extinguish cocaine CPP. No differences were found on locomotor activity between any of the groups (data not shown) The magnitude of extinction (x test/ post-test 1) was calculated as the ratio of post-test 1 cocaine-side preference remaining on post-test 2 (post-reconditioning), on free exploration day 1, or free exploration day 2. A two-way repeated measure ANOVA [conditioning history x time; repeated measures: post-test 1 (preference test), post-test 2, free exploration day 1, and free exploration day 2] was employed to investigate statistical differences. The outcome of extinction training on preference expressed (ratio of seconds spent on the cocaine-paired side during x test/ post-test 1), as the dependent variable, showed significant differences in time [$F(3,59)$ = 32.40, $P < 0.05$ for all days and conditions, **Figure D.3**] but not treatment, and no significant interaction was observed. However, reconditioning with the descending (8, 4, 2, 1 mg/kg) cocaine dosing schedule was equally effective as saline at extinguishing preference (**Figure D.3**) on the initial extinction test, as no significant differences were observed between extinction training conditions in any of the subgroups examined [F_L-Sal , F_H-Sal, Asc-Sal, F_L-Desc, F_H-Desc, and Asc-Desc)].

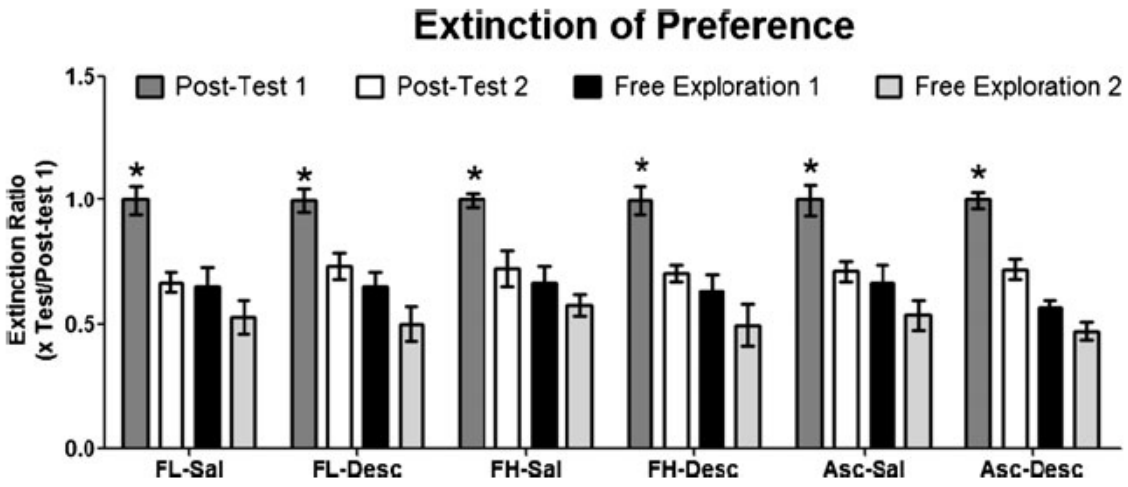


Figure D.3: In extinguishing cocaine CPP, both saline and a descending dosing schedule of cocaine are equally effective. All mice showed a significant within group decrease in preference for the cocaine-paired side on the post-test following reconditioning (Post-test 2) compared to initial preference observed in Post- test 1. No significant differences were found between groups on the ratio of preference remaining for the cocaine-paired side on the post-test following reconditioning (Post-test 2/Post-test 1). Data are represented as mean ± SEM, $*p > 0.05$, n = 7-8 mice per group.

Experiment 3: Extinction training by reconditioning with a descending cocaine dosing schedule attenuates stress-induced reinstatement of cocaine CPP.

Previously, Itzhak and Anderson (2011) demonstrated that a low-dose (3.5 mg/kg) priming injection of cocaine could reinstate cocaine CPP, but only in mice that had been reconditioned with saline, regardless of initial conditioning experience. To assess the impact of reconditioning on stress-induced reinstatement, we utilized a moderate forced swim (FS) stressor to reinstate CPP (stress-induced reinstatement; Kreibach and Blendy 2004; Mantsch *et al* 2010). We hypothesized that reconditioning (with the Desc schedule or saline) and prior conditioning experience (F_L, F_H, and Asc) would affect the magnitude of stress-induced reinstatement.

A 3-way ANOVA was used to analyze the effect of forced swim stress on cocaine CPP with conditioning group, reconditioning group and reinstatement test group as the variables, and with outcome of stress on preference expressed (seconds spent on the cocaine-paired side), as the dependent variable (**Figure D.4**). There was no significant three-way interaction ($P > 0.05$). There was a significant main effect of reinstatement test group (prior free exploration day compared to test immediately following stressor) [$F(1,40) = 23.30$, $P < 0.05$], but no main effect of conditioning group (F_L, F_H, or Asc) or reconditioning group (Desc or Sal). .There was a significant interaction of reconditioning group x reinstatement test [$F(1,40) = 6.91$, $P<0.05$], indicating reduced stress-induced reinstatement of CPP in mice exposed to the descending cocaine schedule during reconditioning. No significant interactions between conditioning and reinstatement test day or conditioning and reconditioning ($P > 0.05$) were observed. No differences were found on locomotor activity between groups (data not shown).

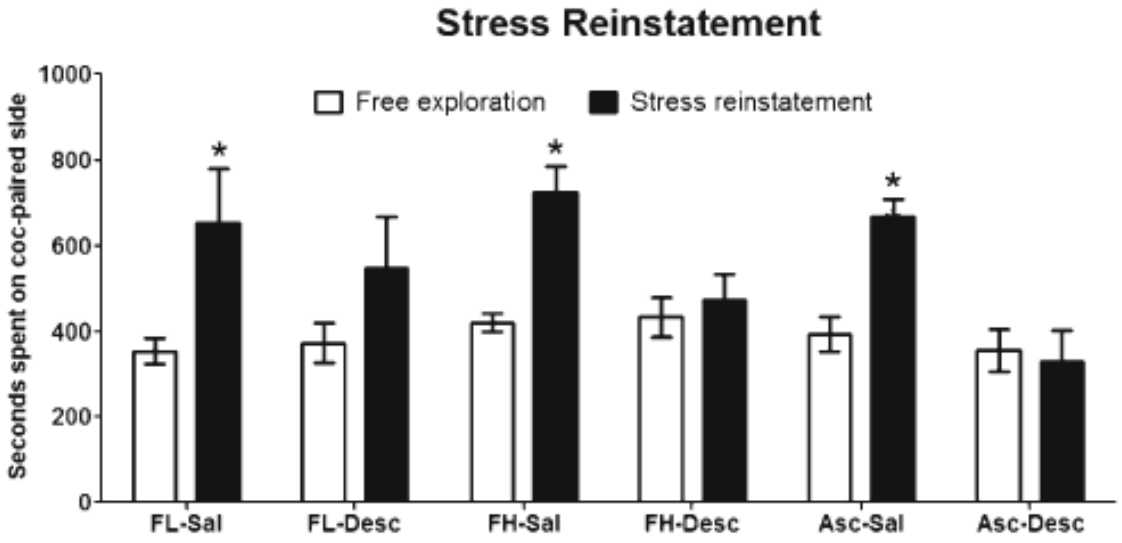


Figure D.4: Reconditioning with descending cocaine doses blocked reinstatement of preference for the cocaine-paired side following a forced swim stressor. Saline reconditioned mice all showed a significant within group increase on time spent on the cocaine-paired side during the stress-induced reinstatement test compared to time spent on the cocaine-paired side during the free exploration session the previous day. None of the Descending schedule reconditioned mice show a significant increase in time spent on the cocaine-paired side during the stress-induced reinstatement test compared to time spent on the cocaine-paired side during the free exploration session the previous day. Data are represented as mean ± SEM, $*p > 0.05$, n = 7-8 mice per group.

Experiment 4: Reconditioning with descending cocaine dosing schedule, but not saline reconditioning, attenuates cocaine-induced reinstatement of CPP.

We used a 3.5 mg/kg cocaine priming dose to induce reinstatement as in Itzhak and Anderson (2011), and we extended their study by (1) also examining a F_L group, and (2) assessing if cocaine-primed reinstatement occurs in mice that had previously undergone a stress-induced reinstatement test.

A 3-way ANOVA was used to analyze the effect of a cocaine priming dose on cocaine CPP with conditioning group, reconditioning group and reinstatement test group as the variables, and with outcome of cocaine prime on preference expressed (seconds spent on the cocaine-paired side), as the dependent variable. There was no significant three-way interaction ($P > 0.05$). There were a significant main effects of reinstatement test group (prior free exploration day compared to test immediately following stressor) [$F(1,40) = 17.14$, $P < 0.05$] and reconditioning (Sal compared to Desc) schedule [$F(1,40) = 3.98$, $P < 0.05$], but there was no significant main effect of conditioning (F_L, F_H, or Asc) schedule. There was a significant interaction of test x reconditioning [$F(1,40) = 6.89$, $P < 0.05$]. No significant interaction of conditioning schedule and testing day or conditioning schedule and reconditioning schedule ($P > 0.05$) was observed. No differences were found on locomotor activity (data not shown). A SNK *post hoc* analysis reveals that reconditioning with a descending dose of cocaine (compared to saline) has a significant effect on cocaine prime-induced reinstatement (**Figure D.5**; $P < 0.05$).

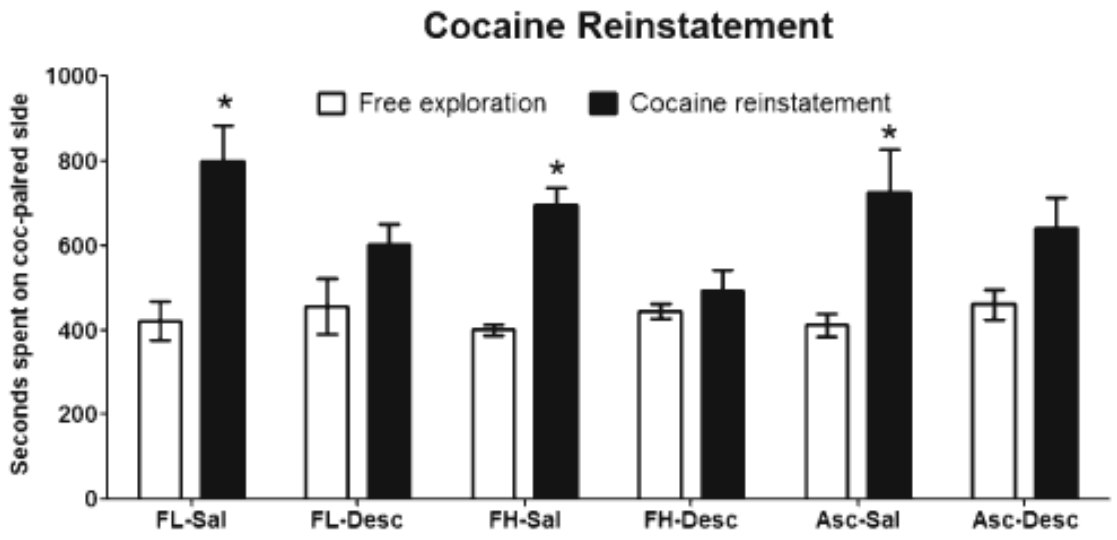


Figure D.5: Reconditioning with descending cocaine doses blocked reinstatement of preference for the cocaine-paired side following a priming injection of cocaine (3.5 mg/kg). Saline reconditioned mice all showed a significant within group increase in time spent on the cocaine-paired side during the cocaine-induced reinstatement test compared to time spent on the cocaine-paired side during the free exploration session the previous day. None of the Descending reconditioned mice show a significant increase in time spent on the cocaine-paired side during the cocaine-induced reinstatement test compared to time spent on the cocaine-paired side during the free exploration session the previous day. Data are represented as mean ± SEM, $*p > 0.05$, n = 7-8 mice per group.

Discussion

In the present study, we found that conditioning with ascending doses of cocaine (Asc; 2, 4, 8, and16 mg/kg) resulted in higher levels of CPP compared to both low (F_L; 7.5mg/kg)- and high (F_H; 16mg/kg)-fixed conditioning doses of cocaine. During reconditioning, administration of either saline or descending doses of cocaine (Desc; 8, 4, 2, 1 mg/kg) lead to similar levels of cocaine extinction, regardless of conditioning history. Our results demonstrate that reconditioning with descending levels of cocaine, but not saline, attenuates cocaine- and stress-induced reinstatement following the reconditioning sessions. Our results are in line with studies showing that human addicts take drugs in which the binge phase involves increasing amounts of cocaine consumption (Angarita et al., 2010; Gawin and Ellinwood, 1989).

This study aimed to replicate and expand on the important recent work by Itzhak and Anderson (2011). Notably, we have expanded these data in four ways: (1) The previous study utilized an intercrossed strain of mouse (C57BL/6J + 129S1/SvlmJ) while all experiments described here used the inbred C57BL/6J strain. (2) As described below, we incorporated a F_LD group, which received equal total cocaine administration as the AscD group. (3) We replicated the effect of a 'priming' dose of cocaine on reinforcement of drug seeking, and demonstrated that the descending dosage schedule of reconditioning also protects against stress-induced cocaine seeking. This is crucial additional data, as priming- versus stress-induced reinstatement of drug seeking involve differential circuitry. (4) Finally, we utilized a slightly biased CPP paradigm in these experiments. While Itzhak and Anderson (2011) did not observe significant bias toward either the smooth-floored or rough-floored compartments of their CPP apparatus, we routinely observe a small but

significant innate preference for the mesh-floored side of our apparatus (57.3% initial preference for this side in the experiments described here, $P < 0.001$). In order to minimize bias, we assigned saline injections to this initially preferred side, and cocaine was administered with exclusive access to the bar-floored side of the apparatus. Thus, mice must overcome this initial bias in order to show CPP. Additionally, we excluded any mice that demonstrated a greater than 33% bias towards one side of the apparatus during the pretest (1/48 mice).

Conditioning mice with an ascending dosing schedule of cocaine results in higher levels of expressed preference for the cocaine-associated environment than either F_LD or F_HD cocaine administration. Consistent with our findings, the Itzhak and Anderson (2011) study demonstrated that even accounting for higher, lower, and similar average daily dosing of cocaine during conditioning could not explain the differences they observed in the magnitude of cocaine CPP. One potential partial explanation of their findings is that the dose employed in their study was relatively high (16 mg/kg) and may be accompanied by more aversive properties. Indeed, other studies have demonstrated that administering fixed doses lower than 16 mg/kg results in more robust CPP (Mantsch et al., 2010). However, we found that even when total cocaine dosing in the F_LD group (30 mg/kg total) was less than half the total dosing in the F_HD group (64 mg/kg total), the magnitude of cocaine CPP was similar. Importantly, we found that although the ascending schedule group had the same total dosing as the F_LD group and less than half that of the F_HD group, the magnitude of CPP observed in the AscD group was significantly higher. Our results suggest that the schedule of dosing, but not the dose itself, may be a more important predictor of the magnitude of the CPP that is expressed. Recent studies have begun to

explore the importance of using different dosing schedules of cocaine to better model addiction (Itzhak and Anderson, 2011; Morgan et al., 2009; Zimmer et al., 2011).

Extinction training with saline is a common procedure in reinstatement studies. Our data, however, demonstrate that reconditioning mice with a descending dose of cocaine during extinction is just as effective as saline in the magnitude of extinction that is achieved, regardless of the original conditioning schedule used. Over time, the rate of extinction is also different between groups; mice reconditioned with a descending dose of cocaine, regardless of conditioning history, and mice conditioned with an ascending dosing schedule of cocaine and reconditioned with saline showed a progressive decrease in preference for the cocaine-paired side over time. Our data suggest the typical model employed in cocaine CPP paradigms of conditioning with a fixed dose of cocaine and extinction training with saline is a less effective approach to extinguishing preference for the cocaine-paired environment. Our results are consistent with a few recent studies (Itzhak and Anderson, 2011; Marks et al., 2010; Panlilio et al., 2007), suggesting that when the expectation of reward is successively lowered (devalued), as may occur over the course of reconditioning with a descending dose of cocaine, an animal can learn and adjust their behavior. Although this idea runs counter to the compulsive and perseverative nature of drug-seeking behavior, to our knowledge no study has directly addressed the hypothesis that operant cocaine-seeking behavior can be attenuated with extinction training by reconditioning with a descending schedule of cocaine administration and future studies should investigate the potential of this paradigm to alter the rate of extinction in contingent self-administration models.

It is important to note that this descending dosage paradigm reduces the length of time between the final drug administration and the post-test when compared to saline-treated animals. Thus, some of the apparent protective nature of the descending paradigm may be due in part to incubation of drug craving within the saline-treated group (Grimm, et al. 2001). Future studies will be needed to match the time of the last descending dose of cocaine with the final cocaine dose in saline-extinguished animals to determine if the protective effect of descending reconditioning is preserved. The descending dosage paradigm also could alleviate withdrawal symptoms, and this decrease in withdrawal may play a role in the observed protective effect. Further studies will be necessary in order to determine if withdrawal severity plays a role in the protective nature of the descending dosage schedule. In addition, further studies will be necessary to isolate whether the descending dose reconditioning paradigm is useful for reduction in reinstatement in an independent context than the initial conditioning cocaine doses.

In mice extinguished with a descending dose of cocaine, some resistance to reinstatement was observed. The ability to extinguish CPP by administering decreasing doses of cocaine in the cocaine-paired context, may prove to be a more useful method of preventing relapse. Indeed, our results replicating and extending the work of Itzhak and Anderson (2011) suggest many new avenues for investigation that may focus on questions of whether recall and reconsolidation of addiction memories are more labile when descending doses of the drug are present. Interestingly, d-amphetamine is clinically being explored as a therapeutic tool for the treatment of cocaine dependence (Rush et al., 2009). Taken together, our results suggest a powerful influence of the pattern of drug exposure, but not the amount of drug, on the magnitude of preference expressed for a conditioned

reward. Our findings may also have significant implications for the development of addiction therapies.

Appendix E

Ketamine Regulation of Corticotropin Releasing Factor within the Bed Nucleus of the Stria Terminalis

E.1 Acute Ketamine Administration has Heterogeneous Effects in Unidentified BNST Neurons

Acute administration of ketamine has been shown to influence excitability of neurons in both the hippocampus (Autry et al., 2011) and the PFC (Li et al., 2011) measured via slice physiology. We had shown that ketamine blunts LTP induction in the BNST when administered *in vivo* 30 minutes prior to slice preparation (Chapter 4, **Figure 4.2**). However the effect of ketamine on individual neurons in this region has not been investigated. Using a whole-cell patch clamp configuration in voltage-clamp mode as previously described, I examined unidentified BNST neurons held at -70mV with $GABA_A$ receptor antagonist picrotoxin (25μM) present in the ACSF (124 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 2 mM Mg_2SO_4, 1.25 mM NaH_2PO_4, 11 mM glucose, and 26 mM $NaHCO_3$) to isolate AMPA sEPSCs (Williams et al., 2014). Access resistance was measured via membrane test every 2 minutes, and recordings in which access resistance changed by greater than ~20% were excluded from analysis. Ketamine (20μM) was bath applied following 10 minutes of stable baseline, and this concentration was chosen based on an earlier study examining ketamine's effect on hippocampal plasticity (Autry et al., 2011). I used slices prepared from both male and female mice (**Figure E.1A**).

A two-way repeated-measures ANOVA of frequency during ketamine bath application (minutes 12-40) revealed significant effect of time ($F(14,196)=1.885$; $P=0.0299$) and significant interaction ($F(14,196)=1.788$; $P=0.0425$), and a trend towards increased frequency in females vs. males ($F(1,14)=4.552$; $P=0.0511$). Given the heterogeneous nature of the BNST, we wondered if we were seeing a dampening of overall effect due to the recording of differential populations that would be typical of patching random unidentified BNST neurons. Indeed, when looking at individual responses to ketamine in female cells (**Figure E.1B**), we saw a variety of response types that hint at heterogeneous responses to ketamine administration.

Given the diversity of responses observed following ketamine administration in unidentified BNST neurons, we felt we needed to narrow our parameters within this region in order to identify ketamine's specific effects. Unlike the hippocampus or prefrontal cortex, which are relatively homogeneous in cell types present, the BNST contains an array of cellular profiles, varying both in protein expression and projection targets. Implications of BNST CRF in depression-related behaviors lead us to examine this population of cells within the region.

E.2 GluN2B is Expressed on the Majority of CRF Neurons

In order to ketamine to exert an effect directly on CRF-containing neurons within the BNST, we needed to first assess whether these cells would be likely to respond to ketamine. Since evidence has indicated that ketamine likely exerts its anti-depressant like effects specifically via interactions with GluN2B-containing NMDARs (Li et al., 2011; Louderback et al., 2013; Miller et al., 2014), we initially wanted to determine if GluN2B and

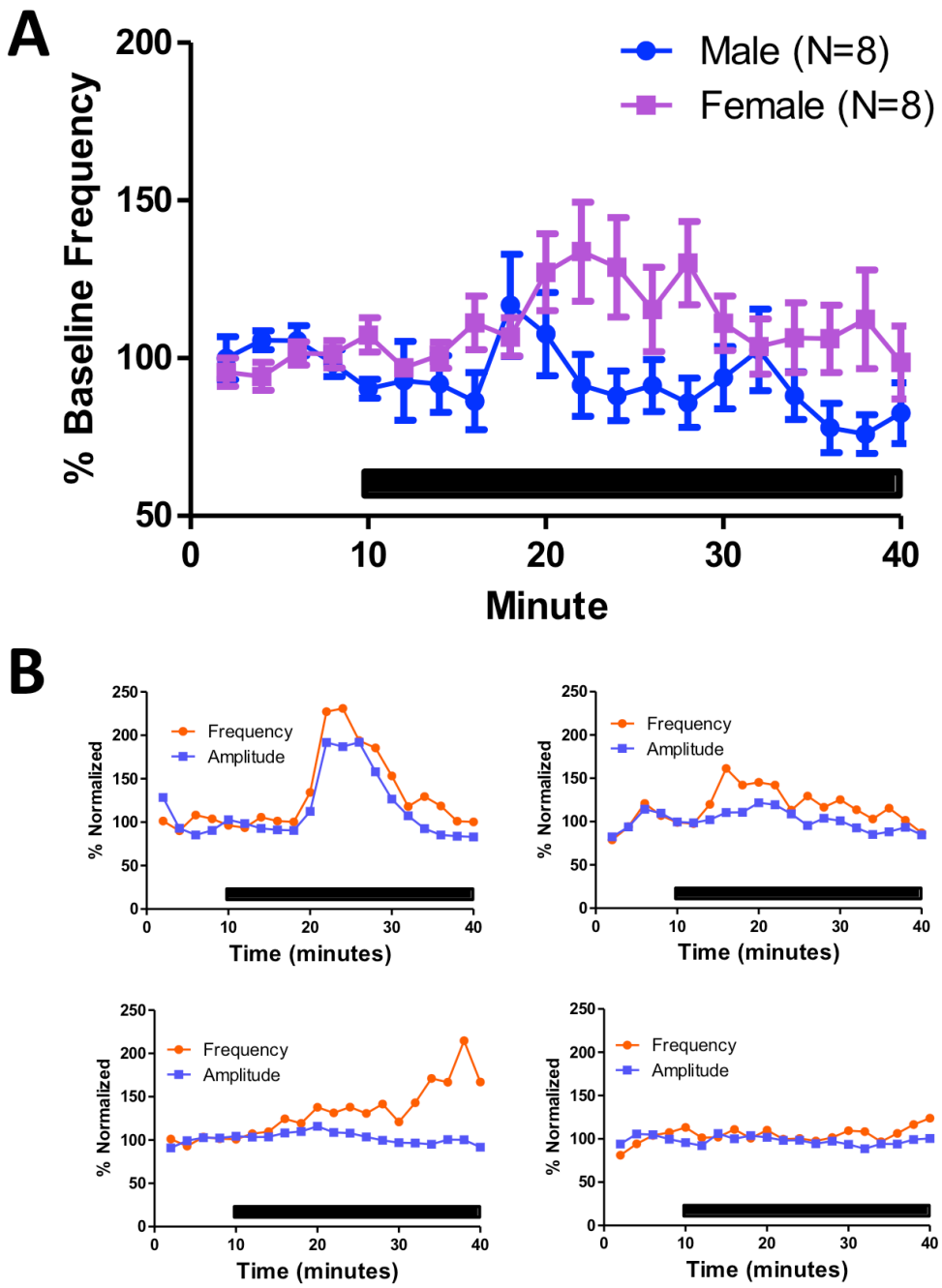


Figure E.1. The effect of bath-applied ketamine on sEPSCs in unidentified BNST neurons. (A) sEPSC frequency in BNST neurons from male and female mice with ketamine application. (B) Frequency and amplitude traces from 4 individual neurons from female mice, indicating heterogeneous responses to ketamine. Black bars indicate 20μM ketamine application.

CRF are co-expressed on the same neurons within the BNST. Previous work attempting to visualize GluN2B in the BNST had been less than fruitful (see Appendix B), but a novel fluorescent ISH technique with higher sensitivity and lower background, RNAscope, had recently been described (Wang et al., 2012). A major benefit of this technique is that every "dot" of fluorescence is thought to correspond to an individual mRNA transcript, thus allowing visualization of low-expressing transcripts that would fall below detection of standard ISH techniques.

We used the RNAscope technique to examine BNST expression of GluN2B and CRF according to detailed procedural instructions provided by Advanced Cell Diagnostics (Hayward, CA), and also stained for DAPI as a rough approximation of cellular boundaries. As seen in **Figure E.2**, clear expression of both GluN2B (red) and CRF (green) mRNA transcripts was detected using this method.

Quantification of co-expression was performed from both 63X and 20X images of the BNST. For a cell to count as "positive" for either transcript, it had to contain a minimum of ~2 dots within the DAPI-determined borders, and this threshold was calculated by dot counts observed with negative control probes. The 20X image allows for regional delineation of expression (not performed here), but tends to under-represent positive cells which only containing near-threshold transcript counts. On the other hand, 63X images allow clear visualization of individual transcripts, thus producing a more accurate count of positive and negative cells for each transcript. However, the zoomed-in nature of such an image only provides insight for a small section of the BNST. Because of the benefits and drawbacks of each magnification, both were analyzed.

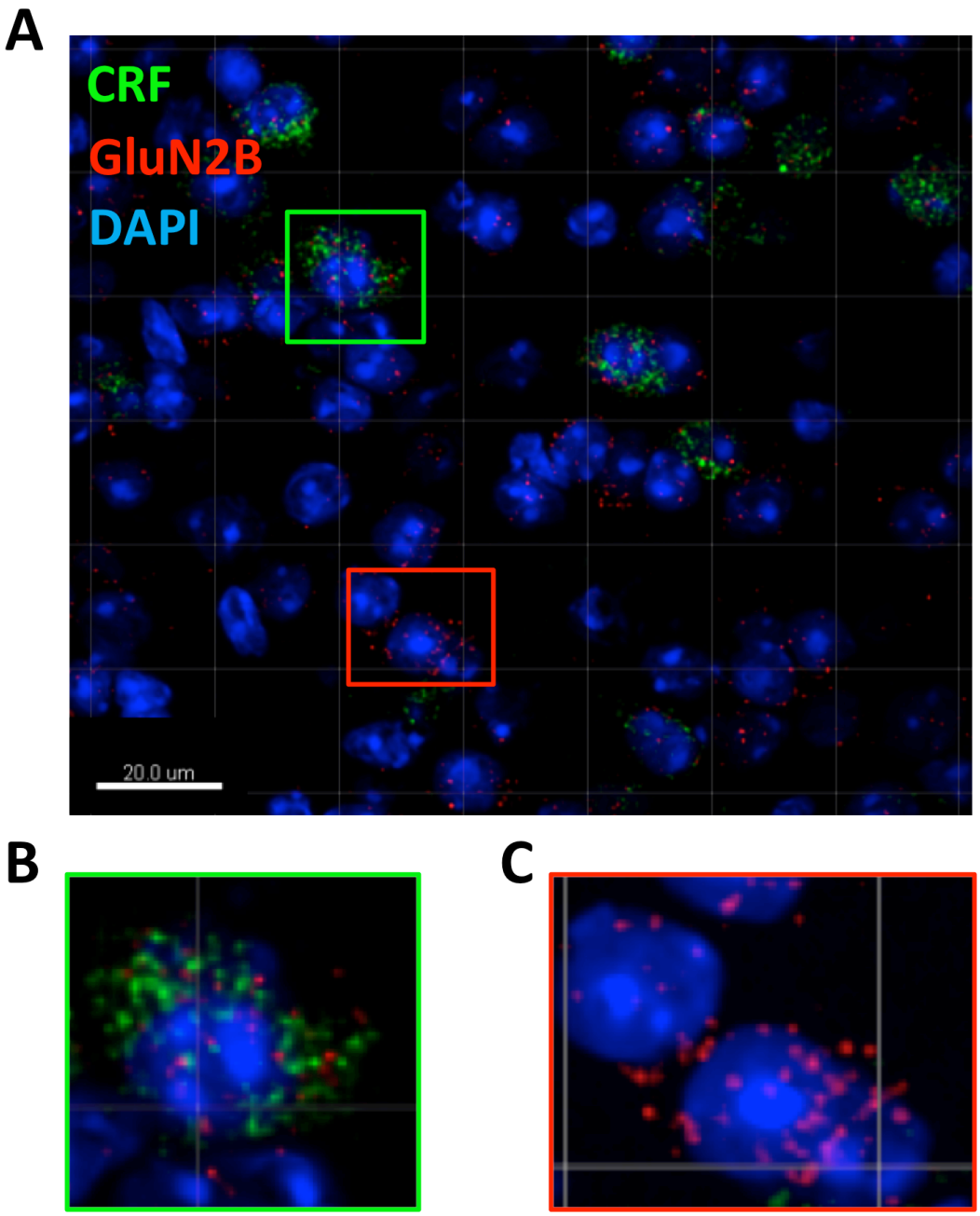


Figure E.2. RNAscope visualization of GluN2B and CRF mRNA expression in the BNST (A) Representative 63X images following RNAscope for CRF and GluN2B in BNST. (B) Example of a cell expressing both GluN2B and CRF, zoom of green rectangle in (A). (C) Example of cells expressing GluN2B only, zoom of red rectangle in (A).

Figure E.3 shows proportion of transcript levels within the BNST averaged from 4 wild type mice. **Figures E.3A** and **E.3A'** show proportion of GluN2B and/or CRF expression in all cells delineated with DAPI under both magnifications. The presence or absence of CRF expression within GluN2B-positive cells is examined in **Figure E.3B** and **E.3B'**. We find that, while most cells expressing GluN2B mRNA do not also express CRF, there is higher proportion of CRF expression in GluN2B cells than in all BNST cells. More importantly, greater than 95% of CRF-positive cells co-express GluN2B (**Figures E.3C** and **E.3C'**), indicating that GluN2B-containing NMDARs are situated in an excellent position to regulate these neurons.

E.3 Ketamine Reduces CRF mRNA expression in the BNST

The presence of GluN2B on CRF neurons provided evidence that ketamine might be able to regulate CRF in the region, and we sought to determine if a behaviorally-relevant ketamine treatment would have any effect on BNST CRF. In order to test this, we used quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR) to assess CRF mRNA levels following systemic treatment with ketamine (3mg/kg; i.p.). We chose 3 time points – 4hrs, 24hs, and 1 week – following ketamine injection to determine onset speed and duration of ketamine's effect on CRF. We examined expression levels in the BNST following these treatments, as well as in the CeA (the other major extra-hypothalamic cite of CRF) and paraventricular nucleus of the hypothalamus (PVN) as a proxy of HPA responses to ketamine. Tissue was collected via circular punch (0.8mm diameter) from 500μm thick coronal sections (bilateral punches collected from BNST and CeA, unilateral

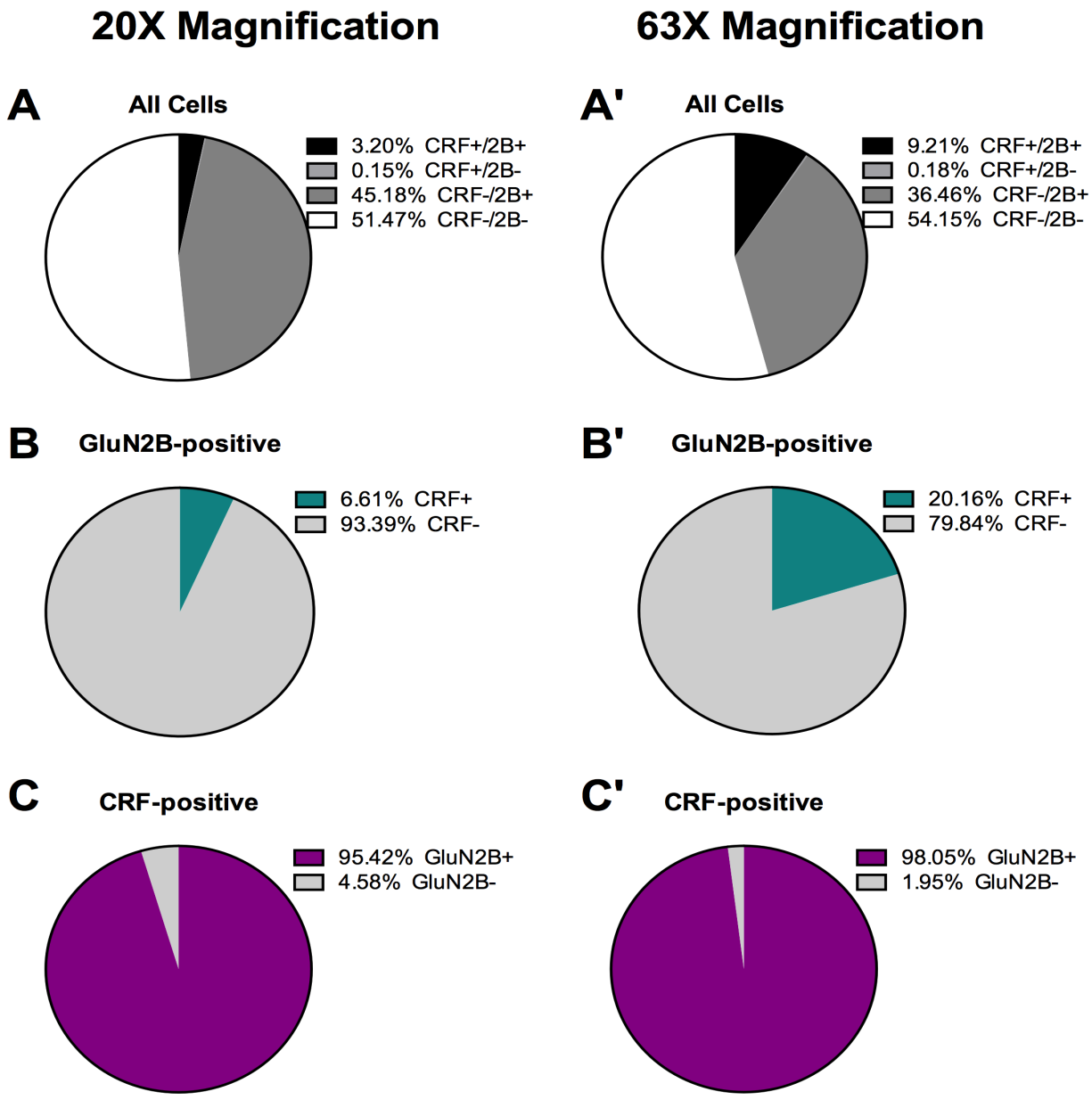


Figure E.3. RNAscope-quantified GluN2B and CRF co-expression in the BNST. Presence of GluN2B and/or CRF transcripts were measured via RNAscope at 20X and 63X magnification. (A,A') Proportion of GluN2B or CRF in all BNST cells labeled with DAPI. (B,B') Proportion of CRF+ cells in cells expressing GluN2B transcripts. (C,C') Proportion of GluN2B+ cells in cells expressing CRF transcripts.

punches from PVN). Tissue was homogenized in TRIzol reagent, and RNA was isolated by chloroform extraction followed by overnight isopropanol precipitation. RNA concentration was measured via NanoDrop, and 1μg total RNA was converted to cDNA using a High Capacity Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific). Alterations in CRF expression were determined using TaqMan gene expression assays (Applied Biosystems) with GAPDH used as an endogenous control. Samples were run in duplicate on the Bio-Rad CFX96, and analysis was performed a using $2^{-\Delta\Delta Ct}$ method with normalization to the average fold change for the control group (24hr saline).

We found that 24 hours after ketamine administration, CRF levels are markedly decreased in the BNST (**Figure E.4**), returning to baseline levels after one week ($P<0.001$ at 24hrs compared to saline control). No effect of ketamine treatment was observed in the CeA, and a non-significant trend for altered CRF expression was found in the PVN ($P=0.0696$).

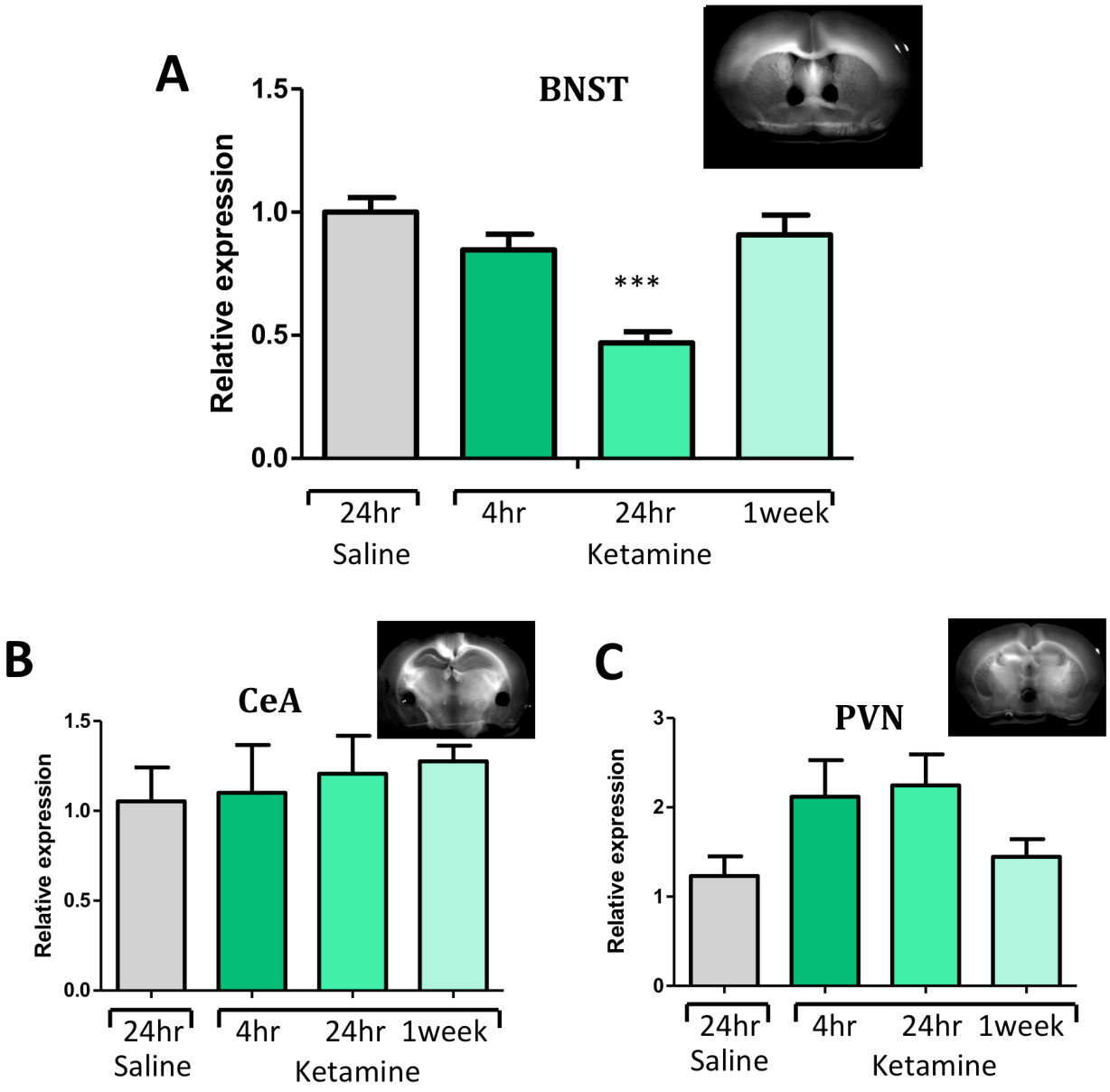


Figure E.4. CRF mRNA expression in the BNST measured by qRT-PCR is significantly reduced 24 hours after systemic ketamine (3mg/kg) injection. Relative expression of CRF in (A) BNST, (B) CeA, and (C) PVN punches following systemic administration of 3mg/kg ketamine at 4hrs, 24hrs, and one week post-injection. Inset images are representative examples of punch location. ***P<0.001

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