Investigating the role of GluN2D-containing NMDARs in BNST excitatory signaling: Implications for regulating cell-specific synaptic function and the modulation of affective behaviors

By

Gregory Joseph Salimando

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

May 31, 2020

Nashville, TN

Approved:

Brad A. Grueter, Ph.D.

Jose Moron-Concepcion, Ph.D.

Terunaga Nakagawa, Ph.D.

Sachin Patel, M.D./Ph.D.

Danny G. Winder, Ph.D.

To the friends and colleagues that I've made in research throughout the years
Support is the heart of good science
Thank you for your optimism, good nature and humor
You're all my inspiration

And

To my mother, father and sister
Thank you for believing in me, and giving me the greatest
examples of compassion and ambition to emulate

ACKNOWLEDGEMENTS

I would first like to acknowledge all the funding sources that have supported the work presented in my dissertation, starting with the Training in Fundamental Neuroscience Grant (T32 MH064913-17) for funding my first two years in my dissertation lab. Funding from grants awarded to Danny Winder from the National Institute on Alcohol Abuse and Alcoholism (R37 AA019455) and the Brain & Behavior Research Foundation (NARSAD Distinguished Investigator Award) also provided generous support for my work. Additionally, I would like to thank the Vanderbilt Brain Institute and Neuroscience Graduate Program for their support (financial and otherwise) throughout my graduate school career, as well as its program manager Roz Johnson and executive assistants Darlene Pope and Beth Sims for always going above and beyond to help the students with anything and everything.

I would next like to thank my mentor, Danny Winder. Your support, guidance and patience have been a constant bright spot throughout the grueling process that can be graduate level research. You have such a wonderful combination of passion for your work, compassion for all those in your lab, and a calm and collected approach to academic research. It truly made my time here more enjoyable than I ever could have thought. Thank you for continuing to foster such an excellent environment for young scientists in your lab, and above all, thank you for continuing to believe in me and push me to believe in myself and walk my own path, even when I had difficulty seeing the way forward. I would also like to thank the members of my thesis committee: Drs. Brad Grueter (chair), Sachin Patel, Teru Nakagawa and Jose Moron-Concepcion, for their support and time. Specifically, I'd like to thank Dr. Grueter for his insight and advice on all things electrophysiology and navigating graduate school and the years beyond, Dr. Patel for his classic wit, Dr. Nakagawa for helping to keep questions of molecular mechanisms in sight, and Dr. Concepcion for bringing an amazing outside perspective to my work. You have all helped shape how I think about and approach science in amazingly positive ways, and I'm honored to have been able to receive guidance from such experts in the field.

Next, I would like to thank the members of the Winder lab, past and present. To Drs. Katie Holleran and Tiffany Wills, thank you for your contributions to the work that provided the spark for my own project in the lab. To Drs. Nick Harris and Tracy Fetterly, thank you for helping me settle into the Winder lab when I first joined and get up to speed on all the things early grad students needed to know at Vanderbilt. To Dr. Yuval Silberman,

thank you for helping to teach me the art of electrophysiology. To Drs. Anel Jaramillo, Oliver Vranjkovic, James Melchior and Sam Centanni, thank you all for serving as excellent examples of postdoctoral researchers. I hope to emulate your enthusiasm and tenacity in my own tenure as a PD. To my fellow students: Kellie Williford, Jordan Brown, Joe Luchsinger, Nick Petersen and Brett Nabit, thank you all for being wonderful and helpful colleagues. I know you are all going to do amazing work in the future with whatever you pursue, inside or outside of science. To Elana Milano and Bridget Morris, thanks for keeping the day to day affairs in our crazy lab running smoothly, and for all your support and help over the years. And lastly, to Rafael Perez, thank you for being one of the best friends and lab mate a guy could have asked for in graduate school. I can't imagine what these past few years would have been like without your constant questions and insightfulness. You're one of the most effortlessly passionate researchers I know, and an inspiration for me to learn how to take a step back and really appreciate all I do and have learned as a scientist, so thank you for that. I look forward to working together and staying in touch for many years to come!

No man (or woman) is an island in science, and I don't think anything I accomplished at Vanderbilt would have been possible without the support of so many amazing friends and colleagues. To my close friends from the first days of the IGP up to the very end: David Marcus, Sean Moran, James Maksymetz, Rafael Perez, Oakleigh Folkes, Tessa Popay, Clayton Wandishin and Francis Prael, thank you. Thank you for being such amazing people and incredibly supportive friends. I don't know how I could have gotten through graduate school without you all, and can't imagine how different my life would be without us meeting. You're all some of the most amazing and intelligent scientists I've ever met. I'm honored to call you all my friends and to be able to look forward to working together with you all in the future as you achieve incredible things in your own lives and careers. To all the collaborators outside of our lab who have helped train me over the years, Dr. Brian Shonesy of the Colbran lab, Drs. Max Joffe and Kevin Manz of the Conn/Grueter labs and Drs. Nolan Hartley, Rita Baldi and Luis Rosas-Vidal of the Patel lab, thank you for taking so much of your time to help contribute to my development as a researcher. To all the new friends and members of the Vanderbilt Center for Addiction Research, especially Veronika Kondev, Kim Thibeault and Nathan Winters, thanks for being such awesome colleagues and keeping me positive and laughing through thick and thin.

Out of all the IGP and Vanderbilt people to thank though, the foremost is my incredible girlfriend, Victoria. Thank you for putting up with me all these years, and for never wavering in your support of me no

matter what. Your ability to relate to me and my experiences over these years made graduate school not seem so bleak, and I appreciate it more than you ever know.

Finally, I would like to thank my family. First to my parents, Donna and Steven, thank you for always supporting my crazy goal of going to grad school to get my Ph.D. Thank you for all the nights listening to me complain, all the advice and insight, and all the commitment to helping me see the merit in my work and myself as a scientist. I am so incredibly lucky to have such amazing people always behind me, and this achievement is as much yours as mine. To my sister Cara, thank you as well for understanding and connecting with me over our mutual industries (music and science) and supporting and recognizing my achievements as we've both inched towards our goals over the years. You're one of my biggest inspirations, and your own tenacity has always inspired me to keep going and keeping working. Thank you all for everything.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	serotonin
5-HTT	serotonin transporter
AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methly-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methly-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
AR	adrenergic receptor
AVP	arginine vasopressin
AVPR	arginine vasopressin receptor
BLA	basolateral nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
Ca^{2+}	calcium
CBT	cognitive behavioral therapy
CeA	central nucleus of the amygdala
ChR2	channelrhodposin-2
CIE	chronic induce ethanol exposure
CMS/CUS	chronic mild/unpredictable stress
CNS	central nervous system
CRH	corticotropin releasing hormone
CRF	corticotropin releasing factor
CRFR1	corticotropin releasing factor receptor 1
CRFR2	corticotropin releasing factor receptor 2
CSDS	chronic social defeat stress
DA	dopamine
DAPI	4’,6-diamidino-2-phenylindole

DAT	dopamine transporter
dlBNST	dorsolateral bed nucleus of the stria terminalis
DR	dorsal raphe
DSM-5	diagnostic and statistical manual 5
DYN	dynorphin
ECT	electroconvulsive therapy
eGFP	enhanced green fluorescent protein
EPM	elevated plus maze
EZM	elevated zero maze
EPSC	excitatory post-synaptic potential
fMRI	functional magnetic resonance imaging
FST	forced swim test
GABA	γ-aminobutyric acid
GABAA	γ-aminobutyric acid receptor A
GABAB	γ-aminobutyric acid receptor B
GC	glucocorticoid
Glu^{+}	glutamate
$GluN2D^{+/+}$	GluN2D-NMDAR wildtype
$GluN2D^{-/-}$	constitutive GluN2D-NMDAR knockout
$GluN2D^{flx/flx}$	conditional GluN2D-NMDAR knockout
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
HAMD	Hamilton depression rating scale
HPA	hypothalamic pituitary adrenal
iGluR	ionotropic glutamate receptor
IHC	immunohistochemistry
i.p.	intraperitoneal
IPSC	inhibitory post-synaptic current

ISH	*in situ* hybridization
KOR	kappa opioid receptor
LC	locus coeruleus
L/D	light/dark box
LTD	long term depression
LTP	long term potentiation
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
MeA	medial nucleus of the amygdala
mEPSC	miniature excitatory post-synaptic current
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
NAc-Sh	shell of the nucleus accumbens
NDRI	norepinephrine-dopamine reuptake inhibitor
NE	norepinephrine
NET	norepinephrine transporter
NIH	novelty-induced hypophagia
NK	neurokinin
NMDA	N-methyl-d-aspartate
NMDAR	N-methyl-d-aspartate receptor
NPY	neuropeptide Y
NRI	norepinephrine reuptake inhibitor
NSFT	novelty-induced suppression of feeding
NTS	nucleus of the solitary tract
OFT	open field test
OXT	oxytocin

OXTR	oxytocin receptor
PAG	periaqueductal grey
PAM	positive allosteric modulator
PBN	parabrachial nucleus
pDYN	prodynorphin
PFC	prefrontal cortex
PNS	peripheral nervous system
PPD	paired pulse depression
PPF	paired pulse facilitation
PPR	paired pulse ratio
PVN	paraventricular nucleus (hypothalamus)
qRT-PCR	quantitative real time reverse transcription polymerase chain reaction
RDoC	research domain criteria
sEPSC	spontaneous excitatory post-synaptic current
sIPSC	spontaneous inhibitory post-synaptic current
SERT	serotonin transporter
SNr	substantia nigra pars compacta
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
STN	subthalamic nucleus
STP	short term potentiation
TCA	tricyclic antidepressant
TRD	treatment resistant depression
TST	tail suspension test
vHPC	ventral hippocampus
VP	ventral pallidum
VTA	ventral tegmental area

Chapter 1

Introduction

Major depressive disorder: overview and current understanding

Depressive disorder epidemiology and pathology

Major depressive disorder (MDD) is one of the most prevalent psychiatric disorders diagnosed in humans, and represents a significant burden on global health. In the United States alone, roughly 7.1% of all adults (ages 18 and older) have reported experiencing at least one major depressive episode in their lifetime, and nearly 4.5% of U.S. adults have reported major depressive episodes accompanied by severe impairments in their day to day lives (SAMHSA, 2018). Indeed, MDD is known to be a highly debilitating condition often due to a constellation of other psychiatric diseases known to be comorbid with depression, such as generalized anxiety and substance abuse, and other associated symptoms including decreased motivation, lack of interest or pleasure, and suicidal ideation (SAMHSA, 2018). While nearly 63.8% of U.S. adults diagnosed with MDD have reported instances of such serious impairments to their wellbeing and mental health, an estimated 35% of all MDD adult patients and 60.1% of all adolescents reporting major depressive episodes go untreated for their condition. This is often attributed to the highly heterogeneous nature of the presentation of MDD and the impact of this on diagnosing and treating the disorder, as no major biomarkers exist for its early detection. Current therapeutics for depression have shown varying levels of efficacy across broad patient populations, as discussed further below (Otte et al., 2016). This highlights a critical need in the field for not only the improvement of our understanding of the pathobiology of MDD, but also the identification of new medications and treatments for managing this serious disorder.

The Diagnostic and Statistical Manual 5 (DSM-5, 2013) classifies MDD as a mood disorder, and requires that five or more of the accepted symptoms of depression be present within a 2 week period for a diagnosis to be made. To meet MDD criteria, at least one of these symptoms must be either depressed mood or anhedonia (a loss of interest or pleasure), while secondary symptoms can include: appetite loss or weight change, difficulty sleeping (insomnia or hypersomnia), psychomotor agitation or retardation, fatigue or loss of energy, diminished concentration, feelings of worthlessness or excessive guilt, and suicidal ideation. These

symptoms are usually scored on an “all or none” scale (0-1), and are collectively used to only determine the presence or absence of major depressive episodes in patients, not to determine the degrees of severity of each symptom or the depressive episodes overall. While considerable effort has been made within the newer iterations of the DSM to subcategorize different types of depression, so as to better account for the heterogeneity of its presentation in patients, the manual’s lack of a consideration for the pathobiology and pathophysiology of psychiatric disorders limit its utility in identifying the most effective treatment paradigms for individuals. Such can also be said of the Hamilton Depression Rating Scale (HAMD), which although adopting a more scaled scoring system to determine the severity of a patient’s depression symptoms, also neglects to consider biological, genetic, molecular and physiological aspects of psychiatric disorders in aiding its diagnostic capabilities.

As mentioned above, while virtually no biomarkers or physiological correlates of MDD have been determined, significant progress in both clinical and preclinical research has been made over the past few decades that has aided in our understanding of the etiology of depressive disorders, which will be touched upon in greater detail later in this chapter. As such, organizations such as the National Institute of Mental Health (NIMH) and others have made strides recently to steer the conversation (and hopefully, the eventual diagnosing) of diseases such as MDD in the direction of these findings. Projects such as the Research Domain Criteria (RDoC) embrace a more global view of the pathology of psychiatric disease states as the product of changes in neural circuity and signaling, genetics and molecular variables, and environmental factors (https://www.nimh.nih.gov/research-priorities/rdoc/index.shtml). As delineated by the RDoC’s provisional matrix, these units of analysis (genes, molecules, cells, circuits, physiology) are taken into account along with the classical behaviors and self-reporting that comprise the majority of the DSM’s diagnostic criteria, and placed in the context of five broad domains that have been determined to be relevant to psychiatric disease through the compilation of years of biomedical research findings, and include: negative valence systems, positive valence systems, cognitive systems, systems for social processing, and arousal/modulatory systems. By attempting to filter the standard symptomology of patients suffering from MDD into these domains, and promoting a stronger focus on how these symptoms may be related to neurobiological factors, it is the hope of the RDoC and other like initiatives to improve not only the successful diagnosis and treatment of depressive disorders, but to also

improve our ability to understand the commonalities across clinical presentations of this disease in order to elucidate new understandings of its biological underpinnings.

To this effect, a convergence of findings from human imaging, genetic and histological studies as well as disease modeling of depressive disorders in rodents in particular has begun to create a better picture of the brain regions and potential circuitry that may be impaired or altered in the disease state, showing promise for identifying potential biomarkers of depression that can enhance diagnostic capabilities and even inform early intervention. Indeed, collective scans from functional MRI (fMRI) studies of MDD patients compared to healthy controls have consistently demonstrated altered blood flow or other inferred measures of metabolic activity changes in regions such as the prefrontal and cingulate cortex, hippocampus, striatum, amygdala, thalamus and hypothalamus (Drevets et al., 2001; Liotti and Mayberg, 2001). Many of these findings of altered functionality in these regions have been corroborated by histological studies of neural tissue samples obtained from depressed patients, showing either atrophy or other abnormalities in many of the regions/structures mentioned above in those suffering from severe and chronic depression (Zhu et al. 1999; Rajkowska et al., 2000; Manji et al. 2001; Drevets et al., 2001). A number of these regions, and evidence of similar dysfunctionality, have consistently been identified in animal studies as well, with a particular focus often being placed on the frontal cortex and hippocampus both for the assumed role these regions may play in regulating the cognitive and introspective aspects of depression, and the alterations in regional activation that have been observed across species (Covington et al., 2010; Milne et al., 2012). Other classical evidence, coupled with newer research findings, have also begun to switch the focus away from solely cortical structures to a number of subcortical regions and circuits that may be key in the regulation of the emotional, motivation, and appetitive symptoms of depression, including limbic structures such as the nucleus accumbens (NAc), amygdala and hypothalamus, key components of the classical reward circuitry of the brain, such as the ventral tegmental area (VTA), and regions implemented in arousal and awareness, such as the locus coeruleus (LC) and the dorsal raphe (DR). Knowledge of the molecular markers and neural circuitry intrinsic to these regions has further assisted in defining our understanding of depression, with numerous studies implementing the importance of the monoaminergic connections from the VTA to NAc (dopamine, DA), LC (norepinephrine, NE) and DR (serotonin, 5-HT) in the pathophysiology of depression, providing a critical basis for the implementation of early pharmacological treatments (Manji et al., 2001; Nester et al., 2002; Morilak and Frazer, 2004). Growing

evidence for the influence of other neuromodulatory systems, such as the neuropeptidergic circuitry/cellular components within the hypothalamic-pituitary-adrenal axis (HPA) often observed in both patient and animals models of depression to be altered, as well as in excitatory and inhibitory circuitry throughout many of the regions mentioned above have begun to further highlight new and important components of the neurobiology of depression that merit further and more focused investigation both clinically and preclinically (Nestler et al., 2002; de Kloet et al., 2005; Thompson et al., 2015; Boku et al., 2017).

While the findings of both human and animal modeling research into depression have begun to provide us with a better understanding of the underlying neural changes and overt pathology of depression, much work remains to be done and to date, no cure or universally effective treatment for depressive disorders exists. How a synthesis of a number of burgeoning areas of study of depression at the molecular, cellular and circuit specific levels may be broaching new horizons in the development of next generation treatments for depression will be discussed in greater detail below, and further in Chapters 2 and 3 in regards to the findings I present in my dissertation research.

Current treatment strategies and therapies for depressive disorders

Over the years, a number of pharmacological, behavioral and experimental treatment paradigms have been developed for MDD and other depressive disorders, with the most prominent and widely applied of these being the use of several classes of pharmacological compounds targeted at the brain's monoamine signaling system (see **Table 1** for a simplified list). The basis for this strategy stems from serendipitous findings dating back to the 1950s showing that patients administered reserpine, an irreversible antagonist of the vesicular monoamine transporter (most commonly at the time prescribed for the treatment of high blood pressure), often displayed many of the core symptoms of depression (Delgado, 2000), and that many of these behavioral changes could also be recapitulated in animal models (Delgado, 2000). This eventually lead to the so called "monoamine hypothesis of depression", which speculated that the disease state arose from a depletion of the levels of key monoamines, specifically 5-HT, NE and DA, in the central nervous system of affected patients (Tran et al., 2003). The pursuit and development of the so-called "first generation anti-depressants" which were chiefly targeted at both increasing monoamine levels at synapses the brain and preventing their breakdown in order to provide relief from depressive episodes, grew from such findings. These included the monoamine

oxidase inhibitors (MAOIs) and the tricyclic anti-depressants (TCAs), and the early successes of MAOIs such as iproniazid and TCAs like imipramine in treating depressed patients provided further credence to the monoamine theory. Many of these early compounds however were later removed from use, owing mostly to a number of detrimental side effects that were found to be associated with long term MAOI or TCA use, including hepatotoxicity, increased hypertension and dependence/withdrawal like effects (Shulman et al., 2013). Today, while more safely formulated MAOIs and TCAs are still in use for the treatment of depression, such as phenelzine and amitriptyline, they are often reserved as a last resort by clinicians for patient cases that prove highly resistant to more standard anti-depressants.

Category	Drug Class	Generic Names	Mechanism of action	Physiological effects
First-generation (typical)	Monoamine oxidase inhibitors (MAOIs)	Tranylcypromine, Phenelzine, Selegiline, Isocaroxazid	Inhibition of MAO A & B, reducing monoamine breakdown	Net increase in all monoamines
First-generation (typical)	Tricyclic antidepressants (TCAs)	Amitriptyline, Clomipramine, Desipramine, Doxepin, Imipramine, Notriptyline, Amoxapine, Protriptyline, Trimipramine	Blockade of SERT & NET; potential antagonism of 5-HT, AR, NMDARs, mAChRs & histamine; agonism of sigma receptors	Elevated NE and 5-HT levels; possible altered SHAM function & synaptic function
Second-generation (typical)	Selective serotonin reuptake inhibitors (SSRIs)	Fluoxetine, Fluvoxamine, Paroxetine, Sertraline, Citalopram, Escitalopram	Selective blockade of SERT	Elevated 5-HT levels; potential anti-inflammatory effects
Second-generation (typical)	Serotonin-norepinephrine reuptake inhibitors (SNRIs)	Duloxetine, Venlafaxine, Mirtazapine, Levomilnacipran	Selective blockade of SERT and NET	Elevated NE and 5-HT levels
Second-generation (atypical)	Norepinephrine-dopamine reuptake inhibitors (NDRIs)	Bupropion, Amineptine	Selective blockade of NET and DAT	Elevated NE and DA levels
Second-generation (atypical)	5-HT2 receptor antagonists	Nefazodone, Trazodone, Agomelatine	Antagonism of the 5-HT2 receptor class (typically the A and B isoforms)	Presumed reduction of 5-HTR autoreceptor function & increased 5-HT signaling
Experimental (atypical)	NMDA receptor antagonists	Esketamine	Competitive antagonism/channel blocker of NMDARs	Presumed increase in excitatory signaling, gene expression and neuroplasticity alterations
Experimental (atypical)	Opioid receptor antagonists	Buprenorphine/samidorphan, Tianeptine, Opipramol (?)	Weak partial agonism and antagonism of kappa, mu and delta opioid receptors; preferential activity at kappa receptors	Presumed alterations in neuroplasticity and neuromodulatory activity induced via opioid signaling
Experimental (treatment resistant)	Electroconvulsive therapy (ECT)	-	Poorly understood; pan increases in neurochemical and neuroplasticity effects	Improved cortical and hippocampal function, blood flow and NT metabolism

Table 1. List of current antidepressants and their physiological effects. Common and currently prescribed antidepressants, along with their drug classifications and known mechanisms of action. Typical antidepressants are accepted to act through increasing monoamine (5-HT, DA and NE) levels throughout the brain, while the atypical antidepressants produce more varied and combinatorial effects on the monoamine, glutamatergic and neuromodulatory signaling systems to produce relief from depressive symptoms. Current experimental approaches and unconventional depression treatments, including ECT and several novel compounds, are listed

as well, primarily for the promise and proven potential they display for aiding patients with forms of depression resistant to known typical antidepressant regiments and psychological counselling/therapy. Further information regarding their precise mechanisms of action and downstream effects are still areas of activity preclinical research.

To this effect, different compounds that could avoid the undesirable side effects of MAOIs and TCAs were developed beginning in the 1980s, often termed the second generation anti-depressants, which focused on selectively blocking the reuptake of certain monoamines at the synapse. Perhaps the most well-known of these compounds are the serotonin selective reuptake inhibitors (SSRIs), in particular fluoxetine (commonly sold under the name Prozac), escitalopram (Lexapro) and sertraline (Zoloft) which represent some of the most successfully marketed and widely prescribed SSRIs (Cipriani et al., 2018). Reuptake inhibitors targeting other catecholamines have also been found to be successful in treating depression, including norepinephrine selective reuptake inhibitors (NRIs) such as bupropion (Wellbutrin) and combined serotonin-norepinephrine reuptake inhibitors (SNRIs) like duloxetine (Cymbalta) and venlafaxine (Effexor). Collectively, these drugs have been shown to produce fewer side effects and toxicity effects than the MAOIs and TCAs across patient populations, and represented a significant leap forward in treatment for those suffering from chronic depression. Despite these successes, however, the vast majority of monoamine targeted compounds have all presented a number of similar shortcomings. Most display a lag of on average several weeks to several months before any therapeutic benefits are clinically reported, and many are also plagued by low rates of efficacy across more broad patient populations, leading to a high incidence of relapse rates for depressive episodes, lack of overall responsiveness to the medication, and patients failing to continue or stick with their treatment plans before the benefits of these compounds can be fully realized (Trivedi et al., 2006; Clevenger et al., 2018). This is particularly concerning when considering that both suicide and self-harm rates among chronically depressed patients are consistently reported at alarmingly high rates (Beck et al., 1985).

Patient cases that show a lack of response to treatment with standard second generation anti-depressants (in isolation and/or combination with therapy) are termed Treatment-Resistant forms of Depression (TRD), and represent a unique problem for medical professionals (Fava, 2003). Aside from the aforementioned pharmacological options available for assisting TRD patients (i.e. MAOIs or TCAs), few other therapeutic avenues exist outside of cognitive behavioral therapy (CBT) in combination with alterations in the dosages and combinations of typical anti-depressants. The use of non-invasive electro-convulsive therapy (ECT), however,

has been found to provide lasting relief and remission from depression for TRD patients, but the exact means by which these effects are achieved are poorly understood, if at all, by the medical and research communities (Singh and Kar, 2017). ECT thus remains another extreme treatment option usually only recommended when all other alternatives have been exhausted.

The combined lack of readily available options for TRD patients and shortcomings of typical second generation antidepressants highlight a critical need for continued study into the molecular and cellular underpinnings of depressive disorders in order to provide new directions for developing novel and more robust therapeutics. Technical advances in the areas of behavioral and systems neuroscience research using animal modeling have begun to make significant contributions to this need in recent years, providing preliminary evidence of potential targets for the treatment of MDD and related neuropsychiatric diseases. This is perhaps best represented by the advent of the use of the drug ketamine, classically believed to only produce anesthetic and psycho-dissociative effects, to treat patients across the spectrum of depressive disorders at low doses (Berman et al., 2000; Krystal et al., 2019). Indeed, the recent approval of the ketamine variants esketamine (sold as Spravato) speaks to the exciting new area of research that the use of ketamine has opened in our understanding of the neurobiology and neurochemistry of depression, and will be discussed in further detail in a later section of this chapter.

Rodent modeling and study of depressive disorders

As previously mentioned, some of the greatest limitations that exists for both improving our understanding of the precise mechanisms of depression's pathology and developing effective treatments lies in its heterogeneous presentation, high comorbidity with other psychiatric diseases, and inherent complexity at the biological level. With a lack of readily available biomarkers to currently aide in diagnosis, and human imaging studies still in their infancy for validating highly specific hallmarks of depression, the use of animal modeling to glean insight to the neural circuity and molecular/cellular changes that underlie depressive disorders has proven to be invaluable for advancing our current understanding of these disease states (Krishnan and Nestler, 2011; Wang et al., 2017; Planchez et al., 2019). Indeed, both rodent and primate models of depression have been developed over the course of such research efforts, and have helped to identify a number of potential indicators of depression and possible treatment avenues at a rapid pace (Planchez et al., 2019). The conception of many

of these models has stemmed from the identification of phenotypes that closely mimic those of human patients (termed face validity), exposure to conditions or factors that produce similar phenotypes in humans (construct validity), and responsiveness to pharmacological and non-pharmacological treatments that have shown efficacy in patient populations (predictive validity, Nestler and Hyman, 2010). Further, animal models allow for the rapid integration of other factors, such as potential genetic manipulations, gene-environment interactions and controlled exposure to stress (a highly cited factor leading to the pathogenesis of depression) either acutely or chronically into such research that are often times difficult or impossible to achieve in human studies. However, as no animal model is able to provide direct insight into the psychological state of a given subject, many of the metrics discussed below serve primarily as proxies of depression, and thus termed observations of "depressive-like" behaviors in animals. Below, I will focus primarily on the modeling efforts undertaken in rodents, which have perhaps the greatest level of throughput and multiplicity of options at the level of transgenic and behavioral modeling that exists for current mood disorder research (see **Fig. 1** for schematics and basic outlines of the tasks discussed below).

Many rodent models have been developed to assess aspects of the emotional or affective components of depression, most commonly through a focus on fear response and anxiety-related behaviors which are readily measurable in both mice and rats. These tests can often also be categorized on their basis of behavioral responses to either acute or chronic stressors, which may help to improve the translation of findings to the human condition on the basis of stress-responsiveness/processing itself, which is often cited as a major precipitating factor of depression. A number of tasks that focus on responses to acute stress typically choose a presentation of stressors under conditions in which the animals cannot escape from them. Perhaps the most well-known of these tasks is the forced swim test (FST), in which a rodent is placed into a cylinder of water and their attempts to escape from the water are scored over time (Porsolt et al. 1977). After an initial period of struggling, swimming and climbing attempts, many animals usually adopt a floating or immobile posture in the water, which is interpreted as an expression of "despair" or "learned helplessness" (Cryan et al., 2005; Lucki et al., 2001). Other iterations of this task exists, such as the tail suspension test (TST) and the inescapable foot shock test, but all focus on the same immobility or "learned helplessness" behavior, primarily due to evidence that has shown that the administration of typical antidepressants significantly increase the amount of time animals will spend struggling to escape in these tasks (Borsini and Meli, 1988) and by contrast, that exposure to

chronic stressors increases the amount of time spent immobile (Strekalova et al., 2004; Petit-Demouliere et al., 2005). The specificity and predictive validity of these tasks, which have shown little to no changes in behavioral phenotypes across studies administering other classes of drugs outside of the typical antidepressants, have long provided the basis of their strength in not only validating new antidepressant-like drugs, but also for the measurement of “depressive-like” behavior across studies focusing on the contribution of novel genetic and environmental factors to the pathology of depression (Krishnan and Nestler, 2011; Steru et al., 1985). Despite this however, these tasks also present a number of inherent issues that diminish their abilities to truly model and examine depressive behavior, due in part to their high reliance on locomotor function in order to be able to interpret results effectively. Criticism of these tasks, and their ability to actually identify new treatments for depression and even serve as a read-out of depressive-like, anxiety-like and other related behaviors, has also been raised in recent years, instead suggesting they may be more useful in studying “coping mechanisms” in the face of highly stressful situations (Molendijk and de Kloet, 2019) and arguing for the need of complementary behavioral analyses to be run in order to better capture multiple facets of depressive behavior in animal models.

One of these additional facets that has been heavily studied in rodents is anhedonia, or the loss of motivation or interest in otherwise pleasurable experiences. This type of behavior is usually examined through the use of food reward paradigms, in which animals are either trained to seek out a highly palatable substance or have been deprived of food for a short period of time, with the most common being the Sucrose Preference Test (SPT). This task early on showed that a rodent’s natural inclination to choose/drink a sweet, caloric substance of assumed hedonic value over water could be disrupted by exposure to stressors in models of anxiety and depression, first suggesting the strength of such approaches in modeling anhedonia as well (Liu et al., 2018; Scheggi et al., 2018). Other tasks have also been developed along similar veins, but with a more built in focus on the direct influences of the effects of stress on the hedonic drive to seek out food, such as the Novelty-Induced Suppression of Feeding test (NSFT) and the Novel-Induced Hypophagia test (NIH). Both tasks center on animals being expose to a stressor or challenge that will test their willingness to seek out these rewards, often with the latency to investigating and imbibing in these foods serving as a read out (Dulawa and Hen, 2005). These tasks are built upon the observation that rodents tend to display an increased reluctance to seek out food when they are placed in a novel environment, thus allowing a change in latency to approach a food reward in a novel/stressful environment to measure “hedonistic drive”. While these tests have more

historically been used to study anxiety-like behaviors in rodents, they have also been shown to be effective in measuring depressive-like behavior (Li et al., 2010; Li et al., 2011; Santarelli et al., 2003), with multiple studies even showing the chronic administration of SSRIs to greatly reduce latency to feeding (Bodnoff et al., 1988; Santarelli et al., 2003). The feeding component of both tasks does introduce the potential for certain confounds in testing as well, particularly in the case of the NSFT which stipulates animals to be food deprived for 24-48 hours prior to the task. The NIH compensates for this in some ways, by removing a fasting period and instead training animals to seek out a palatable food reward, which is then challenged by exposure to a novel environment on testing day. This design may thus better examine the interplay of the stress and reward systems/circuitry in the brain that are thought to be dysregulated in depression (Russo and Nestler, 2013; Nestler, 2015).

In addition to the task mentioned above that focus on anhedonia in the context of acute stressors, anxiety and anxiety-like behaviors that are often observed to be comorbid with depression have a number of tasks dedicated to their appraisal. These most typically consist of the Elevated Plus or Zero Maze tests (EPM and EZM, respectively) and the Open Field Test (OFT). In the EPM and EZM, an apparatus consisting of two open quadrants and two closed quadrants is used to test the willingness of a rodent to spend time in an exposed environment compared with enclosed spaces. Increases in anxiety are inferred by total time spent in the closed quadrants compared to total time spent exploring and moving about in the open quadrants (Pellow et al., 1985). Similarly, the OFT examines the amount of time an animal will spend in the surround region of the apparatus (near the walls and corners) compared with the time spent exploring the more exposed center region, with an increase in center time interpreted as a decrease in anxiety-like behavior (Denenberg, 1969).

The tests discussed thus far are often favored in the study of depression in rodents due to their high throughput and the ease at which phenotyping is believed to be interpretable from them. However, tasks such as these that prioritize a focus on singular or acute stressors are thought by many to fail to accurately model the multitude of stressors and chronic exposure to stress that is typically reported by patients as key in both their development of or relapse into a depressed state. While the work presented in this dissertation will heavily focus on the use of acute stress models and paradigms for phenotyping behavior in mice, it is worth briefly discussing the current chronic stress models that exist for depressive-like behavioral testing due to their high levels of face

and construct validity (Krishnan and Nestler, 2008), and the prospects they present for future behavioral studies that may build upon the results I discuss in Chapters 2 and 3.

Perhaps the most prominently used chronic stress model in rodents is the chronic mild, or unpredicted, stress paradigm (CMS/CUS). This multi-week task typically begins with training rodents to develop a preference for a rewarding substance, such as a highly palatable food source like sucrose or Ensure, after which the animals are exposed to a number of different stressors over the course of several days (Willner, 1991). These different types of stressors typically include, but are not limited to: water or food (chow) deprivation, exposure to intensely bright light, single housing, single bouts of mild foot shock, damp bedding, exposure to white noise, continuous cage changes, or restraint stress (Willner, 2005; Zhang et al., 2014). Following these stress exposure batteries, animals are tested again to gauge their latency to seek out the previously entrained food reward, with increases in both the time taken to initiate drinking, and total amount consumed, interpreted as a decrease in motivation to seeking out a reward, modeling aspects of anhedonia once again. The post-test following CMS/CUS can also be altered to use previously established tasks, such as the FST/TST, in order to gauge altered depressive-like behavior, or simplified further to examine changes in home cage/more passive behaviors that may also be used as a read out of increased anxiety-like or decreased hedonic-like phenotypes (i.e. increased grooming/stereotyped behaviors or decreased mating, respectively). The use of typical anti-depressants has also been shown to reverse increases in these negative affective behaviors in CMS/CUS post-testing (Strekalova et al. 2006), providing future support for the strength of this paradigm for modeling the longer lasting effects of depression.

Apart from CMS/CUS, another task gaining increasing acceptance as a core chronic stress model of depression in rodents is the chronic social defeat stress task (CSDS). Unlike CMS/CUS, which rely on exposure to many physical stressors that may or may not be representative of those experienced by rodents in a more natural setting, CSDS takes into consideration the innate sociability of rodents to examine how “psychosocial” stressors that may be present in the wild influence depressive-like behavior. The task is typically performed by prolonged exposure of test rodents to a more dominant or aggressive rodent that is placed into the test animal’s cage or vice versa, after which changes in submissive behavior are gauged in the test animal (Blanchard and Blanchard, 1977; Malatynska and Knapp, 2005). Continued bouts of these interactions, which in some cases may be amplified by placing animals into a partitioned cage that gives them “sensory exposure” to the aggressor

(Martinez et al., 1998), has been shown to reliably produce increases in depressive-like behaviors, as well as elevated anxiety-/stress-associated behaviors (Tornatzky and Miczek, 1994; Hollis et al., 2010; Duclot et al., 2011). While studies on the effects of anti-depressants to reverse increases in depressive-like phenotypes under these conditions have shown positive outcomes (Venzala et al., 2012), like many of the tests above, the CSDS possesses certain caveats that may not allow it to be viewed as a pure model of depression, and instead capture many more aspects of anxiety-like and social behavior. Despite this, other paradigms that focus on psychosocial stressors as a means to study depressive-like behavior have proved to be useful and relatively unsophisticated, included the use of social isolation stress (or single housing), which is utilized extensively in this dissertation as outlined in the following chapters. Indeed, depressive- and anxiety-like outputs such as decreased center time in the OFT, increased immobility in the FST/TST, and decreased sucrose preference have been shown to increase following chronic isolation (Wallace et al., 2009; Ieraci et al., 2016), and the use of select anti-depressants has been shown to have some efficacy in reversing a number of these phenotypes following prolonged treatment (Wallace et al., 2009; Wilkinson et al., 2009).

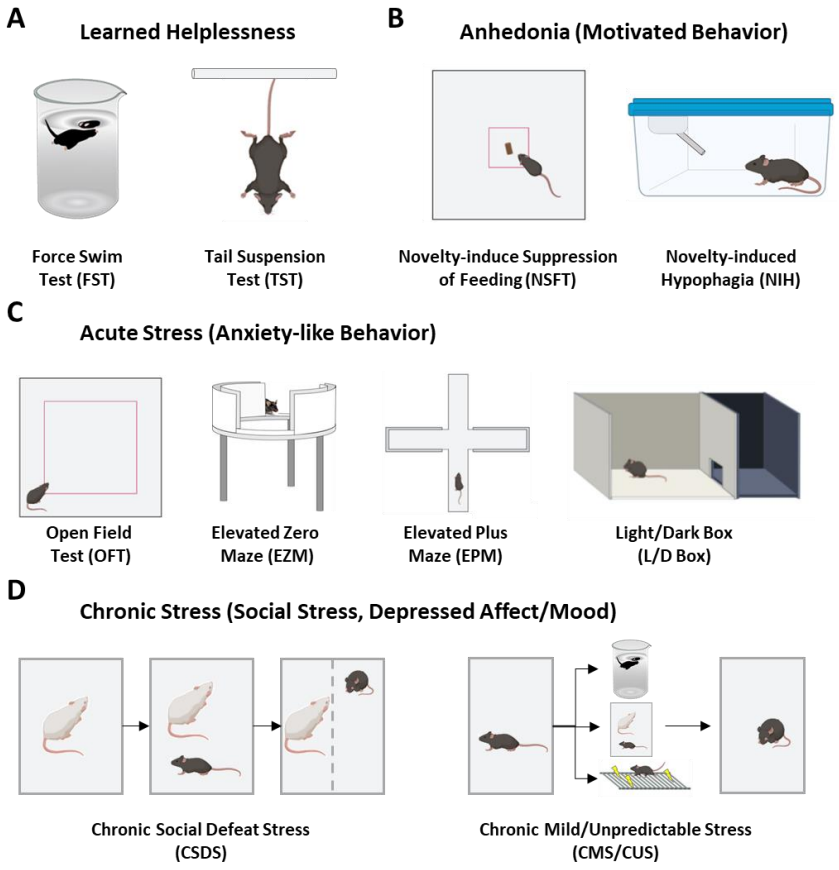


Figure 1. Common rodent behavioral tasks useful in modeling aspects of depressive-like behaviors. Schematics of several popular and highly used tests in rodent studies of depression, anxiety and other mood related disorders, including: **(A)** the forced swim and tail suspension tests (FST, TST) used to examine the phenomenon of "learned helplessness" behavior often seen to be reversed following typical antidepressant administration, **(B)** models of anhedonic behaviors closed tied to depression, such as the novelty-induced suppression of feeding and hypophagia tests (NSFT, NIH), **(C)** the open field (OFT), elevated zero and plus mazes (EZM, EPM) and light/dark box (L/D) used to examine acute changes in anxiety-like and stress induced behaviors often seen as a common comorbidity or precipitating factor of depression, and **(D)** models of chronic or long term exposure to social and environmental stressors such as the chronic social defeat stress (CSDS) and chronic unpredictable stress (CMS/CUS) tasks, which are becoming more widely accepted as useful translational approaches due to the persistent nature of and multitudinous factors that can lead to and exacerbate depression in patients. Adapted in part from Knowland and Lim, 2017. Rodent and apparatus images courtesy of BioRender ®.

Despite the many caveats and lack of introspective insight that is typical of nearly all animal models of depressive disorders, the use of many of the tasks described above has provided invaluable improvements in our understanding of the biology of affective behavior and the pathophysiology underlying the disease state of depression. The speed and ease of replication, combined with the many advances in technologies and transgenic lines that rodent work in particular has seen in the field of neuroscience over the past few decades, has allowed for new and unprecedented glimpses into this pathology at both a cellular and circuit level. This has and continues to prove to be crucial for furthering our understanding of the molecular bases of depression, as well as aiding in the identification of novel targets that may prove to be valuable for both developing new areas of study and future treatments for this disorder.

Classical molecular targets of depressive disorders: insights and shortcomings

The monoamine signaling system

The biomedical community is rapidly approaching the dawn of a new era in depression research that focuses on synthesizing our knowledge on regional, circuit and cell specific functions into a more connected image of a disease to inform a greater understanding of etiology and treatment avenues. It is important, however, to appreciate the influence that early glimpses into the potential molecular underpinnings of conditions like depression provided toward shaping our current knowledge and research goals, and continues to provide in our pursuit of better therapeutics.

Much of this starts with the monoamine signaling system, the first widely accepted target for the treatment of depression and many other similar affective disorders. As mentioned previously, the monoamine

signaling system is comprised of the brain's serotonergic, dopaminergic and noradrenergic circuity and their chief molecules of action: 5-HT, DA and NE, respectively. All three of these neurotransmitters are derived from aromatic amino acids or the products of these amino acids (5-HT from tryptophan, DA from phenylalanine and tyrosine, and NE from DA), and are collectively believed to serve a modulatory role in regards to synaptic transmission and function when compared to the brain's more central neurotransmitters, glutamate and gamma aminobutyric acid (GABA). As such, the projection patterns of each monoamine system extends into many cortical and subcortical structures that have been implicated in cognitive, emotional processing and basal/homeostatic functions. Access to these systems in order to probe the effects of monoaminergic signaling in the context of disease has classically been achieved pharmacologically by targeting the receptors or vesicular/synaptic transporters of each of these molecules, but researchers have now begun to build upon this knowledge by dissecting their core circuitry as well, and determining how monoaminergic inputs/outputs to and between select brain region contribute to the regulation of affective behavior.

Serotonin (5-HT): 5-HT is produced predominantly by neurons in the raphe nuclei of the brainstem, which sends axonal projections to the majority of cortical, subcortical and midbrain structures, the cerebellum, and the spinal cord/spinal ganglia (Nichols and Nichols, 2008). The molecule has a wide variety of receptor targets throughout the central and peripheral nervous systems, consisting of 14-15 known receptors that are broken down into 7 general classes (5-HT1-5-HT7), though the majority are G-protein coupled receptors (GPCRs) that signal through second messenger cascades to drive either broad inhibitory or excitatory modulation at neurons (Nichols and Nichols, 2008). The most consistent target of 5-HT in the CNS for the treatment of depression however has not been any of these receptors, but instead the serotonin transporters (SERT or 5-HTT) that are responsible for driving the re-uptake of 5-HT back into presynaptic terminals at the sites of serotonergic synapses. Much of the focus on 5-HTT stems from clinical studies showing a correlation between decreased levels of the precursors of 5-HT (i.e. tryptophan) in chronically depressed patients (Karege et al., 1994; Ogawa et al., 2014) and others finding that such patients also display decreased levels of 5-HT metabolites such as 5-hydroxyindoleacetic acid or 5-HIAA (van Praag and de Haan, 1979; Asberg et al., 1984; Gjerris et al., 1987). Genome-wide association studies have provided evidence of an association between polymorphisms in 5-HTT and increased reporting of depressive episodes or chronic depressive disorders in patients as well (Caspi et al.,

2003). Collectively, these findings helped to bolster one of the key linchpins of the monoamine hypothesis: that a reduction in 5-HT levels in the brain may serve as either a risk factor or a key underlying cause of the pathology of depression, and the notion that elevating extracellular 5-HT could help to treat patients by reversing this neurochemical imbalance (Albert and Benkelfat, 2013). Indeed, numerous preclinical studies have also demonstrated a clear link between 5-HTT antagonism, increased synaptic 5-HT, and the antidepressant effects of drugs such as the SSRIs (Lemberger et al., 1985; Nakenoff et al., 2016).

The broad elevation of 5-HT via 5-HTT antagonism has remained the primary method of targeting and treating the presumed imbalance in this monoamine in patients, however converging clinical and preclinical data has made a case for the individual 5-HT receptors as other potential targets. While many of the 5-HT receptors have been shown to modulate aspects of depressive-like behavior (Carr and Lucki, 2011), more extensive work has focused on the 5-HT_{1A} and 5-HT_{1B} receptor subtypes, both of which have been shown to have altered expression patterns, associated polymorphisms and contribute to the mechanism of action of select anti-depressants in patient populations (Robinson et al., 1990; Stockmeier et al., 1998; Tatarczynska et al., 2005; Parsey et al., 2010; Murrough et al., 2011; Donaldson et al., 2016). In rodents, pharmacological activation of both receptors has been also been demonstrated to produce acute anti-depressant-like effects in tasks such as the FST (Redrobe and Bourin, 1999; Kennett et al., 1987; Lopez-Rubalcava and Lucki, 2000; O'Neill and Conway, 2001), however differences in the contribution of each receptor to these effects has been reported based on their presumed expression patterns and functionality. Both inhibitory GPCRs (i.e. coupled to $G_{i/o}$), 5-HT_{1A} is shown to be expressed predominantly somatodendritically on neurons, allowing for direct modulation of firing, while 5-HT_{1B} is expressed on axon terminals and its activation has been implicated in the regulation of neurotransmitter release (Boschert et al., 1994; Ghavami et al, 1999; Riad et al., 2000). Key distinctions can also be made regarding the expression of these receptors on serotonergic neurons, where they act as autoreceptors, or non-serotonergic neurons, where they function as heteroreceptors, and the implications this may hold for their role in transducing the effects of altered serotonergic signaling in disease states or in response to treatments with SSRIs. 5-HT_{1A} and 5-HT_{1B} heteroreceptors are heavily expressed throughout the brain's limbic circuitry, particularly in the cortex, hippocampus and basal ganglia, and thought to act in opposition to the autoreceptors found in the raphe nuclei when responding to changes in 5-HT levels brought on by typically SSRIs, contributing to the slow time of onset for anti-depressant actions in many patients

(Maroteaux et al., 1992; Pompeiano et al., 1992; Davidson and Stamford, 2000; Biler and Ward, 2003). As such, the development of new compounds targeted selectively to auto- or heteroreceptors has been proposed as a means to enhance or refine treatments for depression targeting the brain's serotonergic system, with evidence showing that the use of the partial 5-HT_{1A} agonists pindolol or vilazodone (targeting autoreceptors more selectively) can produce robust anti-depressant actions in patients and enhance the effectiveness of classical SSRIs (Tome et al., 1997; Sahli et al., 2016). Similarly, 5-HT_{1A} heteroreceptor preferring agonists have also been shown to greatly reduce depressive-like behaviors in rodents (Newman-Tancredi et al., 2009). A focus on the regional expression patterns of these receptors and their role in intrinsic and extrinsic circuitry has helped to consolidate evidence of 5-HT_{1A} heteroreceptor function in the hippocampus and cortex as crucial in regulating depressive-like behavior (Richardson-Jones et al., 2011), and show that elevated 5-HT_{1B} heteroreceptor expression and function in forebrain/basal forebrain structures such as the cortex and NAc may enhance the effects of antidepressants (Svenningsson et al., 2006; Alexanxder et al., 2010), while stress exposure may increase 5-HT_{1B} autoreceptor expression/activation in the dorsal raphe and drive depressive-like behavior (Neumaier et al., 1997; Clark et al., 2002). Recently, serotonergic inputs from the dorsal raphe to a set of stress-responsive structures known as the extended amygdala have also been shown to drive increases in anxiety and fear behaviors in a 5-HT_{2C} specific fashion, highlighting other aspects of the contributions of 5-HT in the regulation behavioral outputs salient to depression (Marcinkiewcz et al., 2016). The study of the specific circuitry of serotonergic signaling and its molecular substrates continues to support a key role for this monoamine in depression. However, despite advances in more selective receptor targeting, the efficacy of 5-HT focused therapies continue to present slowly in many patients and/or not emerge at all, suggesting that while critical, 5-HT signaling may only represent one of many salient aspects of the underlying pathophysiology of this disorder.

Noradrenaline (NE): The projections of the NE signaling system spread throughout the vast majority of cortical and subcortical structures, originating from a small number of noradrenergic cell populations found primarily in the locus coeruleus (LC) and to a lesser degree in a number of other small, diffuse midbrain and brainstem nuclei (Goddard et al., 2010). Much like the serotonergic signaling system, noradrenergic projections from these regions extend to key centers of cognitive function, such as the cortex and hippocampus, as well as to most of the limbic structures, including the amygdala, extended amygdala and hypothamalus, positioning NE to

significantly contribute to many of the circuits within and between a number of regions that have been implicated in regulating depressive-like behaviors (Stahl, 2003). The NE signaling system extensively innervates the body's peripheral nervous system (PNS), and acts as one of the primary neurotransmitter molecules within the autonomic nervous system, chiefly at the sympathetic division, that is associated with regulating an organism's "flight or fight" responses to external stimuli (Goddard et al., 2010). Prominent amongst these forms of stimuli is stress, and the peripheral responses to stress that are modulated by the sympathetic nervous system and NE signaling are often inextricably tied to a cascade of events that initiates the classical stress response in the CNS at the level of the hypothalamic-pituitary-adrenal axis, or HPA axis (Chrousos, 2009; Goddard et al., 2010). This occurs principally through projections from the LC and nucleus of the solitary tract (NTS) which innervate the paraventricular nucleus (PVN) of the hypothalamus, a key production site of one of the primary signaling stress signaling molecules, adrenocorticotropic hormone (ACTH). The role of the HPA axis and the key modulatory transmitters and peptides that regulate its activity in response to stress, and how its dysregulation may be implicated in depression, will be expanded upon in the next section. For now though, it is sufficient to say to that the close association of NE signaling and HPA axis function has been extensively studied both clinically and pre-clinically in the context of depression and other mood disorders, and has shown enough of a correlation to have early on signaled out NE as a key component of the monoamine system to target for the development of therapeutics (Chrousos, 2009; Goddard et al., 2010).

NE has a number of receptors it acts upon within the CNS and PNS, which are broken down into two main families, the alpha and beta receptors, and are further composed of two to three validated receptors each ($\alpha_{1\text{-}2}$ and $\beta_{1\text{-}3}$). All of these receptors are also GPCRs, with the α_1 receptors associated with a G_q protein, all three β receptors with a G_s protein, and the α_2 with a $G_{i/o}$ protein, giving the α_1 and β receptors a more excitatory modulatory function, while the α_2 receptors are believed to produce more inhibitory effects (Strosberg, 1993). As mentioned above, however, the primary target of NE signaling in regards to the treatment of depression has classically been the norepinephrine transporter (NET). Much like in the case of typical antidepressants targeting the 5-HT system, the majority of NE targeted drugs act at NET to increase the levels of NE in the brain, and as such are often dubbed norepinephrine reuptake inhibitors (NERIs), with many of these drugs formulated to also target 5-HTT, making them combined NE and 5-HT reuptake inhibitors (SNRIs). Early evidence of decreased levels of NE and NE metabolites such as 3-methoxy-hydroxyphenylgycol (MHPG) in serum samples of

chronically depressed patients compared to healthy controls (Lambert et al., 2000) helped to provide initial support for this treatment paradigm, with additional studies suggesting that NET activity is decreased in the LC and limbic structures of depressed or suicidal patients postmortem (Ordway and Klimek, 2001; Brunello et al., 2003) further supporting the monoamine hypothesis's central idea. Genetic studies in patients demonstrating increased susceptibility to depression and depressive episodes have also shown correlative instances of NET mutations that may lead to altered transporter function and overall NE signaling efficiency (Lin and Madras, 2006), again arguing for the continued idea that correcting this decrease in NE levels may lead to depression relief.

More recent work and ideas are beginning to turn their focus away from only studying the role of NE levels and NET function, to now investigate the modulatory effects of noradrenergic receptor signaling and changes in intrinsic NE circuitry that might be implicated in depression pathology. The α_{2a} subtype of the α_2 receptor, specifically, has been found to be elevated in expression in the PFC of suicidal and chronically depressed patients postmortem (Escriba et al., 2004), while rodent studies of mice lacking this receptor show evidence of increased depressive-like behaviors (Schramm et al., 2001). This may suggest a potential protective role for these receptors in response to the initial neurochemical changes associated with depression that may go awry over time, and lead to a suppression of NE signaling via the actions of α_2/α_{2a} auto-receptor function (Chandley and Ordway, 2012; Cottingham and Wang, 2012). Clinical and preclinical studies of the role of the α_{2a} receptor's expression and function in depression have also displayed promising converging data, with the administration of TCAs or the α_2/5-HT_2 receptor antagonist mirtazapine (as well as the use of ECT) all shown to produce a decrease in α_{2a} receptor density in scans from depressed patients (Garcia-Sevilla et al., 1999; Garcia-Sevilla et al., 2004; Cooper et al., 1985; Werstiuk et al., 1996). The use of the subtype-selective α_{2a} antagonist BRL44408 has also been demonstrated to elicit a robust decrease in immobility time in the FST in mice (Dwyer et al., 2010). In addition to this, α_{2a} positive terminals have been observed to be abundant in the dorsal raphe (DR) and the receptor to be prominently expressed on serotonergic neurons in the region as well (Kalsner and Abdali, 2001; Goddard et al., 2010), suggesting a strong cross-talk between serotonergic and noadrenergic circuitry that may be key to the pathology of depression, as well as the mechanisms of action of many typical antidepressants. Apart from the how the circuitry of these two monoamine systems might be intertwined and implicated in the effects of depression, more recent work has also focused on the inputs that

drive both the activation and inhibition of primary noradrenergic cells and sites in the brain, and how this may lead to increased or decreased signaling through the NE modulatory network. Glutamatergic signaling in particular, which has been shown to be elevated in patients of depression (Sanacora et al., 2004; Chandley et al., 2013), has been implicated in driving LC activation and NE signaling, particularly in the context of stress. In rodents, acute stress expose has been shown to enhanced LC excitability (Borodovitsyna et al., 2018), while in postmortem analyses of the LC of chronical depressed or suicidal patients, transcripts analyses of several glutamate receptor subunits were found to be highly elevated when compared with healthy controls (Bernard et al., 2011; Chandley et al., 2014), indicative of altered excitatory activity in the LC of these patients, as well as a dysregulation of glutamate and NE signaling interactions overall. These findings, among others, suggest the possibility that NE and potentially other monoamine signaling systems, can be directly regulated through the more primary neurotransmitter systems, and that through modulating inhibitory or excitatory signaling alone, more universal antidepressant effects can be achieved by targeting a larger variety of the neurochemical imbalances associated with depressive disorders.

Dopamine (DA): DA by contrast has more restricted network in the brain when compared with the other classic monoamines, consisting of five to six primary pathways stemming from the main hubs of DA production in the VTA and the substantia nigra pars compacta (SNc). These include the mesolimbic and mesocortical pathways (often grouped as the mesocorticolimbic projections) that connect the VTA to the ventral striatum (the canonical reward pathway, terminating at the NAc) and the VTA with the PFC, the nigrostriatal pathway that connects the SNc to the dorsal striatum (canonical basal ganglia pathway), the tuberoinfundibular pathway connecting the hypothalamus/arcuate nucleus with the pituitary, the hypothalamospinal pathway between the hypothalamus and spinal cord, and lastly the incertohypothalamic pathway that links the zona incerta with the hypothalamus and brainstem (Malenka et al., 2009). Several different receptors serve as targets for DA in the CNS, and are typically broken down into five subtypes (D_1-D_5) that can be further divided into two broad families: the D_1-like receptors (which consist of D_1 and D_5) and the D_2-like receptors (D_2, D_3 and D_4), all of which are GPCRs. The D_1-like receptors are coupled classically to G_s proteins, giving them an excitatory-like function through the activation of adenylyl cyclase and the cyclic adenosine monophosphate (cAMP) second messenger pathway, while the D_2-like receptors are associated with G_i proteins and exhibit a net inhibitory function when activated

(Beaulieu and Gainetdinov, 2011). Similar to the other monoamine systems discussed here, these receptors have not classically been targeted for the treatment of depression, as initial antidepressants such as the MAOIs were understood to indirectly elevate levels of DA in patients. This unifying theme of lowered or depleted levels of all monoamines that drives one of the initial hypotheses of depression has not been without clinical support, as past and recent studies have also shown levels/activity of the dopamine transporter (DAT) to be significantly lower in chronically depressed patients when compared with healthy controls (Meyer et al., 2001; Sarchiapone et al., 2006; Pizzagalli et al., 2019), and other scans have shown consistent decreases in striatal DA cell activity in similar patients pools (Pruessner et al., 2004). These functional changes in key components of the brain's dopaminergic/reward circuitry have been hypothesized to correlate with one of the key hallmarks of depression: anhedonia, or a loss of motivation to seek out pleasurable or rewarding stimuli (Forbes et al., 2009), an idea that has been supported by animal modeling studies (Papp et al., 2003). As a DAT-specific approach has largely been avoided in the treatment of depression, new studies focusing not only on the changes in intrinsic DA circuitry in depressive disorders, but also on the changes in receptor expression and modulation, have begun to expand upon the possibilities of uncovering a more direct role for DA in the pathology of depression.

In regards to receptors, it has been shown that D_2 levels are highly elevated in the striatum of depressed or suicidal patients when compared with controls (D'Haenen and Bossuyt, 1994) and that both D_2 and D_3 receptor binding is increased in the amygdala of similar patient pools (Pare et al., 1969), suggestive of diminished DA turnover and potentially reduced DA signaling if these two receptors are acting in an auto-receptor-fashion. Indeed, recent work using the D_2/D_3 antagonist amisulpride (which is believed to increase DA signaling by blocking presynaptic auto-receptors) has demonstrated a restoration of activation in striatal-cortical circuitry in depressed patients when compared with placebo groups (Admon et al., 2017) and has previously been shown to promote hedonistic drive in rodent models of depression following chronic administration (Schoemaker et al., 1997). Studies such as these have helped to further promote the idea of directly targeting DA receptors as an alternative treatment to traditional antidepressants, while further advances in animal research modeling and circuit-based neurobiological studies have also placed an emphasis on the role of DA signaling and neuronal activity as important in shaping the affective aspects of depression. CMS/CUS for instance has been shown to produce long term alterations in DA receptor expression in limbic structures (Dziedzicka-Wasylewska et al., 1997; Kram et al., 2002), as well as decreased DA transmission in the NAc (Di

Chiara and Tanda, 1997). VTA lesion studies have also been reported to produce depressive-like behaviors in rats (Winter et al., 2007), suggesting that decreased or altered activity in the DA neurons in this critical region may underlie aspects of depression. This notion has been bolstered by other rodent work showing that the characteristic burst firing of VTA DA neurons in response to rewarding stimuli is blunted in a genetically validated rat model of depression (Friedman et al., 2008), and perhaps most prominently in a tour de force study demonstrating that optogenetic inactivation of VTA DA neurons in mice can drive increases in depressive-like behaviors, all of which could be reversed by selective activation of the same neurons (Tye et al., 2013). Collectively, these pre-clinical data build upon current human imaging work implicating altered activation in the mesolimbic and mesocortical DA pathways as likely associated with the pathology of depression, and have also begun to identify new circuits, such as those between the ventral hippocampus and the NAc (Belujon and Grace, 2014) and the basolateral amygdala (BLA) and the ventral palladium (VP, Chang and Grace, 2014) as potential regions of interest to both screen in patients and further examine for the interplay of other neurotransmitter systems. Indeed, as in the case with NE, the modulation of glutamatergic signaling has been shown to reverse aspects of DA signaling and neuronal activity altered in rodent depression models (Belujon and Grace, 2014), suggesting that broad manipulations of excitatory circuits and molecular targets may also serve to indirectly influence aspects of monoamine signaling in the disease state in an all-encompassing manner.

Neuropeptidergic and neurohormonal modulatory systems

Research focused on expanding the scope of molecular targets for treating depression and other mood disorders beyond that of the monoamines has been a topic of interest for some time, with a number of different potential candidates emerging in the past few decades that have merited more in depth investigation. Some of the most heavily studied classes of molecules to fit this description have been the neurohormones and the neuropeptides, primarily due to a wealth of data implicating many of these molecules in regulating the stress response circuitry of both the PNS and CNS. As mentioned above, perhaps the most well-known of all these circuits is the HPA axis, the dysfunction of which has long been speculated to play a role in the pathogenesis of anxiety and other affective disorders, including recent evidence pointing to depression as well (Tamagno and Epelbaum, 2015; Badhan et al., 2008). Indeed, this master regulator of stress responsiveness hinges on the

signaling achieved through a number of key endocrine molecules that have served as entry points for investigating new means to treat depression that may avoid many of the undesirable side effects of the typical/atypical antidepressants and mood stabilizers on the market today. In addition to this, many research groups have also begun to consider the role of specific neuropeptides in regulating emotional states and behavioral output in response to affective stimuli, owing in large part to work focusing on the role of peptides such as oxytocin and the like in driving positive social interactions seen in model organisms (Insel, 2010). Below, I will briefly outline our current knowledge existing on the connections between several key neurohormones/neuropeptides and depression, including the many challenges that have faced this area of research in finding and developing effective therapeutics.

Corticotropin releasing factor (CRF): Perhaps the most well studied neurohormone in regards to the relation of its signaling and activity in the pathophysiology of affective disorders is corticotropin releasing factor (CRF), also known as corticotropin releasing hormone (CRH). This is primarily due to the key role that CRF plays in regulating the activation of the HPA axis by kicking off the cascade of events that eventually leads to the recruitment of the sympathetic nervous system and the induction of the body's biological stress response, typically comprising of changes in arousal, attention and cognition in the CNS, and increases in cardiovascular tone, respiration, and energy production/consumption peripherally (Chrousos and Gold, 1992). To achieve this, CRF produced within the PVN is released into the anterior pituitary, where it stimulates the release of ACTH, which acts at the adrenal glands to drive the production and release of the glucocorticoid hormones into the bloodstream/periphery. In humans, the chief glucocorticoid is cortisol (corticosterone in rodents), and has long shown to be altered in production and levels in patients suffering from general anxiety and mood related disorders such as depression (Holsboer, 2001). Considering that the entire HPA axis operates as a negative feedback loop, in which enhanced levels of glucocorticoids eventually act to suppress the production/release of CRF and ACTH (Calogero et al., 1988), it has been suggested that inhibiting CRF signaling/release may serve as a more effective way to reduce the chronic affective and stress associated symptoms of depression and promote long term remission (Holsboer and Barden, 1996). This has principally been attempted by targeting the main receptors of CRF within the CNS and PNS: corticotropin releasing factor receptor 1 (CRFR1) and corticotropin releasing factor receptor 2 (CRFR2).

Both CRFR1 and CRFR2 are GPCRs, and are believed to primarily associate with the G_s protein, though evidence has suggested that they are able to associate with both G_q and $G_{i/o}$ proteins as well (Grammatopoulos et al., 2001), making their associated pharmacology and direct manipulation challenging. Despite this, studies of the distribution of the receptors via *in situ* analyses have shown both to be highly abundant in limbic structures such as the prefrontal cortex, ventral hippocampus, amygdala, extended amygdala, VTA and pituitary (Kuhne et al., 2012; Chalmers et al., 1995; Van Pett et al., 2000), providing anatomical evidence for a key role of these receptors in modulating the activity of regions long associated with the pathology of depressive disorders. Similarly, a wealth of preclinical data has shown that animals lacking the CRFR1 receptor exhibit an increase in anxiolytic behaviors and an overall blunted stress response (Smith et al., 1998; Timpl et al., 1998), and that conversely, the activation of these receptors is associated with robust increases in depressive-like behavior (Chen et al., 2018). Other rodent work showing that the administration of CRFR1 selective antagonists such as antalarmin or NBI-27914 can prevent or reduce these behaviors in both mice and rats (Zorrilla et al., 2002; Bale and Vale, 2003; Chen et al., 2018) have further strengthened the arguments for pursuing the development of tolerable compounds to antagonize CRFR1 signaling, which has become broadly accepted to be the key receptor out of the two that drives anxiogenesis (Reul and Holsboer, 2002). Clinical data from depressed patients has also served to galvanize this interest in CRF and the CRFR1, as studies have reported the levels of the neurohormone to be highly elevated in those more susceptible to depression when compared with healthy controls (Nemeroff et al., 1984; Raadsheer et al., 1995), and genome wide association data has additionally identified a correlation between SNPs within the CRFR1 gene and patients suffering from or reporting increased incidence of depressive episodes (Liu et al., 2006; Wasserman et al., 2008). Combining this with studies indicating that long term treatment with typical antidepressants may also serve to reduce CRF levels in patients over time (de Bellis et al., 1993) has further promoted the notation that the regulation of CRF signaling may be critical to achieving lasting relief from depression and normalizing stress responsive behavior in affective disease states.

While preclinical studies using several CRFR1 antagonist compounds have proven promising for the development of novel drugs, few have shown the same efficacious results observed in rodent and monkey/primate models for the relief of anxiety and depressive-like symptoms when tested in human subjects. To date, the majority of all these compounds have failed to meet sufficient criteria during clinical trials to

advance past Phase III testing, leading to many compounds like R121919, CP-316,311, NBI-34041 and others to be abandoned as serious drug candidates when they proved unable to produce statistically robust or long lasting relief from anxiety or depressive symptoms, despite displaying good tolerance across subjects (Zobel et al., 2000; Binneman et al., 2008; Ising et al., 2007; Griebel and Holsboer, 2012). Nevertheless, the evidence implicating CRF signaling in the pathophysiology of depression is compelling, with more recent work suggesting that the direct manipulation of cells that produce CRF and CRFergic circuits in select brain regions may be sufficient to alter affective behaviors in rodents (Asok et al., 2018; Kim et al., 2019a). This suggests that the identification of other molecular targets aside from CRFR1 and CRFR2 to regulate the activity of CRF cells and circuits may prove to be highly useful, and allow for other effective means to correct HPA axis dysfunction at the level of a key modulatory molecule.

Glucocorticoids (GCs): As mentioned, one of the other primary players in the activation of the biological stress response are the glucocorticoids (GCs), chiefly cortisol in human (and corticosterone in rodents), that serve as the final output of the HPA axis. In depressed patients, it has been speculated that dysregulation of the typical negative feedback induced by increasing levels of GCs that leads to an inhibition of HPA axis output can produce long term deficits in affective behavior and lead to HPA axis hyperactivity, a notion supported by studies finding that depressed patients exhibit heightened levels of GCs in saliva, cerebrospinal fluid, plasma and urine when compared with control subjects (Nemeroff et al., 1992; Pariante et al., 2009). Thus, a separate “glucocorticoid hypothesis” of depression has also been speculated in the biomedical community, and has generated an avenue of research into the study of the key receptors of these hormones, the glucocorticoid and mineralocorticoid receptors (GR and MR), and how their function, activity and manipulation may be associated with the pathophysiology of depression as well. Much like in the case of research into CRF and the CRF receptors, the results of these studies have shown spots of promise, but have been ultimately difficult to translate to effective therapeutics at the level of the clinic. Indeed, studies using rodents lacking functional GRs within the nervous system reported decreases in anxiety-like behaviors and stress responsiveness despite elevated levels of CRF and increased secretion of corticosterone (Tronche et al., 1999), while others found that mice expressing either anti-sense GRs or in which GRs were selectively deleted in the hippocampus displayed increased depressive-like behaviors (Pepin et al., 1992; Boyle et al., 2005). Despite opposing results, these

studies highlighted the importance of GR signaling on regulating emotional behavior, and to the initial proposition that blocking GRs could serve as a strategy for combating the effects of heightened GC levels in depression. Early studies with GR antagonists such as RU486 did show some promise both in patient clinical trials and in rodents, specifically in reducing neurocognitive impairments that may be associated with depression (Young et al., 2004; Oomen et al., 2007), but not in directly ameliorating the affective symptoms of depression (Flores et al., 2006), and in some cases, administration of the antagonist led to increased depressive-like behaviors over time in rodents (Papolos et al., 1993) and increased levels of cortisol in clinical trial subjects. Thus, while current attempts to block GR signaling have proven to be somewhat less effective than expected, the collective human and animal studies into GRs/MRs and GCs in relation to depression has uncovered a clear dysfunction in their signaling and activity in the disease states, but may require a better target for regulating the effects of elevated GC levels than the cognate receptors alone.

Oxytocin (OXT): In parallel to studies of the classic molecular targets of depression within the endocrine system, more recent efforts have begun to investigate the role of neuropeptides in both the regulation of emotion and their potential implications in the pathology of mood disorders. One of these peptides is oxytocin (OXT), which apart from its well documented role in social stimuli responsiveness, has also been found to be synthesized and released in response to stress and anxiety-related stimuli as well (Neumann, 2008). Specifically, OXT has been found to exert anxiolytic and antidepressant-like effects in rodents, potentially through its actions at limbic structures such as the PVN and amygdala (Knobloch et al., 2012; Blume et al., 2008). Systemic administration of OXT in rats has also been shown to produce stress protective effects (Windle et al., 2004; Slattery and Neumann, 2010) and mice lacking functional oxytocin receptor (OXTR) have demonstrated increased anxiety-like behaviors (Amico et al., 2004), providing further preclinical evidence for a role of OXT in regulating emotional behaviors implicated in depression pathology, and suggesting that targeting OXTRs may serve as a potential treatment. Despite this, the use of novel OXTR agonists (such as WAY-267464) or antagonists (OXT-A) in rodent studies have failed to directly alleviate or exacerbate depressive-like behaviors across tasks such as the FST and TST, leading to somewhat inconclusive interpretations of the antidepressant potential of OXTR manipulation (Ring et al., 2010; Ebner et al., 2005). Additionally, clinical data from depressed patients has been equally lacking in regards to drawing any clear correlation between OXT levels and increased instances of

depression, with studies showing opposing results at best (Slattery and Neumann, 2010; Parker et al., 2010), and little evidence exists of genetic mutations in OXTRs in patient populations with depressive disorders. Thus, while OXT may be shown to play a clear role in the regulation of affect in response to negative and positive forms of emotional stimuli, how to manipulate this system in order to treat affective disorders such as depression is still uncertain at best.

Vasopressin (AVP): Like OXT, vasopressin (AVP) is another neuropeptide that has recently garnered much interest for its potential role in regulating limbic circuitry and downstream affective behavior. It has been speculated to act in direct opposition to OXT, in that while AVP may also be produced and released in response to anxiogenic, stressful, or negative social stimuli, it is thought that the actions of AVP signaling instead enhance anxiety and depressive-like behaviors (as seen in rodent models) instead of suppressing them (Neumann and Landgraf, 2012). These effects have been supported by a number of transgenic and pharmacological studies in rodents, specifically by targeting the main receptors of AVP: arginine vasopressin receptors 1A and 1B (AVPR1A and AVPR1B), showing that the administration of AVPR agonists such as desmopressin can produce robust anxiogenic effects in rats while AVPR1A or AVPR1B antagonists like atosiban and SSR149415 can directly reverse or suppress such behavior (Mak et al., 2012; Griebel et al., 2002) as well as promote antidepressant-like effects across measures of active coping and immobility in the FST (Griebel et al., 2003; Ebner et al., 2002). AVPR1A knockout mice have also been shown to display an anxiolytic phenotype across metrics like the EPM and OFT compared with wildtype animals (Bielsky et al., 2004), and similar studies in AVPR1B knockout mice also show that the deletion of this receptor can produce a reduction in depressive-like behavior and both AVP and stress hormone circulation in the bloodstream (Tanoue et al., 2004). Clinical data from depressed patients has also demonstrated some promise for the targeting of the vasopressin system, with several studies indicating that AVP levels in both plasma and cerebrospinal fluid may be elevated in chronically depressed subjects when compared with controls (van Londen et al., 1997; de Bellis et al., 1993). Additionally, clinical trials from the use of SSR149415 in depressed patients showed some promise early on to indicate that the drug was not only well tolerated in humans, but that it might be effectiveness in treating the core symptoms of depression, as assessed via HAM-D scoring (Griebel et al., 2012). Despite this though, the drug ultimately failed to reach efficacy expectations and initial trials were ultimately discontinued. These attempts, however,

would not mark the last time that AVPR1B pharmacology was pursued as a treatment for depression, as very recent work using a novel 1B antagonist, ABT-436, showed it to significant reduce GC, CRF and cortisol levels in depressed patients, as well as improve scoring on the Mood and Anxiety Symptom Questionnaire (MASQ), suggesting that further testing in larger subject pools could pull out a more favorable antidepressant-like effect (Katz, et al., 2017). Collectively, while these preclinical and clinical studies are still ultimately unclear as to how effective AVP targeting might be to universally treat symptoms of depression, recent data such as those mentioned above do highlight it as a potentially exciting target warranting further investigation.

Neurokinins (NKs): One of the initial classes of neuropeptides investigated for their potential role in regulating emotional processing and circuitry were the tachykinin family, consisting primarily of substance P, neurokinin A and neurokinin B. Out of these three molecules, the neurokinins and their cognate receptors: tachykinin/neurokinin receptors 1-3 (NK_{1-3}), demonstrated early promise as possible key regulatory switches for the control of cell populations and circuits within both HPA axis and limbic structures, in which all three receptors and the neuropeptides are prominently expressed (Rigby et al., 2005; Griebel and Holsboer et al., 2012). Apart from their distribution patterns across these regions of core emotional processing, early preclinical testing in rodents demonstrated that novel NK_1 antagonists like CP-96,345 and MK-869 were able to consistently produce robust antidepressant-like effects (Herpfer and Lieb, 2005). The role of NK_1 in regulating affect was further bolstered by genetic studies using a NK_1 knockout mouse line, which demonstrated an anxiolytic phenotype along with decreased depressive-like behavior in tasks such as the FST and TST (Bilkei-Gorzo et al., 2002), suggesting that blockade of the receptor in humans could lead to relief from depressive symptoms. Despite this though, most clinical trials using NK_1, and even NK_2 and NK_3, antagonists have been inconsistent and unable to deliver reproducible and highly efficacious reductions in depressive behavior across patient populations and independent trials (Griebel and Holsboer, 2012). Thus, while NK receptor antagonists may still represent a promising alternatives to monoamine targeted antidepressants, studies into their effectiveness has slowed recently in favor of other potential targets.

Neuropeptide Y (NPY): Neuropeptide Y (NPY) represents one of the newer neuropeptides to be investigated for its potential role in the pathophysiology of depression and anxiety related disorders, with a wealth of preclinical

rodent data providing initial support for expanding upon such studies. NPY itself is widely distributed throughout the PNS and CNS, particularly within cortical, hippocampal, thalamic and brainstem structures (Griebel and Holsboer, 2012). To date, there are five known NPY receptor subtypes: Y_1, Y_2, Y_4, Y_5, and Y_6 (all GPCRs), and their distribution through the CNS has also been shown to be widespread, with particularly high levels of enrichment in a number of core limbic structures such as the PFC, amygdala, bed nucleus of the stria terminalis (BNST), PVN, hippocampus, NAc and septum (Redrobe et al., 2002), further purporting a possible role for the peptide in regulating the activity of these regions in response to affective stimuli. Indeed, initial rodent studies which tested the effects of direct NPY administration to mice showed a robust reduction in stress and depressive-like behaviors in the FST (Redrobe et al., 2002), while pharmacological studies using NPY receptor selective agonists demonstrated that the administration of Y_1 and Y_5 preferring compounds can produce similar results (Ishida et al., 2007). Other studies showing that the blockade of Y_1 with selective antagonists could produce opposing phenotypes further helped argue for a key role of Y_1 in transducing the anxiolytic and antidepressant-like effects associated with NPY signaling. Conversely, the Y_2 and Y_4 receptors have been deduced to enhance anxiety and depressive-like behaviors, as genetic deletion or selective inhibition of these receptors has been demonstrated to improve stress resilience and curb depressive-like behaviors (Painsipp et al., 2008; Redrobe et al., 2003; Tasan et al., 2009; Tschenett et al., 2003). Clinical evidence has also supported notions for the involvement of NPY signaling in depression pathology, with several studies demonstrating a decrease in overall NPY levels in cerebrospinal fluid and plasma in depressed patients compared with healthy controls (Widerlov et al., 1988; Hashimoto et al., 1996), and that these levels could be normalized following chronic treatment with typical antidepressants (Ozsoy et al., 2016). These findings, however, have yet to mobilize a concerted effort in the pharmaceutical sector to begin the development of novel NPY receptor targeted compounds, particularly due to anticipated issues with producing highly specific, non-peptide brain-penetrant ligands (Brothers and Wahlestedt, 2010). While these initial hurdles will most likely prove to be surmountable, it has prompted questions as to whether other avenues may exist to endogenously regulate the activity or signaling of neuropeptidergic cells/circuits that may avoid the need to develop and test such potentially problematic compounds.

Collectively, the fields of biomedical and pharmaceutical research into the pathology and treatment of depression have relied heavily on the use of many molecular targets that, while effective in certain patients, may not help to treat the myriad changes in neural chemistry, cellular activity and circuit function that a growing body of evidence seems to indicate as critical in driving many of the core symptoms of the disease. The treatment options mentioned above have been viewed by many in these communities as only able to treat individual aspects of a patient's depression, avoiding other areas of imbalance or dysregulation that are also known to be highly variable on a case by case basis, and that may serve to more effectively treat one patient as opposed to another. The search for a more universal and all-encompassing target for treating depression has thus long been a lofty goal of neuropathological research, and while a definitive answer is still a long way off, current work focusing on *glutamate* and a number of its key receptors and excitatory circuits has become an area of much excitement and promise for potentially identifying of just such a target.

The glutamatergic signaling system: a novel molecular target for treating depression

The glutamate hypothesis of depression

Glutamate is the primary excitatory signaling molecule in the brain, and is essential for the maintenance of a number of structural and functional synaptic processes that are important for learning, memory and general cognitive function. Changes in glutamatergic signaling, particularly in response to stress, have long been suspected to be detrimental to mental health, with numerous studies showing that many of the symptoms of psychiatric illnesses that effect cognition and emotional processing have been linked to the effects of unregulated excitatory signaling, including the modulation of responses to stress-inducing and motivated behaviors that are seen to go awry in depression and anxiety-related disorders. This suggests that the regulation of glutamate is important in areas involved in the reward-seeking and stress-response neurocircuitry, and prompted initial efforts to investigate whether or not overt changes in glutamatergic signaling/activity could be identified in patients suffering from depression.

Glutamate exerts its activity at a number of different receptors located throughout the nervous system, primarily consisting of two main classes: the metabotropic glutamate receptors (mGluRs) and the ionotropic glutamate receptors (iGluRs, see **Fig. 2** for more detail). The mGluR family is composed of eight different types

(mGluRs1-8) collected into three distinct groups (I, II or III), based on their associated second messenger and ligand sensitivity (Willard and Koochekpour, 2013). The group I mGluRs are known to increase excitatory neurotransmission in a Gq GPCR dependent manner, and are primarily located postsynaptically (Willard and Koochekpour, 2013). The groups II and III mGluRs, by contrast, are inhibitory (via Gi dependent GPCR signaling) and tend to be located presynaptically (Willard and Koochekpour, 2013). Likewise, the iGluRs can be divided into four distinct classes based on physiology and ligand responsiveness, and include the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and delta receptors. The AMPA receptors (AMPARs) serve as the primary charge carriers of excitatory neurotransmission, allowing for the initial depolarization of the cellular membrane necessary to bring the cell to threshold for the firing of an action potential (Malenka and Bear, 2004). The NMDA receptors (NMDARs) require an initial depolarization of the membrane in order to respond to further changes in excitatory tone at the synapse, but are highly sensitive to these changes, allowing them to readily modify excitatory signal strength (Malenka and Bear, 2004). Activity dependent glutamatergic signaling through both mGluRs and iGluRs has long been known to be a key mediator of synaptogenesis and synaptic plasticity. Specific forms of plasticity, such as long term potentiation (LTP) and long term depression (LTD), can be modulated by either major class of glutamate receptor, and ultimately result in either increased or decreased activation of the NMDARs and subsequent insertion or removal of AMPARs at synapses to promote stronger or weaker excitatory signaling (Malenka and Bear, 2004). The precise regulation of synaptic structure and function via these means has been shown to be critical for mediating cognition, learning and memory, and behavioral outputs.

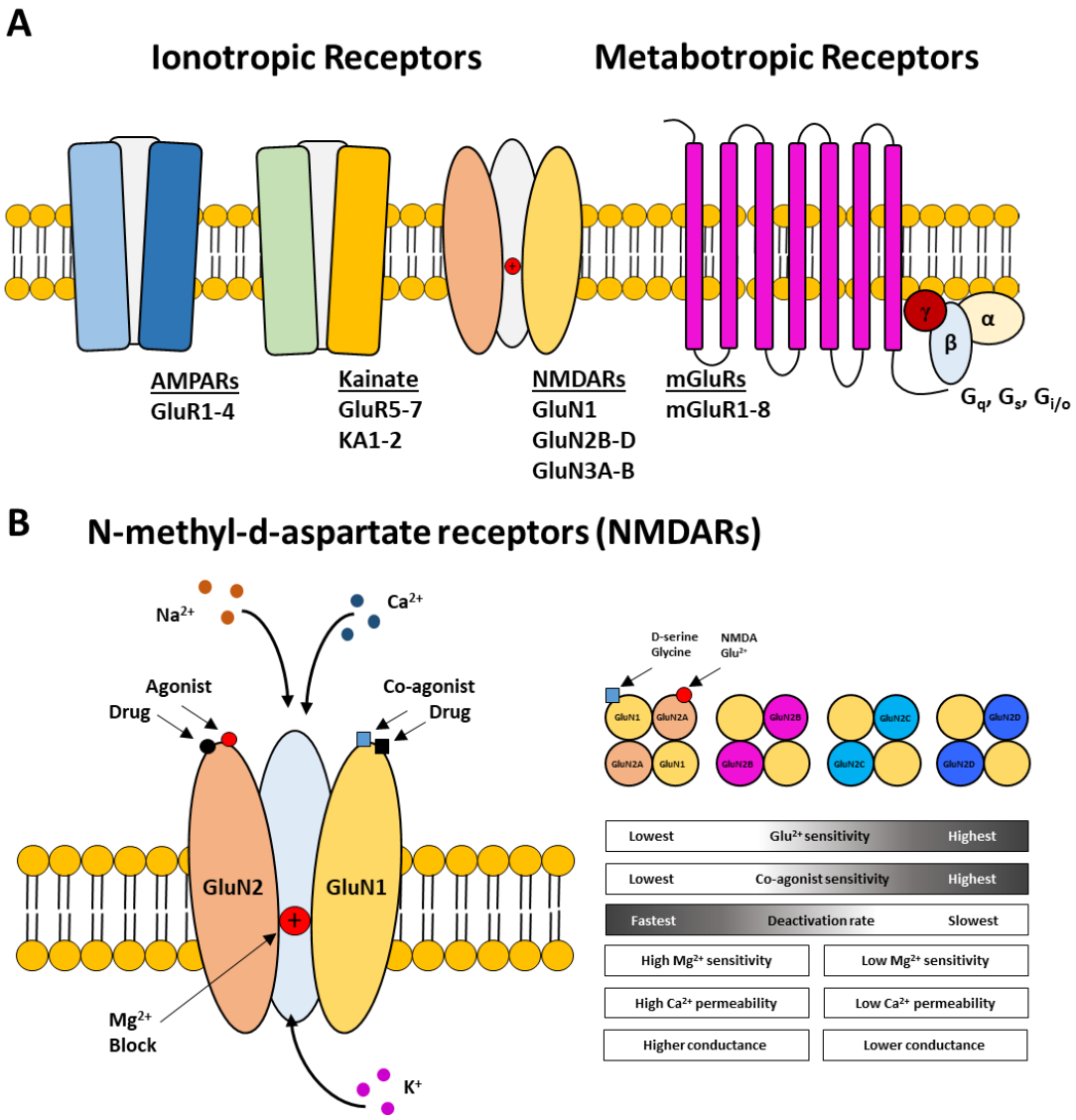


Figure 2. Key glutamatergic receptors classes, and the biophysical attributes of the N-methly-d-aspartate (NMDA) receptors. (A) Schematics of the main ionotropic and metabotropic classes of glutamate receptors found within the CNS and PNS. The ionotropic receptors are ligand gated ion channels which flux cations in/out of the cell following agonist binding and activation, and are chiefly comprised of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-d-aspartate (NMDA) and kainate receptors (though recent evidence also suggests the presence of a fourth class: the delta receptors, as well). AMPARs typical form as heterotetramers consisiting of the GluA1-4 subunits (usually as dimer of dimers of GluA2 and A1,3 or 4) and respond potently to Glu^{2+} release, while the di- and triheteromeric NMDARs form via two obligate GluN1 subunits and a combination of GluN2 or 3 subunits. Kainate receptors also form as tetramers via different combinations of the GluR5-7 and KA1-2 subunits, and function more similarly to the AMPARs. The metabotropic receptors (mGluRs), by contrast, are composed of seven transmembrane domains and signal through their interactions with G proteins and associated G protein signaling cascades, leading to protein changes in gene transcription, protein modifications and altered excitatory signaling. **(B)** Detailed schematic of the basic properties and function of the NMDARs (left), and the physiological attributes imparted on these receptors based on their subunit composition (right), which will be discussed in greater detail in the following section. Adapted in part from Wyllie et al. (2013) and Danysz and Parsons, 2003.

In the early 1990s, converging evidence from early preclinical investigations into the underlying physiology and neurocircuitry of stress-related behaviors often associated with depressive phenotypes in rodents, particularly in regards to hippocampal plasticity and NMDAR function (Shors et la., 1989; McEwen, 1999), led to the discovery that the administration of NMDAR antagonists such as AP-5 and MK-801 could produce robust decreases in behaviors associated with inescapable stress in the FST and related tasks comparable to that of leading typical antidepressants (Trullas and Skolnick, 1990; Papp and Moryl, 1994). Indeed, further testing of the effects of both chronic imipramine and ECT treatment in rodents also indicated long term alterations in the binding properties of glycine and glutamate to the NMDARs in rodents (Nowak et al., 1993; Paul et al., 1993), further suggesting a role for both glutamate signaling, and the NMDARs in particular, as a potential common pathway through which many antidepressants and other accepted treatments for depression ultimately produced their therapeutic effects. These discoveries were quickly followed up on by studies of the effects of chronic administration of many of these antidepressants and ECT treatment on glutamatergic signaling and/or receptor functionality in the brains of rodents (Paul et al., 1994), the results of which strikingly revealed the majority of these treatments to consistently reduce the potency of glycine to inhibit [^{3}H]-5,7-dichlorkynurenic acid binding to strychnine-insensitive glycine receptors (used as a proxy for NMDAR function). Further clinical data revealing that the brains of chronically depressed and suicidal patients postmortem showed evidence of altered NMDARs radioligand binding in the frontal cortex (Nowak et al., 1995) helped to lay the foundations for the burgeoning “glutamate hypothesis of depression” and in particular the emergence of the NMDARs as potentially the key molecular target through which the manipulation of the glutamatergic system could be achieved to produce such promising antidepressant-like effects.

The most definitive evidence for the support of this new hypothesis, however, came from a pilot study conducted in 2000 in the lab of Dr. Johnathan Krystal that tested the effects of administration of the drug ketamine hydrochloride, largely understood to act as a potent noncompetitive antagonist of the NMDAR, in treating the symptoms of patients suffering from MDD (Berman et al., 2000). Using a lower dose of ketamine (0.5mg/kg) in order to avoid the typical anesthetic and dissociative side effects often associated with higher dosing with the drug, the results of the study were striking, with patient reporting near immediate relief from their symptoms as early as a few hours after their initial infusion, and follow up interviews revealing these effects to be felt for days to weeks following the trial. These findings galvanized a number of further clinical trials into the

effectiveness of ketamine as a treatment option for chronically depressed patients, with the majority of these studies not only replicating the robust and rapid results reported by the Krystal lab, but also showing ketamine to be effective in treating TRD patients as well (Zarate et al., 2006; Maeng et al., 2008; Blier et al., 2012; Diaz-Granados et al., 2010; Murrough et al., 2013). The readily reproducible results of low dose ketamine for the treatment of depression represented an enormous leap forward for the mental health community, culminating recently in 2019 with the Food and Drug Administration's formal approval of the S (+) enantiomer derivative of ketamine, esketamine (sold as Ketanest, Spravato and other brand names), for clinical use in the treatment of MDD and TRD.

The mechanism of action underlying ketamine's therapeutic effects is still a topic of debate, however, but numerous studies have been conducted that have begun to hint at several different means through which it's actions on the glutamatergic system can lead to its rapid and robust behavioral changes observed in both patients and animal models. These are believe to center around an acute effect that leads to an initial alteration in excitatory signaling in select regions/cell populations, followed by longer lasting and sustained modifications in protein expression and synaptic plasticity that can promote increased spine formation and synaptogenesis (Zanos and Gould, 2018). Evidence from work in the rodent PFC and hippocampus primarily has provided support for these notions, suggesting that the acute changes may results from the inhibition of GABAergic interneurons that allows for increased glutamate release from excitatory cells and an overall shift in excitatory activity (Moghaddam et al., 1997; Homayoun and Moghaddam, 2007). This increased glutamatergic tone is thought to enhance the activation of AMPARs (Henley and Wilkinson, 2016), a concept that has been buoyed indirectly across rodent and human electroencephalography studies (Sanacora et al., 2014; Kocsis, 2012; Zanos et al., 2016), and by gene expression studies showing the AMPA subunits GluA1 and GluA2 to increase in expression in the hippocampus and PFC mere hours post ketamine injection (Nosyreva et al., 2013; Li et al., 2010). Enhanced AMPAR activation has been correlated with increased production of the growth factor, brain derived neurotrophic factor or BDNF (Jourdi et al., 2009; Clarkson et al., 2011), a molecule that has been proven important in driving synapse formation and plasticity within the CNS, and has been implicated in aspects of the long term antidepressant effects of ketamine (Autry et al., 2011). Additionally, enhanced BDNF production has been speculated to lead to greater downstream changes in gene transcription and protein translation, stemming from increased activation of the BDNF receptor, tropomyosin receptor kinase β (TrkB), which can lead

to the activation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway (Autry et al., 2011; Autry et al., 2012). Coinciding evidence has also supported the notion that these gene transcription effects can be further enhanced via NMDAR blockade by preventing the NMDAR-mediated activation of eukaryotic elongation factor 2 kinase (eEF2K) and its associated phosphorylation of eukaryotic elongation factor 2 (eEF2), which can results in increased protein translation events mediated by eEF2 in regions such as the hippocampus and PFC (Autry et al., 2011; Sutton et al., 2007). Collectively, these studies suggest a complex, two part mechanism though which ketamine is able to drive short term enhancement of glutamate signaling and increases in protein translation that can lead to lasting changes in excitatory plasticity in key brain structures implicated in the regulation of affective behavior.

Despite these findings though, ketamine and its derivatives are still often observed with skepticism in the medical community, owning to the well-known abuse potential and side effects that recreational ketamine use has been shown to produce in regards to cognition, cortical integrity and dissociative/psychotomimetic behaviors (Curran and Monaghan, 2001; Morgan et al., 2004; Liao et al., 2011; Sos et al., 2013). These concerns have led to a continued interest in both the biomedical research and pharmaceutical communities to investigate other means of replicating the effects of ketamine without needing to rely on the drug itself, particularly by targeting the key receptors and downstream effectors though which ketamine is believed to produce it's antidepressant actions. The most prominent of these potential targets to date has without a doubt been the NMDA receptors, due to growing evidence indicating a key role for NMDAR function in mediating excitatory signaling in limbic structures and circuity, and initial pharmacological work implicating their inactivation in mimicking antidepressant effects on salient affective behaviors long before the advent of ketamine.

N-methyl-d-aspartate receptors as an emerging therapeutic target

While recent studies have suggested that drugs like ketamine might actually induce their antidepressant-like effects through AMPARs or other, non-NMDAR actions (Zanos et al., 2016; Chen et al., 2009; Kohrs and Durieux, 1998), separate studies blocking such receptor populations have failed to produce similar sustained and robust changes in depressive-like behaviors to the same degree as those seen with NMDAR antagonists (Maeng et al., 2008). Thus, it is assumed that many aspects of the antidepressant actions

of ketamine, and of the manipulation of the glutamatergic signaling system as well, may be transduced through the NMDARs.

N-methyl-d-aspartate receptors are a class of heteromeric ionotropic glutamate receptors, composed of two obligate GluN1 subunits and a combination of either two GluN2 subunits or GluN3 subunits (Traynelis et al., 2010, **Fig. 2**). The GluN2 and GluN3 subunits come in a number of different isoforms: four for GluN2 (A-D) and three for GluN3 (A-C), with the GluN2 subunits showing much more prominent expression and incorporation *in vivo* into the functional receptors in adult animals (Traynelis et al., 2010; Vyklicky et al., 2014). Activation of the receptor, which leads to the opening of its associated channel and the influx/efflux of mono- and divalent cations, requires binding of both a co-agonist (typically glycine or d-serine) to the GluN1 subunits and the cognate ligand glutamate at the GluN2 subunits, as well as depolarization of the cell membrane in order to allow for the removal of a Mg^{2+} blockade from the center of the channel pore (**Fig.2**). This has often led to the NMDAR being described as a coincidence detector, requiring both a shift in membrane potential and a parallel release of neurotransmitter to activate it, and crucial for its function in driving plasticity phenomena like LTP and LTD (Glasgow et al., 2019). The identity of the GluN2 subunits that compose a specific NMDAR are also considered to be highly important in regards to shaping such events, as they have been shown to directly influence a number of biophysical properties of the receptors such as: channel conductance, open probability, decay kinetics and both ligand and Mg^{2+} block sensitivity (Traynelis et al., 2010; Vyklicky et al., 2014, **Fig. 2**). Physiological profiling of these receptors both *in vitro* and *in vivo* have shown GluN2A-NMDARs to possess the most rapid kinetics, lowest sensitivity to glutamate and highest sensitivity to the Mg^{2+} block, with these metrics shown to skew towards longer decay times, increased glutamate sensitivity and decreased Mg^{2+} block in GluN2B and GluN2C-containing receptors, and even more so in GluN2D-NMDARs, which display the most prolonged decay kinetics, lower open probability and highest sensitivity to glutamate and insensitivity to Mg^{2+} of all four isoforms (Traynelis et al., 2010; Vyklicky et al., 2014, **Fig. 2**). The expression patterns and profiles of these subunits has also been found to be highly variable throughout the brain, as well as developmentally restricted. Early on, GluN2A levels are shown to be very low compared with GluN2B-D, but a large shift in expression occurs during development that drives GluN2A expression up throughout the majority of all cortical and subcortical structures while GluN2B-D expression is reduced. GluN2C and GluN2D in particular have been found to be greatly reduced in the adult brain, as well as highly restricted in their expression patterns to a few

core structures, such as the cerebellum, hippocampus, thalamus, extended amygdala, and brainstem (Sheng et al., 1994; Monyer et al., 1994; Perszyk et al., 2016).

The differences in expression patterns and physiological properties that can be associated with the GluN2 subunits, as well as numerous studies showing that NMDAR-mediated excitatory signaling can vary greatly across different brain regions and neuronal populations, has led many to hypothesize that the therapeutic effects of ketamine may be preferentially achieved through antagonizing a unique subtype of NMDARs as opposed to being the result of pan-NMDAR blockade alone (Zanos and Gould, 2018). Indeed, initial support for this hypothesis came from studies of extra-synaptic NMDARs, the majority of which have been shown to heavily incorporate the GluN2B subunit (Traynelis et al., 2010; Vyklicky et al., 2014). Basal activation of these GluN2B-NMDARs has been speculated to tonically suppress the activation of mTORC1 signaling and allow for the phosphorylation of eEF2 via eEF2K, suppressing protein synthesis and maintaining a level of synaptic homeostasis (Wang et al., 2011). The de-suppression of these translation pathways by the actions of ketamine at GluN2B-NMDARs has thus been strongly posited to drive the majority of its therapeutic effects. Additional studies have supported this notion, principally through the use of GluN2B conditional knockout mice, and shown that deletion of these receptors in regions such as the PFC (Miller et al., 2014) and components of the extended amygdala such as the BNST (Louderback et al., 2013), can mimic the antidepressant effects of systemic ketamine injection into these animals, reducing the incidences of depressive-like behaviors across tasks such as the FST, TST and NIH. Pharmacological studies using the GluN2B-selective antagonist Ro 25-6981 have further bolstered these transgenic findings, showing that systemic administration of Ro can produce robust antidepressant-like effects in rodent models and occlude the effects of ketamine (Miller et al., 2014; Louderback et al., 2013; Maeng et al., 2008; Kiselycznyk et al., 2015). The case for targeting GluN2B-NMDAR had been made even more appealing with the observations from clinical studies of the GluN2B selective antagonists CP-101,606 and MK-0657 that these compounds displayed some level of efficacy for inducing antidepressant-like actions and overall improvements in mood in patients (Preskorn et al., 2008; Ibriham et al., 2012). The initial excitement from these pilot studies was short lived however, as apart from these compounds showing a much more delayed onset of antidepressant-like effects when compared with ketamine, other preclinical tests of CP-101,606 revealed an increased incidence of cognitive impairment following its chronic administration in non-human primates (Weed et al., 2016), and further test with MK-0657 during phase II clinical

trials failed to identify a significant antidepressant effect in subjects. Despite this though, interest into the use of NMDAR subtype selective antagonists has persisted, with several GluN2B-NMDAR antagonists currently under development for the potential treatment of depression and other neurological disorders like epilepsy and chronic pain (Yuan et al., 2015), and studies also focusing on how currently available GluN2B preferring antagonists might be used in combination with typical antidepressants to enhance their efficacy and the speed of onset of their therapeutic effects (Poleszak et al., 2016).

Recent preclinical work has also begun to investigate the benefits of examining other NMDAR subtypes in order to determine whether or not targeting these receptor populations might provide another means for treating depression as well. Indeed, the GluN2C and GluN2D-containing NMDARs appear to sport a number of biophysical features, as well as expression profiles, that suggest them to be ideal targets for the regulation of glutamatergic signaling. These include, but not limited to, their prolonged deactivation kinetics and increased sensitivity to glutamate when compared with GluN2A and GluN2B-NMDARs, along with their highly restricted and reduced expression patterns in the adult brain, which may suggest the prospects of fewer off target effects when manipulating these receptors when compared to the more ubiquitously expressed GluN2A and GluN2B-NMDARs (Monyer et al., 1994; Sheng et al., 1994). Intriguing reports have also shown evidence of ketamine demonstrating increased selective for inhibiting GluN2C and GluN2D-containing NMDARs than GluN2A or GluN2B-containing receptors, in particular the GluN2D-NMDARs, which are speculated to be expressed on inhibitory interneurons in the forebrain, hippocampus and other subcortical structures (Kotermanski and Johnson, 2009; Monyer et al., 1994; Perszyk et al., 2016; Swanger et al., 2015; Volianskis et al., 2013; von Engelhardt et al., 2015; Zhang et al., 2014). While a majority of the current physiological and behavioral studies conducted in rodent models, using a combination of constitutive knockout mouse lines and GluN2C/D preferring agonists/antagonists, have been focused more so on the potential implications of GluN2C-mediated excitatory signaling in regulating the affective and cognitive behaviors associated with schizophrenia as well as select forms of epilepsy (Shelkar et al., 2019; Khlestova et al., 2016; Lozovaya et al.,2014), and the possible involvement of GluN2D-NMDARs in regulating the excitatory activity of interneurons in the striatum in relations to mouse models of Parkinson's disease (Zhang et al., 2014; Zhang and Chergui, 2015), there have been a number of other studies published sporadically over the past few years that seem to implicate GluN2D-NMDAR signaling in the regulation of affective behaviors associated with depression. Indeed, several reports from both

global and conditional GluN2D knockout mouse lines in which the GluN2D protein has been deleted from birth have shown these animals to consistently display increases in both anxiogenic and depressive-like behaviors across multiple tasks (Yamamoto et al., 2016; Shelkar et al., 2019), including work from our lab, which I will discuss in detail in Chapter 2. Although these findings seem to indicate a role for the modulation of GluN2D-NMDAR activity in driving or suppressing affective behavior, no clinically viable compounds exist to effectively target GluN2C or GluN2D-containing NMDARs, as currently available drugs and research-specific compounds lack the specificity to distinguish between the two subtypes (Yi et al., 2020; Swanger et al., 2018; Mullasseril et al., 2010). Similarly, initial tests to see if the antidepressant effects of ketamine are reduced in GluN2D lacking mice have also proved somewhat inconclusive, with some demonstrating a lack of reduction in depressive-like behavior in knockout animals only when administered the (R) enantiomer of ketamine as opposed to the (S) and racemic mixes of the drug (Ide et al., 2017), while others suggest that the subunit is implicated in transducing the negative cognitive effects often associated with ketamine administration instead (Ide et al., 2019; Sapkota et al., 2016). Nevertheless, the data coming from continued preclinical study on selective deletion and pharmacological manipulations of NMDAR subtypes has been largely positive and promising, suggesting that the development of newer and more specific compounds might serve as a powerful method for not only achieving many of the same therapeutic benefits of more classical NMDAR antagonists without the side effects, but also for uncovering new avenues of treatment for neurological disease in general.

Apart from the promise of these subunit specific studies for newer therapeutics, the advent of a NMDAR/glutamate centric focus in regards to the pathology of depression has also opened new areas of research into the underlying circuitry of the disease. Indeed, the neural substrates of early ketamine work and NMDAR subunit antagonist studies in both rodents and humans have highlighted a number of potential brain regions and pathways that may prove to be central to the underlying neurobiology of depression when dysregulated. Further study of these regions and circuits, particularly those in which some of these unique NMDAR subtypes are expressed (either on synaptic terminals or specific neuronal populations) has thus been speculated to provide further insights into developing new biomarkers as well as access points for testing the effectiveness of novel compounds for treating the pathophysiology of depression and other mood disorders.

Regional and circuit-based studies of excitatory signaling and NMDAR function in depression

Much of the work investigating the regional substrates underlying the antidepressant effects of NMDAR manipulation has historically focused on the PFC and hippocampus, due to both early evidence from rodent and human studies showing correlative data between excitatory signaling dysregulation in these areas and increases in depressive behavior, as well as the ease that examining structures such as these were afforded with early imaging technologies. Improvements in functional imaging, along with other early evidence from histological studies, have shifted some of this focus away from these cortical structures in recent years, and placed more of an effort on understanding how excitatory signaling in subcortical limbic structures may also be altered and implicated in aspects of depression, in the hopes of providing a more holistic view of the disorder outside of the PFC and hippocampus alone (i.e. focusing on the amygdala, extended amygdala, hypothalamus, and so on, as outlined in **Fig. 3** below). In regards to the PFC, however, evidence as early as the 1990s obtained from chronically depressed patients showed a decrease in its overall excitatory function (Biver et al, 1994; Baxter et al., 1989). More contemporary studies have also elegantly provided correlative histological evidence in both human patients and rodent models of depression to show that the PFC does indeed demonstrate a reduction in overall activity in depressed states (Covington et al., 2010), and that optogenetic activation of the medial PFC is able drive a robust antidepressant-like effect in rodents. Combining this with previously mentioned evidence indicating that the deletion of GluN2B-NMDARs from the medial PFC in mice was also able to replicate the antidepressant effects of both ketamine and Ro 25-6981 suggests that excitatory signaling in the PFC is indeed a determinant of depressive-like behaviors and antidepressant responses (Miller et al., 2014). These findings are also significant in regards to the glutamatergic circuitry of the PFC and cortex in general, which is known to send numerous excitatory projections to a number of limbic structures such as the NAc, BNST, amygdala, hippocampus and VTA, as well as extensive connect back on itself (Del Arco and Mora, 2009). Additionally, many of these limbic structures send reciprocal excitatory projections back to the PFC (i.e. VTA, ventral hippocampus, amygdala), further suggesting that these direct connections between regions of higher cognitive functionality and centers of emotional and reward processing may play a critical role in regulating many of the behavioral outputs often known to be altered in depression (Knowland and Lim, 2018).

The hippocampus has long been considered important in regulating aspects of emotion and emotional processing that may be implicated in the pathology of depression, owing in part to a wealth of human imaging

studies that have consistently shown hippocampal volume to be reduced and its overall activation suppressed in chronically depressed patients (Castanheira et al., 2019; Kempton et al., 2011). More recently, however, a key distinction has been emerging between the dorsal region of the hippocampus and the ventral region, with the dorsal now more canonically accepted to be associated with the learning and memory functions of the hippocampus, while the ventral with its role in emotional processing and HPA axis regulation (Gulyaeva, 2018; Fanselow and Dong, 2010). Excitatory signaling within the ventral hippocampus (vHPC) has also been demonstrated across a growing body of preclinical literature to be highly implicated in the pathology of depression (Marrocco et al., 2012). As in the case of the PFC, the vHPC is also known to send and receive glutamatergic projections to/from multiple limbic regions, including the mPFC, NAc, amygdalar structures such as its basolateral nucleus (BLA), other extended amgydalar structures such as the BNST and components of the HPA axis as well. Many recent studies in rodent have directly shown each of these circuit to drive aspects of affective and stress related behavior upon selective manipulation (Bagot et al., 2015; Chang and Gean et al., 2019; Knowland and Lim, 2018; Glangetas et al., 2017; Ortiz et al., 2019), suggesting that not only might these pathways serve as possible biomarkers of depression in patients through the use of functional imaging, but that their regulation may be key in treating the symptomology of depression. The manipulation of NMDARs has also been speculated as a means to regulate excitatory signlaing in the hippocampus and influence downstream behavioral output. Indeed, initial studies in rats have shown that infusion of the NMDAR antagonist AP-5 into the ventral hippocampus specifically can produce a robust decrease in anxiogenic behavior (Nascimento Hackl and Carobrez, 2007). Gene expression of NMDAR subunits in the ventral hippocampus of rats has also been observed to sharply increase following chronic stress in models of depression, a change that can be normalized following the administration of typical antidepressants (Calabrese et al., 2012). More recently, it has been reported that ketamine administration in mouse models of depression can produce similar changes in NMDAR expression and function in the hippocampus, driving both increases in LTP and NMDAR-mediated excitatory signaling (Yang et al., 2018), while projections from the ventral hippocampus to the extended amygdala that have been implicated in driving anxiety-like behavior have been demonstrated to do so in an NMDAR-specific fashion (Glangetas et al., 2017), further suggesting an important role for these receptors in mediating the effects of excitatory signaling in key limbic structures that regulate affective behavioral output.

Outside of the PFC and hippocampus, two other structures have also received more selective focus in the literature in regards to their implication in the pathology of depression, one of these being the VTA and the other the amygdala. As anhedonia is typically considered one of the core symptoms of chronic depression, it stands to reason that deficits in the functionality of the brain's reward pathway would be associated with the changes in motivated behaviors that are often observed in patients. Similarly, the long held notion of the amygdala representing one of the core centers of emotional processing in the brain also highlighted it as another likely region implicated in depression pathophysiology. Imaging studies have supported these hypotheses, with fMRI and PET imaging studies of the amygdala in depressed patients showing a decrease in both volume and overall activation when compared with healthy controls (Sacher et al., 2012; Hamilton et al., 2008), while more recent work has also revealed evidence of decreased VTA connectivity and an overall reduction in its activation in reward-related learning tasks in depressed patients as well (Geugies et al., 2019; Anand et al., 2019). This work has supported the notion that a further study of the basal and circuit level function of these regions may provide key insights into the physiological bases of depression. As mentioned above, both of these structures are known to be highly interconnected with a host of limbic structures, including each other, via a number of key circuits that have been extensively dissected and validated for their influence upon affective behavior. The mesolimbic pathway between the VTA and NAc for instance has been heavily implicated in regulating depressive-like behaviors, with work from separate groups showing that differing patterns of activation of this circuit may increase or suppress these behavior in rodent models of chronic stress and depression (Chaudhury et al., 2013; Tye et al., 2013). Within the amygdala, in particular the BLA, rodent studies have also identified salient inputs into the VP that allow for the indirect regulation of VTA DA neuronal activity (Chang and Grace, 2014), and is speculated to lead to decreased DA firing in depressive states (Belujon and Grace, 2017). Excitatory signaling in both of these regions has also been speculated to modulate much of the intrinsic reward and stress related functionality associated with the VTA and amygdala. While many of these circuits represent a complex mix of modulatory, inhibitory and excitatory signaling, the role of glutamate within both regions has also been significantly vetted and found to have possible implications for the regulation of both the VTA and amygdala's reward and stress related functions, respectively. CRF signaling within the VTA, for example, has been found to occur primarily at glutamatergic synapses and to drive increases in NMDAR-meditated currents on VTA cells following endogenous CRF release or bath application *ex vivo* (Ungless et al.,

2003). Glutamate has also been shown to be prominently enhanced in the BLA following stress exposure in mice (Reagan et al., 2012), and has been postulated to lead to increased plasticity within the BLA and central nucleus of the amygdala (CeA) microcircuitry that is often associated with chronic stress, and that may go awry as the pathophysiology of depression progresses (Zhang et al., 2018).

Taken together, these findings again suggest the potential for the regulation of glutamatergic signaling in these regions, specifically through the NMDA receptors, as a mean to manipulate a number of different modulatory systems and circuit level functions that have collectively been viewed as altered across multiple different models and presentations of depressive disorders. While this has begun to provide insight into not only the potential mechanism of action of current therapeutics targeted at the glutamatergic signaling system, it has also helped lead to the identification of other important regions implicated in the circuitry of depression, in particular a number of subcortical structures that are often overlooked despite their well-documented contributions to the regulation of stress, addiction and affective related behavior.

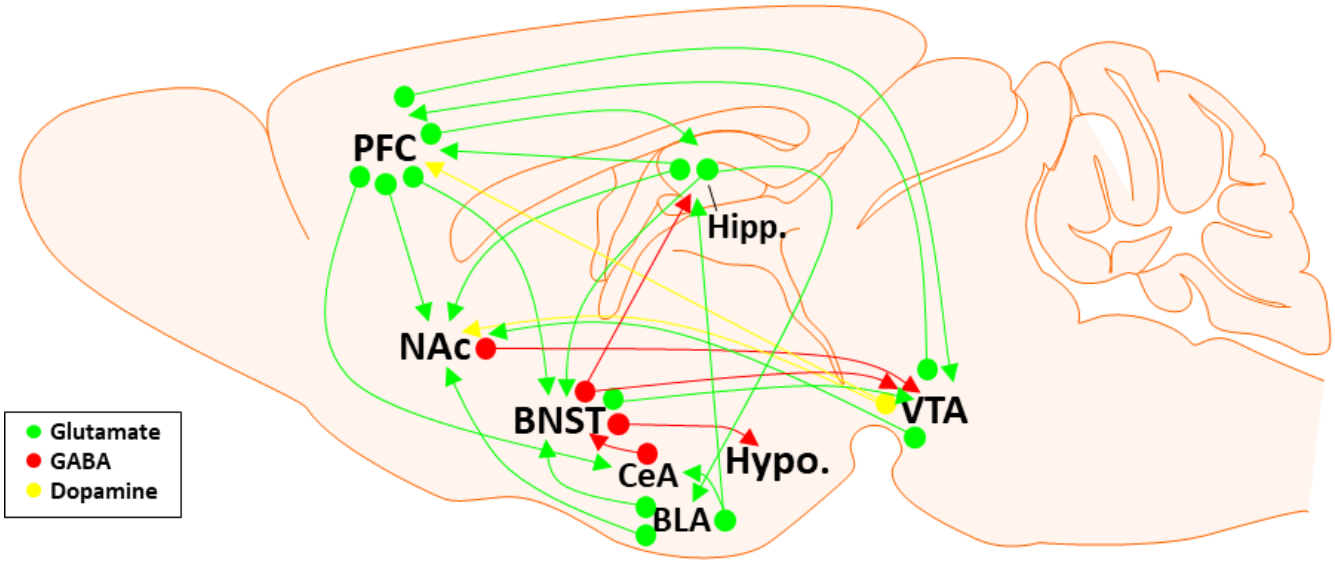


Figure 3. Simplified circuitry of depression: key excitatory, inhibitory and modulatory regions and pathways. Sagittal schematic of the key brain regions, glutamatergic, GABAergic and dopaminergic circuits implicated in the pathophysiology of depression. A vast body of literature has implicated excitatory signaling/processing in the PFC and Hipp. as central to the regulation of affective behavior in preclinical models, and patient studies have classically shown deficits in the function of both in depression. More recent preclinical work has gone on to identify the canonical VTA-NAc reward pathway, as well as excitatory and inhibitory connections between amygdalar, extended amygdalar and hypothalamic regions implicated in stress-responsiveness as altered within the disease state, with improvements in modern imaging studies now also providing evidence in patients of similar disruptions within components of the HPA axis and limbic system as well. The extended amygdala (BNST, NAc-Sh, CeA) in particular has gained an increase focus due to its high interconnectivity with other excitatory, inhibitory and modulatory stress circuits, and recent literature indicating

that individual nuclei/cell populations germane to this macrostructure can differential modulate affective behaviors associated with depression. BNST – bed nucleus of the stria terminalis; BLA – basolateral nucleus of the amygdala; CeA – central nucleus of the amygdala; Hipp. – hippocampus; Hypo. – hypothalamus; NAc – nucleus accumbens; PFC – prefrontal cortex; VTA – ventral tegmental area. Sagittal mouse brain image courtesy of Motifolio (Biology Bundle Toolkit).

The extended amygdala: excitatory signaling and the modulation of affective behavior

Perhaps one of the most intriguing regions that has recently emerged as an important subcortical network involved in the pathology of depression and other mood related disorders is the extended amygdala. The extended amygdala is comprised of three core structures: the bed nucleus of the stria terminalis (BNST), the CeA and the shell of the nucleus accumbens (NAc-Sh; Alheid, 2003; Cassell et al., 1999) and is speculated to play a key role in the processing and response to stressful and rewarding stimuli within the brain. The three main structures of the extended amygdala are highly interconnected with one another, as well as with a number of other regions that also participate in the responses to stress and motivated behaviors, including components of the HPA axis and VTA (Georges and Aston-Jones, 2001; Georges and Aston-Jones, 2000; Herman et al., 1994). Reciprocal connections exist between the BNST and CeA, with the CeA exerting inhibitory regulation over the BNST's activity (Davis et al., 2010; Dong et al., 2001b; Choi et al., 2007). Responses to stressful stimuli are thought to be mediated not only by these connections, but also through projections the BNST sends to the paraventricular nucleus of the hypothalamus (PVN), where it can influence the release of stress hormones like cortisol (Herman et al., 1994; Herman et al., 2003). Rewarding stimuli, by comparison, are thought to be regulated through the BNST's projections to the NAc-Sh, its excitatory and inhibitory projections to the VTA, and its aforementioned hypothalamic connections (Koob and Volkow, 2010; Georges and Aston-Jones, 2001; Georges and Aston-Jones, 2002). The extended amygdala is also densely populated by a number of cells that produce and respond to many of the key stress responsive neurohormones and neuropeptides that have been previously characterized as heavily participating in the pathology of depression, including CRF, NPY and several others (Kash and Winder, 2006; Hauger et al., 2009; Aborelius et al., 1999). As discussed above, much interest has been given to the modulatory effects of the neurohormone/neuropeptide systems on glutamatergic signaling following HPA axis activation, and in response to neuroadaptive changes occurring in disease, with several studies indicating that these cell populations may exert a degree of bidirectional control over the activation or inhibition of the extended amygdala's stress and reward related circuitry (Kash and Winder, 2006; Silberman and Winder, 2003). CRF is prominently expressed in the BNST and CeA, and both regions send

CRFergic projections to one another, as well as to other limbic structures (Dabrowska et al., 2013; Silberman and Winder, 2013). Within the CeA specifically, glutamatergic signaling has also been shown to be heavily influenced by CRF, with studies showing that activation of CRFR1 in the CeA strongly potentiate its glutamatergic connection with the BLA (Fu et al., 2007), and that direct injection of CRF into the CeA produces an increase in extracellular glutamate levels, similarly to the changes in CeA glutamate concentration seen in response to external stress. It is unsurprising thus that signaling via CRF within the extended amygdala has been linked to the regulation of a number of other behavioral responses and disease models salient to anxiety and depressive-like disorders (Regev et al., 2011; Smith and Aston-Jones, 2008).

While the majority of neurons found within the components of the extended amygdala are GABAergic interneurons, and thus send and receive many inhibitory connections (Lein et al., 2007; Oh et al., 2014), the region also possesses numerous excitatory afferent and effect projections. Glutamatergic inputs to the extended amygdala come from multiple cortical and limbic regions, including the BLA, ventral hippocampus, PFC, insular cortex and the limbic cortex, while its glutamatergic outputs principally project to the VTA, as well as reciprocal projections to several of the structures mentioned previously (Dong et al, 2001a; Dong and Swanson, 2006, **Fig. 3**). Given its interconnectivity with numerous sites of emotional, cognitive and functional processing, and its participation in regulating the circuitry of stress, anxiety and drug-seeking behaviors, it is not surprising that altered excitatory activity within the extended amygdala has been suggested to occur in affective disorders. Indeed, direct inhibition of AMPAR-dependent glutamatergic signaling in the BNST (but not CeA) via the injection of the channel blocker NBQX has been shown to decrease normal acquisition of fear conditioning in rodents, while injection into regions of the BLA that send excitatory projections to the BNST enhances fearful and anxiety-like responses (Davis et al., 2010). The CeA is also known to serve as one of the primary site of intra- and extra-amygdalar glutamatergic inputs, receiving afferents from the BLA as well as the thalamus, cortex and brainstem at its lateral subdivision (McDonald, 1998; McDonald et al, 1999). Additionally, it is one of the main output nuclei of the amygdalar complex, and projects via its medial subdivision to multiple brain structures involved in fear, arousal, and stress responses, pointing to an important role for the CeA in the regulation of these behaviors (Davis, 1997). A number of studies have shown that extracellular glutamate increases in the CeA in response to stress, resulting in enhanced anxiogenic behaviors and fear conditioning in rodent models (Reznikov et al., 2007) and suggesting that the glutamate signal within the CeA is particularly

sensitive to prolonged exposure to external stressors. This has led to overarching speculation that the neuroadaptation of the excitatory circuitry of the CeA resulting from either dysregulation of stress hormone signaling or chronically altered glutamatergic tone may underlie many of the changes in stress responses noticeable in patients suffering from depression and anxiety related disorders.

The emergence of deficits in motivated behaviors, which as mentioned above may be implicated in the anhedonia of depressed patients, has also been implied to depend on changes in the strength of glutamatergic projections innervating the VTA and HPA axis, and several lines of research have purported that the extended amygdala is an important source of this glutamatergic signal (Graybeal et al., 2012; McElligott et al., 2009). The NAc is an important regulator of the VTA (VTA-NAc, primary mesolimbic pathway) and integrates excitatory information from the mPFC, BLA, ventral hippocampus and VP (Britt et al., 2012; Smith et al., 2011; Sesack and Grace, 2010), with multiple studies emerging that have shown that stress is able to alter the glutamatergic signaling in the NAc that can result in deficits in associated hedonistic and stress-related behaviors, particularly via altered excitatory activity at select populations of medium spiny neurons (MSNs) (Golden et al., 2013; Christoffel et al., 2011). Indeed, animals exposed to chronic stress have shown decreases in AMPAR mediated excitatory drive onto MSNs positive for the D1 dopamine receptor (D1-MSNs) in electrophysiological recordings, as well as associated decreases in reward-seeking behaviors (Lim et al., 2015). Chronic stress specifically has been shown to produce similar decreases in the frequency of D1-MSN excitatory postsynaptic currents (EPSCs) and overall cell excitability in *ex vivo* recordings (Francis et al., 2015). *In vivo* experiments examining the bidirectional control of excitatory drive onto the MSNs via optical stimulation of either channelrhodopsin 2 (ChR2) or injection of the designer drug clozapine-N-oxide (CNO) to activate the inhibitory designer receptor exclusively activated by designer drug (DREADD) hM4(Gi) has also been shown to respectively diminish or exacerbate anxiety- and depressive-like behavior in the awake, behaving mice (Francis et al., 2015). In models of chronic depression in mice, synaptic transmission has been found to be decreased and increased, respectively, at excitatory mPFC-NAc and vHPC-NAc pathways (Knowland and Lim, 2018; Bagot et al., 2015), as well as expression patterns of monoamine receptors in the NAc to be significantly altered when compared with control animals (Bagot et al., 2017). Intriguingly, many of these physiological deficits within the NAc were found to be reversed following ketamine treatment (Bagot et al, 2017; Zanos et al., 2016), suggesting that the modulation of NMDAR-mediated excitatory signaling may also serve as a means to target and treat the

pathophysiology of depression in structures closely tied to the hedonistic aspects of the disorder in a broad but effective manner.

Much of the work discussed above has discovered glutamatergic signaling within the extended amygdala to be highly tied to modulating behavioral responses to stressful and hedonistic stimuli, with excitatory inputs and intrinsic activity within CeA and NAc-Sh in particular shown to be implicated in the regulation of many of the acute anxiogenic behaviors and longer term changes in motivated behavior that are hallmarks of depressive disorders. Recent evidence, however, has emerged that suggest that the other core component of the extended amgydala, the BNST, may be uniquely sensitive to the effects of NMDAR antagonists such as ketamine and highly tied to their antidepressant-like actions. The unique connectivity patterns with limbic structures, highly heterogeneous and specialized cell populations, and glutamatergic receptor expression profile within the BNST also suggest that it may play an important role in the pathophysiology of depression, warranting a more critical and in depth level of interest from the research community that I will expand upon below and in the coming chapters.

The bed nucleus of the stria terminalis (BNST) and depression

The BNST: structure, circuitry and function

The BNST is considered to be a key integrator of stress and reward related stimuli, with its positioning in the brain, anatomy and overarching projection patterns strongly supporting this notion. These anatomical features have been comprehensively defined in rodent models through a series of anterograde mapping studies (Dong et al., 2001a; Dong and Swanson, 2004a; Dong and Swanson, 2006; Dong et al., 2001b; Dong and Swanson, 2004b) and by massive bioinformatics undertakings which have helped to define many of the molecular features of the region, including key receptor populations, neurotransmitter classes and synaptic proteins and transporters (Bota et al., 2012). Studies such as these have led to the identification of 18 accepted BNST subregions (or subnuclei) that are often organized into more general anterior and posterior divisions. Considerable more work (including that presented in this dissertation) has gone into dissecting and studying the anterior portion of the BNST in rodents, and thus I shall primarily focus on this division throughout the rest of this section.

The anterior BNST, which itself is often defined as possessing dorsal and ventral regions separated by the anterior commissure, is heavily interconnected with many of the major limbic structures mentioned above, permitting it express participation in regulating stress and anxiety related stimuli (see **Fig. 4** below). These consists of dense GABAergic inputs from the VTA, lateral hypothalamus, and amygdalar structures (CeA and MeA), glutamatergic inputs from the vHPC, PFC, limbic and insular cortices, parabracial nucleus (PBN) and BLA, and dopaminergic inputs from the VTA and PAG (Dong et al., 2001a; Dong and Swanson, 2004a; McDonald et al., 1998; Hasue and Shammah-Lagnado, 2002; Kim et al., 2013; Dobolyi et al., 2005; Phelix et al., 1992). In addition to this, the ventral division of the anterior BNST is also known to receive one of the densest noradrenergic innervations within the entire brain, coming primarily from the NTS via the ventral noradrenergic bundle and also from the dorsal noradrenergic bundle via the LC to a lesser degree (Forray and Gysling, 2004; Banihashemi and Rinaman, 2006; Woulfe et al., 1988). The anterior BNST also sends numerous reciprocal projections back to many of these regions, including glutamatergic efferents to the VTA speculated to participate in the regulation of reward oriented behaviors (Georges and Aston-Jones, 2001; Dong and Swanson, 2004a; Kim et al., 2013), GABAergic efferents to the VTA, amygdala (BLA and CeA), VP, and NAc implicated in anxiety, stress and other aspects of reward centric activity (Lebow and Chen, 2016), and a highly prominent GABAergic output to the PVN (Cullinan et al., 1993). This pathway in particular has been shown to potently regulate HPA axis function in response to chronic and acute stressors within rodents, with additional studies of the posterior BNST also revealing this division to chiefly innervate the PVN and participate in the regulation of defensive and other stress-related behaviors (Choi et al., 2007; Dong and Swanson, 2004a). Taken together, these findings further suggest a role for the BNST in controlling specific forms of affective behavior, particularly through its connectivity to key structures implicated in the neurocircuitry of stress responsiveness.

The degree to which the BNST is able to contribute to the regulation of these behavioral modalities has also been speculated to be owed in large part to the highly heterogeneous cellular makeup of the region, which is comprised of a number of specialized populations that either secrete or respond to key modulatory molecules that have been previously implicated in the pathology of anxiety and depressive disorders. Indeed, while the BNST is primarily known to be composed of GABAergic interneurons, many of these inhibitory cells can be further subdivided and studied based on the expression of these signaling molecules. A particular distinction has been made for the neurohormones and neuropeptides, many of which have been found to be abundantly

expressed within the BNST (Kash et al., 2015), with the most well documented and well-studied of these being CRF. Indeed, the BNST has been found to express some of the highest levels of CRF outside of the PVN (Swanson et la., 1983), and it has been shown across multiple preclinical studies that CRF within the BNST acts as a peptide neurotransmitter to influence both circuit function and downstream behaviors (Kash and Winder, 2006; Silberman et al., 2013; Heisler et al., 2007; Funk et al., 2006). The actions of CRF in the BNST have also been shown to be complex and differ based on the intrinsic activity of BNST-CRF (+) neurons, which have been found throughout multiple sub-nuclei and to vary in their physiological and projection profiles (Silberman, et al., 2013), and changes in regional signaling induced by the activation of CRFR1 and CRFR2 receptors that have also been shown to be heavily expressed through the region (Botas et al., 2012). While this suggests that BNST-CRF cell activity and CRF signaling may influence a wide range of the behavioral outputs, cross species studies have consistently shown their effects to correlate most strongly with anxiety and stress related behavior (Sahuque et al., 2006; Sink et al., 2013; Giardino et al., 2018; Fetterly et al., 2019; Tran et al., 2014).

Additional neuropeptidergic cell populations have also been found to be highly abundant in the BNST, including NPY, pituitary adenylate cyclase-activating polypeptide (PACAP), and dynorphin, all of which have also been heavily implicated in regulating similar behaviors via their actions within the region. NPY, as mentioned previously, has been speculated to act in opposition to CRF signaling in order to decrease stress related behavior and endocrine signaling (Heilig et al., 1994), and evidence to this effect has been demonstrated in the BNST. *Ex vivo* physiology studies have shown NPY washed onto BNST slices decreases GABAergic transmission that can be enhanced by CRF signaling (Kash and Winder, 2006), while more recent studies using a convergence of pharmacological and chemogenetic approaches have shown that selective activation of BNST Y1R cells can suppress maladaptive behaviors found in rodent models of chronic ethanol drinking (Pleil et al., 2015). Correlative evidence has also indicated that increased activation of Y1R and increased NPY expression in the BNST may also be associated with reductions in anxiety and depressive-like behaviors (Desai et al., 2014; Hawley et al., 2010). PACAP signaling in the BNST has conversely been more closely linked to CRF signaling and viewed as promoting stress like behavior, with rodent studies indicating both PACAP and one of its primary receptors, PAC1, are elevated in expression in the BNST following exposure to chronic stress (Hammack et al., 2009; Lezak et al., 2014). Pharmacological inactivation of this receptor has further been demonstrated to reduce increases in anxiety-like behaviors and BNST corticosterone levels in typical models of

anxiety-related disorders (Roman et al., 2014). The role of dynorphin in the BNST and the regulation of affective behavior is less clear; however, transcript expression of pro-dynorphin has been found to be elevated in the BNST following exposure to major stressors such as forced swim (Chung et al., 2014) and more recently it has been suggested that dynorphin signaling through kappa opioid receptors (KORs) expressed on BLA-BNST terminals may play a principle role in regulating the anxiety-related behaviors that are often seen to be enhanced following the activation of this pathway, as deletion of (KORs) from this circuit was found to produce an anxiolytic effect (Crowley et al., 2016).

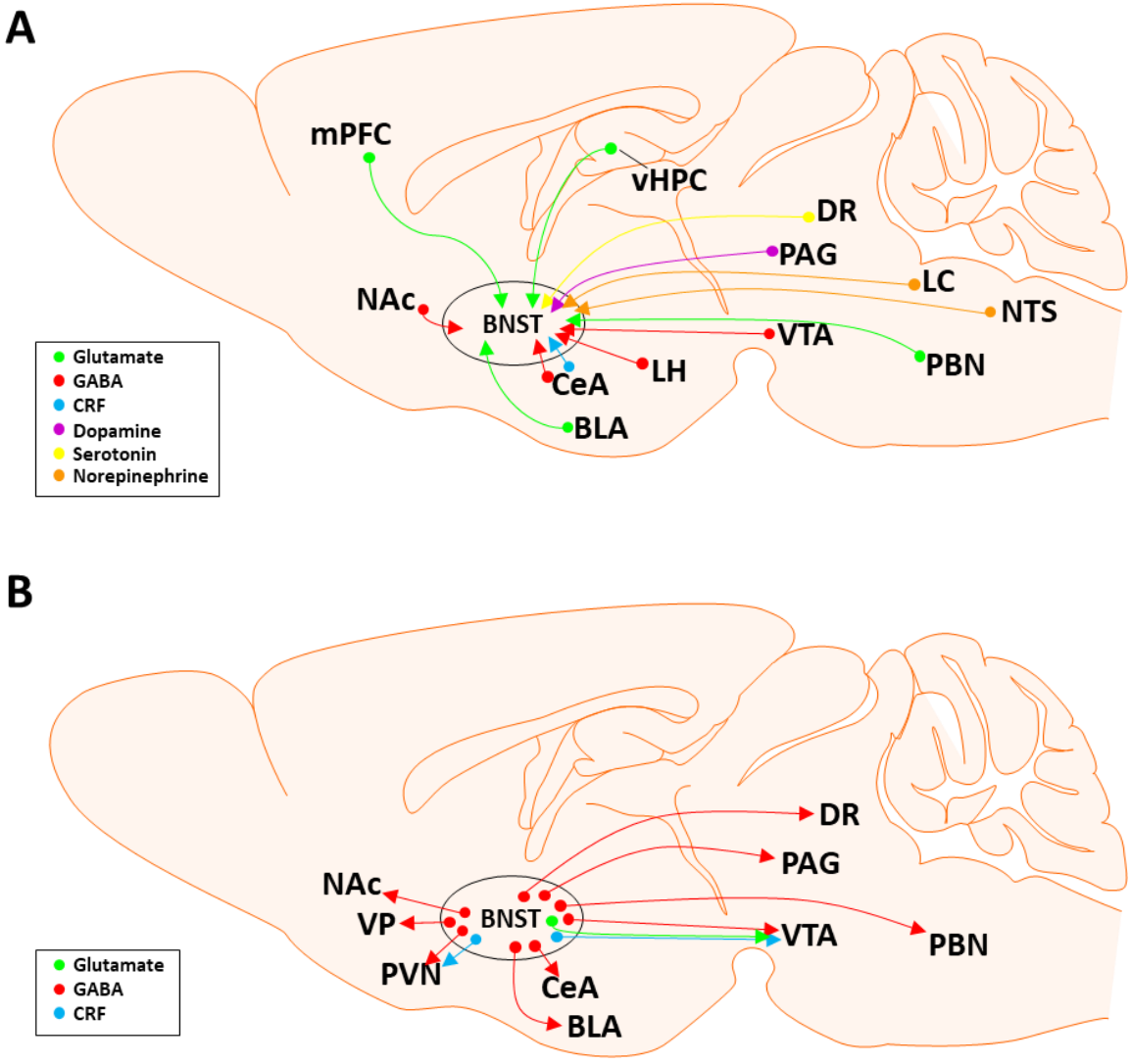


Figure 4. Key BNST excitatory, inhibitory and modulatory circuitry of affective behavior. Sagittal schematics of the main BNST (A) afferents, and (B) efferents as identified across multiple neuroanatomical and functional studies of BNST signaling. The BNST both sends and receives widespread innervation to/from many structures heavily involved in the processing of and response to stimuli related to stress, reward, arousal, social interaction and valance. Many of these excitatory and inhibitory circuits have been functionally implicated in

affective behavioral output salient to depression and other related anxiety and mood disorder models, while key modulatory molecules and circuits known to regulate HPA axis also show high expression or pass through the BNST. BLA – basolateral nucleus of the amygdala; BNST – bed nucleus of the stria terminalis; CeA – central nucleus of the amygdala; DR – dorsal raphe; LC – locus coeruleus; LH – lateral hypothalamus; mPFC – medial prefrontal cortex; NAc – nucleus accumbens; NTS – nucleus of the solitary tract; PAG – periaqueductal grey; PBN – parabrachial nucleus; PVN; paraventricular nucleus of the hypothalamus; vHPC – ventral hippocampus; VP – ventral pallidum; VTA – ventral tegmental area. More details in text. Adapted in part from Ch'ng et al. (2018). Sagittal mouse brain images courtesy of of Motifolio (Biology Bundle Toolkit).

BNST function and glutamatergic signaling in models of depression

Given the underlying circuitry and high levels of stress-related neurohormone and neuropeptide expression within the BNST, it is perhaps unsurprising that it has historically been linked to regulating affective behaviors in rodents. This activity, however, has also been shown to be divergent from that of the CeA and NAc, which have been shown to respond more phasically to acute stressors and salient reward cues (LeDoux, 2007; Herman and Cullinan, 1997). By contrast, the BNST is preferentially engaged by chronic stress exposure (Walker and Davis, 2008), suggesting a means by which maladaptive activation of the BNST can lead to states of constant and heightened anxiety often associated with a number of psychiatric disorder, including depression. Additionally, it has also been suggested that the BNST may participate in the processing and assignment of emotional valence (i.e. negative or positive) to specific stimuli and/or changes in physical or social environments and interactions that may carry stressful or rewarding connotations to them (Lebow and Chen, 2016). The positioning of BNST function at the intersection of stress and reward, the processing and distinction between which can often be thought to be imbalanced in depressive disorders, further suggests a key role of this region in the pathophysiology of such disorders, and has been supported across numerous preclinical studies and hinted at from emerging clinical data as well.

The regulation of anxiety has been the most consistently studied within the BNST and through its many connections to key regions involved in the regulation of fear, stress and reward related stimuli. While many of these circuits, as mentioned above, are known to be GABAergic or modulatory, increasing evidence has suggested that the regulation of select BNST glutamatergic circuits, as well as the intrinsic BNST glutamatergic signal, can drive robust changes in anxiety-related behaviors (Dong et al., 2001a, Dong et al., 2001b, Dong and Swanson, 2006). Indeed, studies examining the effects of expose to mild chronic stress and/or intermittent injection of the stress hormone corticosterone have reported significant increases in anxiety-like behaviors and an associated reduction in BNST plasticity (Conrad et al., 2011a), suggesting that an increased anxiety-like

response to stress may correlate with decreased intercellular BNST excitatory drive (Conrad et al., 2011b). More recent work using a combination of electrophysiological, pharmacological and optogenetic techniques has also demonstrated the effects of glutamatergic regulation in the BNST on anxiety-like behavior as well, showing that both direct infusion of glutamate receptor specific antagonists into the dorsal region of the anterior BNST and optically invoked inhibition excitatory signaling in this region as able to reduce anxiety-like behaviors observed in the elevated plus maze and the open field test (Kim et al., 2013). CRF within the BNST has also been linked to the modulation of its glutamate signaling (Aborelius et al., 1999; Dabrowska et al., 2013; Silberman and Winder, 2013; Regev et al., 2011; Sink et al., 2013), and in particular has been demonstrated to enhance the frequency of excitatory post synaptic currents at BNST neurons projecting into the VTA (Silberman et al., 2013). Such increase in BNST-VTA glutamatergic signaling in response to CRF suggests that chronic stress may enhance the BNST's excitatory input to the regions like the VTA, potentially inducing maladaptive changes in rewarding and aversive motivational states. Several studies have supported this notion, including work using *in vivo* recording to show that excitatory signaling within the glutamatergic BNST-VTA pathway specifically was enhanced during stressful events, while the activation of its GABAergic inputs to the VTA was suppressed (Jennings et al., 2013) Selective activation of this GABAergic projection was also found to decrease avoidance behaviors in real time conditioned place preference assays, while activation of the glutmatergic projections produced adverse and anxiogenic behaviors reminiscent of the effects of CRF application, as well as direct activation of BNST CRF cells (Giardino et al., 2018; Jennings et al., 2013). This suggested dysregulation of activity within the BNST that has been found quite consistently in rodent models has also been shown to have potential human correlates as well, with recent imaging and functional connectivity studies suggesting recruitment of the BNST in response to chronic stress and the anticipation of stressful stimuli (Straube et al., 2007; Mobbs et al., 2010), as well as validating similar circuitry between the human BNST and many of the correlative limbic structures that are often implicated in the regulation of stress-related behavior in animal models (Avery et al., 2014; Avery et al., 2016).

While much focus has been placed on the role of the BNST in regulating anxiety-like behaviors in response to chronic stress and changes in social/environmental stimuli, preclinical studies have also indicated a clear role for the BNST in regulating depressive-like behaviors as well. In particular, many of these indicated that the direct suppression BNST activity may be correlated with enhanced antidepressant-like effects observed

across a number of tasks, such as the FST (Crestani et al., 2010; Hammack et al., 2004). This connection of altered BNST activity within the context of depressive-like behaviors has also been shown to be highly associated with the actions of CRF within the region. Indeed, early studies in rodent models of depression observed that increased concentrations of CRF in the BNST were correlated with anhedonic behavior in rats (Stout et al., 2000) and that lentiviral mediated overexpression of CRF specifically within the BNST was able to increase depressive-like behaviors in mice in both the FST and TST as opposed to anxiety-like behaviors measured across other tasks (Regev et al., 2010). This, coupled with additional studies that have identified few changes in CRF expression following exposure to inescapable stressor or depressive-like behaviors in response to lesioning in regions such as the PVN and CeA, supports the notion of a unique role for the BNST and BNST CRF signaling in regulating aspects of depressive behavior related to chronic stress that are separable from fear and acute stress responsiveness (Maier et al., 1993; Helmreich et al., 1999; Hammack et al., 2015). Lesion studies across the BNST have also indicated that these CRF specific signaling affects may also be more prominently restricted to the anterior portion of the BNST, where CRF expression is the most highly concentration and the lesion of which specifically has been shown to produce decreases in anxiety and depressive-like behaviors (Hammack et al., 2015). Outside of the effects of CRF alone, alterations in glutamatergic signaling as a whole within the BNST and at many of its principle outputs has also been demonstrated to alter hedonistic behavior, particularly in relation to the activation of BNST-VTA pathway mentioned above, as well as the selective deletion of specific NMDAR subunits within the BNST (Jennings et al., 2013; Louderback et al., 2013). This later study was unique in particular, in that NMDAR subunit deletion was only found to reduce depressive and anhedonic-like behavior across the FST and NIH, respectively, but not anxiety-like behaviors in tasks such as the EPM. The implications of this will be expanded upon below.

Much like in the discussion of human correlates for BNST function in aspects of anxiety above, clinical data has also indicated that similar changes have been observed in case of depression as well (Avery et al., 2016). Indeed, histochemical analyses of tissue samples taken from suicidal or chronically depressed patients postmortem revealed an increase in CRF mRNA both amygdala and BNST (Merali et al., 2006), while a recent clinical trial using deep brain stimulation of the BNST in depressed patients reported promising antidepressant-like effects over time (Blomstedt et al., 2017). Despite this, human studies of the BNST and depression are still within their infancy, and may require an increased focus at the level of preclinical studies to not only investigate

the avenues of most promise for potential translation in regards to our current knowledge regarding the underlying pathology of depression and emerging treatment methods. It is here that recent work implicating a role for not only ketamine, but for the function of NMDARs specifically within the BNST as a means for modulating aspects of depressive behavior has started to become an increasingly intriguing area for future investigations.

NMDAR-mediated excitatory signaling in the BNST: implications for modulating affect

As previously mentioned, NMDA receptors are known to play a critical role in regulating the plasticity events that are essential for alterations in synaptic strengths within neuronal circuits. Exposure to specific kinds of external stimuli, both of negative and positive connotations, has also been demonstrated to prominently influence changes in synaptic strength in many of the limbic structures and affective circuitry discussed above, with the BNST serving as no exception. As a general phenomenon, changes in plasticity within the BNST have been shown across multiple sets of *ex vivo* experiments to be readily inducible for the purposes of physiological study (Weitlauf et al., 2004; Weitlauf et al., 2005; Wills et al., 2012) and as evidence of potential alterations in response to specific stimuli or treatment paradigms either in slices or model organisms prior to performing electrophysiological recordings. Indeed, as mentioned above, it has been demonstrated that exposure to stressful stimuli in particular can produce a potent reduction in long term potentiation (LTP) within the BNST, suggestive of an overt disruption in glutamatergic function in response to such conditions (Conrad et al., 2011a). Similarly, changes in the potentiation of excitatory signaling within the BNST via LTP, as well as the de-potentiation of select synapses via long term depression (LTD), have been implicated in shaping both the positive and maladaptive responses of the BNST to reward salient stimuli as well (Harris and Winder, 2018). This suggests these phenomenon, and the function of NMDARs within the region in general, are critical for shaping the BNST's output and influence on downstream behavior. More recent studies assessing alterations in the potentiation of specific glutamatergic BNST circuits has attested to this as well, with evidence indicating that the disruption of an NMDAR-mediated form of LTP produced at vHPC-BNST inputs can produce increases in anxiogenic behaviors in mice (Glangetas et al., 2017).

How plasticity events are shaped by NMDARs has also been shown to be highly dependent on the make-up and biophysical properties of the receptors themselves, with the GluN2 subunits in particular shown to

play a pivotal role in this. As previously discussed, the expression patterns of the four GluN2 subunits are developmentally regulated and restricted in the adult brain, with the GluN2A subunit becoming more prominently incorporated into most NMDARs within the many cortical and subcortical structures, while levels of GluN2B, GluN2C and GluN2D are all shown to be downregulated by comparison. A number of subcortical structures, however, continue to show high levels of GluN2B expression in the adult brain (Monyer et al., 1994; Watanabe et al., 1992), and as data from our own lab has indicated, expression of GluN2B transcripts within the anterior BNST is shown to be prominently localized on ~95% of all cells (Katie Holleran and Greg Salimando, data not shown), suggesting them as a critical subunit within the region. This has been supported from additional studies demonstrating that GluN2B-containing NMDARs (or at least the GluN2B subunit) are critical for the maintenance of BNST LTP, while the more ubiquitously expressed GluN2A subunit was not found to be required to maintain such plasticity events (Weitlauf et al., 2005). Indeed, bath both bath application of the GluN2B selective antagonist Ro 25-6981 and conditional deletion of the GluN2B subunit from the BNST via the use of a transgenic *Grin2b* floxed mouse demonstrated a lack of LTP induction in response to patterns of high frequency stimulation (two bouts of 100 Hz tetanus) typically shown to drive robust potentiation within the region (Wills et al., 2012; Weitlauf et al., 2004). While these studies initially provided informative physiological data regarding excitatory signaling and NMDAR function within the BNST, perhaps more intriguingly where the implications such findings held for the potential role of NMDAR-mediated excitatory signaling in the regulation of depressive-like behaviors and a region specific sensitivity to the effects of ketamine.

Indeed, previous work had suggested a potential role for ketamine to induce its antidepressant effects more selectively through GluN2B-containing NMDARs (Miller et al., 2014), and reports from our lab provided evidence to suggest that these effects may be partially induced through ketamine's actions at GluN2B-NMDARs within the BNST. Using a combination of conditional knockout mouse and pharmacological studies, it was demonstrated that the conditional deletion of the GluN2B subunit from the anterior BNST was able to produce a robust antidepressant-like effect that mimic the actions of systemic ketamine or Ro 25-6981 administration across multiple behavioral tasks (Louderback et al., 2013). Indeed, conditional knockout mice showed decreased immobility time in the FST when compared with controls, as well as increased hedonistic drive as measured in the NIH. Most interestingly, however, the effects of this deletion were only found to affect depressive-like behavior, as Cre-injected mice showed no differences in anxiety-like behaviors when compared

with GFP-injected controls. These findings not only provided further support for the hypothesis of ketamine transducing the majority of its therapeutic effects through GluN2B-NMDARs, but also demonstrated NMDAR-mediated excitatory signaling within in the BNST to be necessary in regulating specific aspects of depressive-like behaviors in rodent models.

Despite this though, BNST NMDARs are not speculated to be exclusively composed of GluN2B subunits as obligate diheteromers, with several *in situ* hybridization studies suggesting that another subunit, GluN2D, may also be expressed in the region (Monyer et al., 1994; Watanabe et al., 1993; Sheng et al., 1994; Cull-Candy et al., 2001). Previous proteomic work from our group provided initial confirmations of this, showing the GluN2D subunit to be pulled down along with GluN2B more selectively within tissue punches from the anterior BNST when compared with other structures within the adult mouse brain (**Fig**. **21** below), suggesting both the functional expression of GluN2B and its possible incorporation into triheteromeric NMDARs within the BNST (Yi et al., 2019). Considering the aforementioned findings suggesting that ketamine may more effectively inhibit NMDARs containing this subunit when compared with GluN2A or GluN2B containing receptors alone (Khlestova et al., 2016) as well as several behaviors studies showing depressive, anxiety and anhedonic-like behaviors to be enhanced in constitutive GluN2D knockout animals (Yamamoto et al., 2016; Shelkar et al., 2019) and the antidepressant effects of (R) ketamine to be significantly diminished in these animals as well (Ide et al., 2017), the discovery of GluN2D expression within the BNST warranted further confirmation and investigation in regards to its contribution to the behavioral effects that appear to be driven through NMDAR-mediated excitatory signaling within the BNST. The initial pursuit of this prospect will thus comprise the bulk of the dissertation work presented in the coming chapters.

Summary

Major depressive disorder is a common and devastating mood disorder that affects a significant portion of the global population, necessitating a critical demand for the development of both a greater understanding the neural mechanisms underlying its pathophysiology and new therapeutics for more rapidly and efficaciously providing relief from this disease. Though deficits in many neuromodulatory and neurotransmitter systems have been found to be implicated in depression and a number of molecular targets identified for treating these

aspects of the disease, the majority of these have not proven to successfully lead to the overarching treatment of the highly heterogeneous symptoms and maladaptive changes intrinsic to depression, highlighting a need for the identification of novel targets to more effectively achieve these goals. Recently, the glutamatergic signaling system has emerged as just such a potential target, specifically through the manipulation of excitatory signaling mediated by the ionotropic N-methyl-d-aspartate class of glutamate receptors (NMDARs). The implication for these receptors in achieving these effects was first demonstrated via the use of the drug ketamine, which is widely accepted to act as a noncompetitive antagonist of the NMDARs that blocks the channel pore of the receptor to reduce its overall activity. This has been speculated to lead to a number of downstream changes in both gene expression and overall synaptic function that can overturn deficits in excitatory signaling throughout the brain, including at key limbic structures and neural circuits implicated in the regulation of affective behavior and stress-responsiveness.

The therapeutic mechanism of action of drugs such as ketamine, and the overall means by which modulation of excitatory signaling through NMDARs can achieve such robust antidepressant-like effects, is still poorly understood. Additionally, many of the key structures and circuits that may be implicated in these effects are also relatively understudied, with a more disproportionate amount of research focusing on the contribution of higher cortical and subcortical structures and more canonical stress and reward salient circuits that are thought to be dysregulated in depression and lead to the development of anhedonia, chronic stress and other core symptoms of this disease. While a wealth of preclinical and clinical data has indicated that at a molecular level, these effects may be produced by the modulation of select subtypes of NMDARs (in particular those containing the GluN2B subunit) and that the manipulations of these receptors in regions such as the PFC and hippocampus can lead to the amelioration of depressive symptoms, conflicting evidence has also suggested that the GluN2B-containing NMDARs may be a poor target that do not avoid the many side effects of ketamine. It has also become increasingly apparent that that regions outside of those typically studied in the context of depression (PFC, hippocampus, amygdala, etc.) also contribute significantly to the regulation of depressive-like behaviors. This has indicated that regions known to play a pivotal role in integrating and processing emotional stimuli, such as the extended amygdala, may warrant further investigation in the context of the diesease.

The BNST in particular is a key structure of interest within the extended amygdala in these regards, as it is known to integrate information salient to stress and reward related stimuli from multiple limbic structures

previously tied to depression pathology. Additionally, the BNST is a highly heterogeneous structure that contains a number of cell populations that express many of the stress-related neurohormones, neuropeptides and other modulatory molecules implicated in the modulation of depressive-like behaviors and the core stress responsive circuitry (i.e. the HPA axis) of the body, chiefly CRF. Furthermore, manipulation of NMDAR-mediated function within the BNST has been found to drive antidepressant-like effects, while emerging data has demonstrated that lower abundant subtypes of NMDARs show unique expression profiles within the BNST, particularly in regards to those expressing the GluN2D subunit, which have been suggested to play a potential role in regulating depressive-like behavior as well. Whether or not their function in the BNST is salient to driving these effects, however, is unknown.

In this dissertation, I will investigate the contribution of GluN2D-containing NMDARs within the BNST to the regulation of anxiety- and depressive-like effective behaviors and overt excitatory signaling within the region. Chapter 2 will describe these efforts via the use of a previously validated GluN2D constitutive knockout mouse line, and several transgenic lines designed for the study of cell-specific GluN2D-NMDAR function in the BNST. This will include a more direct validation of the expression of functional GluN2D within the BNST, as well as an examination of its overt expression profile throughout the region and on certain specialized cell populations, with a specific focus on BNST-CRF (+) cells. The effects of GluN2D knockout on regional synaptic plasticity and the excitatory/inhibitory synaptic profile of the BNST-CRF cells will also be investigated, in order to determine a significant contribution of GluN2D to these physiological properties. Chapter 3 will continue these efforts in a more targeted and selective fashion via the use of a GluN2D conditional knockout line generated for the express examination of the effects of GluN2D deletion from the BNST alone on the behavioral modalities and aspects of excitatory signaling at BNST-CRF discussed above. Overall, this dissertation will seek to address the following hypothesis and primary aims:

<u>Hypothesis</u>: The deletion of inhibition of GluN2D-containing NMDARs increases anxiogenic and depressive-like behaviors in mice, and increases excitatory signaling in the anterior BNST.

<u>Aim 1</u>: Test the hypothesis that GluN2D is expressed on specific BNST cell populations.

<u>Aim 2</u>: Test the hypothesis that GluN2D deletion increases the excitable membrane properties and synaptic physiology of BNST cells.

<u>Aim 3</u>: Test that hypothesis that loss of GluN2D-NMDARs in the BNST promotes an increase in anxiety and depressive-like behaviors.

Chapter 2

Effects of constitutive GluN2D-NMDAR deletion on BNST function and affective behavior

Adapted from: Salimando et al., "BNST GluN2D-containing NMDA receptors influence anxiety- & depressive-like behaviors and modulate cell-specific excitatory/inhibitory synaptic balance"

Excitatory signaling mediated by N-methyl-d-aspartate receptors (NMDARs) has been shown to regulate mood disorders. However, current treatments targeting NMDAR subtypes have shown limited success in treating patients, highlighting a need for alternative therapeutic targets. Here, I identify a role for GluN2D-containing NMDARs in modulating emotional behaviors and neural activity in the bed nucleus of the stria terminalis (BNST). Using a GluN2D knockout mouse line (GluN2D$^{-/-}$), I assessed behavioral phenotypes across tasks modeling emotional behavior. I then used a combination of *ex vivo* electrophysiology and *in vivo* fiber photometry to assess changes in BNST plasticity, cell-specific physiology and cellular activity profiles. GluN2D$^{-/-}$ male mice exhibit evidence of exacerbated negative emotional behavior, and a deficit in BNST synaptic potentiation. I also find that GluN2D is functionally expressed on corticotropin-releasing factor (CRF) positive BNST cells implicated in driving negative emotional states, and recordings in mice of both sexes revealed increased excitatory and reduced inhibitory drive onto GluN2D-/- BNST-CRF cells ex vivo, and increased activity *in vivo*. Taken together, this study provided initial evidence of the expression of GluN2D-containing NMDARs within the BNST of adult animals and to display a more exclusive expression profile on a stress-salient population of neurons. Further, this work also replicated the findings of previous studies using the GluN2D$^{-/-}$ line, demonstrating more consistent agreement that the global loss of these receptors can produce robust increases in anxiety and depressive-like behaviors. This may suggest a key role for GluN2D-NMDARs, and specifically GluN2D-NMDARs within the BNST, in shaping aspects of affective behavior associated with the pathology of depression.

Introduction

Dysregulated excitatory signaling is proposed to underlie a number of mood-related disorders (Graybeal et al., 2012; Sanacora et al., 2012). Despite this, the molecular underpinnings of how excitatory signaling influences emotional behavior are poorly understood, underscoring a critical need for continued study of the

receptor and cell types involved in shaping the physiological changes that drive them. The N-methyl-d-aspartate receptors (NMDARs) in particular have emerged as targets both associated with the pathobiology of these disease states and for developing treatments for their more intractable forms (Sanacora et al., 2008; Javitt et al., 2011; Ghasemi et al., 2014).

NMDARs are heteromeric receptors composed of two GluN1 subunits and a combination of two GluN2 or GluN3 subunits, with GluN2 subunits being more prominent in the adult brain (Hansen et al., 2018). GluN2 subunits are comprised of four isoforms (GluN2A-D) each conferring distinct physiological properties (Paoletti et al., 2013). NMDARs containing the GluN2D subunit (GluN2D-NMDARs) represent an interesting potential target due to their unique biophysical attributes, including prolonged deactivation kinetics, weakened Mg^{2+} sensitivity, and enhanced glutamate sensitivity (Vicini et al., 1998; Qian et al., 2005). Recent rodent studies have also reported that genetic deletion of GluN2D-NMDARs can alter emotional behavior; however, opposing phenotypes have been observed, warranting further investigation (Miyamoto et al., 2002; Yamamoto et al., 2017; Shelkar et al., 2019).

While GluN2D-NMDARs may represent an interesting therapeutic target due to their distinctive properties and lower expression in the adult brain (Monyer et al., 1994; Wenzel et al., 1996), little is known about their functional role in regions known to drive emotional behavior. The restriction of GluN2D expression in the forebrain and select limbic structures that occurs during development (Sheng et al., 1994; Cull-Candy et al., 2001) suggests that their influence might be significant in extended amygdalar regions. The bed nucleus of the stria terminalis (BNST) is one such region that has been heavily implicated in modulating emotional states, serving as a key integrator of negative valence and stress-related stimuli (Dong et al., 2001; Lebow and Chen, 2016). Excitatory signaling in the BNST has also been implicated in driving emotional behavior, with previous studies reporting that disruptions of inter- and intra-BNST excitatory synaptic function can lead to increases or decreases in anxiety- and depressive-like behaviors (Jennings et al., 2013; Kim et al., 2013; Glangetas et al., 2017). Moreover, the BNST is known to contain dense populations of neuropeptidergic cells that have been linked to regulating emotional behavior, in particular those expressing corticotropin-releasing factor (CRF, *Crh*). These cells (BNST-CRF) have been shown to drive negative valence, with numerous preclinical studies linking increases in both BNST and extended amgydalar CRF cell signaling to depression- and stress-related states/disorders (Lebow et al., 2012; Sink et al., 2013; Pleil et al., 2015; Fetterly et al., 2019; Kim et al., 2019b).

Using behavioral testing and electrophysiological recording techniques, we demonstrate here that mice constitutively lacking GluN2D (GluN2D$^{-/-}$) exhibit increases in anxiety- and depressive-like behaviors, and associated disruptions in BNST synaptic potentiation. Closer examination of GluN2D expression across the BNST revealed the subunit to be present on BNST-CRF neurons, suggesting a role for GluN2D-NMDARs in regulating their activity. *Ex vivo* whole cell electrophysiology and *in vivo* recordings of calcium transients from these neurons in GluN2D$^{-/-}$ mice confirmed this, showing converging evidence of increased basal excitatory activity in these BNST-CRF cells that may correlate with alterations in emotional behavior and stress processing.

Materials and Methods

Animals

Male and female mice of at least 8 weeks of age were used throughout this study. GluN2D constitutive knockout mice (GluN2D$^{-/-}$) were purchased from the RIKEN Experimental Animal Division Repository (Tsukuba, Japan; RBRC no. 01840), while Crh-IRES-Cre, Ai14 and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME; stock no. 012704, 007908 & 00064). Crf-*Tomato* mice were generated as previously reported (Chen et al., 2015; Silberman et al., 2013). To generate reporter lines for CRF cells lacking the GluN2D subunit, GluN2D$^{-/-}$ mice were crossed with Crf-*Tomato* mice for constitutive deletion studies (GluN2D$^{-/-}$/Crf-*Tomato*). GluN2D$^{-/-}$ mice were also bred to the Crh-IRES-Cre line in order to generate the mice necessary for conducting *in vivo* fiber photometry studies (GluN2D$^{-/-}$/Crf-Cre). GluN2D$^{-/-}$ and Crf-*Tomato* mice were genotyped using protocols reported for each respective line on the Jackson Laboratory's website.

For behavioral studies using the GluN2D$^{-/-}$ line, male mice were primarily used in order to replicate the conditions of previously published behavioral work with these animals (Ikeda et al., 1995; Miyamoto et al., 2002; Obiang et al., 2012; Yamamoto et al., 2017; Shelkar et al., 2019). For *ex vivo* electrophysiological and *in vivo* fiber photometry studies using the GluN2D$^{-/-}$/Crf-*Tomato* and GluN2D$^{-/-}$/Crf-Cre lines, mice of both sexes were used to minimize the total number of animals. No sex differences were observed in these studies, and as such, all data are compiled across groups into values representative of both sexes. All mouse lines were maintained

on a C57BL/6J background and backcrossed as needed. Mice were group housed with 2-5 individuals per cage and maintained on a 12 hour light/dark cycle (lights on at 0600 hrs) under controlled temperature (20-25°C) and humidity (30-50%) levels. Mice were given access to food and water *ad libitum*. All treatments and interventions were approved by the Vanderbilt Animal Care and Use Committee.

Behavioral Testing

All experiments took place during the light phase of the cycle. Group housed male GluN2D$^{-/-}$ mice were transferred into the vivarium 3 weeks prior to testing and allowed a minimum of 1 week for acclimation. Following acclimation, mice were singly-housed for 2 weeks. 5 days prior to the start of behavioral testing, mice were handled daily to help reduce experimenter-induced stress as previously described (Olsen and Winder, 2010). On test days, mice were brought into procedure rooms approx. 1 hour prior to the start of any experiment to allow for acclimatization, which was performed under full light (~300-400 lux) in rooms while mice were provided food and water *ad libitum*. All equipment used during testing was cleaned with a 70% ethanol solution prior to start, and in between each animal run, in order to mask odors and other scents.

Open Field Test (OFT): Mice were run in ENV-S10S open field activity chambers (Med Associates Inc.) fitted with IR photo-beam arrays for 60 minutes under full light in the chambers (~200-300 lux). Total locomotor activity, and zone analyses for total time spent in the designated center and surround zones of the activity arena were performed using Med Associates software.

Light/Dark Box (L/D Box): Mice were run using the same activity chambers described above, with inserts made of blacked out Plexiglas fitted into the left side of each chamber, occupying ~1/2 of the total chamber area. Mice were placed into the dark side to begin the test and run for 10 minutes. Total movement between and activity within each side of the chamber was automatically recorded via Med Associates software based on IR beam breaks. Zone analyses for total time spent in each side of the chamber, as well as total zone entries and overall locomotion (distance travelled) were later performed using Med Associates software.

Elevated Zero Maze (EZM): Mice were run using a custom EZM apparatus measuring 34 cm inner diameter, 46 cm outer diameter, placed 40 cm off the ground on four braced legs, with two open quadrants and two closed quadrants. Testing was administered under illuminated conditions (~200-300 lux for open quadrants and ~100-

150 lux for closed quadrants), with mice initially placed into one of the open quadrants, after which they were run for 5 minutes in the maze and filmed continuously. Videos were analyzed automatically via AnyMaze software (Stoelting Co.) for total time spent in the designated open quadrants compared to total time spent in the closed quadrants, and presented as percent total time in open quadrants overall. Total locomotion and quadrant entries were similarly assessed via AnyMaze.

Forced Swim Test (FST): The FST was always administered on the final day of all behavioral testing batteries. Mice were placed in Plexiglas cylinders containing room temp. (~22-23°C) tap water for 6 minutes while being continuously filmed. Videos were then analyzed to determine the total immobility time (i.e. lack of swimming/struggling while in water) observed in mice during the last 4 minutes of the test (first 2 mins were designated as a habituation period to the water/test). All results were hand-scored by individuals blinded to the genotype of test mice.

Slice Preparation for Electrophysiology

Male and female mice were transported from animal colony housing facilities to the laboratory and placed in a sound-attenuated chamber for 1 hour before *ex vivo* brain slice preparation as previously described (Harris et al., 2018; Fetterly et al., 2019; Centanni et al., 2019). Mice were anesthetized with isoflurane gas and then decapitated, after which brains were quickly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF) containing the following: 194mM sucrose, 20mM NaCl, 4.4mM KCl, 2mM CaCl2, 1mM MgCl2, 1.2mM NaH2PO4, 10mM glucose, and 26mM NaHCO3. For slices prepared for field potential recordings, 0.9mM of ascorbic acid was added to help preserve cell health in the interface chamber. Dissecting solution was saturated with 95% O2 / 5% CO2 (vol./vol). Coronal slices 300μm in thickness were prepared using a VT1200S vibratome (Leica). Slices containing anterior portions of the dlBNST (Bregma, 0.26-0.02mm) were selected using the internal capsule, anterior commissure and stria terminalis as landmarks.

Field Potential Recordings: After dissection, slices were transferred to an interface recording chamber (Fine Science Tools), where they were perfused with heated (~29°C) and oxygenated (95% O2 / 5% CO2, vol/vol) ACSF (124mM NaCl, 4.4mM KCl, 2mM CaCl2-2H2O, 1.2mM MgSO4, 1mM NaH2PO4, 10mM glucose, and 26mM NaHCO3; pH 7.2-7.4; 295-305 mOsm) at a rate of approx. 2 mL/min. Slices were allowed to equilibrate in ACSF for at least 1 hour before recording began. A bipolar Ni-chrome wire stimulating electrode and a

borosilicate glass recording electrode (1-2 MΩ) filled with ACSF were placed in the dlBNST to elicit and record extracellular field responses, which were amplified using an AxoClamp 2B amplifier (Molecular Devices). Baseline responses to electrical stimulus at an intensity that produced approx. 40% of the maximum response were recorded for 20 mins at a rate of 0.05 Hz. After acquisition of a stable baseline, long term potentiation (LTP) was induced with two trains of 100 Hz, 1 sec tetanus delivered with a 20 sec inter-train interval at the same intensity as the baseline stimuli. The N1 (an index of sodium channel-dependent firing) was also monitored, and experiments in which it changed by more than approx. 20% were discarded. Analyses were made from the percent change of the N2 (an index of the extracellular population response) from baseline 0-10 min after tetanus and 51-60 min after tetanus. Electrical signals were low-pass filtered at 2 kHz, digitized at 20 kHz and acquired with a Digidata 1322A and pClamp 9.2 system (Molecular Devices). All relevant analysis work was conducted with Clampex 10.6 software (Molecular Devices).

Whole Cell Recordings: Following dissection, slices were transferred to a holding chamber containing heated (~29°C) and oxygenated (95% O2 / 5% CO2, vol/vol) ACSF. Slices were allowed to equilibrate in ACSF for at least 1 hour, after which they were transferred onto a submerged recording chamber on a BX51WI upright microscope (Olympus). In the chamber, slices were continuously perfused with oxygenated and heated ACSF at a rate of 2mL/min. Recording electrodes (3-6 MΩ) were pulled on a Flaming/Brown Micropipette Puller (Stutter Instruments) using thin walled borosilicate glass capillaries, and filled with a cesium gluconate internal solution (117mM Cs-gluconate, 20mM HEPES, 0.4mM EGTA, 5mM TEA-Cl, 2mM MgCl2, 4mM ATP and 0.3mM GTP; pH 7.2-7.4; 285-290 mOsm). A bipolar Ni-chrome stimulating electrode was placed in the stria terminalis approx. 100-300um dorsal to the recorded cell, and electrical responses were evoked via local fiber stimulation (5-15 V at a duration of 100-150 us) at 0.1 Hz. After achieving whole cell configuration, cells were allowed to dialyze and equilibrate for 2-5 mins prior to recording. Postsynaptic parameters were continuously monitored during all experiments, and cells were excluded from final analysis if the access resistance (Ra) changed by >20% in either direction. For spontaneous EPSC measurements made in voltage clamp mode, responses were isolated by adding 25μM picrotoxin ($GABA_A$ receptor antagonist, Tocris) into the ACSF and bath applying over slices while recording at a holding potential of -70mV. Spontaneous IPSC measurements were performed by adding 10μM NBQX (pan-AMPA and kainate receptor antagonist, Tocris) and 25μM AP-V (pan-NMDA receptor antagonist, Tocris) into bath applied ACSF, while maintaining a holding potential of -70mV. Miniature EPSC

recordings were also made at a holding potential of -70mV, while ACSF containing 1μM of tetrodotoxin (sodium channel blocker, Abcam) and 25μM picrotoxin was perfused over the slice. In paired pulse ratio (PPR) experiments, paired evoked 100- to 200-pA responses at 0.05 Hz were elicited and inter-stimulus intervals of 30, 50 and 100ms were utilized. For measuring the evoked, isolated NMDAR EPSCs used for analyzing differences in NMDAR kinetics, the holding potential for the cells was adjusted to +40mV and ACSF containing 10uM NBQX and 25uM picrotoxin was perfused over the slice. Evoked, isolated NMDAR EPSCs were also elicited for GluN2C/D antagonist wash-on experiments, but this time using a modified, 0 Mg^{2+}-based ACSF (124mM NaCl, 4mM KCl, 1mM NaH2PO4 and 3.7mM CaCl2-2H2O; pH 7.4-7.2; 295-305mOsm) containing 25μM picrotoxin, 10μM NBQX and 40μM of the selective GluN2C/2D antagonist DQP-1105 (selective GluN2C/D antagonist, Tocris) and utilizing a -70mV holding potential. Slices were prepared as described above for these experiments, but allowed to recover and equilibrate in heated, normal ACSF before being transferred to the rig and perfused with the modified 0 Mg^{2+} ACSF. Signals were acquired with a Multiclamp 700B amplifier (Molecular Devices), digitized via a Digidata 1322A and analyzed with pClamp 10.6 software (Molecular Devices). Spontaneous and miniature voltage clamp recordings were analyzed via Clampfit 10.6 (Molecular Devices) by measuring the peak amplitudes and frequencies of events over a 6 minute period (in three, 2 min bins). Paired pulse ratios were analyzed by dividing the value of the amplitude of the second response over the amplitude of the first (P2/P1), and the resulting ratios plotted out for each inter-stimulus interval period. Evoked NMDAR EPSCs decay kinetics were interrogated by examining the amplitude of the current trace at half of the tau (τ) value. Evoked NMDAR EPSCs isolated in 0 Mg2+ ACSF for DQP-1105 wash-on studies were analyzed by measuring the percent change in the amplitude of the response from baseline during the last 8 mins of the recording (i.e. following DQP application and drug wash out).

RNAscope In Situ Hybridization

RNAscope studies were performed as previously described (Ghamari-Langroudi et al., 2015). Three mRNA species expressed by neurons in the dlBNST were visualized across separate sets of experiments using the enhanced fluorescent *in situ* hybridization technique RNAscope® (ACD, Advanced Cell Diagnostics). RNAscope® cDNA probes and detection kits were purchased from ACD and used according to the company's online protocol for fresh frozen tissue. The probe sets directed against *Crh* and *Grin2d* were designed from

sequence information from the mouse RefSeq mRNA IDs NM_205769.2 and NM_008172.2, respectively. C57BL/6J male mice, aged 8-10 wks, were anesthetized using isoflurane and the brains were quickly removed and frozen in Tissue Tek® O.C.T. compound (Sakura) using Super Friendly Freeze-It Spray (Fisher Scientific). Brains were stored at -80°C until cut on a CM3000 cryostat (Leica) to produce 16µm coronal sections. Sections were adhered to warm, charged glass microscope slides and immediately refrozen before being stored at -80°C until ready to undergo the RNAscope® procedure. In brief, following the ACD protocol for fresh frozen tissue, slides were fixed for 15 mins in ice cold 4% paraformaldehyde and then dehydrated in a sequence of ethanol serial dilutions (50%, 70% and 100%, twice each). Slides were briefly air-dried and then a hydrophobic barrier was drawn around the tissue sections using a Pap Pen (Vector Labs). Slides were then incubated with ACD's Pretreat 4 solution for 30 mins at room temperature in a humidified chamber. Following protease treatment, sections were incubated with RNAscope® cDNA probes (2 hours), and then with a series of signal amplification reagents provided by the Multiplex Fluorescence Kit from ACD: Amp 1-FL (30 mins), Amp 2-FL (15 mins), Amp 3-FL (30 mins) and Amp 4-FL ALT A (15 mins). Two minutes of washing in RNAscope® wash buffer (1x from 50x stock) were performed between each step, and all incubation steps with the cDNA probes and amplification reagents were preformed using a HybEZ oven (ACD) at 40°C. cDNA probe mixtures were prepared at a dilution of 50:1 of the C1 channel probe (Grin2d) and C2 channel probe (Crh) for C57 studies. Sections were also stained for DAPI using the reagent provided by the Fluorescent Multiplex Kit. Immediately following DAPI staining, sections were mounted and cover-slipped using Aqua-Poly Mount media (PolySciences) and left to dry overnight in a dark, cool place. Sections from the dlBNST were collected in pairs, using one section for incubation with the cDNA probes, and another for incubation with a probe for bacterial mRNA (DapB, ACD) to serve as a negative control. Sections were imaged using a 710 scanning confocal microscope (Zeiss) at 20x (20x/0.50 N.A. lens) and 63x magnification (63x/0.50 N.A. oil immersion lens), and composite images of the dlBNST were generated as TIF files and analyzed in Fiji/ImageJ. Images from sections treated with the negative control probe for each pair of slides were used to determine brightness and contrast parameters that minimized observation of bacterial transcripts and auto-fluorescence, and these adjustments were then applied to the images from experimental sections treated with the cDNA probes. Adjusted experimental images were then analyzed in a designated region of interest around the dlBNST. Cells in these regions of interest were identified using both DAPI stained nuclei and the borders present between cells identified with the help of gray scale

differential interference contrast (DIC) overlays, and the total number of cells in each region were counted. Cells were appraised for the presence of *Grin2d* and *Crh* signal in order to determine the total number of cells showing probe expression either alone or in combination in C57 studies. Transcripts were readily identified as round, fraction delimited spots over and surrounding DAPI-labeled nuclei.

Stereotaxic Surgery Procedures

Adult mice (~8 weeks of age) were anesthetized with isoflurane (initial dose = 3%, maintenance dose = 1.5%) and surgery was performed using an Angle Two stereotaxic frame (Leica) to intracranially inject adeno-associated virus (AAV) into the dorsolateral BNST based on the Franklin and Paxinos (2004) mouse brain atlas (from Bregma: AP=0.14, ML=+/-0.88, DV=-4.24, 15.03° tilt) at a rate of 100nl/min. For photometry studies, GluN2D$^{-/-}$/Crf-Cre mice of either sex were injected with ~300nl of AAV9-Syn-FLEX-jGCaMP7f-WPRE (Addgene) into the right dlBNST, and then given a 3 week recovery period to allow for full expression of the virus. Following this period, mice underwent a second round of surgery to place a fiber optic cable implant (4.1mm fiber, Doric) just above the injection site of the virus within the dlBNST (~0.1mm above DV coordinate listed above). After placing the fiber optic into position, a system consisting of a light curable Optibond primer and adhesive agent (Henry Schein) and Herculite XRV dental composite enamel (Kerr) was applied to the mouse's skull in order to firmly affix and hold the fiber optic in position. Briefly, after exposure of the skull, gel etchant was used to clean the skull, a screw was placed rostral to the craniotomy hole, and the implant was slowly lowered through the previously made craniotomy hole. Optibond FL Primer was applied around the implant, followed by Optibond FL light curable adhesive, and lastly the light curable Herculite enamel. Mice were given a minimum 1 week recovery period following implant surgery in order to allow for the surgery sites to fully heal prior to the start of *in vivo* calcium signal recording.

In Vivo Fiber Photometry Recordings

Optical recordings of GCaMP7f fluorescence were acquired using a RZ5P fiber photometry detection system (Tucker-Davis Technologies, TDT), consisting of a processor with Synapse software (TDT), and optical components (Doric Lenses & ThorLabs). Excitation wavelengths generated by LEDs (470nm blue light and 405nm violet light) were relayed through a filtered fluorescence minicube at spectral bandwidths of 460-495nm and 405nm to a pre-bleached mono fiber-optic cable connected to the implant on top of each animal's head.

Power output for the primary 470nm channel at the tip of the fiberoptic cable was measured at ~25-30uW. Single emissions were detected using a femtowatt photoreceiver with a lensed fiber cable adapter. Signal in both 470nm and 405nm channels were monitored continuously throughout all recordings, with the 405nm signal used as an isobestic control for both ambient fluorescence and motion artifacts introduced by movement of the fiber optic implant. Wavelengths were modulated at frequencies of 210-220 and 330 Hz, respectively, and power output maintained at 20mA with a DC offset of 3mA for both light sources. All signals were acquired at 1kHz and lowpass filtered at 3Hz. Mice were housed and handled as described above, with the addition of a 5 min session each handling day during which the mice were hooked up to the fiberoptic cable to allow them to become accustomed the tethered cable. Mice were transported to the procedure room and habituated for 1 hour. Mice were then connected to the photometry system, and following a 1-2 min period to adjust to these manipulations, a 30 min recording session was conducted to observe basal activity in the dlBNST. Mice were placed in a custom arena and allowed to explore for the duration of the recording. Following testing, all mice were perfused and tissue was assessed for both proper targeting of both initial viral injections and optic fiber placement via immunohistochemistry.

Analysis of the GCaMP signal was done with a custom-written MATLAB code (can be provided upon request), with the bulk fluorescent signal from both the 470 and 405 channels normalized in order to compare differences in calcium-mediated event frequencies across groups using the 405 as a control channel. Linear regression was utilized to correct for the bleaching of signal for the duration of each recording, using the slope of the 405nm signal fitted against the 470nm signal. Detection of GCaMP-mediated fluorescence is presented as a change in the 470nm/fitted 405nm signal over the fitted 405 signal ($\Delta F/F$). Peak analysis of calcium-mediated events to determine frequency across subjects was performed by running the normalized, filtered signals produced by our MATLAB code through Clampfit 10.6 software and performing a template matching event detection analysis on each file. The template was set to match the average width and amplitude of an imputed, positive-going calcium-mediated event, with a template matching variable set at ~2.5.

Immunohistochemistry

Immunostaining for the GluN2D protein in neural tissue sections was performed using a modification of the protocol previously described (Yamasaki et al., 2014). In brief, mice were anesthetized using isoflurane and

underwent transcardial perfusion using ice-cold phosphate-buffered saline (PBS) and a 4% paraformaldehyde solution (PFA) made in PBS. Following perfusion, brains were quickly removed and placed in ice-cold 4% PFA over night for post-fixation, and then into an ice-cold 30% sucrose solution prepared in PBS for 2-3 days to cryo-protect the tissue. Brains were then embedded in paraffin wax and sectioned at 5µm on a microtome. Sections containing the dlBNST were baked in a hybridization oven at 50°C for 1-2 hours to melt wax, before going through a series of washes in citrisol solution, 100%, 95%, 70% and 50% ethanol, PBS and 1% hydrogen peroxide. Sections were next digested in a pepsin solution (Dako, 1mg/ml) at 39°C for ~20 mins, and then washed with PBS and a PBS/Triton-X100 (PBS-T, 0.1%) solution. A hydrophobic barrier was drawn using a PAP-Pen. Sections were then blocked with a buffer containing 10% normal donkey serum (Jackson Immuno) in PBS for 1 hour at room temp. in a humidified chamber. Primary antibody for GluN2D (generously provided by the lab of Masahiko Watanabe) was applied at a concentration of 1mg/ml in a solution of 10% normal donkey serum and PBS-T, and the sections were stored in a humidified chamber at 4°C for ~ 72-96 hours. Primary antibody was removed with a series of four washes in ice-cold PBS-T buffer before a biotin conjugated secondary antibody (Jackson Immuno) prepared in 10% normal donkey serum and PBS-T was applied at a concentration of 1:2000 for ~3 hours at room temp in a humidified chamber. Secondary antibody was removed with four washes in ice-cold PBS-T, and then the tissue underwent amplification for the GluN2D signal using a Cy3-Tyramide Signal Amplification (TSA) kit (PerkinElmer). In brief, horse radish peroxidase reagent provided by the kit was applied to sections at a concentration of 1:100 in room temp. TNB buffer overnight at 4°C in a humidified chamber, and washed off the following day via three, 5 min washes using TNT buffer. Cy3 fluorophore was then prepared at a 1:50 dilution and applied to the sections for 5 mins at room temp before being washed off with three, 5 min washes in TNT buffer. Lastly, sections were treated with a DAPI stain (1:10,000) for ~30 secs at room temp before being mounted and cover-slipped using warm, Aqua-Poly Mount media. Once dried, sections were then imaged using the same confocal microscope described above. Endogenous eGFP signal was used to validate the expression of GCaMP7f in the dlBNST, and DAPI staining combined with DIC imaging was utilized in order to validate the position of implanted fiber optic cables for mice used in photometry studies.

Western Blotting

Bilateral tissue punches (~0.8 mm) of the dlBNST collected from GluN2D$^{-/-}$ or GluN2D$^{+/+}$ mice, were homogenized in lysis buffer containing 2% SDS and 1X protease inhibitor cocktail (Roche) via pipetting and passage through a 27 gauge syringe and an insulin syringe. Samples were centrifugated at 1000x g for 10 mins and total protein concentration was then determined using a BCA protein assay kit (Thermo Scientific). Whole homogenate was used for analysis, and all samples were diluted to an equal concentration by mixing with sample buffer containing 62.5 mM Tris-HCl (pH 6.8), glycerol, 5% SDS, 0.5% bromophenol blue and 5% β-mercaptoethanol. Diluted samples were boiled at 90°C for 3 mins, and then 10-20µg of each were run on a 7% polyacrylamide-resolving gel. Protein was then transferred onto a single nitrocellulose membrane, and then stained for total protein using Ponceau S. Membranes were cut at specific molecular weights and probed with different antibodies for select products. Primary antibodies used included mouse anti-NMDAR2D (1:5000, MAB5578, Millipore), mouse anti-beta-3 tubulin (1:1000, 4466S, Cell Signaling Technologies) and mouse anti-GAPDH (1:10,000, MAB374, Millipore). All primary Ab dilutions were prepared in 5% powdered milk solution in 1x Tris-buffered saline/Triton-X100 (TBS-T), and applied to blots overnight at 4°C. Blots were washed four times in 1x TBS-T the following day, and then probed with an anti-mouse, horse radish peroxidase conjugated secondary antibody (1:8,000, W402B, Promega) in 5% milk, 1x TBS-T for ~2 hours at room temp. Blots were then washed an additional four times with fresh 1x TBS-T and then soaked in Western Lighting Plus-ECL solution (PerkinElmer) for 1-2 mins before imaging on X-ray film. Blots were appraised as needed for changes in total protein expression via densitometry analysis using Fiji/ImageJ software.

Experimental Design and Statistical Analysis

The number of animals used in each experiment were predetermined based on analyses of similar experiments in the literature and supplemented as needed based on observed effect sizes (Silberman et al., 2013; Louderback et al., 2013; Ghamari-Langroudi et al., 2015; Holleran et al., 2016; Fetterly et al., 2019; Centanni et al., 2019). All data are represented as means ± the standard error of the mean (SEM) for each group, and all statistical analyses were performed using GraphPad Prism 8 software (Graphpad Software, San Diego, CA). We utilized both male and female mice within this study. When sex was not found to be a statistically significant factor, we combined male and female data for analysis. For behavior experiments, data comparing metrics between knockout and wildtype groups across tasks were analyzed using unpaired, two-

tailed Student's *t*-tests, while analyses of changes in locomotive behavior between both groups across multiple time points were performed using two-way ANOVAs along with Sidak's multiple comparison post hoc test, and corrected *p*-values are reported in the text as needed. Two-tailed *t*-test analyses were also performed on LTP data from knockout and wildtype mice, total cell counts and CRF (+) for *in situ* hybridization studies, drug effects of DQP on NMDAR eEPSC amplitude and decay kinetics, sEPSC, sIPCS and mEPSC amplitude and frequency values in *ex vivo* recordings, and frequency values for imputed calcium mediated events in *in vivo* recordings (all of which consisted of comparisons between only two groups). For PPR data examining changes in the ratio across 3 separate inter-stimulus intervals, a two-way ANOVA was utilized, along with Sidak's multiple comparison post hoc test, to assess effects of genotype and the PPR over time, with corrected *p*-values presented in the text. For all analyses, significance levels were set at α=0.05. Detailed statistics are provided within the text and figure legends.

Results

GluN2D knockout mice exhibit enhanced negative emotional phenotypes.

Previous behavioral studies in GluN2D knockout ($GluN2D^{-/-}$) mice have reported opposing phenotypes, with an initial study suggesting a decrease in anxiety- and depressive-like behaviors, while subsequent studies found these behaviors to be significantly increased in the knockouts (Miyamoto et al., 2002; Yamamoto et al., 2017; Shelkar et al., 2019). In order to address these conflicting findings, we employed a battery of four tests to assess different aspects of negative emotional behavior between $GluN2D^{-/-}$ and wildtype ($GluN2D^{+/+}$) littermates (**Fig. 5A**). Age-matched male mice (~12 wks) underwent open field (OFT), light/dark box (L/D), elevated zero maze (EZM) and forced swim (FST) testing over four consecutive days. $GluN2D^{-/-}$ mice displayed a significant decrease in the percent total center time in the OFT when compared with $GluN2D^{+/+}$ controls (**Fig. 5B**, $2D^{+/+}$=47.8±2.3% time in ctr, $2D^{-/-}$: 28.1±3.1% time in ctr, t[19]=5.05, p=<0.0001, unpaired t-test). Additionally, percent total time in the open quadrants of the EZM was also significantly decreased in the $GluN2D^{-/-}$ mice compared with controls (**Fig. 5D**, $2D^{+/+}$: 34.2±2.4% time open quad., $2D^{-/-}$: 26.8±2.6% time open quad., t[21]=2.10, p=0.048, unpaired t-test). Differences in behavior were not observed between groups in the L/D (**Fig. 5C**, $2D^{+/+}$: 21.3±2.5% time in light, $2D^{-/-}$: 22.1±1.7% time in light, t[20]=0.27, p=0.793, unpaired t-test). Total

immobility time on the FST was significantly increased in the GluN2D$^{-/-}$ compared with GluN2D$^{+/+}$ mice (**Fig. 5E**, 2D$^{+/+}$: 97.1±11.0 sec immobile, 2D$^{-/-}$: 134.5±10.6 sec immobile, t[21]=2.45, p=0.023, unpaired t-test). Collectively, our findings from the OFT, EZM and FST suggest an overt increase in anxiety- and depressive-like behaviors in the GluN2D$^{-/-}$ mice, generally consistent with the results presented in Yamamoto et al. (2017) and Shelkar et al. (2019). While no apparent anxiety-like behavior was observed in the L/D, it is possible that differences in our experimental design for the task (black insert = 1/2 area of activity chamber as opposed to 1/3 area, Crawley and Goodwin, 1980) could have increased the possibility of false negatives.

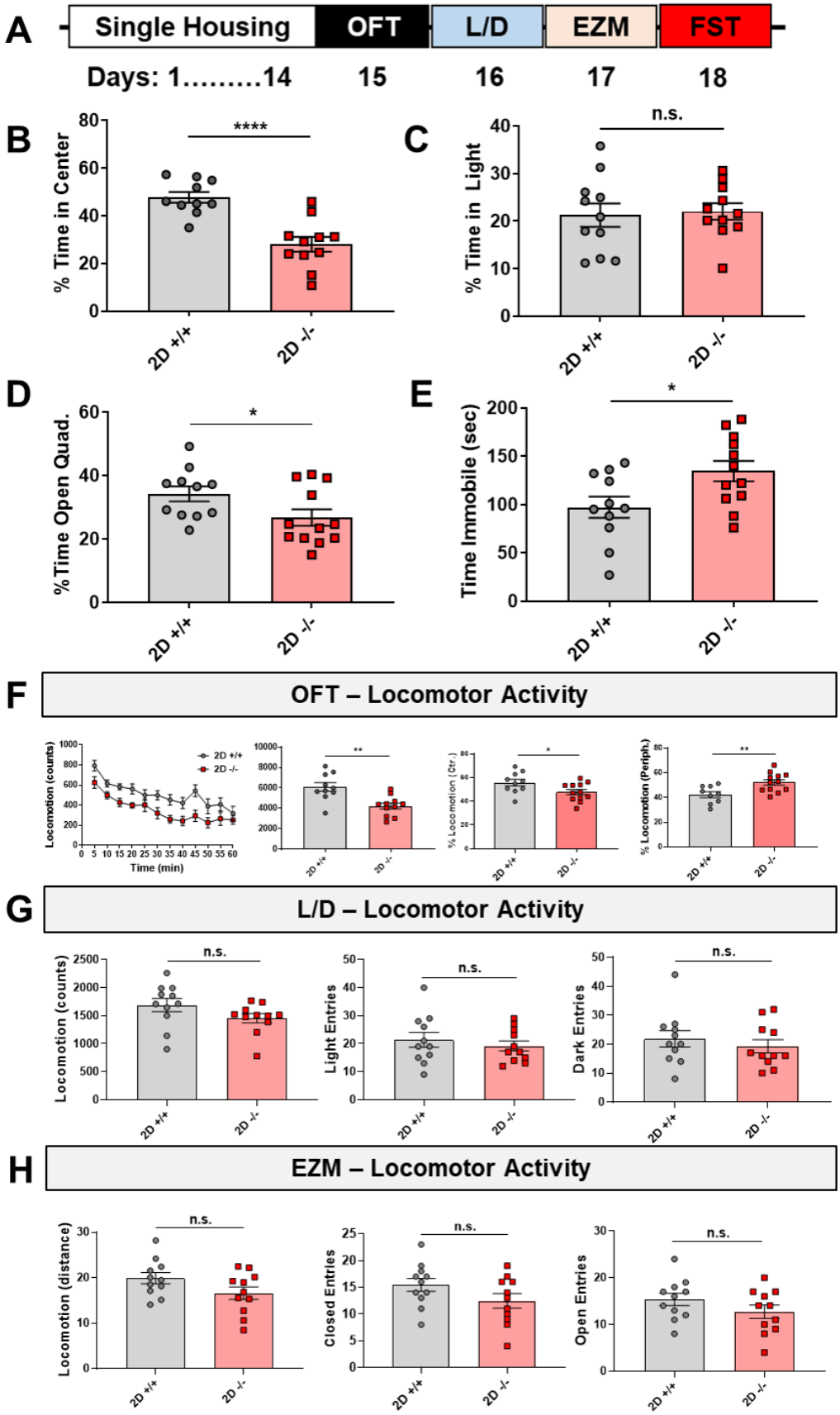

A
Single Housing
OFT
L/D
EZM
FST
Days: 1.....14
15
16
17
18
B
% Time in Center
****
2D +/+
2D -/-
C
% Time in Light
n.s.
D
% Time Open Quad.
*
E
Time Immobile (sec)
*
F
OFT – Locomotor Activity
Locomotion (counts)
Time (min)
% Locomotion (Ctr.)
% Locomotion (Periph.)
G
L/D – Locomotor Activity
Locomotion (counts)
Light Entries
Dark Entries
H
EZM – Locomotor Activity
Locomotion (distance)
Closed Entries
Open Entries

Figure 5. GluN2D knockout mice exhibit altered affective phenotypes. (A) Design schema and timeline for the 4 day behavioral testing battery, consisting of open field (OFT), light/dark box (L/D), elevated zero maze (EZM) and forced swim testing (FST). **(B)** Percent total time spent in the center zone of the OFT arena was shown to be significantly decreased in GluN2D knockout ($2D^{-/-}$) mice when compared with GluN2D wildtype ($2D^{+/+}$) littermates ($p< 0.001$). **(C)** No difference in the percent total time spent on the light side of the L/D box was found between $2D^{-/-}$ and $2D^{+/+}$ mice ($p=0.793$). **(D)** Percent total time spent in the open quadrants of the EZM was however found to be significantly decreased in the $2D^{-/-}$ ($p=0.048$). **(E)** Total time spent immobile during the course of the FST was also found to be significantly increased for the $2D^{-/-}$ when compared with the $2D^{+/+}$ ($p=0.023$). **(F)** Analysis of total locomotion during the OFT via a plot of minute to minute time course for activity (counts, i.e. IR beam breaks) during the 60 min recording (left) and summary graph of total locomotion throughout (right). $2D^{-/-}$ show a significant decrease in total locomotion on in the OFT ($p=0.002$), as well as a significant decrease in percent distance travelled in the center, and an increase in percent distance travelled in the periphery of the arena ($p=0.033$ & 0.008, respectively). The $2D^{-/-}$ also show no overt deficits in locomotor activity in the **(G)** L/D or the **(H)** EZM. Analysis of distance travelled and total entries into the light and dark sides of the chamber in the L/D are not significantly different between genotypes ($p=0.117$, 0.503 & 0.490, respectively), and similar measures of total distance travelled and entries into the open or closed quadrants in the EZM ($p=0.089$, 0.192 & 0.117, respectively) also revealed no indication of a locomotor phenotype. Data presented as means ± SEM overlain with individual points ($N_{2D-/-} = 11$, $N_{2D+/+} = 12$). $^{*}p\leq0.05$, $^{****}p\leq0.0001$, n.s. = not significant.

As an important control, we also assessed changes in total locomotion across all relevant tasks. Total locomotion was found to be decreased in $GluN2D^{-/-}$ mice when compared with $GluN2D^{+/+}$ controls in the OFT, consistent with previous findings (Ikeda et al., 1995; Obiang et al., 2012; Shelkar et al., 2019). However, further analysis of changes in percent total distance travelled in the center and periphery of the chamber between both groups showed more modest differences in locomotion (**Fig 5F**, total locomotion: $2D^{+/+}$: 6093±420.2 beam breaks, $2D^{-/-}$: 4219±295.4 beam breaks, $t[19]=3.70$, $p=0.002$; % distance ctr: $2D^{+/+}$: 55.8±2.8%, $2D^{-/-}$: 47.7±2.2%, $t[20]=2.29$, $p=0.033$; % distance periphery: $2D^{+/+}$: 42.4±2.4%, $2D^{-/-}$: 53.3±2.2%, $t[19]=2.99$, $p=0.008$, unpaired t-tests). Additionally, measurements of differences in locomotor activity and zone/quadrant entries compared between the two groups in the LD and EZM tasks revealed no difference in overall locomotion (**Fig. 5G-H**, LD total locomotion: $2D^{+/+}$: 1690±117.9 beam breaks, $2D^{-/-}$: 1455±82.0 beam breaks, $t[20]=1.64$, $p=0.117$; light entries: $2D^{+/+}$:~21±3, $2D^{-/-}$: ~19±2, $t[20]=0.68$, $p=0.503$; dark entries: $2D^{+/+}$: ~22±3, $2D^{-/-}$: ~19±2 $t[20]=0.70$, $p=0.490$; EZM total locomotion: $2D^{+/+}$: 19.9±1.2 ms travelled, $2D^{-/-}$: 16.6±1.4 ms travelled, $t[20]=1.79$, $p=0.089$; open quad. entries: $2D^{+/+}$: ~15±1, $2D^{-/-}$: ~13±1, $t[20]=1.35$, $p=0.192$; closed quad. entries: $2D^{+/+}$: ~15±1, $2D^{-/-}$: ~12±1, $t[20]=1.64$, $p=0.117$, unpaired t-tests). Our observations here are thus in close agreement to those of previously published datasets that suggest that the effects of constitutive GluN2D deletion are more in line with altered emotional behavior and not gross locomotor function.

GluN2D deletion produces deficits in BNST excitatory synaptic potentiation.

While GluN2D-NMDARs have been shown to regulate synaptic function in the cerebellum, basal ganglia and hippocampus (Harney et al., 2008; Zhang et al., 2014; Swanger et al., 2015; von Engelhardt et al., 2015; Dubois et al., 2016), their role in extended amygdalar circuits regulating emotional behaviors has been unexplored. Deletion or pharmacological blockade of select NMDAR subtypes in the BNST can produce both alterations in synaptic function and negative emotional behavior (Wills et al., 2012; Louderback et al., 2013), and previous studies suggested the potential presence of GluN2D in this region (Watanabe et al., 1992; Watanabe et al., 1993; Wenzel et al., 1996; Yamasaki et al., 2014). To confirm this, we collected tissue punches from the dorsolateral BNST (dlBNST) of GluN2D$^{+/+}$ and GluN2D$^{-/-}$ mice to test for the presence of GluN2D protein. Western blot analysis of whole tissue lysates revealed a band for GluN2D in dlBNST lysates, as well as in lysates taken from regions known to express high levels of the subunit such as the thalamus and hypothalamus. These bands were absent from all regions in samples collected from GluN2D$^{-/-}$ mice (**Fig. 6A**). Additional means of validation were also performed via immunohistochemistry and yielded similar results for GluN2D expression and absence in the BNST across GluN2D$^{+/+}$ and GluN2D$^{-/-}$ tissue samples, as well as thalamus sample as an additional control (**Fig. 6B**).

We next set out to examine whether GluN2D-NMDARs contribute to shaping synaptic function in the BNST by performing extracellular field potential recordings in the dlBNST to assess differences in NMDAR-dependent long-term potentiation (LTP) of evoked field potentials in acute *ex vivo* brain slices from GluN2D$^{+/+}$ and GluN2D$^{-/-}$ mice. After establishing a stable baseline recording, we applied two brief trains of tetanizing electrical stimulation to induce LTP (**Fig. 6C**, arrow insert) and observed a robust potentiation of the evoked responses in GluN2D$^{+/+}$ slices comparable to previously reported findings by our lab (Wills et al., 2012). In the GluN2D$^{-/-}$ slices, we observed a marked decrease in the amplitude of responses immediately following tetanus compared to GluN2D$^{+/+}$ controls, an effect that was maintained for up to 10 minutes (**Fig. 6C-D**, % increase over baseline 2D$^{+/+}$: 173.6±9.0%, 2D$^{-/-}$: 133.3±5.0%, t[17]=3.52, p=0.003, unpaired t-test). While there was a clear reduction in the early phase of the response to tetanus in GluN2D$^{-/-}$ slices, LTP at later time points in the dlBNST was not altered, as a comparison of the overall amplitude of the field potential during the final 10 minutes of a 60 minute post-tetanus recording revealed no significant difference between GluN2D$^{-/-}$ or GluN2D$^{+/+}$ responses (**Fig. 6D**, % increase over baseline 2D$^{+/+}$: 139.3±6.8%, 2D$^{-/-}$: 123.5±5.3%, t[17]=1.72,

p=0.105, unpaired t-test). Overall, these data suggest that GluN2D-NMDARs may contribute to the induction of short-term synaptic potentiation (STP) in the dlBNST, consistent with pharmacological studies of hippocampal STP (Volianskis et al., 2013; Volianskis et al., 2015; France et al., 2017).

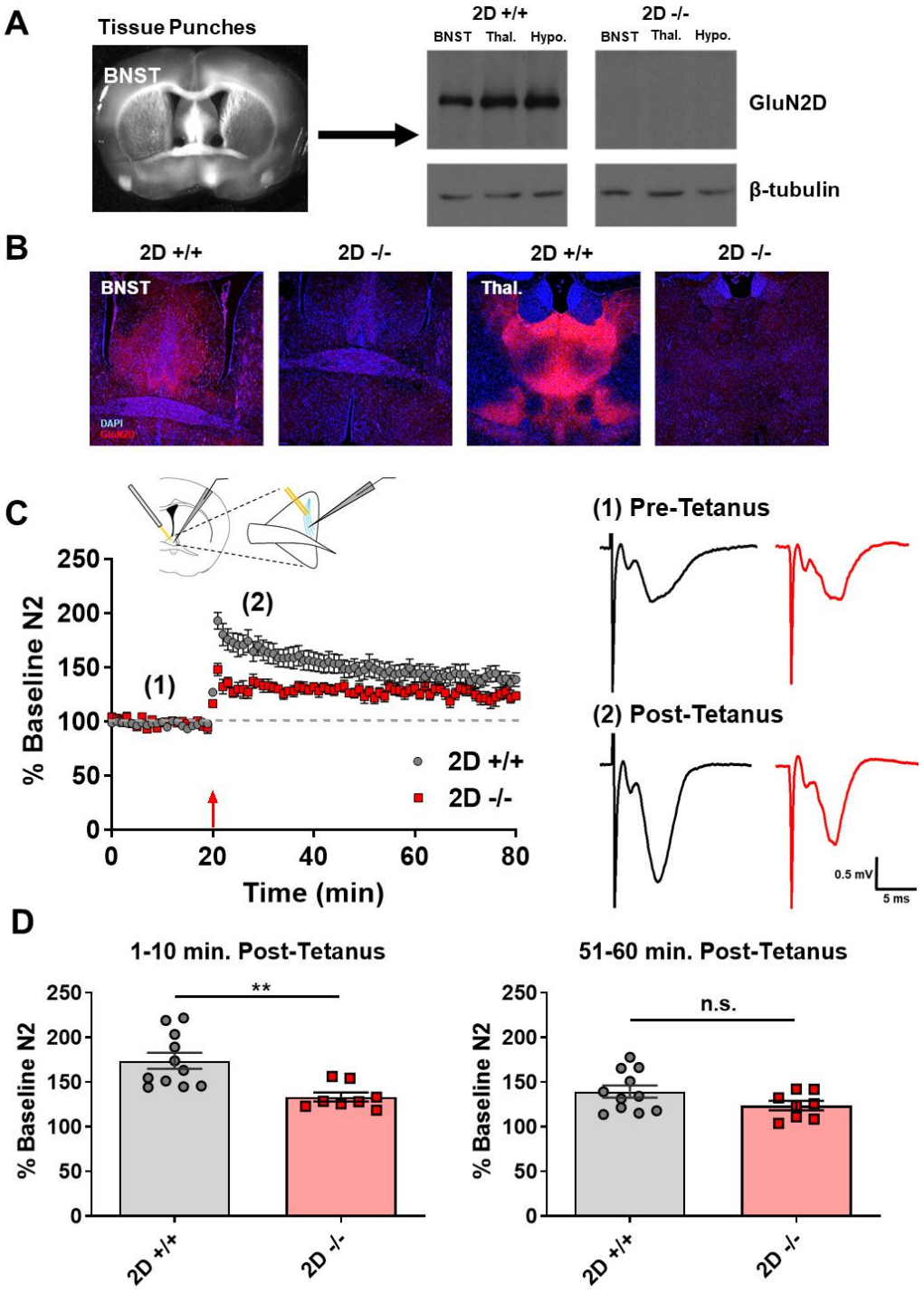


Figure 6. GluN2D deletion produces deficits in BNST excitatory synaptic potentiation. (A) Western blot analysis of total protein lysate taken from tissue punches (0.8mm) of the dlBNST, medial thalamus (thal.) and hypothalamus (hypo.) from GluN2D wildtype ($2D^{+/+}$) and GluN2D knockout ($2D^{-/-}$) mice. Representative image of punch locations for the dlBNST is shown on the left, and a representative blot of bands for both GluN2D protein and control β3-tubulin is shown on the right. **(B)** Representative 5x images of the BNST and the medial thalamus taken from $2D^{+/+}$ and $2D^{-/-}$, stained for the GluN2D protein (red) and counterstained with the nuclear marker DAPI (blue). **(C)** Averaged time courses of excitatory post-synaptic field potentials recorded from the dlBNST of $2D^{+/+}$ and $2D^{-/-}$ acute, *ex vivo* brain slices after high frequency stimulation (arrow; two, 1-s trains at 100 Hz). Representative traces from both $2D^{+/+}$ (black) and $2D^{-/-}$ (red) slices before tetanus (1) and ~10 mins after tetanus (2) are shown to the right. A schematic of the recording set-up is shown as an insert above the time course plot. **(D)** Summary graphs of the averaged field potential responses 0-10 mins after tetanus (left) and 51-60 mins after tetanus (right). A significant difference in the amplitude of responses recorded from $2D^{-/-}$ slices was noted for up to 10 mins following tetanus when compared with $2D^{+/+}$ controls (p=0.003), but not during the final 10 mins of recording (p=0.105). Data presented across summary graphs as means ± SEM with individual points overlain ($n_{2D+/+}$ = 11 slices from $N_{2D+/+}$ = 9 mice, $n_{2D-/-}$ = 8 slices, from $N_{2D-/-}$ = 7 mice). $^{**}p \leq 0.01$, n.s. = not significant.

Grin2d mRNA co-localizes with corticotropin-releasing factor (CRF) transcripts in dlBNST.

Given the alteration in field potential-level synaptic plasticity that we observed in the BNST of $GluN2D^{-/-}$ mice, we next performed whole cell patch-clamp recording experiments to assess whether genetic deletion of GluN2D altered basal synaptic function. However, when using unbiased selection and patching only visually identified cells throughout the dlBNST, we observed no significant differences in the physiological profiles of cells recorded from in $GluN2D^{+/+}$ and $GluN2D^{-/-}$ brain slices when analyzing spontaneous excitatory postsynaptic current events (sEPSCs, **Fig. 7A** freq: $2D^{+/+}$: 1.5±0.3 Hz, $2D^{-/-}$: 1.4±0.2 Hz, t[34]=0.02, p=0.982; amp: $2D^{+/+}$: -24.5±0.9 pA, $2D^{-/-}$: -25.6±0.9 pA, t[34]=0.88, p=0.383, unpaired t-tests) or the kinetics of isolated NMDAR-mediated evoked postsynaptic currents (eEPSCs, **Fig. 7B** $2D^{+/+}$: 72.2±10.5 ½ tau [ms], $2D^{-/-}$: 67.9±9.5 ½ tau [ms], t[17]=0.30, p=0.767, unpaired t-test).

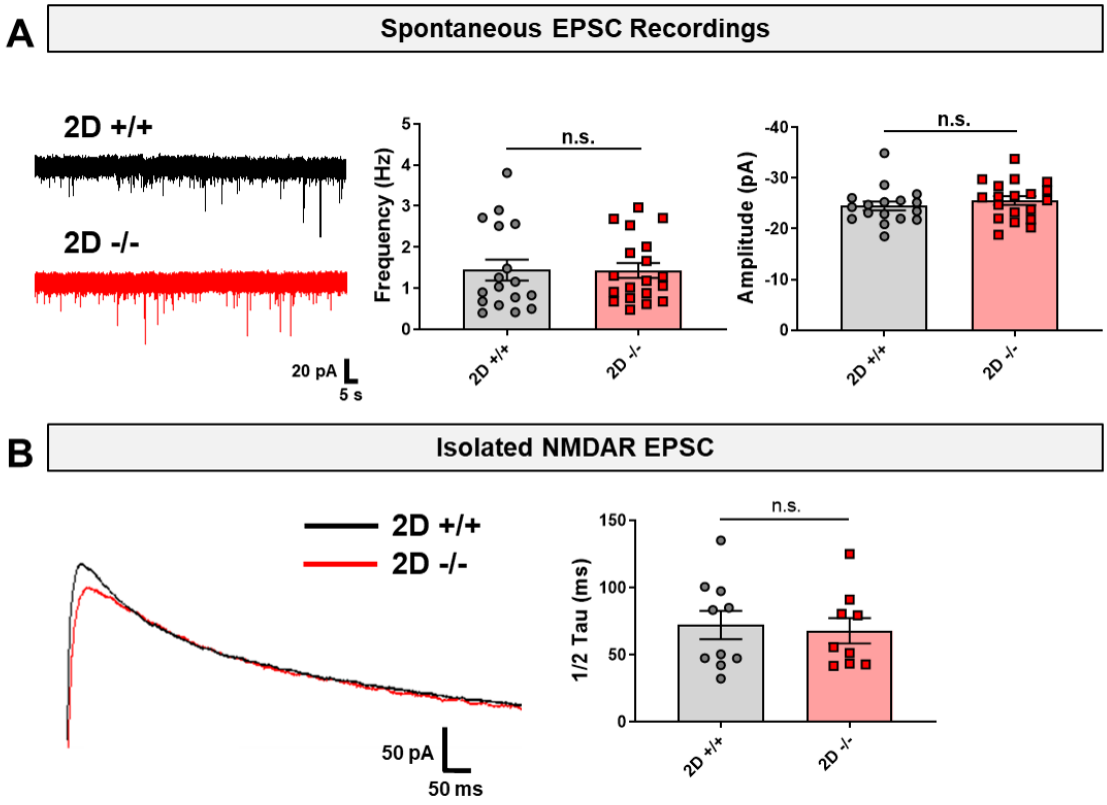


Figure 7. Unbiased examination of dlBNST neurons in the GluN2D-/- does not identify overt differences in basal excitatory physiology. (A) Representative traces of sEPSC recordings from dlBNST neurons in GluN2D$^{+/+}$ (2D$^{+/+}$, black) and GluN2D$^{-/-}$ (2D$^{-/-}$, red) brain slices (left). Summary graphs of both frequency and amplitude analyses of recorded sEPSCs revealed no differences in either metric across groups when sampling cells with an unbiased approach (freq: p=0.981, amp: p=0.383; $n_{2D+/+}$ = 17 cells from $N_{2D+/+}$ = 4 mice, $n_{2D-/-}$ = 19 cells from $N_{2D-/-}$ = 7 mice). **(B)** Representative traces of electrically isolated NMDAR-EPSCs (holding potential: +40mV) from 2D$^{+/+}$ and 2D$^{-/-}$ dlBNST neurons (left). Summary graph to the right shows analysis of changes in the decay kinetics (milliseconds, ms) of NMDAR-isolated currents from both groups revealed no difference when using an unbiased approach to identify and patch dlBNST neurons (p=0.767; $n_{2D+/+}$ = 10 cells from $N_{2D+/+}$ = 5 mice, $n_{2D-/-}$ = 9 cells from $N_{2D-/-}$ = 5 mice). All data presented as means ±SEM with individual data points overlain. n.s. = not significant.

The BNST contains a number of highly heterogeneous and specialized cell populations (Kash et al., 2015; Lebow and Chen, 2016), such that the lack of observable differences in synaptic function in individual neurons could be due to a restricted pattern of expression of GluN2D within these subpopulations. To determine the expression profile of GluN2D in dlBNST we utilized an enhanced form of fluorescent *in situ* hybridization (RNAscope®) to examine the expression of GluN2D transcripts throughout the region and on select cell populations. We focused in particular on those cells expressing the stress-related peptide corticotropin-releasing factor (CRF), as this population has been previously implicated in the regulation of emotional behaviors (Sink et

al., 2013; Silberman and Winder, 2013; Pleil et al., 2015; Marcinkiewcz et al., 2016; Fetterly et al., 2019). Using custom cDNA probes designed for *Grin2d* (GluN2D) and *Crh* (CRF) transcripts, we examined transcript expression patterns and co-localization across the left and right dlBNST of 3 separate adult male C57BL/6J mice (**Fig. 8A**). Consistent with our hypothesis, we found 2D transcripts to be diffusely expressed throughout the dlBNST and present on a minority of BNST cells when comparing the number of *Grin2d* transcript-positive cells with total unlabeled cells across both the right and left dlBNST (**Fig. 8B** *Grin2d* [-]=1661.7 cells, ~67.5% of total cells, *Grin2d* [+]=773.3 cells, ~32.5% of total cells; lower insert: t[4]=7.17, p=0.002, unpaired t-test). Upon closer inspection of the stratification of the *Crh* labeled population, we observed that signal for this transcript was highly co-localized on cells labeled with probes for the *Grin2d* transcript (**Fig. 8C** *Crh* [+] only=19 cells, ~22.8% of total *Crh* [+] cells, *Crh+Grin2d* [+]=61 cells, ~77.2% of total *Crh* [+] cells; lower insert: t[4]=5.11, p=0.007, unpaired t-test), suggesting the potential of increased expression of GluN2D-NMDARs on this subset of BNST neurons.

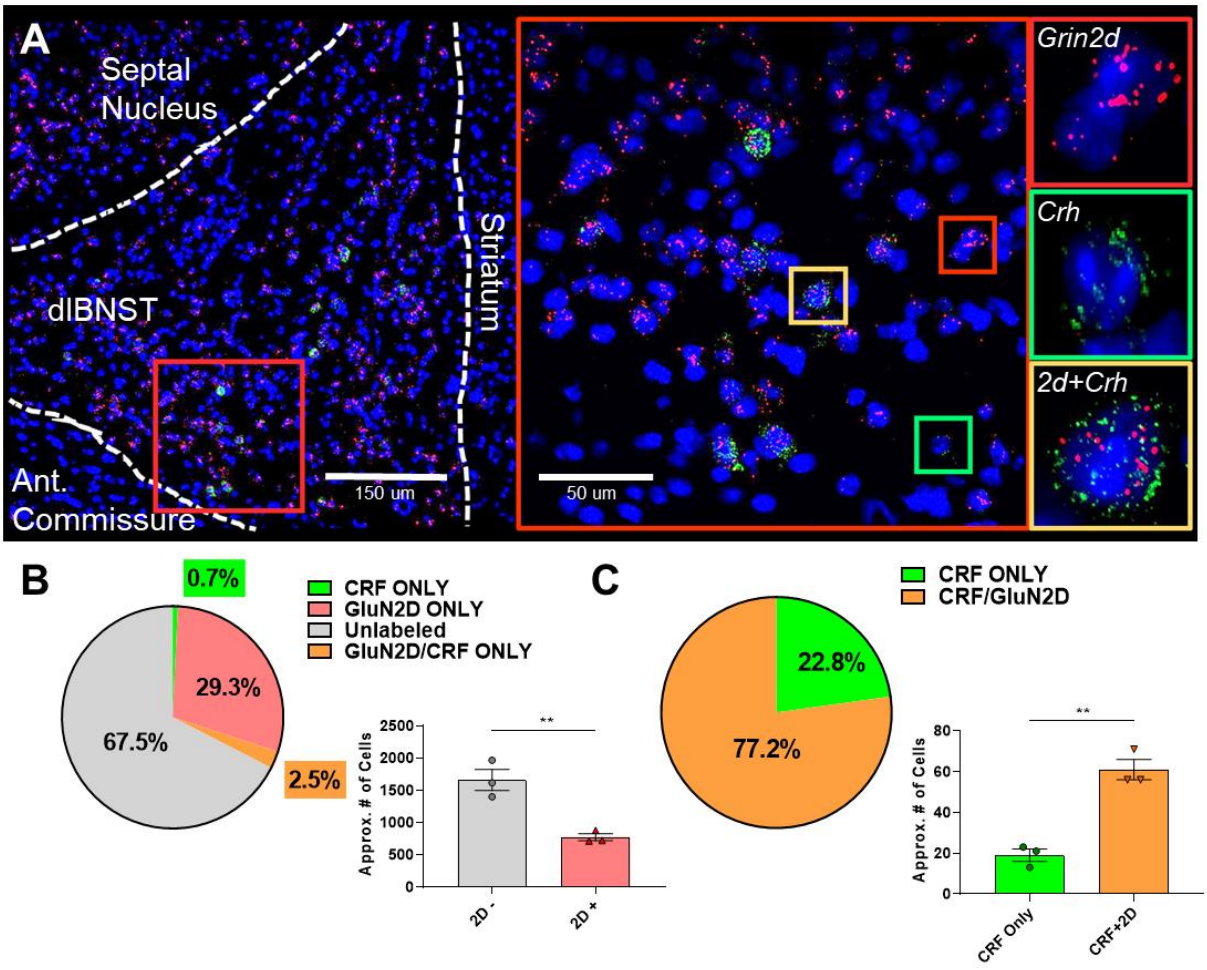


Figure 8. Grin2d mRNA co-localizes with corticotropin-releasing factor (CRF) transcripts in dlBNST. (A) Representative image of the dlBNST (outlined via dashed white lines) at 20x magnification after undergoing RNAscope® (top left), showing individual cells with DAPI (blue) counterstained nuclei labeled for *Grin2d* (red) or *Crh* (green) mRNA transcripts. The red boxed area (top right) shows a representative high magnification image of the dlBNST at 63x, along with example images to the far right of cells labeled for each transcript of interest, and in combination. **(B)** Summary graph showing the portion of total counted dlBNST cells (left & right dlBNST, N = 3 C57BL/6J mice) labeled for the *Grin2d* and *Crh* transcripts alone or in combination. When comparing the total of cells negative for the *Grin2d* transcript (2D [-]) with those labeled for the transcript (2D [+]), cells positive for GluN2D mRNA are shown to represent a lower population within the dlBNST (p=0.007). **(C)** Summary graph of the *Crh* transcript positive cell populations in the dlBNST. As a portion of these cells, those co-labeled for *Grin2d* are shown to make up a greater overall percentage. Additional comparison of the cells labeled for the *Crh* transcript alone with those labeled for *Crh* and *Grin2d* in combination shows the number of co-labeled cells to be significantly higher (p=0.002). All data are presented as means ± SEM with individual data points overlain. $^{**}p \leq 0.01$.

Functional synaptic GluN2D-containing NMDARs are expressed on adult mouse BNST-CRF neurons.

Given the concentration of *Grin2d* transcript on *Crh* transcript positive neurons, we next sought to assess whether functional GluN2D-NMDARs were present on these cells (BNST-CRF), and how the loss of this subunit altered their synaptic physiology. To identify BNST-CRF cells in *ex vivo* brain slices for whole-cell patch clamp electrophysiology, we crossed the GluN2D$^{-/-}$ mice to a previously validated CRF reporter line (Crf-*Tomato*, Chen et al., 2015; Silberman et al., 2013, **Fig. 9A**). We then isolated electrically-evoked synaptic NMDAR-mediated EPSCs at BNST-CRF neurons via the use of a modified, Mg^{2+}-free artificial cerebrospinal fluid (Wills et al., 2012) while holding the cells at -70mV; after which, the GluN2C/2D selective antagonist DQP-1105 (Acker et al., 2011) was bath applied (40uM, **Fig. 9B-C**, baseline: 8 mins, DQP wash-on: 10 mins, wash-out: 10 mins). In slices from GluN2D$^{+/+}$ mice, DQP-1105 wash-on produced a ~36% decrease in the amplitude of the evoked NMDAR EPSC, an effect that was absent in slices prepared from GluN2D$^{-/-}$ mice (**Fig. 9C**, CRF-2D$^{+/+}$ pre-DQP: 103.1±2.0% baseline, post-DQP: ~67.9±8.2% baseline, t[8]=4.21, p=0.003, unpaired t-test; CRF-2D$^{-/-}$ pre-DQP: 100.0% baseline, post-DQP: ~100.0±7.3% baseline, t[6]=0.003, p=0.997, unpaired t-test). In *in vitro* systems, GluN2D-NMDARs have been shown to exhibit substantially slower kinetics relative to NMDARs containing the GluN2A/B or C subunits (Vinci et al., 1998; Paoletti et al., 2013; Hansen et al., 2018). Consistent with these previously reported differences, we noted that the decay kinetics of electrically isolated NMDAR EPSCs (+40mV holding potential) on BNST-CRF neurons in slices from the GluN2D$^{-/-}$ mice were substantially accelerated when compared with those observed in slices from the GluN2D$^{+/+}$ mice (**Fig. 5D**, CRF-2D$^{+/+}$: 134.8±10.1 ½ tau [ms], CRF-2D$^{-/-}$: 101.0±11.9 ½ tau [ms], t[16]=2.18, p=0.045, unpaired t-test).

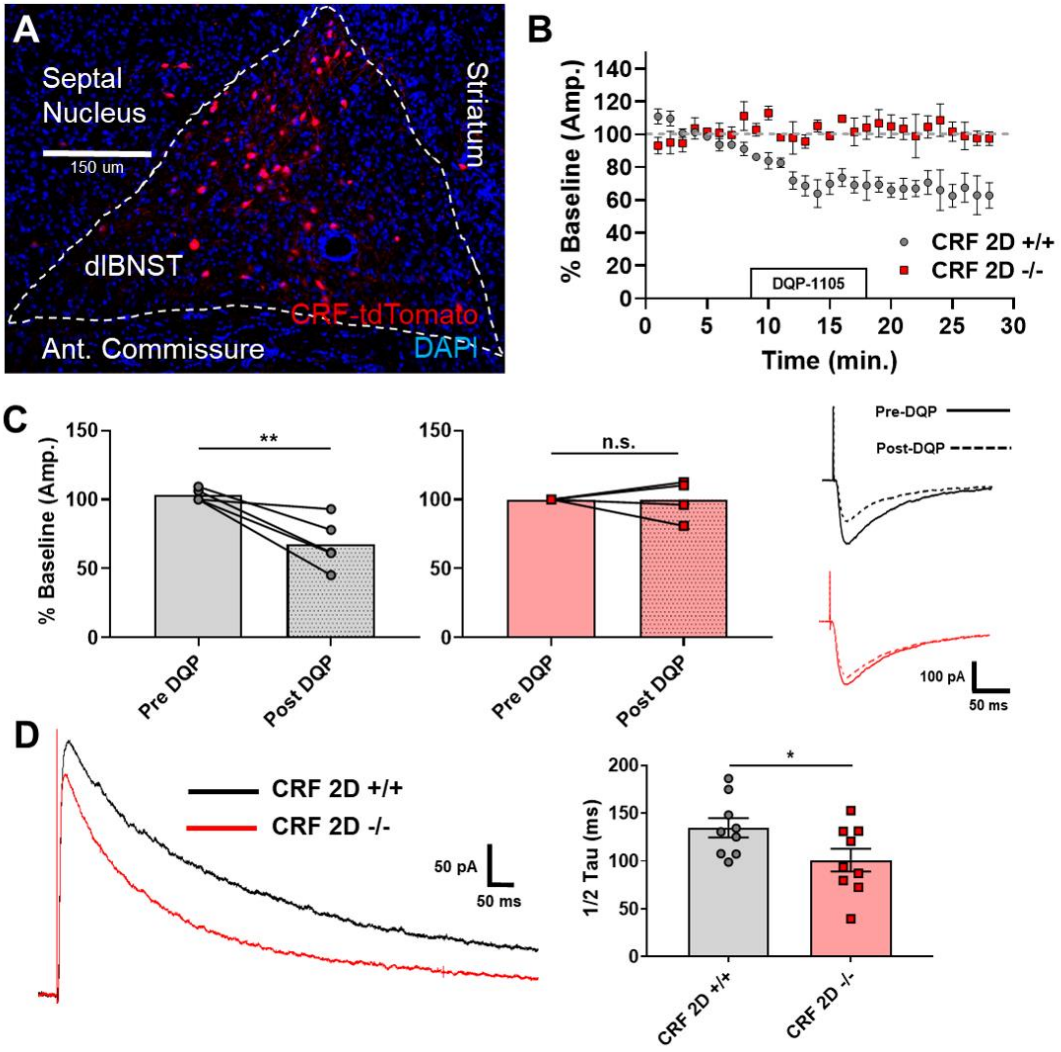


Figure 9. Functional synaptic GluN2D-containing NMDARs are expressed on adult mouse BNST-CRF neurons. (A) Representative image of the dlBNST (outlined via dashed white lines) from the GluN2D$^{-/-}$/Crh-IRES-Cre/Ai14 reporter line (CRF 2D$^{-/-}$) taken at 20x magnification. CRF positive cells labeled with tdTomato are shown in red, and cell nuclei denoted by DAPI counterstaining are in blue. **(B)** Averaged time courses of isolated NMDAR excitatory postsynaptic currents (NMDAR-EPSC, holding potential: -70mV, modified 0 Mg^{2+} ACSF) recorded from CRF 2D$^{+/+}$ and CRF 2D$^{-/-}$ cells in the dlBNST. Bath application of the GluN2C/GluN2D selective antagonist DQP-1105 (40uM) reduced the amplitude of the NMDAR-EPSC only in BNST-CRF 2D$^{+/+}$ cells (35.3% ±8.4% decrease from baseline). **(C)** Summary graphs for NMDAR-EPSC amplitude from BNST-CRF 2D$^{+/+}$ (p=0.003) and 2D$^{-/-}$ (p=0.997) cells comparing the first 8 mins of recordings prior to bath application of DQP-1105 (baseline, pre-DQP) and the last 8 mins of the recording following both 10 min wash-on of the drug, and a 10 min wash-out (post-DQP). Representative traces pre- (solid line) and post-DQP (dashed line) are shown to the right. Data presented as means ±SEM with individual points overlain ($n_{CRF\ 2D+/+}$ = 5 cells from $N_{CRF\ 2D+/+}$ = 3 mice, $n_{CRF\ 2D-/-}$ = 4 cells from $N_{CRF\ 2D-/-}$ = 2 mice). **(D)** Representative traces of electrically isolated NMDAR-EPSCs (holding potential: +40mV) recorded from BNST-CRF 2D$^{+/+}$ and 2D$^{-/-}$ cells (left). Analysis of the overall decay kinetics of the NMDAR-EPSC across both groups revealed a significant decrease in ½ tau (milliseconds, ms) value for BNST-CRF 2D$^{-/-}$ cells compared with 2D$^{+/+}$ (right, p=0.045). Data presented as means ±SEM with individual points overlain ($n_{CRF\ 2D+/+}$ = 9 cells from $N_{CRF\ 2D+/+}$ = 2 mice, $n_{CRF\ 2D-/-}$ = 9 cells from $N_{CRF\ 2D-/-}$ = 2 mice). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, n.s. = not significant.

Excitatory and inhibitory transmission on BNST-CRF neurons are divergently controlled by GluN2D.

Next, we examined spontaneous excitatory and inhibitory transmission onto BNST-CRF cells in brain slices from GluN2D$^{-/-}$ mice. Spontaneous EPSC (sEPSC) frequency and amplitude were both found to be increased in BNST-CRF neurons from GluN2D$^{-/-}$ slices when compared with GluN2D$^{+/+}$ mice (**Fig. 10A**, freq: CRF-2D$^{+/+}$: 0.6±0.1 Hz, CRF-2D$^{-/-}$: 1.2±0.2 Hz, t[28]=2.73, p=0.011; amp: CRF-2D$^{+/+}$: -23.8±1.3 pA, CRF-2D$^{-/-}$: -29.5±1.2 pA, t[28]=3.29, p=0.003, unpaired t-tests). To probe these results further, we repeated this analysis in the presence of the sodium channel blocker tetrodotoxin (TTX, 1uM) to remove presynaptic action potential firing and isolate miniature EPSCs (mEPSCs) generated by singular vesicle release events. We observed that mEPSC amplitude, but not frequency, was increased on BNST-CRF cells from GluN2D$^{-/-}$ slices in comparison with GluN2D$^{+/+}$ slices (**Fig. 10B**, freq: CRF-2D$^{+/+}$: 0.4±0.01 Hz, CRF-2D$^{+/+}$: 0.6±0.1 Hz, t[17]=1.45, p=0.167; amp: CRF-2D$^{+/+}$: -22.5±1.1 pA, CRF-2D$^{-/-}$: -27.6±1.2 pA, t[17]=3.01, p=0.008, unpaired t-tests). In addition, we analyzed paired-pulse ratios (PPR) of electrically-evoked EPSCs at -70mV across 3 separate inter-stimulus intervals (ISI) on BNST-CRF cells in the GluN2D$^{-/-}$, with a two-way ANOVA revealing main effects of ISI time, genotype and subject (**Fig. 10C**, ISI time: $F_{(2,32)}$=5.62, p=0.008, genotype: $F_{(1,16)}$=34.54, p<0.0001, subject: $F_{(16,32)}$=3.58, p=0.001, ISI time x genotype: $F_{(2,32)}$=2.70, p=0.083). Post-hoc analysis with Sidak's multiple comparisons test showed significantly decreased paired pulse ratios (<1) in cells from the GluN2D$^{-/-}$ when compared with GluN2D$^{+/+}$ cells at all time points (30ms: p=<0.0001, 50ms: p=<0.0001, 100ms: p=0.005), indicative of paired pulse depression. Collectively, these results suggest an enhancement of excitatory drive onto BNST-CRF cells in the knockout, likely through a combination of pre- and post-synaptic mechanisms.

In addition to an analysis of excitatory drive onto BNST-CRF neurons, we assessed the impact of GluN2D deletion on spontaneous inhibitory postsynaptic currents (sIPSCs) as well. We observed that, in contrast to our sEPSC findings, sIPSC amplitude was decreased onto BNST-CRF neurons in slices from the GluN2D$^{-/-}$ mice when compared with the GluN2D$^{+/+}$, while frequency was not altered (**Fig. 10D**, freq: CRF-2D$^{+/+}$: 1.4±0.2 Hz, CRF-2D$^{-/-}$: 1.6±0.3 Hz, t[28]=0.77, p=0.447; amp: CRF-2D$^{+/+}$: -24.6±1.7 pA, CRF-2D$^{-/-}$: -19.3±0.8 pA, t[30]=2.79, p=0.009, unpaired t-tests).

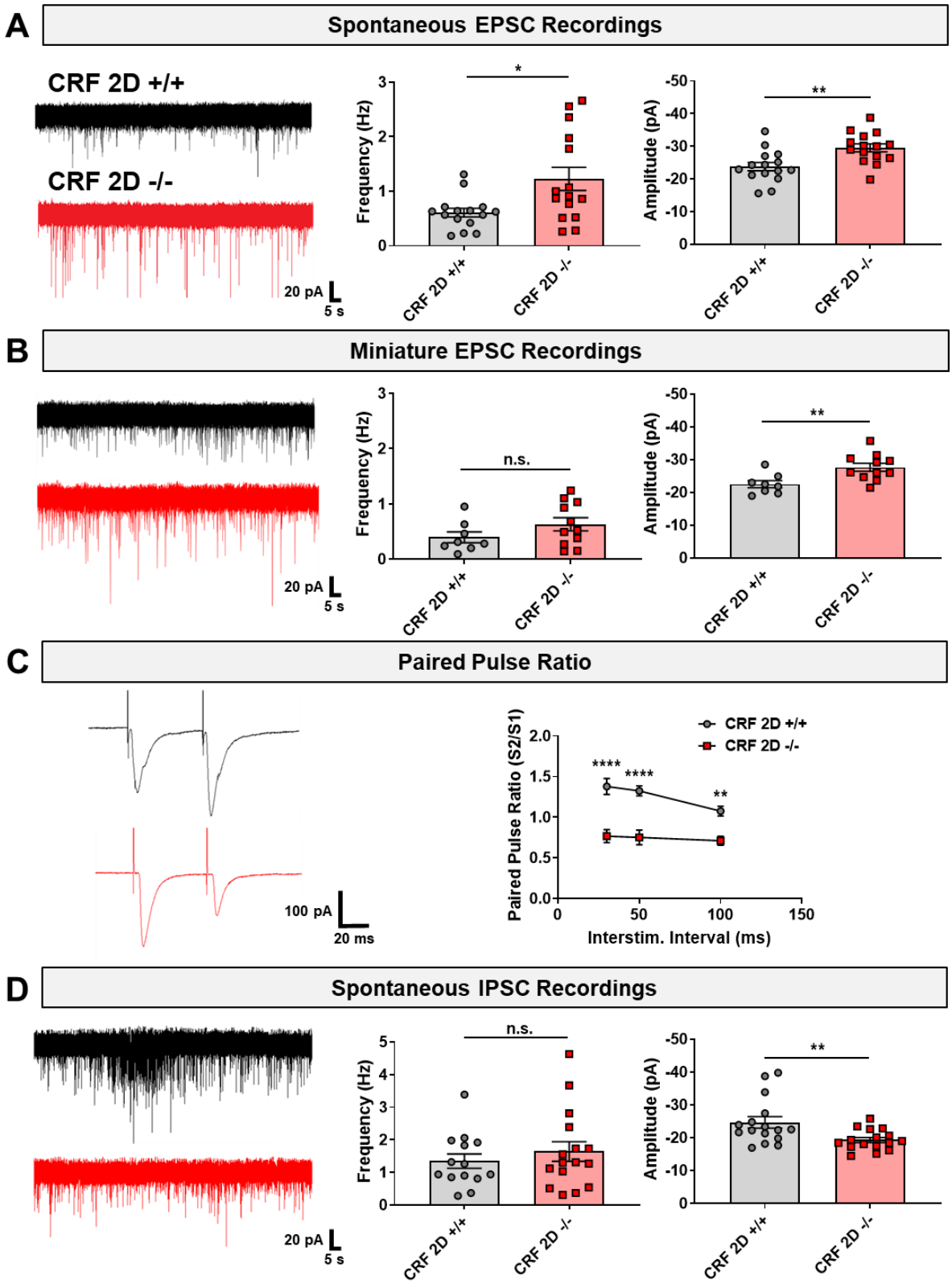

A
Spontaneous EPSC Recordings
CRF 2D +/+
CRF 2D -/-
20 pA
5 s
Frequency (Hz)
Amplitude (pA)
*
**
CRF 2D +/+
CRF 2D -/-
B
Miniature EPSC Recordings
n.s.
**
C
Paired Pulse Ratio
100 pA
20 ms
Paired Pulse Ratio (S2/S1)
Interstim. Interval (ms)
****
****
**
D
Spontaneous IPSC Recordings
n.s.
**

Figure 10. Excitatory and inhibitory transmission on BNST-CRF neurons are divergently controlled by GluN2D. (A) Representative traces of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from BNST-CRF $2D^{+/+}$ (black) and $2D^{-/-}$ (red) cells (left). Both sEPSC frequency and amplitude were shown to be significantly increased in BNST-CRF $2D^{-/-}$ cells when compared with $2D^{+/+}$ cells (freq: p=0.011, amp: p=0.003; $n_{CRF\ 2D+/+}$ = 15 cells from $N_{CRF\ 2D+/+}$ = 5 mice, $n_{CRF\ 2D-/-}$ = 15 cells from $N_{CRF\ 2D-/-}$ = 5 mice). **(B)** Representative traces of miniature excitatory postsynaptic currents (mEPSCs) recorded from BNST-CRF $2D^{+/+}$ (black) and $2D^{-/-}$ (red) cells (left). No differences were noted in the frequency of mEPSCs in BNST-CRF $2D^{+/+}$ or $2D^{-/-}$ cells, but amplitude was found to be significantly increased in the $2D^{-/-}$ cells (freq: p=0.167, amp: p=0.008; $n_{CRF\ 2D+/+}$ = 8 cells from $N_{CRF\ 2D+/+}$ = 4 mice, $n_{CRF\ 2D-/-}$ = 11 cells from $N_{CRF\ 2D-/-}$ = 4 mice). **(C)** Representative traces of paired EPSCs at an inter-stimulus interval (ISI) of 50 ms from both BNST-CRF $2D^{+/+}$ (black) and $2D^{-/-}$ (red) cells (left). Paired pulse ratios (PPR) of evoked 100 to 200 pA responses elicited at ISI of 30, 50 and 100 ms are plotted on the right, and show significant decreases in the ratio at all 3 time points for the CRF $2D^{-/-}$ cells when compared with metrics from $2D^{+/+}$ cells ($F_{2,32}$=5.62, p=0.008; $n_{CRF\ 2D+/+}$ = 10 cells from $N_{CRF\ 2D+/+}$ = 4 mice, $n_{CRF\ 2D-/-}$ = 8 cells from $N_{CRF\ 2D-/-}$ = 4 mice). **(D)** Representative traces of spontaneous inhibitory post-synaptic currents (sIPSCs) recorded from BNST-CRF $2D^{+/+}$ (black) and $2D^{-/-}$ (red) cells (left). No differences were noted in sIPSC frequency between BNST-CRF $2D^{+/+}$ and $2D^{-/-}$ cells upon comparison, but the amplitude of these currents was found to be significantly increased in the $2D^{-/-}$ (freq: p=0.447, amp: p=0.009; $n_{CRF\ 2D+/+}$ = 14 cells from $N_{CRF\ 2D+/+}$ = 3 mice, $n_{CRF\ 2D-/-}$ = 16 cells from $N_{CRF\ 2D-/-}$ = 4 mice). All data across graphs presented as means ±SEM with individual points overlain. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{****}p\leq0.0001$ n.s. = not significant.

BNST-CRF cells in the GluN2D$^{-/-}$ show evidence of increased activity in vivo.

Our observations that excitatory and inhibitory drive onto BNST-CRF neurons are altered in opposing fashions by the loss of GluN2D-NMDARs suggested the possibility that these cells may exhibit increased activity *in vivo* in response to such changes in intra-BNST circuit dynamics. We tested this hypothesis by conducting *in vivo* recordings of neuronal activity from awake, behaving mice via fiber photometry. To generate the necessary transgenic animals, we crossed our GluN2D$^{-/-}$ mice to the an Crh-IRES-Cre line, then injected Cre-dependent virus transducing the expression of the calcium-sensor GCaMP7f under control of the hSyn promoter unilaterally into the dlBNST, and lastly implanted the mice with a fiber optic cable to allow for the detection of GCaMP fluorescence (**Fig. 11A**). We then examined the basal activity of BNST-CRF cells in both GluN2D$^{+/+}$ and GluN2D$^{-/-}$ mice after placing them in a novel, open arena (**Fig. 11B-D**). We recorded GCaMP7f signal for 30 minutes, after which the collected signal was processed and normalized to produce traces showing changes in net fluorescence over time ($\Delta F/F$). Neuronal activity was interpreted as imputed calcium-mediated transients (i.e. events) detectable above noise in a trace, and total events were quantified over the course of each recording to examine event frequency. Under basal conditions when exploring a novel environment, we observed a significant increase in the frequency of events recorded from the BNST-CRF cells of GluN2D$^{-/-}$ mice compared with GluN2D$^{+/+}$ (**Fig. 11C-D**, CRF-2D$^{+/+}$: 0.09±0.01 Hz, CRF-2D$^{-/-}$: 0.11±0.004 Hz, t[14]=2.54, p=0.024, unpaired t-test), consistent with our hypothesis of increased activity of these cells in the knockout *in vivo*. To validate the fidelity of the recorded GCaMP7f signal observed during this task, we placed mice into an

anesthesia chamber following a brief recovery period in their home cages and recorded activity in the BNST during isoflurane exposure (**Fig. 11B, E**). A one minute exposure of isoflurane decreased imputed Ca^{2+} transients drastically, and persisted after the removal of the mice from the chamber until they regained consciousness, after which the signal was restored to basal levels.

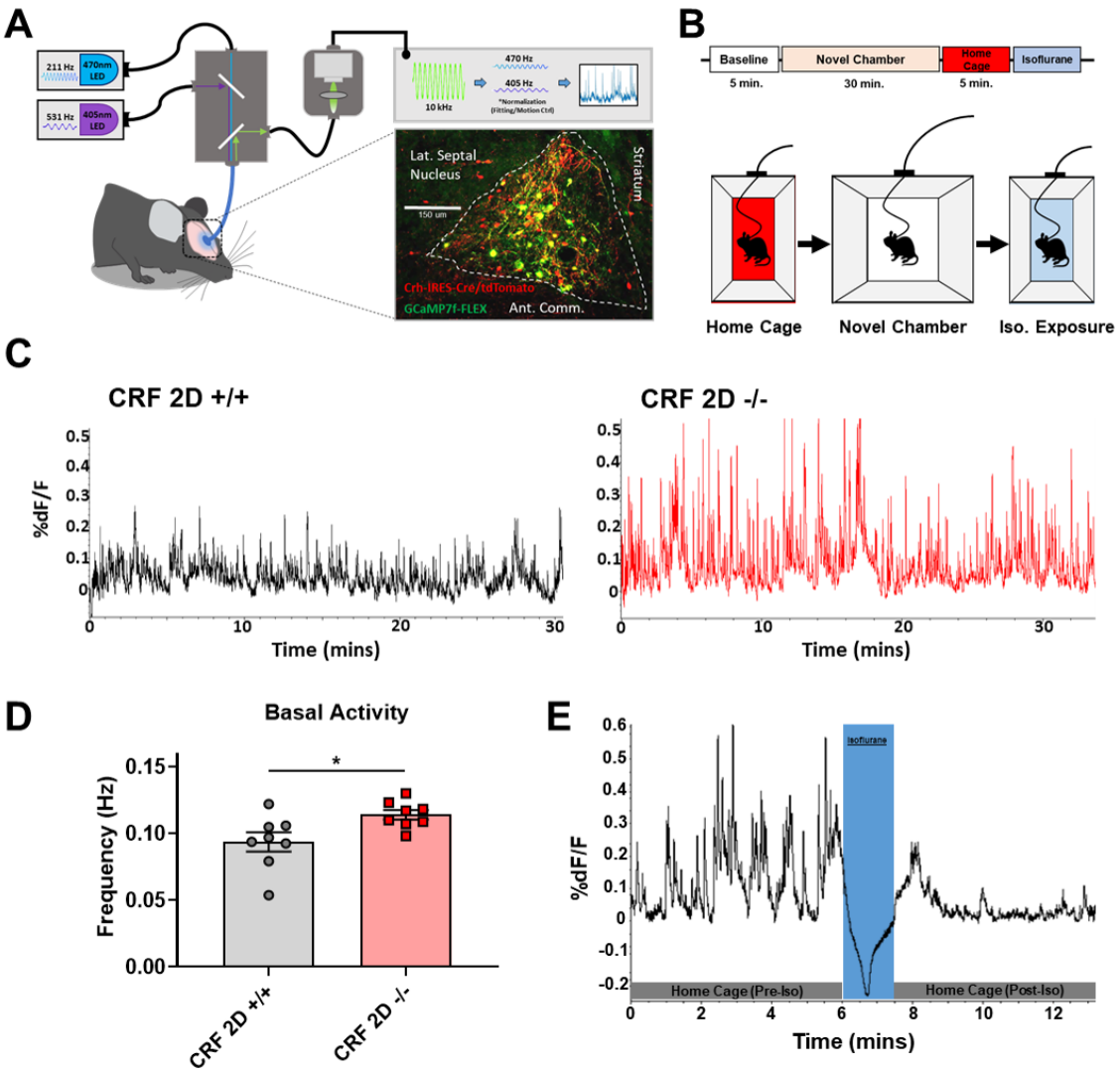


Figure 11. BNST-CRF cells in the GluN2D$^{-/-}$ show evidence of increased activity in vivo.
(A) Schematic showing setup of *in vivo* fiber photometry system utilized for detecting and recording GCaMP7f signaling from CRF-Cre (+) cells in the dorsolateral BNST (dlBNST) of awaking, behaving GluN2D$^{-/-}$/Crh-IRES-Cre mice. Representative 20x magnified image in the lower right shows the general expression patterns of Crh-IRES-Cre in the dlBNST and co-expression of a Cre-dependent virus encoding GCaMP7f. **(B)** Outline of behavioral testing for recording basal activity of BNST-CRF cells in GluN2D$^{+/+}$ (2D$^{+/+}$) and GluN2D$^{-/-}$ (2D$^{-/-}$) when placed in a novel environment. A baseline of initial activity in home cages were observed for validating signal output. Select individuals were briefly exposed to isoflurane gas to assess specificity of signal in awake, behaving mice. **(C)** Representative fiber photometry traces showing changes in the basal activity of BNST-CRF

cells from both the $2D^{+/+}$ (left, black) and $2D^{-/-}$ (right, red) mice over a 30 mins recording. **(D)** Summary graph comparing the frequency of recorded GCaMP7f events from BNST-CRF $2D^{+/+}$ and $2D^{-/-}$ cells under basal conditions for 30 mins. Event frequency was found to be significantly increased in the BNST-CRF cells of the $2D^{-/-}$ animals when compared with $2D^{+/+}$ controls ($p=0.024$). Data are presented as means ±SEM, with individual data points overlain ($N_{BNST\text{-}CRF\ 2D+/+}$ = 8 mice, $N_{BNST\text{-}CRF\ 2D-/-}$ = 8 mice). Imputed calcium transients are presented as changes in dF/F. $^{*}p \leq 0.05$.

Discussion

In Chapter 2, we demonstrate the role of GluN2D-NMDARs in both modulating emotional behavior and regulating BNST neuronal activity. We find that GluN2D deletion promotes an increase in anxiety- and depressive-like behavior, consist with previous studies (Yamamoto et al., 2017; Shelkar et al., 2019). Using *ex vivo* electrophysiology and *in vivo* photometry, we also identified adaptations in the BNST that correlate with these behaviors via enhanced excitatory drive and basal activation of BNST-CRF positive cells. Collectively, these findings suggest the GluN2D-NMDARs as an intriguing target for modulating glutamatergic signaling in key stress-responsive cell populations, as well as a receptor population that may require a more refined examination of its specific role within the BNST using methods aimed at more direct pharmacological manipulation or targeted deletion.

GluN2D-NMDAR expression in the BNST and emotional behavior

The BNST receives glutamatergic projections from several structures implicated in the regulation of emotional behavior, and deficits in BNST excitatory signaling have consistently been shown to be linked with increases in negative emotional behavior (McElligott and Winder, 2009; Conrad et al., 2011; Conrad and Winder, 2011; Lebow and Chen, 2016; Glangetas et al., 2017; Ch'ng et al., 2018). Studies have also implicated NMDAR-mediated signaling in the BNST as important to the regulation of these emotional states, with Glangetas et al. (2017) demonstrating that the inhibition of an NMDAR-mediated form of long term potentiation (LTP) in the vHPC-BNST circuit promotes anxiogenic behavior in the elevated plus maze. Previous studies have shown GluN2D to be enriched in select forebrain, midbrain and hindbrain structures in adult rodents (Monyer et al., 1994; Wenzel et al., 1996), and we provide evidence here for the subunit's expression in the BNST of adult mice. We further show a basal disruption of short term potentiation (STP) in the BNST of the $GluN2D^{-/-}$, resulting in an acute blunting of synaptic potentiation. GluN2D deletion or pharmacological inhibition has previously been

found to produce similar reductions in forms of hippocampal STP, demonstrating a conserved role for GluN2D-NMDARs in the control of short term plasticity (Volianskis et al., 2013; Volianskis et al., 2015; France et al., 2017). A correlation may thus exist between the deficits in BNST plasticity and enhanced negative emotional state we observed in the GluN2D$^{-/-}$, and is supported by work from Conrad et al. (2011) in which mice that presented with a heightened anxiogenic state following chronic stress were shown to have a blunting of LTP in the BNST. Whether such parallel changes in both behavior and BNST plasticity are predicated on reduced GluN2D-NMDAR function will require more directed and in depth study, specifically in regard to whether real time disruption of receptor function can drive changes in emotional behavior in awake, behaving mice. Nevertheless, our work here presents important initial evidence for both the expression and function of GluN2D-NMDARs in the BNST of adult mice, and positions this receptor population as a potential key player in the maintenance of plasticity events germane to emotional behavioral output.

GluN2D-NMDAR-mediated signaling and CRF neuronal activity

Direct and indirect studies of the activation (or deactivation) of CRF positive neurons within the BNST have implicated a role for these cells in driving negative emotional behaviors and/or responding to stress-related or aversive stimuli (Pleil et al., 2015; Butler et al., 2016; Marcinkiewcz et al., 2016; Giardino et al., 2018; Lin et al., 2018; Fetterly et al., 2019). These findings suggest that greater activation of BNST-CRF neurons underlies aspects of the anxiety- and depressive-like behaviors observed in animal models of mood related disorders. The GluN2D$^{-/-}$ line presents similar phenotypes, and we found GluN2D-NMDARs to be functionally expressed on a large majority of BNST-CRF neurons. Further, we found excitatory drive onto these cells to be enhanced, while inhibitory drive was diminished in the GluN2D$^{-/-}$, suggestive of a hyperactive state that has consistently been implicated in both clinical and preclinical literature with the pathology of multiple mood related disorders (Arborelius et al., 1999; Keck and Holsboer, 2001; Bale and Vale et al., 2004; Ronan and Summers et al., 2011). Our *in vivo* recordings of the basal activity of these neurons in the GluN2D$^{-/-}$ demonstrated a robust increase in the frequency of calcium-mediated events, further supporting this conclusion and the possibility of the GluN2D-NMDARs emerging as a unique target for modulating the activity of the CRF system within the BNST.

BNST, GluN2D-NMDARs in regulating downstream behavior and cell-type specific physiology.

Previous studies of NMDAR function in regions shown to contain synaptic or extra-synaptic GluN2D-NMDARs (subthalamic nucleus, hippocampus and cerebellum) have shown the subunit to exert unique control over the deactivation time course of NMDAR-EPSCs (Swanger et al., 2015; von Englehardt et al., 2015; Dubois et al., 2015), an effect we also show to be true at BNST-CRF cells in the GluN2D$^{-/-}$. The slower deactivation kinetics characteristic of GluN2D-NMDARs are thought to be imparted by the receptors' unique physiological properties (Monyer et al., 1994; Qian et al., 2005; Erreger et al., 2007), and may contribute to a greater temporal summation of excitatory signaling at cell containing these receptors (Bourne and Nicoll, 1993; Edmonds et al., 1995). Properties such as these have previously been shown to result in stronger and more robust plasticity (Carmignoto and Vinici, 1992; Malenka, 1994; Edmonds et al., 1995), and thus might explain the blunting in BNST STP we observe in the GluN2D$^{-/-}$.

GluN2D-NMDAR localization both pre- and post-synaptically has been speculated to allow these receptors to uniquely regulate glutamatergic signaling, with recent work in the cerebellum demonstrating that presynaptic GluN2D-NMDARs can act as detectors of glutamate spillover at the synapses of inhibitory interneurons to regulate GABA release and excitatory/inhibitory transmission cross-talk (Dubois et al., 2015). GluN2D-NMDARs have also been speculated to be extra-synaptically localized (Brickley et al., 2003; Harney et al., 2008; Costa et al., 2009), which may allow for these receptors to participate in the regulation of tonic glutamatergic currents at specific synapses (Papouin and Oliet, 2014). While similar extra-synaptic mechanisms may be in place at BNST-CRF cells, our data indicate the presence of synaptic GluN2D-NMDARs on these neurons via the changes in NMDAR-isolated EPSC amplitudes noted in GluN2D$^{-/-}$ or DQP-1105 treated slices. The presence of both synaptic and extra-synaptic GluN2D-NMDARs has been supported by physiological studies of dopaminergic neurons in the substantia nigra (Morris et al., 2018), and suggests the idea of these receptors serving as both detectors of presynaptic glutamate release and ambient changes in extracellular glutamate levels to control excitatory input at the cell.

Overall, our data show that GluN2D-NMDARs provide an important role in modulating excitatory activity both in key stress-responsive regions and cell populations implicated in mood and anxiety-related disorders. The implications of functional GluN2D-NMDARs both within the BNST and more exclusively on BNST-CRF

neurons suggests a potential means through which NMDAR antagonism may induce it's antidepressant-like effects as well, by directly regulating and altering the excitatory signaling of this region and of a modulatory system shown to be crucial in driving both PVN CRF cells activation and CRF release onto the HPA axis. These findings also open to door to a number of different question as well, principally the effects of GluN2D-NMDARs signaling exclusively within the BNST and also exclusively on the BNST-CRF cells studied here. The findings presented in Chapter 2, while insightful and novel in regards to dissecting the contribution of GluN2D function to excitatory signaling within the BNST, make use of a constitutive knockout line and animals that lack functional GluN2D protein expression from birth. This suggests that potential compensation effects could occur in regards to receptor subunit expression profiles and NMDAR function/regulation of excitatory signaling overall within the brain and the BNST of these animals that could instead precipitate the behavior changes and physiological effects we observed. Preliminary data from our lab suggests this might occur as well, with preliminary biochemical studies indicating that the expression of GluN2B within the BNST of GluN2D$^{-/-}$ mice may increase by two fold (see **Fig. 12** below). Thus, future studies would benefit from the use of either a conditional knockout line, or the administration of drugs such as DQP-1105 directly into the BNST via cannulation, in order to observe the acute effects of GluN2D-NMDAR manipulation or dysfunction in the fully developed adult mice. Considering the findings of previous studies in our lab showing that conditional GluN2B deletion from the BNST of adult mice produces a robust antidepressant-like effect, it would be interesting and informative to gauge how conditional deletion of the GluN2D subunit may contribute to this phenotype, and/or the presentation of other changes in anxiety or depressive-like behavior that more closely mimics our findings in GluN2D$^{-/-}$ mice.

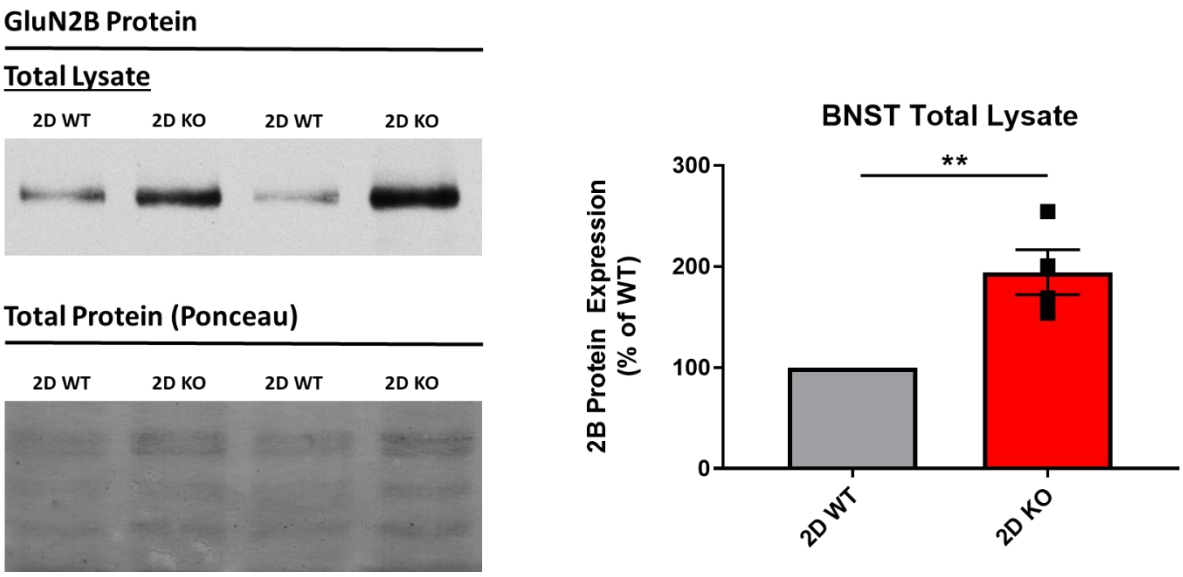


Figure 12. GluN2B protein expression is upregulated in the BNST of GluN2D$^{-/-}$ mice. (A) Western blotting results for total GluN2B protein expressed in BNST tissue taken from 2D WT and 2D KO mice (n = 4 separate sets of bilateral punches from male mice, ~10 wks of age). Upper images show GluN2B immunoblots (1:3000, BD Biosciences, #610417) and lower images show Ponceau total protein staining. (B) Comparison of changes in optical density of protein bands in 2D KO samples compared to the 2D WT ($p = 0.0054$). Percent change in optical density is compared to WT. Data presented as averages normalized to WT ± SEM, and analyzed via unpaired t-test, $^{**}p \leq 0.01$.

Chapter 3

Behavioral and cell-specific effects of targeted GluN2D-NMDAR deletion within the BNST

Adapted from: Salimando et al., "BNST GluN2D-containing NMDA receptors influence anxiety- & depressive-like behaviors and modulate cell-specific excitatory/inhibitory synaptic balance."

Introduction

In Chapter 2, a GluN2D constitutive knockout line was used to examine the effects of the deletion of this NMDAR subunit on anxiety and depressive-like behavior, and to a larger degree, the regulation of excitatory function within the BNST, a region in which NMDAR-mediated excitatory signaling has been consistently shown to influence affective behavioral output (Glangetas et al., 2017; Louderback et al., 2013; Conrad et al., 2011a). The downregulation and subsequent restricted expression of GluN2D known to occur during early development (Monyer et al., 1994; Wenzel et al., 1996), suggests a complex role for GluN2D-NMDARs in the adult brain however, and one that may not be fully captured through the use of a constitutive knockout. Indeed, GluN2D expression has been shown to be much more prominent throughout the majority of the brain prenatally, particularly within the diencephalon, cerebellum, brainstem and select cortical/subcortical structures (Sheng et al., 1994; Wyllie et al., 2013), which *in situ* studies indicate may include the BNST and our data presented in Chapter 2 verifies (**Fig. 6A**). In both the mature and developing brain, GluN2D has also been demonstrated to show higher expression patterns on GABAergic interneurons (Monyer et al., 1994; Standaert et al., 1996; Yamasaki et al., 2014; von Engelhardt et al., 2015; Perszyk et al., 2016), with recent studies also suggesting the expression of this subunit early on in interneurons profoundly influences their maturation and functional integration into surrounding circuits (Hanson et al., 2019). Developmental loss of GluN2D-containing NMDARs could be hypothesized to significantly alter synaptic function and overt glutamatergic signaling in within structures primarily composed of GABAergic interneurons like the BNST (Nguyen et al., 2016; Sheng et al., 1994). Thus, the selective manipulation or acute deletion of the GluN2D subunit within this region may be necessary in order to develop a clearer picture of its contribution to NMDAR function and cell excitability, as well as downstream affective behaviors known to be mediated by the BNST.

To address this, I utilized a GluN2D conditional knockout line (GluN2D$^{flx/flx}$) to examine the effects of BNST-GluN2D loss in adult mice on anxiety and depressive-like behaviors across standard behavioral testing paradigms. Further, I generated a transgenic line that expressed Flp recombinase under the control of the *Crh*-promoter to study the cell-type specific effects of regionally-restricted GluN2D deletion on BNST-CRF cell physiology. Using these transgenic tools, I found that BNST GluN2D deletion produces increased depressive-like behavior, as well as increases in excitatory drive onto the BNST-CRF neurons similar to what I observed in GluN2D$^{-/-}$ mice in Chapter 2 (**Fig. 10**). Overall, this study expands upon the findings presented in Chapter 2, and more directly suggests that GluN2D-NMDARs play an important role in shaping excitatory signaling in the BNST and specifically at BNST-CRF cells. The correlations found between the changes in BNST activity following GluN2D deletion and deficits in affective behavior further supports a role for GluN2D-NMDARs in regulating such outputs through their influence on excitatory signaling in a region-specific manner, and suggests that these NMDARs may serve as a novel target for selectively modulating changes in glutamate signaling in stress-responsive structures and cell populations implicated in the pathophysiology of disorders such as depression.

Materials and Methods

Animals

Male and female mice of at least 8 weeks of age were used throughout this study. GluN2D conditional knockout mice (GluN2D$^{flx/flx}$) were purchased from MRC Harwell (Grin2d$^{tm1c[EUCOMM]Wtsi}$, EMMA ID: 04857) and bred as outlined by the Wellcome Trust Sanger Institute guidelines for their suite of conditional ready mice. Crh-IRES-FlpO mice were generously provided for our use by the lab of Bernado Sabatini (Jackson Laboratory stock no. 031559). To generate lines for the identification of BNST CRF cells lacking the GluN2D subunit via a combination of virally encoded Cre recombinase and a reporter fluorophore, GluN2D$^{flx/flx}$ mice were crossed with hemizygous Crh-IRES-FlpO mice (GluN2D$^{flx/flx}$/Crf-Flp). Crh-IRES-FlpO mice were genotyped using protocols reported for each respective line on the Jackson Laboratory's website, while custom primers were designed for end-point PCR to genotype the GluN2D$^{flx/flx}$ mice (forward primer: GTG TGA CCA GGA AGC CAC TT, reverse primer: TCC TTG ATC CCG TCC CTC AA).

For behavioral studies using the GluN2D$^{flx/flx}$ mouse line, male mice were primarily used in order to replicate the conditions of previously published behavioral work examining the behavioral effects of GluN2D deletion (Ikeda et al., 1995; Miyamoto et al., 2002; Obiang et al., 2012; Yamamoto et al., 2017; Shelkar et al., 2019). For *ex vivo* electrophysiological studies using the GluN2D$^{flx/flx}$/Crf-Flp lines, mice of both sexes were used to minimize the total number of animals. No sex differences were observed in these studies, and as such, all data are compiled across groups into values representative of both sexes. All mouse lines were maintained on a C57BL/6J background and backcrossed as needed. Mice were group housed with 2-5 individuals per cage and maintained on a 12 hour light/dark cycle (lights on at 0600 hrs) under controlled temperature (20-25°C) and humidity (30-50%) levels. Mice were given access to food and water *ad libitum*. All treatments and interventions were approved by the Vanderbilt Animal Care and Use Committee.

Behavioral Testing

All single housing, handling, and habituation of GluN2D$^{flx/flx}$ mice used in behavioral studies was performed as described above in Chapter 2. The set-up and cleaning of equipment was also conducted in a similar fashion, unless outlined differently below.

Open Field Test (OFT): Mice were run on the OFT as described above in Chapter 2. In brief, animals were run in ENV-S10S open field activity chambers (Med Associates Inc.) fitted with IR photo-beam arrays for 60 minutes under full light in the chambers (~200-300 lux). Total locomotor activity, and zone analyses for total time spent in the designated center and surround zones of the activity arena were performed using Med Associates software.

Elevated Zero Maze (EZM): Mice were run using on the EZM as described above in Chapter 2. In brief, animals were run using a custom EZM apparatus measuring 34 cm inner diameter, 46 cm outer diameter, placed 40 cm off the ground on four braced legs, with two open quadrants and two closed quadrants. Testing was performed under illuminated conditions (~200-300 lux for open quadrants and ~100-150 lux for closed quadrants), with mice initially placed into one of the open quadrants, after which they were run for 5 minutes in the maze and filmed continuously. Videos were analyzed via ANY-Maze software (Stoelting Co.) for total time spent in the designated open quadrants compared to total time spent in the closed quadrants, and presented as percent total time in open quadrants overall. Total locomotion and quadrant entries were assessed via AnyMaze.

Forced Swim Test (FST): The FST was also administered on the final day of behavioral testing in GluN2D$^{flx/flx}$ mice, and was conducted as described above in Chapter 2. Mice were placed in Plexiglas cylinders containing room temp. (~22-23°C) tap water for 6 minutes while being continuously filmed. Videos were then analyzed to determine the total immobility time (i.e. lack of swimming/struggling while in water) observed in mice during the last 4 minutes of the test (first 2 mins were designated as a habituation period to the water/test). All results were hand-scored by individuals blinded to the genotype of test mice.

Novelty-induced Hypophagia (NIH): Testing was performed as previously described (Louderback et al., 2013). In brief: mice were subjected to 4 days of training on drinking a highly palatable food (liquid Ensure, vanilla flavor) in their home cages, and a final testing day in a novel cage. Training consisted of 30 mins of access to Ensure in the home cage under low red lighting conditions (~40-50 lux), and mice were timed for latency (secs) to first lick of sipper bottles containing Ensure and then for total consumption of Ensure (grams) at the end of each session. On test day, mice were placed in a new cage devoid of bedding under bright lights (~400-500 lux) immediately prior to Ensure access, after which latency to first lick and total consumption of Ensure at the end of the 30 min session were measured. Results were scored by individuals blinded to treatment groups, and total consumption of Ensure was measured across the entire experiment to rule out potential issues of a feeding phenotype.

Whole Cell Electrophysiology

Male and female mice used in whole cell recording underwent a similar procedure for slice preparation as described above in Chapter 2. Slice transfer and recovery procedures were similarly conserved, as were the types of external and internal (Cs-Gluc) solution and recording parameters described above. Cells deemed viable for patching were identified via expression of the red fluorescent protein tdTomato (for CRF+ cells) and eGFP (for Cre+ cells) in order to identify CRF neurons that either underwent GluN2D conditional deletion via Cre transfection, or were wildtype for the subunit.

For spontaneous EPSC measurements made in voltage clamp mode, responses were isolated by adding 25μM picrotoxin ($GABA_A$ receptor antagonist, Tocris) into the ACSF and bath applying over slices while recording at a holding potential of -70mV. Spontaneous IPSC measurements were performed by adding 10μM NBQX (pan-AMPA and kainate receptor antagonist, Tocris) and 25μM AP-V (pan-NMDA receptor antagonist,

Tocris) into bath applied ACSF, while maintaining a holding potential of -70mV. In paired pulse ratio (PPR) experiments, paired evoked 100- to 200-pA responses at 0.05 Hz were elicited and inter-stimulus intervals of 30, 50 and 100ms were utilized. For measuring the evoked, isolated NMDAR EPSCs used for analyzing differences in NMDAR kinetics, the holding potential for the cells was adjusted to +40mV and ACSF containing 10uM NBQX and 25uM picrotoxin was perfused over the slice. Signals were acquired with a Multiclamp 700B amplifier (Molecular Devices), digitized via a Digidata 1322A and analyzed with pClamp 10.6 software (Molecular Devices). Spontaneous and miniature voltage clamp recordings were analyzed via Clampfit 10.6 (Molecular Devices) by measuring the peak amplitudes and frequencies of events over a 6 minute period (in three, 2 min bins). Paired pulse ratios were analyzed by dividing the value of the amplitude of the second response over the amplitude of the first (P2/P1), and the resulting ratios plotted out for each inter-stimulus interval period. Evoked NMDAR EPSCs decay kinetics were interrogated by examining the amplitude of the current trace at half of the tau (τ) value.

RNAscope In Situ Hybridization

RNAscope studies were performed as previously described (Ghamari-Langroudi et al., 2015). mRNA species expressed by neurons in the dlBNST were visualized across separate sets of experiments using the enhanced fluorescent *in situ* hybridization technique RNAscope® (ACD, Advanced Cell Diagnostics). RNAscope® cDNA probes and detection kits were purchased from ACD and used according to the company's online protocol for fresh frozen tissue. The probe sets directed against *Crh* were designed from sequence information from the mouse RefSeq mRNA IDs NM_205769.2, and custom probes for the Flp recombinase were designed by ACD (Cat. No. 448191). Crh-IRES-FlpO male mice, aged 8-10 wks, underwent the same procedure for RNAscope described above in Chapter 2. cDNA probe mixtures were prepared at a dilution of 50:1 of C1 probe for Crh and C2 probe for Flp. Cells within the BNST were appraised for the presence of *Crh* and Flp signal signal in order to determine the total number of cells showing probe expression either alone or in combination. Transcripts were readily identified as round, fraction delimited spots over and surrounding DAPI-labeled nuclei.

Stereotaxic Surgery Procedures

Adult mice (~8 weeks of age) were anesthetized with isoflurane (initial dose = 3%, maintenance dose = 1.5%) and surgery was performed using an Angle Two stereotaxic frame (Leica) to intracranially inject adeno-associated virus (AAV) into the dorsolateral BNST based on the Franklin and Paxinos (2004) mouse brain atlas (from Bregma: AP=0.14, ML=+/-0.88, DV=-4.24, 15.03° tilt) at a rate of 100nl/min. Male GluN2D$^{flx/flx}$ mice used in behavioral studies were bilaterally injected with ~300nl of recombinant AAV5-CMV-eGFP or AAV5-CMV-Cre-eGFP (UNC Vector Core), and given a 4 weeks recovery period. GluN2D$^{flx/flx}$/Crf-Flp mice of either sex used for physiology studies were bilaterally injected with ~300nl of a 1:1 mixture of an AAV9-Ef1a-fDIO-tdTomato virus (Stanford Vector Core) and the AAV5-CMV-Cre-eGFP virus, and similarly given 4 weeks recovery prior to producing slices for *ex vivo* recordings.

Immunohistochemistry

IHC and imaging of Cre and eGFP expression introduced via viral injections into the dlBNST, was conducted as described above in Chapter 2. Floating tissue sections were then incubated with mouse anti-Cre (1:1000, MAB3120, Millipore) or chicken anti-GFP (1:1000, ab13970, Abcam) primary antibodies prepared in a solution of 10% normal donkey serum and PBS-T for ~72 hours at 4°C as necessary. Cy3 donkey anti-mouse (1:400, Jackson Immuo) or Cy2 donkey anti-chicken (1:400) secondary Abs prepared in PBS-T were applied next for 24 hours at 4°C, after which sections were counterstained with DAPI (1:10,000) and then mounted and cover-slipped. Slides were imaged using an Imager M2 upright fluorescent microscope (Zeiss) at 5x and 10x magnification.

Western Blotting

Bilateral tissue punches (~0.8 mm) of the dlBNST collected from from GluN2D$^{flx/flx}$ mice injected with either a virus encoding Cre or eGFP, and were processed for protein extraction and Western blotting as described above in Chapter 2. Primary antibodies used included mouse anti-NMDAR2D (1:5000, MAB5578, Millipore) and mouse anti-GAPDH (1:10,000, MAB374, Millipore). All primary Ab dilutions were prepared in 5% powdered milk solution in 1x Tris-buffered saline/Triton-X100 (TBS-T), and applied to blots overnight at 4°C. Blots were washed four times in 1x TBS-T the following day, and then probed with an anti-mouse, horse radish

peroxidase conjugated secondary antibody (1:8,000, W402B, Promega) in 5% milk, 1x TBS-T for ~2 hours at room temp. Blots were then washed an additional four times with fresh 1x TBS-T and then soaked in Western Lighting Plus-ECL solution (PerkinElmer) for 1-2 mins before imaging on X-ray film.

Experimental Design and Statistical Analysis

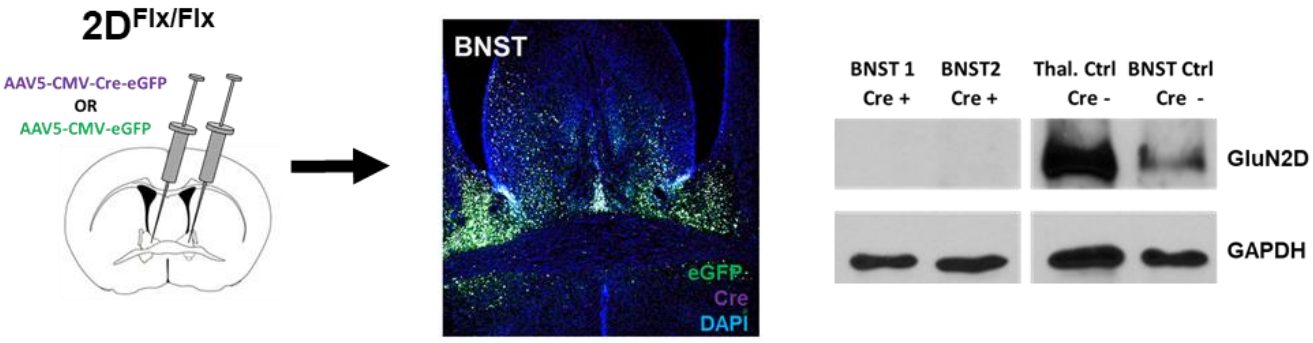
The number of animals used in each experiment were predetermined based on analyses of similar experiments in the literature and supplemented as needed based on observed effect sizes. All data are represented as means ± the standard error of the mean (SEM) for each group, and all statistical analyses were performed using GraphPad Prism 8 software (Graphpad Software, San Diego, CA). We utilized both male and female mice within this study. When sex was not found to be a statistically significant factor, we combined male and female data for analysis. For behavior experiments, data comparing metrics between knockout and wildtype groups across tasks were analyzed using unpaired, two-tailed Student's *t*-tests, while analyses of changes in locomotive behavior between both groups across multiple time points were performed using two-way ANOVAs along with Sidak's multiple comparison post hoc test, and corrected *p*-values are reported in the text as needed. Two-tailed *t*-test analyses were also performed on total cell counts for *in situ* hybridization studies, NMDAR eEPSC amplitude and decay kinetics, sEPSC, sIPCS and mEPSC amplitude and frequency values in *ex vivo* recordings, and frequency values for imputed calcium mediated events in *in vivo* recordings. For PPR data examining changes in the ratio across 3 separate inter-stimulus intervals, a two-way ANOVA was utilized, along with Sidak's multiple comparison post hoc test. For all analyses, significance levels were set at $\alpha=0.05$. Detailed statistics are provided within the text and figure legends.

Results

BNST specific deletion of the GluN2D subunit produces an enhanced depressive-like phenotype in mice.

Our findings in Chapter 2 suggest that the constitutive loss of the GluN2D subunit from NMDARs in adult mice leads to increased negative emotional behavior and associated increases in the activity profiles of BNST-CRF neurons. To more specifically manipulate BNST GluN2D subunits, and control for potential compensatory effects resulting from the loss of a key subunit from birth (Balu and Coyle, 2011), and the differing

role in signaling and level of expression of GluN2D-NMDARs, particularly in interneurons, noted in the early stages of development (Monyer et al., 1994; Wenzel et al., 1996; Hanson et al., 2019), we utilized a GluN2D conditional knockout line (GluN2D$^{flx/flx}$) to selectively ablate the subunit from the BNST in adult mice. We first tested the efficiency of the GluN2D$^{flx/flx}$ for knocking down the protein by performing intracranial injections of viruses encoding either Cre or eGFP bilaterally into the dlBNST (**Fig. 13**, middle panel) and then collecting



tissue punches from BNST for Western blot analysis using whole tissue lysates. Samples from Cre-injected mice revealed a robust knockdown of GluN2D, as evidenced by the lack of a band for the protein across all relevant samples (**Fig. 13**, right panel). By comparison, BNST samples from eGFP-injected controls still displayed a prominent band for GluN2D, while additional samples taken from the thalamus of BNST-Cre injected mice also showed a robust band, further confirming the selectively of the GluN2D$^{flx/flx}$ line.

Figure 13. Validation of region specific deletion of GluN2D using the GluN2D$^{flx/flx}$ line. Schematic of mouse surgery and viral injection paradigms for driving either Cre or eGFP expression bilaterally in the dlBNST of GluN2D$^{flx/flx}$ mice (left). Representative 5x image (center) showing expression patterns of Cre (magenta), eGFP (green) and DAPI counterstaining (blue) in the dlBNST roughly four to five weeks after surgery. Western blot analysis of total protein lysate taken from tissue punches (0.08mm) of the dlBNST following either Cre (Cre [+]) or control/sham injection (Cre [-]). Representative blot of bands for both GluN2D and control GAPDH for Cre-injected mice (left) and controls (right) show robust knockdown of subunit expression in the conditional line when Cre is present. Control punches were taken from the medial thalamus of BNST-injected mice as well to verify specificity of targeted deletion.

From here, we moved onto repeating the behavioral studies we ran on our GluN2D$^{-/-}$ mice across cohorts of age-matched (~12 wks) male GluN2D$^{flx/flx}$ following intracranial injection of either Cre or eGFP encoding virus bilaterally into the dlBNST (**Fig. 14A-C**). Analysis of total center time in the OFT revealed no difference in the percent total time spent in the center of the chamber between Cre or eGFP injected mice (**Fig. 14B**, left & left center panels, 2D$^{flx/flx\text{-}eGFP}$: 57.2±7.2% time in ctr, 2D$^{flx/flx\text{-}Cre}$: 46.6±4.4% time in ctr, t[21]=1.29, p=0.212, unpaired t-test), as well as no overt differences in locomotor activity across all time points (**Fig. 14B**,

left panel, locomotion: $F_{(1,21)}$=1.28, p=0.271, two-way ANOVA with Sidak's repeated measures post hoc test). We also noted no difference in the total percent time spent in the open quadrants of the EZM when running both Cre and eGFP injected mice in this task (**Fig. 14B**, right center panel, $2D^{flx/flx\text{-}eGFP}$: 40.8±7.1% time in ctr, $2D^{flx/flx\text{-}Cre}$: 32.7±3.9% time in ctr, t[21]=1.03, p=0.317, unpaired t-test), indicating along with the results of the OFT an apparent lack of increased anxiety-like behavior. By contrast, the FST revealed a significant increase in the total immobility time for Cre injected mice when compared with eGFP injected controls (**Fig. 14B**, right panel, $2D^{flx/flx\text{-}eGFP}$: 62.9±10.7 sec immobile, $2D^{flx/flx\text{-}Cre}$: 108.2±13.2 sec immobile, t[21]=2.63, p=0.016), suggesting a possible increase in depressive-like behavior similar to what we observed in the constitutive knockouts. To further assess this phenotype, we tested a new cohort of mice on the novelty-induced hypophagia task (NIH) in order to assess the latency to consume a highly palatable food (**Fig. 14C**, Dulawa and Hen, 2005; Louderback et al., 2013). Cre-injected mice demonstrated an increase in their overall latency to first approaching and licking sipper bottles containing Ensure when compared with eGFP-injected control littermates (**Fig. 14C**, left center panel, $2D^{flx/flx\text{-}eGFP}$: 167.8±45.7 sec [latency to lick], $2D^{flx/flx\text{-}Cre}$: 417.6±99.4 sec [latency to lick], t[19]=2.21, p=0.04, unpaired t-test). Analysis of the overall acquisition of the task and total Ensure consumption were also tracked across groups for the duration of the NIH, and showed no differences in the initial ability of the mice to learn to drink the Ensure (**Fig. 14C**, left panel, latency: $F_{(1,19)}$=2.135, p=0.16, subject: $F_{(19,76)}$=1.472, p=0.121, two-way ANOVA with Sidak's repeated measures post hoc test) or amount of Ensure consumed across all days (**Fig. 14C**, right and right center panels, consumption time course, latency: $F_{(1,19)}$=0.02, p=0.90, day x latency: $F_{(4,76)}$=0.69, p=0.601, two-way ANOVA with Sidak's multiple comparisons post hoc test; test day consumption: $2D^{flx/flx\text{-}eGFP}$: 0.97±0.1 consumed [g], $2D^{flx/flx\text{-}Cre}$: 0.9±0.1 consumed [g], t[19]=0.95, p=0.354, unpaired t-test).

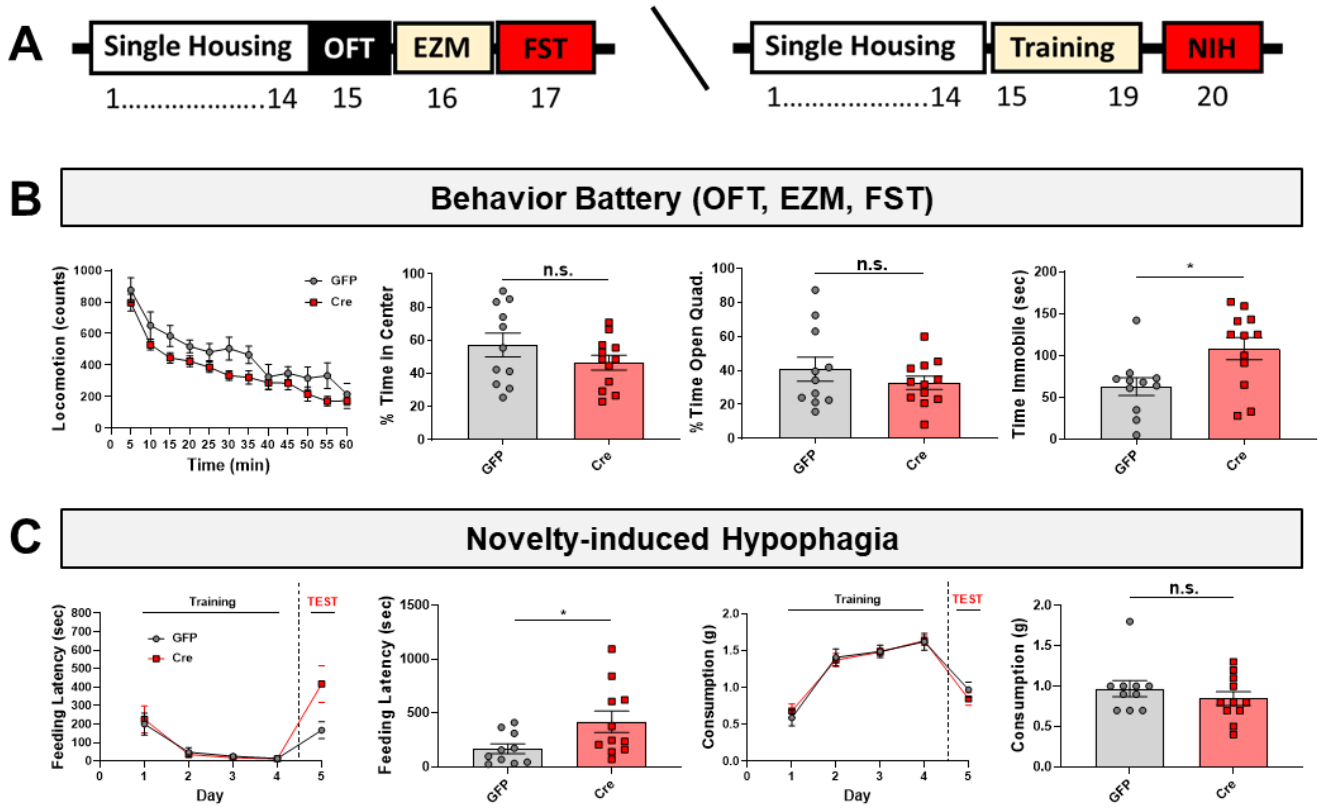


Figure 14. Conditional deletion of GluN2D in the BNST produces an increase in depressive-like behaviors in mice. (A) Design schema and timeline for modified 3 day behavioral testing battery, consisting of the open field test (OFT), elevated zero maze (EZM) and the forced swim test (FST), and a separate timeline for the novelty-induced hypophagia task (NIH). **(B)** Behavioral testing results comparing anxiety- and depressive-like behaviors of GluN2D$^{flx/flx}$ mice following bilateral BNST knockout of GluN2D (Cre) or control virus injection (eGFP). Analysis of percent total center time in the OFT revealed no significance difference in behavior in either Cre or eGPF injected individuals (left center), as well as no observable differences in locomotor activity between groups during the 60 min task (left, locomotor activity: $F_{(1,21)}$=1.28, p=0.271; center time: p=0.212). Analysis of percent total time in the open quadrants of the EZM in both Cre and eGFP injected mice also showed no significant differences in anxiety-related behaviors (right center, p=0.317). Total immobility time in the FST was however found to be significantly increased in Cre injected individuals compared with eGFP injected controls (right, p=0.016). **(C)** A separate cohort of mice were run on the NIH as an additional measure of depressive-like behaviors. No overt differences in ability to acquire the drinking paradigm was observed between groups (left, latency: $F_{(1,19)}$=2.14, p=0.16), but Cre injected mice displayed a significant increase in the total time (sec) taken to first approaching and drinking sippers of Ensure in a novel, brightly lit cage when compared with eGFP injected controls (left, p=0.04). Both groups also showed no difference in total Ensure consumption across days or amount of Ensure consumed on test day (latency course: $F_{(1,19)}$=0.02, *p* = 0.902, day x latency: $F_{(4,76)}$=0.69, p=0.601; test day consumption: p=0.354). Data are presented as means ±SEM, with individual data points overlain ($N_{GluN2D^{flx/flx\text{-}eGFP}}$ = 11 mice, $N_{GluN2D^{flx/flx\text{-}Cre}}$ = 12 mice). $^{*}p \leq 0.05$, n.s. = not significant.

BNST specific deletion of GluN2D produces increased excitatory drive onto BNST-CRF neurons.

To better clarify the effects we observed on BNST-CRF neuron excitability in the GluN2D$^{-/-}$/Crf-*Tomato* line, we crossed the GluN2D$^{flx/flx}$ mice with a line expressing Flp recombinase (Crh-IRES-FlpO) under the control of the *Crh* promoter (GluN2D$^{flx/flx}$/Crf-Flp) to allow for CRF cell-specific recordings in the BNST following GluN2D

deletion. Using RNAscope®, we examined transcript expression patterns and co-localization across the left and right dlBNST of 7 separate adult Crh-IRES-FlpO mice for both *Crh* and Flp cDNA to validate the fidelity and penetrance of Flp expression in BNST-CRF cells. We found the probes to show a high level of co-localization with one another in the dlBNST, with minimal ectopic expression of signal for the Flp probe on cells negative for the *Crh* probe (**Fig. 15** penetrance: *Crh*[+] only =227 cells, ~25.8% of total *Crh*[+] cells, *Crh*[+]/Flp[+]=654 cells, ~74.2% of total *Crh*[+] cells; fidelity: Flp[+] only =44, ~6.3% of total Flp[+] cells, Flp[+]/*Crh*[+]=654 cells, ~93.7% of total Flp[+] cells; lower insert left, *Crh* cell average totals: t[12]=2.48, p=0.029; lower insert right, Flp cell average totals: t[12]=3.87, p=0.002, unpaired t-tests), indicating that the majority of BNST-CRF positive cells in these mice are Flp positive as well.

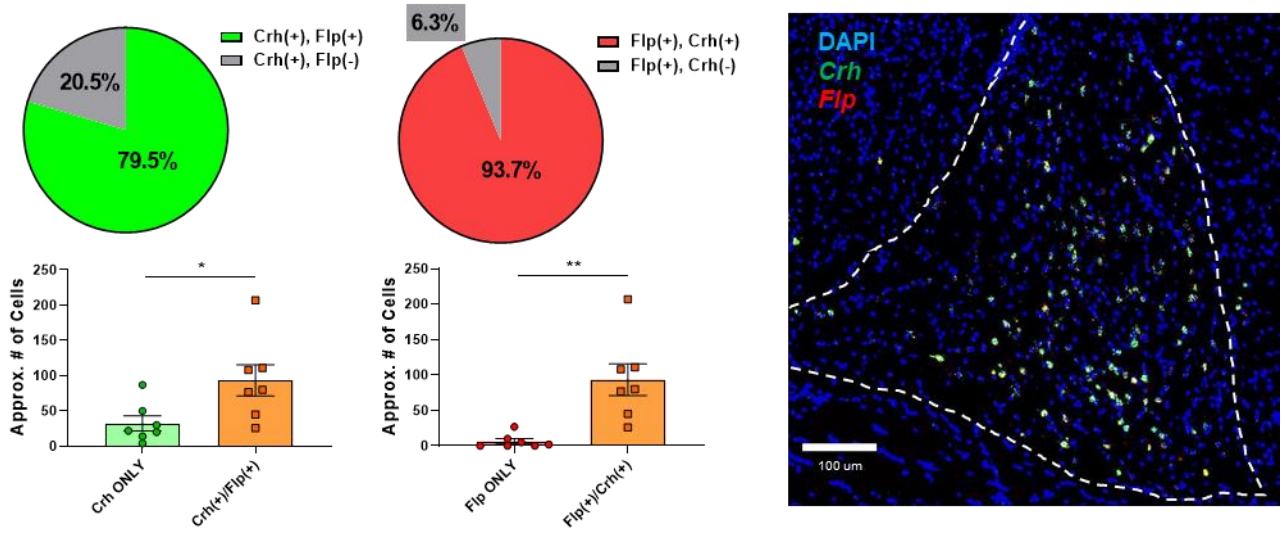


Figure 15. Flp expression is restricted to CRF(+) neurons in a Crh-FlpO transgenic line. Representative image of the dlBNST (outlined via dashed white lines) at 20x magnification after undergoing RNAscope® (right), showing individual cells with DAPI (blue) counterstained nuclei labeled for Crh (green) or Flp (red) mRNA transcripts. Summary graphs (left) showing the portion of total counted dlBNST cells (left & right dlBNST, N = 7 Crh-IRES-FlpO mice) labeled for the Crh and Flp transcripts alone or in combination. When comparing the total of cells negative for the Crh transcript (Crh Only) to those labeled for both transcripts (Crh[+]\Flp[+]), cells positive for both are shown to represent the majority of Crh(+) cells labeled within the dlBNST and averaged counts of cells from both left and right dlBNST across all mice confirms the high penetrance of Flp expression in this population (p=0.029). Similar results are also shown for Flp(+) cells when confirming the fidelity of Flp expression in Crh(+) cells, showing only minimal ectopic Flp expression outside of Crh(+) cells (p=0.002). Data are presented as means ±SEM, with individual data points overlain. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

Following this confirmation, GluN2D$^{flx/flx}$/Crf-Flp mice were intracranially injected with viruses encoding Cre tagged with an eGFP reporter and Flp-dependent tdTomato into the BNST, allowing us to both identify and patch tdTomato positive (i.e. CRF positive) Cre positive (Cre[+]) or Cre negative (Cre [-]) cells in *ex vivo* slices (**Fig. 16A**). Using this approach, we first confirmed NMDAR function to be altered in Cre(+) cells when compared with Cre(-) cells by examining isolated NMDAR eEPSC decay kinetics, and noted a significant increase in the decay rate in Cre(+) comparable to what we found when recording from BNST-CRF cells in the GluN2D$^{-/-}$ line (**Fig. 16B**, Cre[-]=58.8±5.8 ms, Cre[+]=39.2±5.4 ms, t[13]=2.48, p=0.028, unpaired t-test). We then recorded sEPSCs for both Cre(+) and Cre(-) cells, and observed a significant increase in the frequency of these events in Cre(+) cells, as well as a significant increase in the sEPSC amplitude (**Fig. 16C**, freq: Cre[-] =1.3±0.4 Hz, Cre[+]=3.6±0.8 Hz, t[18]=2.59, p=0.019, unpaired t-test; amp: Cre[-]=-26.5±1.4 pA, Cre[+]= -32.6±2.0 pA, t[20]=2.38, p=0.027, unpaired t-test). When examining the paired pulse ratio between Cre (+) and Cre (-) cells, however, we did not find any significant difference between the groups, or evidence of PPD in the Cre (+) CRF cells comparable to that observed in recordings from BNST-CRF cells in the GluN2D$^{-/-}$ (**Fig. 16D**, ISI time: $F_{(1,18)}$=1.01, p=0.351, 2D deletion: $F_{(1,14)}$=4.10, p=0.063, ISI time x deletion: $F_{(2,28)}$=0.87, p=0.432), although this may be explained by potential difference in intrinsic BNST synaptic physiology following acute GluN2D deletion as opposed to developmental deletion of the subunit. When taken together overall though, these data demonstrate an enhancement of excitatory drive onto BNST-CRF cells that occurs following acute deletion of GluN2D-NMDARs in the BNST similar to what we reported in the case of the GluN2D$^{-/-}$. This further indicates a key role for these receptors in regulating intra-BNST excitatory signaling, specifically on select subpopulations of BNST cells.

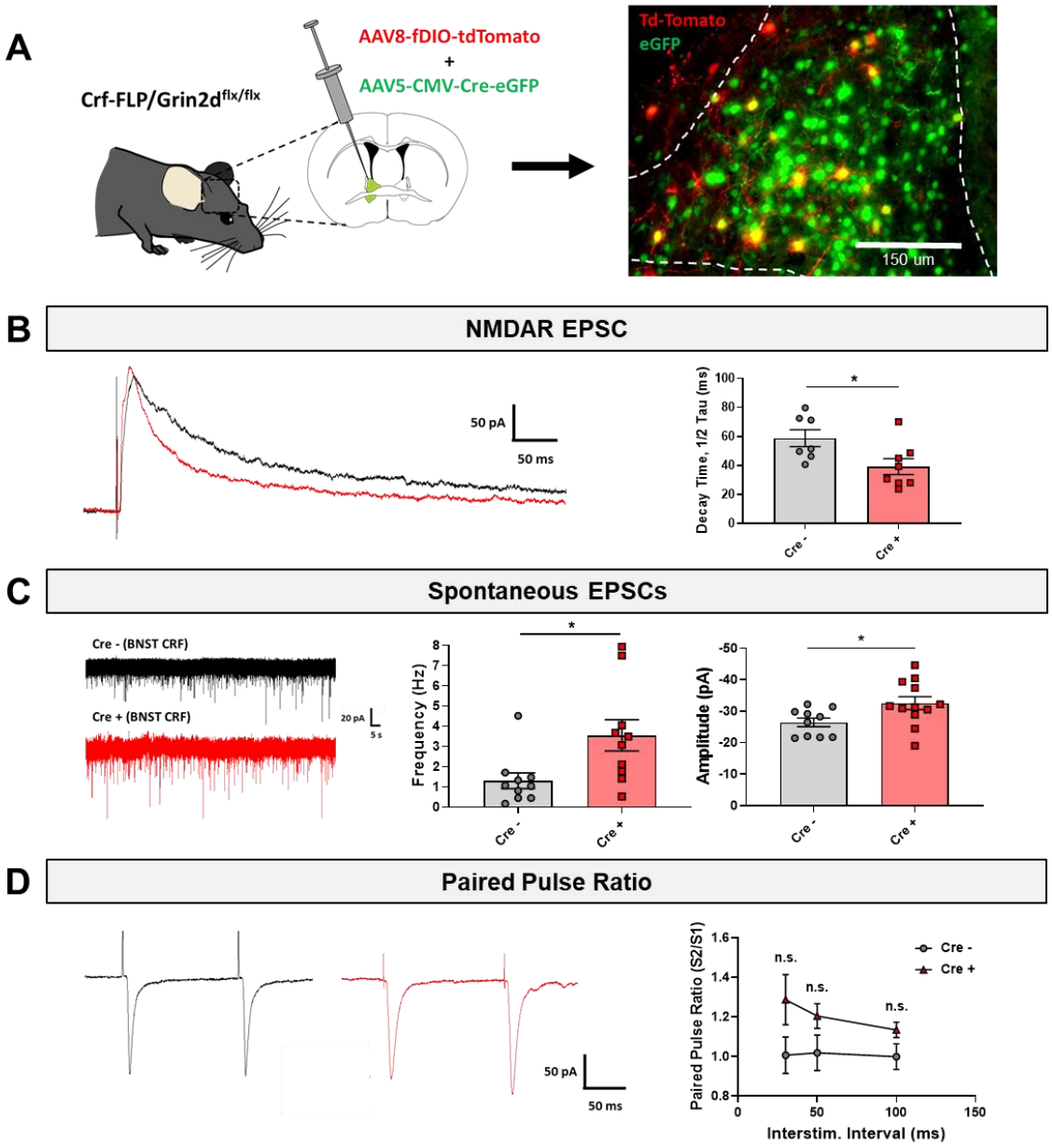


Figure 16. Region-specific deletion of GluN2D in dlBNST produces increased excitatory drive onto CRF cells and altered NMDAR kinetics. (A) Schematic outlining surgery and viral injection strategy for region specific ablation of GluN2D in the dlBNST and identification of BNST-CRF cells in slices during whole cell patch clamp recordings using a co-injection of Flp dependent tdTomato and Cre virus (1:1 mix). A representative 20x image of both eGFP-labeled Cre positive cells (green) and tdTomato labeled cells (red) in the dlBNST ~four to five weeks after surgery. **(B)** Representative traces of isolated NMDAR-EPSCs recorded from GluN2D$^{flx/flx}$ BNST-CRF Cre/tdTomato positive cells (Cre[+], red) and CRF tdTomato positive, Cre negative cells (Cre[-], black) are shown on the left. Comparison of decay kinetics observed between Cre(-) and Cre(+) cells revealed a significant decrease in ½ tau (ms) measurements for Cre(+) cells compared with Cre(-), indicative of altered NMDAR function (p=0.028). **(C)** Representative traces of sEPSC recordings from Cre(-) and Cre(+) BNST-CRF cells are shown on the left. Summary data of comparisons of both frequency and amplitude metrics in Cre(-) and Cre(+) cells revealed a significant increase in sEPSC frequency in the Cre(+) group (p=0.019). A trending, but non-significant increase in sEPSC amplitude was also observed between groups (p=0.053). **(D)** Representative traces of paired EPSCs at an inter-stimulus interval (ISI) of 50 ms from both Cre (-) cells (black) and Cre (+) cells

(red). Paired pulse ratios (PPR) of evoked 100 to 200 pA responses elicited at ISI of 30, 50 and 100 ms are plotted on the right, and revealed no significant in the ratio across any of the 3 time points between groups, despite apparent indications of a trend toward an increase observed in Cre (+) cells. Data are presented as means ±SEM, with individual data points overlain. ($N_{GluN2D}^{flx/flx\text{-}Cre}$ = 3 mice, NMDAR-EPSCs: $n_{BNST\text{-}CRF\ Cre(-)}$ = 7, $n_{BNST\text{-}CRF\ Cre(+)}$ = 8, sEPSCs: $n_{BNST\text{-}CRF\ Cre(-)}$ = 10, $n_{BNST\text{-}CRF\ Cre(+)}$ = 10, PPR: $n_{BNST\text{-}CRF\ Cre(-)}$ = 8, $n_{BNST\text{-}CRF\ Cre(+)}$ = 8). $^{*}p \leq 0.05$, n.s. = not significant.

Discussion

Selective deletion of BNST GluN2D-NMDARs and depressive-like behavior

To expand upon our work in Chapter 2, we utilized a previously validated $GluN2D^{flx/flx}$ line (Shelkar et al., 2019) in order directly interrogate the role of BNST GluN2D-NMDAR mediated excitatory signaling in the regulation of downstream behaviors. While we observed that the constitutive loss of GluN2D was able to produce an increase in both anxiety- and depressive-like behaviors in male mice, when replicating the same battery of behavioral testing in mice following BNST GluN2D deletion, these animals only appeared to show deficits in depression-related behaviors (i.e. center time in the OFT, immobility time in the FST, and feeding latency in the NIH), as opposed the differences in the anxiety-salient behaviors observed in the constitutive knockout (open quadrant time in the EZM). Although this finding is interesting on its own purely based off of the replication of a specific behavioral phenotype across lines, it is also particularly so when considering that previous BNST NMDAR subunit conditional deletion studies that targeted GluN2B found the opposite phenotype in regards to depressive-like behavior(Louderback et al., 2013). Indeed, BNST GluN2B deleted mice showed evidence of a *decrease* in depressive-like behaviors when compared which control mice. Intriguingly, no differences in anxiety-related behaviors measured via the EZM were noted in these animals as well. These findings along with those we present in this chapter seem to support a more significant role for NMDAR-mediated excitatory signaling in the BNST in regards to the regulation of depressive-like behaviors preferentially over anxiety-like behaviors, however, the exact mechanisms by which these effects are exerted has yet to be fully elucidated. Further, while we have isolated these GluN2D-NMDAR specific effects more exclusively to the BNST, our data may indicate that this relatively understudied receptor population could serve as a putative target for regulating glutamatergic signaling salient to the pathophysiology of depression. Additional behavioral testing, as well as more systemic and region-specific pharmacological studies of the effects of GluN2D-NMDAR manipulation on the emergence of depression-related phenotypes both within and outside of the BNST will be

required to more definitively demonstrate this connection, and are the topics of potential future studies that I will outline in the next chapter.

Region-specific deletion of GluN2D-NMDAR produces similar alterations in excitatory drive onto BNST-CRF cells

Apart from our interest in studying the behavioral effects of the deletion of BNST GluN2D-containing NMDARs, we were also curious to see if the acute ablation of this subunit from this region alone within the brains of adult rodents was able to replicate the BNST cell specific physiological effects observed in the constitutive knockouts discussed in Chapter 2. The use of our GluN2D$^{flx/flx}$ line presumably afforded us the ability to avoid any potential development changes in glutamatergic signaling that may have arouse from life-long loss of the GluN2D subunit, as previous studies have implicated these receptors as critical in shaping the maturation and proper function of GABAergic interneurons early in life (Hanson, et al., 2019). Focusing again on the CRF cells due principally to the functional incorporation of GluN2D into NMDARs expressed on them we observed above (**Fig.9**), we generated a transgenic line expressing the Flp recombinase under the control of the *Crh* promoter crossed to our conditional knockout mice in order to identify CRF (+) cells in the BNST of animals injected with either a Cre expressing virus or an eGFP control virus to probe the region-specific effects of acute GluN2D loss on the physiology of these cells. Additionally, due to the wealth of literature linking changes in the excitatory activity of BNST-CRF (+) neurons with increased negative emotional behaviors as mentioned previously (Pleil et al., 2015; Butler et al., 2016; Marcinkiewcz et al., 2016; Giardino et al., 2018; Lin et al., 2018; Fetterly et al., 2019), we wished to see if similar changes occurred following BNST GluN2D-NMDAR deletion that thus may correlate with the behavioral effects we reported above.

Regional deletion of GluN2D from the BNST produced similar increases in the decay kinetics of NMDAR-isolated currents in BNST-CRF Cre (+) cells to what was observed in this same population in our constitutive studies, further suggesting the presence of functional, post-synaptic GluN2D-NMDARs on them (**Fig.10B**). Similar significant increases in both the frequency and amplitude of spontaneous EPSCs were also found at BNST-CRF cells positive for Cre as compared with BNST-CRF cells that were not transfected by the recombinase, suggesting that acute deletion of GluN2D from the BNST lead to similar increases in excitatory drive onto these neurons (**Fig.10C**). PPR metrics, however, did not show a similar PPD effect at Cre (+) cells

following regional GluN2D deletion when compared with the effects found in the GluN2D constitutive knockout, though these studies may require further replication in order to fully validate this effect (**Fig.10D**). Regardless though, the changes observed in spontaneous excitatory signaling are intriguing, both in that they pheno-copy our results from our Chapter 2 studies, and that they show what may again appear to be evidence of a pre- and post-synaptic mechanism that underlies these effects. Further, these findings suggest that changes in GluN2D-NMDAR mediated excitatory signaling within the BNST contributes directly to the enhanced excitatory tone we observed at BNST-CRF (+) neurons across both our constitutive and conditional studies, potentially excluding any major influence of compensatory changes in NMDAR subunit expression or developmental deficits owing to life-long GluN2D loss (although, such alterations can still not be completely disregarded).

Additional examination of the physiological properties of BNST-CRF (+) cells under these conditions may prove important to further elucidating the role of GluN2D-NMDARs in regulating other aspects of their intrinsic membrane properties and excitatory profile, and further implicate these receptors as a means to selectively manipulate these cells in order to produce the changes in excitatory activity and downstream behavioral deficits we hypothesize as correlated with our physiological findings. It is important to note that we cannot rule out that regional deletion of GluN2D in the BNST also affects BNST-CRF (-) cells, and may result in potential changes in the surrounding BNST micro-circuitry that could factor into the control of excitatory-inhibitory tone at BNST-CRF (+) cells (Gracy and Pickel, 1995; Paquet and Smith, 2000; Turesson et al., 2013). Thus, further cell-specific manipulations of GluN2D-NMDAR function in the BNST are required to gain a more complete mechanistic understanding of its role in mediating regional excitatory signaling. Apart from extending these studies to the BNST-CRF (-) cells that also express functional GluN2D-NMDARs, determining if several of the key regions providing glutamatergic inputs to the BNST express the receptor (somatically, axonally, or dendritically) will also be essential to providing a more definitive understanding of GluN2D-mediated signaling in relation to stress-responsive or negative emotional behavior. Taken together though, the data presented in this chapter further demonstrates that GluN2D-NMDARs provide an important role in modulating excitatory activity both in key stress-responsive regions and cell populations implicated in mood- and anxiety-related disorders, and may position them as attractive targets for the development of novel therapeutics.

Chapter 4

Conclusions and Future Directions

The collective work presented in this dissertation establishes a relevant role for GluN2D-NMDAR-mediated signaling in driving and shaping complex emotional behavior, and suggests an important need to develop a better understanding of these receptors' functional and modulatory properties at key stress-salient neuronal populations, neural circuits and structures, particularly in regions such as the BNST which are shown to express high levels of GluN2D in the adult brain. Considering the unique biophysical properties of GluN2D-NMDARs and their restricted expression patterns, our findings here suggest that their manipulation may avoid the undesirable side effects of current treatments aimed at both the glutamate and CRF signaling systems in affective disorders (Preskorn et al., 2008; Binneman et al., 2008; Coric et al., 2010; Iadarola et al., 2015; Weed et al., 2016; Pomrenze et al., 2017), and pave the way for further studies of novel targets informed at the level of region-, circuit- and cell-centric physiology. Although this dissertation work presents important insights into the function of GluN2D-containing NMDARs within the BNST, and their potential influence in mediating depressive-like behavior, a number of questions remain regarding the specific mechanisms through which these receptors exert the observed physiological effects reported here, as well as the means by which their manipulation could aide in the treatment of depression and other related mood disorders. Below, I will speculate on some of these key questions and discuss the future studies that may be undertaken to further dissect how GluN2D-NMDARs contribute to the regulation of depressive-like behaviors and excitatory signaling in salient stress-responsive structures such as the BNST.

Role of GluN2D-NMDARs at synapses within the BNST: a proposed model

One of the primary foci of these studies was to investigate the role of GluN2D-NMDARs in the regulation of excitatory signaling in the BNST. As shown above in **Fig.6**, our work presented the first direct evidence that the GluN2D protein is abundantly expressed within the BNST, with previous *in situ* studies only hinting at this possibility indirectly (Monyer et al., 1994, Wenzel et al., 1996). The tight developmental regulation of GluN2D expression patterns also discussed above, also hints at a unique role for these receptors in regards to

influencing excitatory activity in the few areas that they remain functionally expressed in the adult brain (Sheng et al., 1994; Cull-Candy et al., 2001). Initially, the role of these receptors was speculated to be chiefly extra-synaptic, with early studies indicating little evidence of GluN2D expression at the synapse (Cull-Candy et al., 2004; Momiyama et al., 1996; Misra et al., 2000, Momiyama et al., 2000; Brickley et al., 2003). More recent work has brought this classification of GluN2D-NMDARs as exclusively extra-synaptic to task, however, with evidence from physiological studies in the substantia nigra (SNr), subthalamic nucleus (STN), cortex, spinal cord and hippocampus suggesting the presence of GluN2D-NMDARs at the synapse (Morris et al., 2018; Swanger et al., 2015; Hanson et al., 2019; Hildebrand et al., 2014; Lozovaya et al., 2004; von Engelhardt et al., 2015), with latter studies in the hippocampus in particular indicating that extra-synaptic GluN2D-containing NMDARs may be recruited to the synapse following the induction of LTP (Harney et al., 2008). These findings collectively argue for the expression of GluN2D-containing NMDARs post-synaptically on inhibitory interneurons across several distinct structures. Additionally, equally convincing physiological evidence in the cerebellar recordings and extensive transcript expression analyses of GABAergic cell types demonstrate the expression of GluN2D-NMDARs pre-synaptically as well, be it axonally or on the body of afferent cell projections (DuBois et al., 2015; Yamamoto et al., 2013; Watanabe et al., 1992). It is thus feasible that GluN2D-NMDARs are able to exert effects both pre- and post-synaptically within a given synapse, and our findings within the BNST, in particular at BNST-CRF (+) neurons, indicates just such a possibility. In recordings from BNST-CRF (+) cells in both the constitutive and conditional GluN2D knockout lines, we consistently observed spontaneous EPSC frequency and amplitude to be increased, and NMDAR-mediated currents to show alterations in their decay kinetics when the GluN2D subunit was deleted or pharmacologically inhibited with the GluN2C/D preferring antagonist DQP-1105 (**Figs.9-10**). Similarly in our constitutive knockout studies, short term potentiation within the BNST was found to be attenuated, suggesting again that GluN2D-NMDARs may participate in regulating changes directly at synapses within the region, either through intrinsic expression at the synapse or recruitment to the synapse from an extra-synaptic location (**Fig.6**). We also found that GluN2D deletion affected spontaneous inhibitory currents in the BNST as well, producing a significant decrease in their amplitude indicative again of an effect on post-synaptic neurotransmission. We interpreted this findings to indicate that an excitatory-inhibitory balance at the synapse of select (presumably GluN2D expressing) BNST cells was disrupted following the manipulation of GluN2D, leading to both increased excitatory drive onto these cells and

an enhanced intrinsic excitability as well. Further PPR and miniature EPSC recordings lead more support to this mixed pre- and post-synaptic hypothesis of GluN2D-NMDAR function in the BNST, and building off of our work elucidating the changes in BNST-CRF excitatory signaling in both GluN2D knockout lines, we generated a hypothetical model to speculate on the how the loss of GluN2D may produce the physiological changes we measured (**Fig. 17** below).

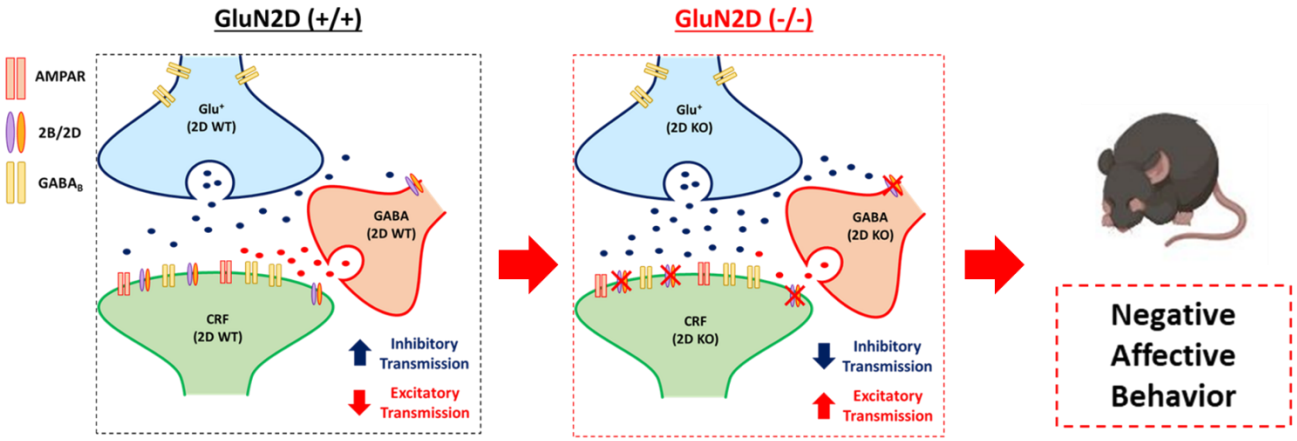


Figure 17. Proposed model of the regulatory function of GluN2D-NMDARs on synaptic function in BNST CRF neurons and implications for behavioral deficits. Cartoon showing a potential model for GluN2D-containing NMDAR function at the BNST synapses. Under basal conditions (left), functional GluN2D-NMDARs may be expressed on presynaptic GABAergic cell terminals/body, allowing for the detection of increased Glu^{2+} and/or spillover from glutamatergic inputs onto target BNST neurons, driving increased GABA release and the establishment of tonic inhibitory currents to balance out changes in excitatory transmission. In instances of GluN2D deletion from the BNST (right), loss of GluN2D-containing NMDARs from terminals, leading to possible increases in GluN2B/B diheteromers or otherwise, disrupts this balance excitatory-inhibitory balance in favor of greater excitatory drive onto target neurons (i.e. BNST-CRF [+]) and decreased sensitivity for Glu^{2+} spillover detection. This may result in the increased excitability and/or activity of select cell populations, and in the case of BNST-CRF (+) cells, may produce changes in downstream behaviors resulting in the anxiety and depressive-like phenotypes observed in the studies presented here.

It is possible that when endogenously expressed (both pre- and post-synaptically), the GluN2D-NMDARs within the BNST function in both the detection of glutamate release synaptically and extra-synaptically, as evidenced by our own findings and those outlined by previous studies. In reference to the proposed expression of GluN2D axonally on GABAergic presynaptic neurons (Watanabe et al., 1992), GluN2D-NMDARs may be similarly localized on pre-synaptic sites within the BNST, where they may serve as detectors of glutamate spillover following the activation of excitatory terminals within the region. This function of GluN2D-NMDARs has been highly speculated due to their increased sensitivity to glutamate (Wyllie et al., 2013; Hess et

al., 1998), as well as directly supported via studies at cerebellar stellate cells showing that prolonged stimulation of parallel fiber inputs is capable of producing a stimulation-induced, lasting increase in GABA release that was GluN2D dependent (DuBois et al., 2015). In our proposed model within the BNST, GluN2D-containing NMDARs perform a similar function: acting to detect prolonged or increased activation of glutamatergic inputs that can produce an increase in glutamate concentration at the synapse, eventually lead to spillover and the activation of GluN2D-NMDARs located on the terminals of presynaptic GABAergic interneurons to drive a complementary increase in GABA release (**Fig.17**). This may serve as a braking mechanism to reduce excessive excitatory activity/activation of specific circuits/cells within in the BNST. The maintenance of such an excitatory-inhibitory balance at the synapse, in particular the regulation of inhibitory transmission at interneurons, has been shown to be highly influenced by glutamatergic signal (Castillo et al., 2011; McBain and Kauer, 2009), with specific implications for the role of the NMDARs in mediating this having been demonstrated across several brain regions (Duguid and Smart, 2004; Lachamp et al; 2009; Lien et al; 2006; Liu et al., 2007). It is thus possible to hypothesize that a similar role for NMDARs, specifically GluN2D-NMDARs, could be at play within the BNST. In the case of GluN2D deletion then, we would assume this breaking mechanism to be released off of such synapses, diminishing the sensitivity of glutamate spill-over detection and allowing for both increased excitatory drive onto post-synaptic neurons, and an unchecked dysregulation of presynaptic transmitter release as well. In the context of our findings regarding BNST-CRF (+) cell activity, this could thus lead to an enhanced activation of these neurons, which may correlate in part with the behavioral phenotypes that we observed in both constitutive and conditional GluN2D knockout animals.

The mechanism described above is purely speculative, but it does present a potential framework for future experiments to further investigate the localization and function of GluN2D-NMDARs within the BNST. Localization studies into the prospective extra-synaptic BNST GluN2D-NMDARs could be performed via whole cell electrophysiological recordings prior to and in the presence of the glutamate uptake inhibitor DL-theo-β-benzyloxyapartic acid (TBOA) to induce the spillover of glutamate while measuring isolated NMDAR-EPSCs. An increase in NMDAR-EPSC amplitude, rise time, and decay kinetic time has previously been demonstrated to correlate with the expression of extra-synaptic NMDARs on the post-synaptic neuron (Harney et al., 2008), and could similarly be validated by the wash-on of DQP-1105 or other GluN2C/D preferring antagonist such as UBP141 or PPDA to test for any reduction in the aforementioned metrics. Similarly, it may be possible to test for

the presence of GluN2D-NMDARs localized on pre-synaptic GABAergic inputs to such BNST interneurons in a similar fashion: via the isolation and measurement of inhibitory currents/tone at the post-synaptic cell both in the absence and presence of TBOA. An increase in inhibitory current amplitude or frequency recorded at the post-synaptic site could potentially indicate an increased release of GABA onto the target neuron, and/or an increase in overall inhibitory drive. This function of GluN2D-containing NMDAR-mediated responses to glutamate both at pre- and post-synaptic sites to regulate GABA release has been supported by several studies based on physiological findings and the localization of GluN1 expression and GluN2D mRNA to at the terminals and axons of inhibitory interneurons (Akazawa et al., 1994; Monyer et al., 1994; Paquet and Smith, 2000; Thompson et al., 2000), and thus could help to explain a potential mechanism of action for GluN2D-NMDARs within the BNST.

Another possible explanation could be a more preferential function of GluN2D-NMDARs at either pre- or post-synaptic sites in assessing aspects of our physiological findings, and that one effect or the other may be better unmasked at specific BNST cell populations or circuits via more rigorous testing. Future studies could thus expand upon the work presented in these studies above by examining alterations in pre-synaptic release onto BNST cells via the isolation of miniature IPSCs as well, in order to determine whether or not the release of presynaptic GABA onto cells following either GluN2D ablation or pharmacological inhibition is blunted. Our current efforts into changes in inhibitory signaling at the level of whole cell physiology in the BNST have only extended to measuring spontaneous inhibitory currents, and only on BNST-CRF (+) cells. Thus, it is possible that a more robust examining of inhibitory metrics (mIPCSs, PPR at inhibitory synapses, etc.) may reveal further changes in GABAergic signaling in the BNST as a result of GluN2D manipulation, and possibly more germane to the presumed dominate form of signaling within the BNST given its high GABAergic cellular makeup. In addition to this, a further investigation into possible post-synaptic changes, as well as intrinsic excitability, could also be informative to not only examine specific synaptic changes more succinctly, but to further enhance the findings of our photometry studies, which suggest that the loss of GluN2D on specific BNST cells may lead to greater activation (**Fig.11**). A survey of membrane potential readings in both GluN2D deleted and wildtype cells basally, or in response to the wash-on of GluN2C/D preferring antagonists, could provide such initial information, indicating whether cells lacking GluN2D or exposed to one of these antagonist could lead to a more positive resting membrane potential and thus greater firing probability. Further current clamp analyses, including

current step injections to measure rheobase and average action potential firing events in response to a depolarizing current, would provide additional clarity, specifically if firing was found to be enhanced in response to knockout or acute pharmacological inhibition. A final means to access the localization of GluN2D-NMDARs at BNST synapses could also be achieved by ultrastructural analyses with a combination of electron microscopy and immunogold labeling. Though our lab has only previously shown the successful application of this technique with a transgenic reporter line labeled with a human influenza hemagglutinin (HA) tag (Flavin et al., 2014), primary antibodies for GluN2D exist and have been validated for the immunolabeling of individual cells (Yamasaki et al., 2014). With the use of proper gold-labeled secondary antibodies, this technique could be utilized to examine synapse structures in ultrathin BNST slices, and provide more qualitative insights to the localization of GluN2D-containing receptors within the region.

As outlined above, there is still a wealth of basic information to be uncovered in regards to the physiological function of GluN2D-NMDARs within the BNST. Our efforts have attempted to provide the first glimpses into the role of these unique receptors on excitatory signaling in the region, but have only extended to a single population of cells, despite our data shown in **Fig.8** suggesting that this does not capture the fully extent of other GluN2D positive neurons. Tools do exist to expand our research to these other BNST-CRF (-) populations, in the form of an eGFP-tagged GluN2D receptor reporter line developed by the laboratory of Dr. Hannah Monyer (Meyer et al., 2002; von Engelhardt et al., 2015), that could allow for the quick and straightforward identification of GluN2D (+) cells within *ex vivo* BNST slices for the profiling of basal inhibitory and excitatory physiology in many cells across all regions of the BNST. This line does present potential drawbacks though, as the generation and use of an eGFP-GluN2D BAC inserted into the mouse genome could induce the expression of tagged-GluN2D receptors at cells that also expressed endogenous GluN2D, possibly leading to an overexpression of GluN2D (Maue, 2013) and potentially misleading findings regarding the “basal” influence of GluN2D-NMDARs. eGFP tagging of an ionotropic receptor/channel could also impact the biophysical properties of said channel, possibly resulting in deficits in its function (Maue, 2013). Despite this though, the expression of this reporter as presented in von Engelhardt et al. (2015) is robust and readily visible by standard fluorescent microscopy, and the study of these eGFP-tagged neurons within the hippocampus revealed no overt changes in morphological or standard physiological properties consistent with GluN2D-containing NMDARs, as well as an expression profile highly restricted to interneurons, typical of the distribution

patterns for GluN2D protein in the adult brain. It would thus be interesting to utilize this transgenic line to expand on our current findings here and generate a more general consensus for the contributions of GluN2D-NMDARs to BNST physiology at the whole cell level.

Receptor stoichiometry and other relevant physiological contributions of GluN2D in the BNST

The incorporation of GluN2D subunits into functional NMDARs has been shown across multiple studies to profoundly impact it's biophysical properties (Traynelis et al., 2010; Wyllie et al., 2013), however it has rarely been reported that GluN2D-containing NMDARs are found as diheteromers (GluN1-GluN2D-GluN2D) *in vivo*, with the majority of work conducted on the physiology of such receptors occurring predominantly *in vitro* under more artificially induced circumstances (Vance et al., 2012; Wyllie et al., 2013). Based on an abundance of evidence from both *in vivo* and *ex vivo* studies of GluN2D-NMDARs in rodents, a prevailing notion has emerged that the majority of GluN2D expression within receptors found in the adult brain is restricted to triheteromer receptors also containing the GluN2B subunit (Dunah et al., 1998; Pina-Crespo and Gibb, 2002; Brickley et al., 2003; Jones and Gibb, 2005; Harney et al; 2008; Brothwell et al., 2008; Suarez et al., 2010; von Engelhardt et al., 2015; DuBois et al., 2015; Swanger et al., 2015; Morris et al., 2018). This has been functionally demonstrated across many of these studies at interneuron populations within the cerebellum, hippocampus, STN and SNr, revealing that the decay kinetics and overall strength isolated NMDAR excitatory currents at putative GluN2D (+) cells within these structures show sensitivity to both GluN2C/D preferring antagonists like DQP-1105 and UBP141, as well as to highly selective GluN2B antagonist such as ifenprodil and Ro 25-6981. These findings propose that the GluN2D subunits contribute to the channel open time, rise time and current propagation through these NMDARs, leading to prolonged decay kinetics. These properties are significant when considering the subunit's increased sensitivity to glutamate, and suggest that GluN2D incorporation into NMDARs leads to greater establishment of a baseline current flow in response to ambient glutamate levels, as well as in the genesis of plasticity changes in response to higher/continuous stimuli/glutamate release at select synapses. Indeed, it has been suggested that the presence or movement of GluN2D-containing NMDARs into the synapse is critical to the maintenance of certain forms of LTP (Harney et al., 2008; Volianskis et al., 2013) and that the loss or inhibition of these receptors can lead to LTD (Harney and Anwyl, 2012) or otherwise a

blunting of synaptic potentiation (Volianskis et al., 2013), much in line with our findings report above in Chapter 2 (**Fig.6**). This may also explain a key function of GluN2D-NMDARs within the BNST: as essential for driving plasticity at certain synapse within the region, and/or in upregulating the strength/propagation of current flow through NMDARs on select cells (Hrabetova et al., 2000). This last point may be of particular consequence to the BNST-CRF (+) neurons focused on in our study, as an increase in the open time of NMDARs on such cells could hypothetically allow for increased Ca^{2+} influx to occur through these receptors as well to drive up intracellular Ca^{2+} concentrations (Erreger et al., 2005), an important requirement for the release and fusion of dense core vesicles known to contain neurohormones/neuropeptides within such cells (Zhang et al., 2011). While other studies have called into question how prominently GluN2C or GluN2D subunits contribute to calcium permeability at NMDARs compared to GluN2A or GluN2B (Evans et al., 2012), the prospect of GluN2B/GluN2D triheteromer suggests that a combination of the two subunits could lead to greater calcium influx overall due again to GluN2D imposes increases in decay kinetics and channel open time.

Correlative evidence from our lab suggests the possibility that within the BNST, GluN2D may also be incorporated into GluN2B/GluN2D triheteromeric receptors, as the expression of the GluN2B subunit has been shown to be robust within the BNST (Katie Holleran and Greg Salimando, unpublished data). Studies of synaptic plasticity and excitable cell properties within the BNST as well have also shown the region to be highly sensitive to GluN2B specific manipulations, with both Ro, 25-6981 and ifenprodil wash-on studies in *ex vivo* BNST slices producing robust decreases in sEPSC and isolated NMDAR-EPSC kinetics, as well as the complete ablation of LTP within the BNST when applied prior to tetanus, an effect that mimicked conditional GluN2B knockdown in the BNST (Wills et al., 2012). Perhaps most convincing, however, are the results we have obtained from immuno-pulldown and mass spectrometry assays of GluN2B associated proteins in BNST tissue punches, which showed GluN2D protein to be associated with GluN2B within BNST more exclusively when compared with other brain regions (see **Fig. 21** below). While these results strongly contribute to the prospect of the triheteromeric nature of GluN2D-containing NMDARs in the BNST, further measures should be taken to test this, as a greater insight into the precise stoichiometry of GluN2D-containing NMDARs within the BNST would be highly informative not only from the standpoint of enhancing our knowledge of basic BNST physiology, but also to further clarify how GluN2D and its manipulation made lead to the downstream behavioral effects reported in this dissertation work. Electrophysiological recordings similar to those reported in the studies

mentioned above could thus be performed with BNST cells already known to be positive for GluN2D, such as the BNST-CRF (+) cells we discuss here, to begin these investigations. This could include using *ex vivo* slice pharmacological recordings to test the sensitivity of NMDAR-isolated currents at these cells to tiered wash-on of both GluN2D and GluN2B selective antagonists. The use of the aforementioned eGFP-GluN2D reporter line for these studies would provide a greater means to generalize testing more broadly throughout the BNST, and provide more definitive evidence of the homogeneity of GluN2B/GluN2D triheteromers on BNST cells following repeated testing across slices from both anterior and posterior portions of the region. The implications for the identification of such a triheteromeric population within a stress-responsive region would be equally informative to the understanding of the role of these receptors in the actions of select NMDAR antagonists such as ketamine, and thus their influence in driving specific antidepressant-like effects, with recent literature suggesting this classes of receptors may be highly specific pharmacological targets (Yi et al., 2019). Thus, further interrogation of the presence of GluN2B/GluN2D triheteromers in the BNST and at BNST-CRF (+) neurons, could further identify such NMDAR population as unique and promising for modulating the pathophysiological changes associated with depression as observed across preclinical and clinical studies.

GluN2D-NMDAR-mediated regulation of BNST-CRF cell activity: implications for disease

In regards to the role of CRF signaling and CRF cell function in the BNST in relation to models of stress, depression and other affective disorders, a clear connection has been drawn between increased activation of this population and greater levels of anxiety and depressive-like behavior (Fetterly et al., 2019; Giardino et al., 2018; Butler et al., 2016). Given our data presented above in Chapters 2 and 3, we proposed that the activity of these cells (and thus potentially, the emergence of these anxiety and depressive-like phenotypes) could be controlled in part by GluN2D-NMDAR mediated excitatory signaling in the BNST. Indeed, as we show in **Fig.11**, BNST-CRF (+) cells activity is basally enhanced in GluN2D$^{-/-}$ mice when compared with GluN2D$^{+/+}$ littermates, suggesting a potential connection with the behavioral profiles we observe in these animals and in the BNST GluN2D deletion animals in regards to increased negative affect. Despite this though, these two approaches can be considered somewhat incomplete, as one utilizes a constitutive knockout and the other a region restricted deletion of GluN2D to draw correlative connections with observed deficits in behavior and the contribution of

BNST-CRF (+) cell activity specifically. A more refined next step to directly link these two effects would be through the use of novel viral strategy to selectively delete the GluN2D protein from BNST-CRF (+) cells alone, and could be achieve via the use of the GluN2D$^{flx/flx}$/Crf-Flp we developed in the pursuit of our work presented here. Mice positive for the Flp transgene could be stereotaxically injected into both the left and right BNST with a virus encoding a Flp-dependent Cre recombinase, with successful transfection driving Cre expression exclusively in CRF (+) cells. These animals could then be tested across the same behavioral tasks outlined in Chapters 2 and 3, and the differences in their anxiety and depressive-like behaviors assessed to determine the significance of the contribution of BNST-CRF (+) cell function to the increases in negative affective behaviors we have observed in over the course of this dissertation research.

Additional measures could also be made to determine the real time changes in BNST-CRF (+) cell activity in response to negative stimuli across tasks modeling anxiogenic and depressive behaviors. These would serve as a natural extension of the aforementioned work presented in **Fig.11**, which did not extend beyond observations of basal changes in BNST-CRF (+) cells activity when animals were placed in a novel environment, and could thus be further refined via the use of our GluN2D$^{flx/flx}$/Crf-Flp line and a Flp-dependent GCaMP6f virus that is commercially available. Injection of this virus bilaterally into the BNST, along with fiber optic implantation, would allow for the real time measurement of Ca^{2+} activity in BNST-CRF (+) cells exclusively, and when paired with either Flp-dependent Cre or a Flp-dependent reporter virus injections, these measurements could be scored against the performance of both BNST-CRF GluN2D deleted or BNST-CRF GluN2D wildtype animals in tasks such as the EPM/EZM, OFT, TST and NIH. The performance of salient changes in anxiety and depressive-like behavior could also be time-locked to measured outputs of Ca^{2+} dynamics, allowing for a more robust and powerful interpretation in the changes in BNST-CRF (+) activity than is usually afforded via post hoc identification of changes in neuronal activity typically relying on altered expression of the immediate early gene, *Fos* (Hoffman et al., 1993; McReynolds et al., 2018). Our lab has already generated a bulk of yet published data that has demonstrated the effectiveness of this approach in measuring changes in the Ca^{2+} dynamics at BNST-CRF (+) cells in response to restraint stress, and found that robust increases in this signal are time locked to bouts of struggle (i.e. active stress coping) in animals undergoing this task (Joseph Luchsinger, Samuel Centanni, Kellie Williford and Gregory Salimando, unpublished data). Measures such as these would provide key insights to the responses of the BNST-CRF (+) cells to acute

stressors often utilized in models of depression, and investigate the impact of GluN2D deletion on these responses. The prolonged access to these cell populations for continuous measurements that a technique such as fiber photometry allows would also make studies of the effects of chronic stress both on BNST-CRF (+) activity and the emergence of depressive-behaviors much more feasibly and informative to conduct as well. Thus, in addition to our acute measurements, these cohorts could also be tested on CMS/CUS and CSDS in order to more incorporate studies that model other aspects of depressive phenotypes known to emerge in rodents over time. The development of newer tools in the arena optical and cable neuroscience also promises the potential to directly investigate the effects of BNST-CRF (+) cells activation on the *release* of CRF both in the BNST and at regions known to receive CRFergic inputs from the BNST such as the VTA and PVN that have been implicated in both changes in stress and reward responsiveness often implicated in depression pathology. While these prospects are still conceptual, the advent of GPCR-based genetically encoded sensors has recently been developed and functionally validated in the detection of monoamine release (Zeng et al. 2020). This GPCR-Activation-Based (GRAB) sensor technology has been shown to be highly adaptable to many different GPCR variants, thus suggesting that the development of sensors based on the CRFR1 and CRFR2 receptors may be feasible and even underway. Until then though, the current optical approaches afforded to us will still allow for unparalleled detection of changes in BNST-CRF (+) cell activity which can greatly bolster our previous physiological findings in a way more salient to behavioral output. While it may seem unlikely that changes in the activation of such a relatively small population of BNST neurons could exert the level of control of total BNST activity or output that may be presumed necessary to drive the behavioral changes we report here following total or regional deletion of GluN2D, previous reports have revealed that BNST-CRF (+) neurons are extensively connected with the rest of the BNST, as demonstrated in both rat and mouse anatomical mapping studies of BNST micro-circuitry (Phelix and Paull, 1990, Debrowska et al., 2016), and in particular have been shown to display higher incidence of connections with BNST-CRF (-) neurons (Patridge et al., 2016). This suggests that BNST-CRF (+) cells may indeed exert a substantial level of control over the activity of other neurons across multiple BNST sub-nuclei, and contribute significantly to the regulation of regional activity overall. The future studies suggested above, particularly in the context of manipulations to excitatory signaling at these cell via GluN2D-NMDAR genetically or pharmacologically, could be of particular importance in regards to improving our knowledge of overall BNST physiology and the link between BNST excitatory function and behavioral regulation.

BNST intrinsic/extrinsic circuitry and GluN2D expression

The majority of the data collected for this dissertation focused on the role of GluN2D-NMDARs expressed on post-synaptic neurons within the BNST. A natural extension of our work here could thus extend to the expression of GluN2D-NMDARs on the numerous inputs and/or outputs from the region in order to investigate the role of these NMDARs in the regulation of key BNST circuits that have been implicated in the regulation of affective behaviors. These include GABAergic, glutamatergic and neuromodulatory inputs at limbic structures heavily implicated in driving these behaviors as well, including several that are of particular note due to their direct connect with stress responsive cell populations both in the BNST and in other structures (Silberman et al., 2013; Stamatakis et al., 2014; Fetterly et al., 2019; Ch'ng et al., 2017; Centanni et al., 2019), as well as those that have been suggested to express the GluN2D subunit, albeit indirectly (Monyer et al., 1994, Wenzel et al., 1996; Sheng et al., 1994). Determining if GluN2D-NMDARs may be preferentially expressed on specific BNST afferents or efferents could greatly inform future work with regards to the role of these receptors in regulating BNST function and affecting downstream behavior. In relation to our focus here on the impact of BNST glutamatergic signaling and CRF (+) cell function regarding affective behavior, perhaps some of the most intriguing inputs to the BNST to investigate would be those from the insular cortex and PBN. Recent work from our group identified these inputs as able to directly regulate the excitatory activation of BNST-CRF (+) neurons, and specifically to enhance the activity of this population in response to stressors (Fetterly et al., 2019; Centanni et al., 2019). To test for the expression of GluN2D on these inputs, a retrograde tracer approach could be implemented via the injection of a retro-virus encoding a tdTomato reporter bilaterally into the BNST. Following sufficient time for expression of the virus in rodents, the brain could be removed, sectioned and processed via a method such as RNAscope, in which specific oligo probes *Grin2d* and for the tdTomato reporter could be applied to sections containing the insular cortex and PBN in order to examine co-localization of *Grin2d* probe signal on tdTomato positive cells. Functional validation studies could follow from here, using a similar viral strategy but instead of processing tissue for immunostaining, the brains would be sectioned to prepare acute *ex vivo* brains slices for pharmacological recording studies. Using the tdTomato marker to once again identify BNST projecting cells from the insula or PBN in slices, whole cell patch clamp could be performed to measure the effects of GluN2C/D preferring antagonists on the amplitude and kinetics of isolated NMDAR-EPSCs. This

approach could also be applied more broadly to other BNST projecting structures that would presumably undergo transfection from the tdTomato reporter, including the CeA, BLA, vHPC, PFC and PVN (Stamatakis et al., 2014; Ch'ng et al., 2017).

In addition to the examination of many of these core circuits that were described in detail in Chapter 1, as mentioned previously, the BNST is also known to send and receive several CRFergic projections as well, with efferent projection consisting of inputs to the PVN-CRF (+) cells (Champagne et al., 1998; Dong et al., 2001a) and to the VTA (Rodaros et al., 2007; Silberman et al., 2013a), and the primary afferents being projections from CeA-CRF (+) cells into the BNST that have been shown regulated by NMDAR-mediated signaling (Sakanaka et al., 1986; Jasnow et al., 2004; Beckerman et al., 2013). These circuits have each been shown to contribute to the regulation of different forms of stress-related behaviors implicated in models of social defeat, stress induced drug seeking, and overall changes in hedonistic drive (Jasnow et al., 2004; Vranjkovic et al., 2014; Pleil et al., 2015), thus heavily implicating them in the pathophysiology of affective disorder like depression. An examination of whether or not these circuits could be regulated by changes in GluN2D-NMDAR function, and thus contribute to the behavioral phenotypes we have outlined here, would be an interesting next set of steps for circuit dissection studies. A similar viral and staining approach could be implemented to the one described above, with the additional use of the Crf-Tomato reporter line that we made use of in Chapter 2. Administration of a commercially available Cre-dependent retrovirus encoding the fluorescent reporter eGFP into the PVN of Crf-Tomato mice would allow for the specific transfection of tdTomato-tagged, Crf-Cre positive cells, and for the virus to then travel back to the BNST to label neurons directly connected with these PVN-CRF (+) cells. Treatment of tissue sections containing the BNST via RNAscope could be performed once again to label cells positive for *Grin2d* transcript, tdTomato (CRF), and the eGFP reporter protein from the retrovirus in order to determine if BNST-CRF GluN2D (+) neurons do indeed synapse on PVN-CRF (+) neurons. Concrete evidence of this would suggest that, by proxy, the regulation of BNST-CRF (+) cell activity via manipulation of GluN2D-NMDARs could produce downstream effects on the activation of PVN-CRF (+) cells, possibly leading to increased activation of the HPA axis. The dysregulated excitatory activity of BNST-CRF (+) cells and associated increased anxiety and depressive behaviors we observed in both GluN2D$^{-/-}$ and GluN2D$^{flx/flx}$ mice could thus be explained in part by such an effect, and would be intriguing to pursue further with more advanced techniques that would allow for the optogenetic manipulation of this circuit in the presence or absence of GluN2D preferring

agonist and antagonists. The BNST-VTA CRFergic inputs, as well as the glutamatergic inputs extensively characterized in Jennings et al. (2013), represent additional circuits that would be interesting to analyze for the expression of GluN2D-NMDARs on the BNST projection cells, due to the role they have been demonstrated to play in regards to motivated behaviors and the enhancement of stress-related responses, respectively. The same set of strategies outlined for the analysis of the BNST-PVN circuit could be applied here as well, with follow-up functional validation studies at the level of whole cell recordings and behavior. Physiological studies could include the injection of an anterograde propagating virus encoding Cre-dependent ChR2 into the BNST of Crf-Tomato animals, after which *ex vivo* slices of the VTA could be obtained and recordings on neurons in proximity to labeled ChR2 terminals performed in the presence of either GluN2D preferring antagonists to determine whether manipulation of putative GluN2D-NMDARs on these terminals could exert an effect on neuronal activation or excitability in the VTA. In regards to behavioral testing, similar experiments to those outlined for potential BNST-PVN projection studies, and those performed in Jennings et al. (2013), could be replicated, with the additional use of newly developed fiber optics probes such as the optofluidic system generated by Jeong et al. (2015) to allow for dual optical activation of BNST-VTA projecting glutamatergic or CRFergic cell along with the delivery of a GluN2D preferring antagonist to the same site, to observe how the manipulation of the GluN2D-NMDARs in the BNST could go on to induce behavioral changes measured in real time. Collectively, these proposed studies could help to further expand our knowledge of BNST circuit function and influence over salient affective behaviors in rodent models of depression, as well as further indicate the GluN2D-NMDARs as promising targets for the development of new therapeutics.

BNST excitatory activity mediated by GluN2D-NMDARs: relevance to affective behavior & disease

As mentioned above, much of the work presented in this dissertation aimed at investigating the effects of alterations in GluN2D-NMDAR function in the BNST and its correlation to changes in affective behaviors has been achieved via behavioral modeling of anxiety and depressive-line behaviors and physiological studies in transgenic mouse line lacking functional GluN2D receptors. While this has provided much new insight into the influence of these receptors at BNST synapses and suggested that dysregulating in BNST activation achieved via the deletion of this subunit may be salient to the behavioral changes we have reported, we have yet to

examine both of these modalities at the same time. Thus, the use of *in vivo* fiber photometry again presents itself as a powerful means by which we can start to achieve this, and measure changes in BNST activation in real time during behavior challenges under conditions in which the GluN2D subunit has been selectively deleted from the region (via use of the GluN2D$^{flx/flx}$ line). Using stereotaxic injections, virus encoding a somatically expressed variant of GCaMP7f can be mixed 1:1 with additional viruses encoding either Cre recombinase tagged with a mCherry fluorophore or the fluorophore on its own and administered bilaterally into the BNST. This will allow for pan expression of both the genetically encoded calcium sensor and Cre in the majority of BNST neurons, and thus the recording of changes in *in vivo* Ca^{2+} dynamics broadly throughout the entire structure in awake, behaving mice. Animals can then undergo behavioral testing across a number of tasks focused on either acute stress or chronic stress models of depression, while simultaneously recording Ca^{2+} signal time-locked to salient behaviors observed throughout testing. In this way, we will be able to glean a broad understanding of the changes in BNST activation in animals lacking the GluN2D subunit when compared with controls. Changes in Ca^{2+} event dynamics are often used as proxy for action potential firing (and thus, activation) of a population of cells as measured by a net change in GCaMP-mediated fluorescence over background signal (Grienberger and Konnerth, 2012), and thus while not a substitute for the temporal precision and physiological relevant that can be achieved via whole cell recordings, we can make use of this method to gain more broad, generalized insights to the changes in the activity of a given region. Indeed, previous work from our lab has implicated this strategy successfully when measuring changes in total BNST Ca^{2+} signal during the EPM task (Harris et al., 2018), and has shown evidence indicating that BNST activation may be increased during open quadrant entry more prominently then during center quadrant or closed quadrant exploration. Based on our knowledge of acute signaling within the BNST in response to stressors (Conrad et al., 2011a; Fetterly et al., 2019), and our findings presented in Chapters 2 and 3, we would hypothesize that the loss of GluN2D-NMDARs within the BNST could produce similar but more robust and frequent changes in fluorescence in BNST GluN2D deleted mice during tasks utilized in our GluN2D$^{-/-}$ and GluN2D$^{flx/flx}$ studies than what would be observed in animals wildtype for the GluN2D subunit. This would indicate that increases in overall BNST activation in response to such behavioral challenges directly correlates with increases in negative affective behaviors. How this occurs could be speculated to be due to either unchecked increases in glutamatergic signaling within the BNST, or alterations in inhibitory signaling with the region's extensive GABAergic micro-

circuitry, leading to a potential dis-inhibition of inhibitory drive (as speculated in Kim et al., 2013) onto select cell populations and induce an overall increase in their activation and downstream signaling effects. Some of our results in Chapter 2 however do present a potential counterpoint to this theory, as we observed no overt changes in the intrinsic excitatory activity of the majority of unspecified BNST cells we patched in *ex vivo* slices from both GluN2D$^{+/+}$ and GluN2D$^{-/-}$ animals, indicating that such overt changes in total BNST activation/excitability may not occur following the deletion of a subunit that is only present on roughly one third of all cells within the anterior BNST (**Fig.7**). Despite this though, as mentioned above, previous work has indicated that BNST-CRF (+) cells are extensively connected with surrounding BNST-CRF (-) cells, and thus may exert a robust level of control over the many other GABAergic cells within the BNST (Patridge et al., 2016). While changes in basal excitatory properties at GluN2D lacking BNST CRF (+) cells may have thus been readily observed when recording from this population, the effects of their potential regulation of other BNST neurons they synapse onto, and by which they may produce activity dependent changes, may not have been easy to detect without the use of a dual patching approach (onto both BNST-CRF [+] cells and BNST-CRF [-] directly connected with them) that would allow for the stimulation of these BNST-CRF (+) cells and measurements of responses in the paired CRF (-) neurons. Under these conditions, it is possible that deficits in the activation or firing patterns of other unspecified BNST cells could be observable following GluN2D knockout or acute pharmacological manipulation. Further physiological study into the overarching role of GluN2D-NMDAR signaling within the BNST is thus necessary to develop a better understanding of their function and mechanism of action in the regulation of excitatory activity within the region, as well as the connection that exists between this function and behavior.

While many of these circuit based approaches are informative to our understanding of the BNST, as well as how interconnected limbic structures may orchestrate changes in complex behaviors in response to changes in excitatory signaling, the approaches that we have implemented and suggested thus far to study these topics do not necessarily translate well to treating human patients (i.e. optogenetics, conditional deletion, viral transfection, etc.). However, there is still much basic pharmacological studies that could be conducted to examine the effects of direct and indirect manipulation of GluN2D-NMDAR signaling in the BNST with regards to changes in depressive-like behaviors, and that may suggest a greater potential for the implementation of GluN2D targeted pharmacology in human patients as a therapeutic avenue for treating depression. Such direct

assessments regarding whether acute pharmacological antagonism of GluN2D-NMDARs replicate the behavioral effects we have observed in both the GluN2D$^{-/-}$ and GluN2D$^{flx/flx}$ mice, two basic approaches could be attempted: i.p. administration of GluN2D preferring compounds to observe the systemic effects of receptor manipulation, and/or the bilateral cannulation of the BNST to directly administer compounds into the region and test whether phenotypes observed similar to those we report in Chapter 3 for BNST, GluN2D deleted animals. Systemic administration studies present a greater translational value, as similar approaches would be attempted in humans via the use of easily injectable or ingestible formulations of GluN2D-regulatory compounds, and a previous study has shown that both DQP-1105 and UBP-141 are readily able to penetrate the blood brain barrier at viable concentrations for drug action at either GluN2C or GluN2D containing receptors when administered to mice via i.p. injection (Lozovaya et al., 2014). The injection of these drugs was also demonstrated to produce observable changes in rodent behavior, albeit in regards only to seizure and epliptoform related behavior in the context of this study, suggesting that similar approaches could be successfully implemented in our own lab in order to the determine the effects of such drugs on the presentation of anxiety and depressive-like behaviors. Given the enhanced negative affective behavioral profiles that we and others (Shelkar et al., 2019; Yamamoto et al., 2016) have observed in the GluN2D$^{-/-}$ mouse line, we would hypothesis that systemic injection of DQP-1105 or UBP-141 would produce similar increases in anxiety-like metrics across tasks such as the OFT and EPM/EZM, as well as in depressive metrics in the FST/TST and NIH.

While an approach such as this would of course target multiple structures within the brain known to possess both GluN2C/D containing NMDARs, it is a bit coarse for drawing more than generalizations about the feasibility of pan GluN2C and GluN2D NMDAR antagonism as a means to regulate affective behavior. Thus, in order to determine if the actions of these antagonists would extend to impacting BNST excitatory function, separate sets of behavioral and physiological experiments could be performed in Crf-tdTomato or GluN2D-eGFP tagged animals. Correlations could then be drawn between observations in behavioral changes in drug injected mice (compared with vehicle injected controls) with metrics obtained from whole cell recordings performed in *ex vivo* slices containing the BNST from animals administered antagonist 30-60 mins prior to preparing slices (a similar amount of time that would be allowed to pass before performing behavioral experiments) in order to determine if similar enhancements of excitatory drive and alterations in NMDAR-isolated currents at unspecified or BNST-CRF (+) cells could be gleaned, and whether these effects of acute

pharmacological inhibition replicated our findings in recordings from both constitutive and conditional knockout slices. To draw more direct correlations between behavioral effects paired with drug administration, similar i.p. injections could also be performed on rodents from the same mouse lines prior to an exposure to restraint stress, a task that we have previously found to greatly enhanced BNST activity and specifically BNST-CRF (+) cell activation (Fetterly et al., 2019; Centanni et al., 2019). Drug and vehicle injected animals could be sacrificed shortly after restraint and then either prepped for physiological recordings or immunostaining with antibodies targeted to *Fos*, both metrics that would be able to identify increases in BNST excitatory activity and gross activation in response to these compounds. Additionally, more extensive wash-on studies could also be performed in *ex vivo* slices with DQP-1105 or UBP-141 to further determine if short term application of these drugs was also sufficient to produce noticeable changes in BNST neuron excitability and/or excitatory drive onto post-synaptic neurons.

In order to avoid some of the potential drawbacks of systemic injection studies, as mentioned above a different set of experiments that would be promising to undertake would involve the cannulation of both the left and right BNST for the direct administration of GluN2C/D preferring compounds into the region. Similar behavioral and post hoc immunostaining studies could also be performed under these test conditions, but the initial strength of this approach would involve the selective and acute pharmacological manipulation of the BNST GluN2D-NMDARs in order to see if such measures could produce the same changes observed in our conditional knockout animals, but with compounds that would eventually wash-out and their effects diminish (as opposed to the chronic deletion of the subunit from the adult brain). Based off our conditional knockout studies, we would hypothesize that DQP-1105 or UBP-141 administration into the BNST would similarly disrupt regional excitatory function and produce an enhancement of depressive-like behavior. Evidence of this would provide a significant boost of support for our previous work, but also enhanced the translational feasibility of the use of GluN2C/D preferring drugs to target stress-responsive structures implicated in depressive and mood disorders to alter their excitatory activity in a way that may lead to either an exacerbation or suppression of negative affective behavior. Certain caveats exist for all the pharmacological studies proposed here though, primarily due to the fact that as of yet, no GluN2D specific or GluN2D preferring compounds alone exist, with the vast majority of them showing similar levels of selectively for GluN2C and GluN2D-containing NMDARs over GluN2A or GluN2B (Mullasseril et al., 2010; Swanger et al., 2018; Yi et al., 2020). This may complicate interpretation of

systemic injection studies, despite additional analysis of changes in BNST function under these conditions via post hoc *Fos* immunostaining or recordings. The same may not be the case for cannulations studies, however, as we have generated preliminary evidence indicating that GluN2C protein shows little to no detectability at all within the BNST (data not shown), indicating that any potential effect of manipulation to the region with currently available GluN2C/D preferring compounds may occur exclusively through GluN2D-containing NMDARs. Cannulation studies do carry their own caveats though, specifically that injected compounds are not guaranteed to remain only within targeted structures. Considering GluN2D and GluN2C expression in known to be high in several regions flanking the BNST such as the septal nucleus and striatum (Monyer et al., 1994; Wenzel et al; 1996; Sheng et al., 1994), which have both also been implicated in the regulation of affective behaviors (Singewald e al., 2011; Der-Avakian And Markou, 2012), the interpretation of the results of these studies may be difficult without control experiments targeting these other areas. The emergence of similar behavioral deficits to what we observed in our conditional knockout work again, however, would probably go a long way to support a replication of such effects pharmacological via cannulation, providing stronger evidence to suggest that GluN2D-NMDAR inhibition and/or deletion in the BNST can promote depressive-like phenotypes.

The next obvious question would of course be: can we reduce these effects if we *enhance* GluN2D-NMDAR-mediated excitatory signaling in the BNST as opposed to inhibiting it? The work presented within this dissertation would lead to the hypothesis that selective potentiation of BNST GluN2D-NMDARs would indeed potentially suppress the emergence of negative affective behaviors. This notion could be readily tested using the cannulation strategies described above, and make use of a number of compounds that have been shown to selectively potentiate the activation of GluN2C/D NMDARs. Work over the past decade has led to the development of a number of positive allosteric modulators (PAMs) that show higher preferring for binding at the GluN2C and GluN2D subunits over GluN2A and GluN2B, allowing them to increase the functional kinetics of these channels and their responsiveness to cognate ligands (Perszyk et al., 2020; Perszyk et al., 2018; Burnell et al., 2019), with the first being compound CIQ from the lab of Stephen Traynelis (Mullasseril et al., 2010) and a paper released this year outlined the development of an even more potent GluN2C/D PAM, PTC-174 (Yi et al., 2020). These compounds allow for the exciting first looks into the physiological and behavioral effects of enhancing the activation of these receptors populations, with recent studies showing that both systemic administration of CIQ and intracranial injection into the amygdala can reverse increases in pre-pulse inhibition of

startle and enhance the extinction of learned fear response in mouse models of mood disorders, respectively (Ogden et al., 2014; Suryavanski et al., 2013). These results suggest that the potentiation of GluN2C/D containing NMDARs, particular in limbic structures like the amygdala, may indeed be critical in the regulation of stress and affective related responses that have been thought to be governed by such structures, and overt changes in excitatory signaling that may be implicated in depression and/or mood disorders. We would propose to test this hypothesis via both systemic and BNST intracranial administration of PTC-174, which has also shown an increased affinity at GluN2B/GluN2D triheteromers, prior to testing rodents across several tasks modeling acute stress exposure salient to anxiety and depression-related disorder, including the OFT, EZM, FST and NIH. Similar post hoc immunohistochemical and physiological experiments to those discussed above could be performed as well, in which we would predict PTC-147 treatment to promote both a reduction in *Fos* expression in the BNST (particularly in BNST-CRF [+] cells), as well as a restoration in GABAergic tone onto post-synaptic BNST neurons, similar to those we observed in our BNST GluN2D$^{+/+}$ slice recordings (**Fig.10D**).

The idea of potentiating a class of NMDARs as opposed to inhibiting them may seem somewhat paradoxical in light of our current theories regarding the antidepressant mechanism of action ascribed to most NMDAR antagonists, in which the inhibition of NMDARs is often thought to lead to an *increase* in excitatory signaling and a reduction of depressive-like symptoms (Iadarola et al., 2015). However, given the BNST's predominantly GABAergic make-up, it is possible to speculate that alterations in excitatory activity within the region and/or excitatory drive onto specific cell populations/projection pathways may lead to a net dysregulation of excitatory-inhibitory balance, releasing a brake that may keep these components in check and lead to enhanced anxiety and depressive-like behavior. The work from Kim et al. (2013) mentioned above has lent some support to this idea, with data suggesting that at the level of the oval nucleus in the BNST that increased activation of its inhibitory inputs to the anteromedial BNST can enhance anxiogenic behavior. This may have broad implications for the activation of BNST-CRF (+) neurons, which show enhanced responsiveness to stress and even drive stress-like behaviors (Giardino et al., 2018; Fetterly et al., 2019), and that we have already shown to present with increased excitability in BNST GluN2D deletion models. In thinking about the place of GluN2D-NMDARs in responding to this, another study mentioned above from DuBois et al. (2015) has suggested that the activation of GluN2D-NMDARs, particularly in response to glutamate spillover/increased glutamatergic tone, may be key for driving an increase in the release of GABA from presynaptic neurons.

GluN2D-NMDARs may thus be essential at specific synapses and cell populations within the BNST for the maintenance of tonic inhibitory currents that aide in balancing out changes in the regional excitatory-inhibitory signaling balance. More extensive physiological studies would need to be conducted to investigate such hypotheses, but nevertheless, our work presented here provides an initial starting point from which to build out these future investigation, and more rigorously investigate the roles of both GluN2D-NMDARs and BNST excitatory activity in the context of depression and other mood disorders.

The GluN2D-NMDARs as a target for the treatment of depression: insights gleaned from BNST studies

In our efforts to further dissection the contribution of excitatory signaling to the pathophysiology and symptomology of depression, one of the main questions that developed over the course of this dissertation work was whether or not the dysfunction of GluN2D-NMDARs could contribute to the development of this disorder. By association, and as our findings appeared to indicate that a correlation between altered GluN2D-NMDAR-mediated excitatory signaling and depression does indeed exist, one of the other key questions that arouse was whether or not manipulation of these receptors could reverse these behavioral and physiological deficits and provide relief from the symptoms of chronic depression. To date, possibly the best and most well studied means of NMDAR antagonism to produce antidepressant-like effects is via the administration of ketamine at sub-anesthetic concentration (Berman et al., 2000; Krystal et al., 2019), with numerous studies emerging in recent years to suggest that these effects might be transduced specifically through ketamine's actions at select subtypes of NMDARs as opposed to their actions across all NMDARs throughout the brain (Zanos and Gould, 2018). The most prominent class of NMDARs tied to these effects, as mentioned above, have been those containing the GluN2B subunit, with several preclinical and clinical studies showing great promise for the use of GluN2B-NMDAR selective antagonist for the treatment and long term relief of the negative affective aspects of depression while avoiding the psychotomimetic effects often found to occur with the use of ketamine (Ibrahim et al., 2012; Louderback et al., 2013; Miller et al., 2014). These effects have been validated in rodent models across multiple studies, including those investigating the effects of GluN2B deletion or GluN2B antagonist administration on limbic structures implicated in depression pathology like the PFC (Miller et al., 2012) and the hippocampus (Graef et al., 2015) on the incidence of negative affective phenotyping or disruptions in synaptic

plasticity often observed in the disease state (Martella et al., 2018). In our own research group as well, we have generated compelling data showing that the selective deletion of GluN2B from the BNST in mice produces a robust antidepressant-like effect mimicking those of systemic ketamine administration prior to testing on the EZM and FST (Louderback et al., 2013), suggesting that GluN2B-NMDAR action within the BNST alone may be sufficient to influence aspects of depressive-like behavior.

Despite the initial success of many GluN2B selective antagonists in preclinical studies, the majority that have moved onto clinical trials have yet to produce a robust enough effect in patients to warrant full scale approval. While it is not outside the realm of possibility that more efficacious compounds may be developed in the future, the more robust expression of GluN2B throughout many cortical and subcortical structures in the adult brain suggests that off target effect of antagonists targeting these receptors may persist throughout patient populations, highlighting a need to continue investigating whether other means of NMDAR antagonism could produce the same antidepressant-like effects as ketamine and GluN2B selective compounds while avoiding the cognitive and dissociative effects that have been reported in select studies. As mentioned in a previous section, recent physiological evidence has indicated that ketamine appears to have an even higher selectivity for NMDARs containing the GluN2D subunit when compared to those containing GluN2B (Kotermanski and Johnson, 2009; Khlestova et al., 2016), and furthermore that GluN2B/GluN2D triheteromeric receptors also show a greater binding efficiency for ketamine when compared with other diheteromeric and triheteromeric NMDARs subtypes (Yi et al., 2019), suggesting the tantalizing prospect that the antidepressant effects of NMDAR antagonism may also be exerted in part through actions at these receptors as well. Pairing this with the highly restricted and reduced expression patterns of the GluN2D subunit in the adult brain (Monyer et al., 1994; Sheng et al., 1994; Wenzel et al., 1996), which may indicate a decreased likelihood of overt negative side effects following their manipulation, GluN2D-containing NMDARs appear to be a target of potentially great therapeutic value warranting further study at the level of circuit and behavioral neuroscience relevant to depression.

In light of these findings, as well as our own regarding the antidepressant effects of GluN2B deletion in the BNST (Louderback et al., 2013) and identification of prominent GluN2D expression within the region (**Fig.6**), we undertook these studies using the BNST as a proxy for investigating the effects of GluN2D-NMDAR manipulation within stress-responsive structures implicated within the overarching circuity and pathology of

depression. As demonstrated in Chapters 2 and 3, our results appear to bolster a growing consensus that GluN2D deletion can produce increases in negative affective behaviors (Ikeda et al., 1995; Yamamoto et al., 2017; Shelkar et al., 2019), specifically behaviors more in line with models of depression when the subunit is selectively deleted from the BNST (**Figs 5 & 14**). These findings are particularly interesting when considering that the deletion of GluN2B within the BNST produces the opposite behavioral effect: driving *increases* in antidepressant-like behaviors instead. Though these two findings do appear to support a clear role for the BNST in mediating behaviors salient to depression, the reason for the disparity between these two manipulations is unclear. Data from other studies of the GluN2D$^{-/-}$ line (including our own proteomic work in **Fig.12**), however, has suggested that the loss of GluN2D either from a specific region or the entire brain during development and onward with drive compensatory changes in NMDAR stoichiometry, typically resulting in increased incorporation of GluN2B in receptors (von Engelhardt et al., 2015; Brickley et al., 2003). As such, it is possible that some of the effects we have noted in both our behavioral studies and physiological analyses of changes in excitatory signaling in the BNST could be due to enhanced GluN2B diheteromer expression as opposed to the direct loss of GluN2D. Considering the relation that has been drawn between GluN2B manipulation and enhanced antidepressant behavior, the converse (i.e. overexpression of GluN2B-NMDARs) may disrupt excitatory signaling to produce increases in depressive-like behaviors instead. Studies in GluN2D$^{-/-}$ mice administered racemic or (S)-ketamine (the variant currently sold for clinical use) also showed no differences in anxiety or depressive-like behaviors in the TST or FST when compared with GluN2D$^{+/+}$ mice, suggesting that ketamine may still be transducing it's effects through the GluN2B-NMDARs (Ide et al., 2017). Intriguingly though, administration of (R)-ketamine, a enantiomer variant also found to produce antidepressant-like effects, did not produce a decrease in depressive behaviors in the GluN2D$^{-/-}$ animals but did attenuate these responses in wildtypes. While these results do little more than suggest that some of ketamine's actions may still yet be exerted through GluN2D-containing NMDARs, much work is needed to more directly prove a role for an antidepressant effect of acute manipulation of these receptors, particularly at the level of neuropharmacology, as it is yet unknown if the deletion of GluN2D that we induced in the BNST of adult GluN2D$^{flx/flx}$ mice could eventually lead to an upregulation in GluN2B expression. The insights we stand to gain via the study of GluN2D-NMDAR potentiation at the level of both the BNST and the whole brain may thus greatly assist in the

development of novel therapeutic means for treating depression in a more selective manner that avoids the problems facing current NMDAR antagonists on the market today.

Overall Conclusions and Final Remarks

Taken together, this dissertation aimed to examine the role of GluN2D-containing NMDARs in regulating excitatory signaling in the BNST in a region and cell-specific fashion, while also providing insight into the overarching implications for the manipulation of GluN2D-NMDAR signaling in the pathophysiology of depression. To achieve this, we utilized a combination of techniques to address the effects of GluN2D deletion, which had previously been shown to drive increases in negative affective behaviors, on BNST synaptic function, excitatory-inhibitory balance, and downstream behavioral output, with a particular focus on the stress-responsive population of BNST neurons expressing the neuropeptide CRF. Numerous studies have shown excitatory activity within the BNST and at CRF (+) cells to be altered in rodent models of depression, with the BNST additionally showing sensitivity to GluN2B-NMDAR antagonism associated with driving antidepressant effects, and that the manipulation of CRF (+) cells and CRF signaling at CRFR1 receptors can result in either the exacerbation or suppression of depressive-like behaviors. Despite this, both GluN2B and CRFR1 antagonists have failed to produce lasting and consistent relief from the symptoms of depression in clinical trials, suggesting a need for more selective means of regulating the changes that occur at the level of both modulatory and excitatory signaling systems in the disease state. Through our work's focus on the BNST, we identified the GluN2D-NMDARs as a physiologically relevant receptor population in regards to regulating the overt activity of the BNST and its participation in driving anxiety and depressive-like behavior and as highly abundant on BNST CRF (+) neurons.

While pan NMDAR antagonists such as ketamine have recently gained FDA approval for the widespread treatment of depression and other mood disorders, whether it achieves this effect through overarching NMDAR inhibition or at a specific NMDAR subtype is unknown. The prospect of NMDAR subtype specific antidepressant actions is intriguing, and could promise more effective treatment of this disorder across larger patient populations while avoiding the negative side effects of ketamine that may be associated with its broad off target activity at receptors and moieties aside from the NMDARs (Panos et al., 2018). Similarly, the

regulation of the CRF signaling system via CRFR1 antagonists may fail to success treat depression due to a greater need to regulation the cellular sources of CRF secretion and participation in stress-salient circuitry as opposed downstream receptors responsive to the neuropeptide alone. Our findings here suggest that the GluN2D-NMDARs serve as a unique receptor for controlling the activation of CRF (+) neurons and key structures implicated in affective behavioral output such as the BNST, and may thus represent a more efficacious target for treating excitatory and modulatory signaling dysfunction in depression. Future studies into the physiological function of GluN2D-NMDARs in other limbic regions and neural circuits implicated in driving anxiety and depressive-like behaviors and as expressing some level of GluN2D (Monyer et al., 1994; Sheng et al., 1994; Wenzel et al., 1996) will be important to build upon our work here and further support this notion. Ultimately though, we anticipate such work to provide key insights into excitatory signaling function, and in the development of novel and more highly efficacious treatment for depression and other affective disorders.

APPENDIX A

Evaluating the Expression Profile of the GluN2D Subunit across Additional BNST Neuropeptidergic Cell Populations

As outlined in Chapter 2, after being unable to determine any notable differences the excitatory physiological profiles of unspecified BNST neurons patched in acute *ex vivo* brain slices from GluN2D$^{+/+}$ and GluN2D$^{-/-}$ mice, we turned to the use of RNAscope *in* situ hybridization assays to identify potential specific populations of cells within the BNST that may more selectively express GluN2D-NMDARs. These initial efforts identified BNST cells positive for *Crh* mRNA transcripts as one such unique population that extensively co-labeled with *Grin2d* mRNA as well, suggesting to use the potential that the GluN2D-NMDARs within the BNST may be expressed on neurons that have been shown to contribute to the regulation of stress-related behaviors. Given the relatively low abundance of *Grin2d* (+) cells within the BNST overall, and their presence on ~75% of all BNST-CRF cells, we hypothesized that these receptors may be more preferentially expressed on neuropeptidergic cells within the BNST when compared with other interneurons spread throughout the region. To test this, we decided to run additional RNAscope assays using custom cDNA probes designed to mark mRNA transcripts for NPY (*Npy)* and pro-dynorphin (*Pdyn*), two neuropeptides that have been similarly implicated in drive stress-responsive behavior. NPY expressing neurons within the BNST in particular (as discussed above) have been shown to act in direct opposition to BNST CRF neurons in regards to regulating BNST inhibitory transmission (Kash and Winder, 2006), and their activation has been suggested to reduce anxiogenic behaviors as opposed to driving or exacerbating them (Desai et al., 2014; Ide et al., 2013; Tasan et al., 2009; Pleil et al., 2015). Several studies have also suggested that dynorphin (DYN) signaling and/or DYN (+) cell activity within the BNST may also be associated with its regulation of stress and affective behaviors, with examinations of prodynorphin transcript levels shown to increase in the BNST following forced swim stress (Chung et al. 2014) and more recent work showing that the activation of DYN (+) neurons within the BNST via a BLA circuit is able to drive increases in anxiety-like behavior that can be reversed via kappa opioid receptor deletion (Crowley et al., 2016). Systemic CRF administration has also been shown to enhance kappa opioid receptor binding in the extended amgydala and elevated extracelluar dynorphin concentrations in the CeA following intra-amygdalar injections (Land et al., 2008; Lam and Gianoulakis, 2011), further linking dynorphin not only to the stress response, but specifically to the CRF signaling system as well. Using the same procedures

and conditions for RNAscope listed above in Chapters 2 and 3, we tested for the presence and co-labeling of *Npy, Pdyn, Crh* and *Grin2d* probes in sections containing the anterior (dorsolateral) BNST taken from two separate cohorts of 3-4 C57Bl6/J male mice.

In regards to our studies of *Npy* transcript expression in the BNST (**Fig. 18A-E**), we were surprised to find that *Npy* and *Grin2d* showed a highly level of co-localization on cells within this region than we have had observed for *Crh* and *Grin2d* co-localization, with nearly ~90% of all *Npy* (+) cells showing co-labeling with *Grin2d* probes as well. Upon further analysis of the total number *Npy/Grin2d* (+) cells compared with those labeled for only *Npy* (+), we observed that this increased co-localization between the two transcript was highly significant as well (**Fig. 18D-E** *Npy* [+] only=9 cells, ~11.4% of total *Npy* [+] cells, *Npy+Grin2d* [+]=68 cells, ~86.1%, *Npy+Crh* [+]=0 cells, *Npy+Crh+Grin2d* [+]=2 cells, ~2.5%; $t[4]=17.73$, $p<0.0001$, unpaired t-test), suggesting that functional GluN2D-NMDARs may also be highly localized on this subset of BNST neurons as well. We also analyzed the co-localization patterns of *Npy* and *Crh* probes on across all *Grin2d* (+) cells in the BNST, as well as the co-localization of *Npy* and *Grin2d* on *Crh* (+) neurons in isolation as well (**Fig. 18A-B**). When examining total *Grin2d* (+) cells, we found that both *Grin2d/Npy* and *Grin2d/Crh* co-labeled cells compromised similar, but small, proportions of the total *Grin2d* (+) cells across samples (**Fig. 18A** *Grin2d* [+] cells=644, ~83.3% of total *Grin2d* labeled cells, *Grin2d+Crh* [+] cells=59, ~7.6%, *Grin2d+Npy* [+]=68, ~8.8%, *Grin2d+Crh+Npy* [+] cells=2, ~0.26%). We also noticed little to no co-labeling of cells for both *Npy* or *Crh* transcripts, as well as little to no co-labeling of cells for all three transcripts in combination. This we also observed this pattern of labeling when examining the *Crh* (+) cells on their own, however we found very similar patterns of *Crh/Grin2d* co-localization once again, bolstering our previous *in situ* findings presented above in Chpater 2 (**Fig. 18B** *Crh* [+] cells=18, ~22.8% of total *Crh* [+] cells, *Crh+Grin2d* [+] cells=59, ~74.7%, *Crh+Npy* [+] cells=0, *Crh+Npy+Grin2d* [+] cells=2, ~2.5%). These results appear to indicate the BNST CRF (+) and NPY (+) cells exist as largely separate populations, with little to no cells within the anterior (dorsolateral) region of the nucleus at least showing co-expression of the two neuropeptides.

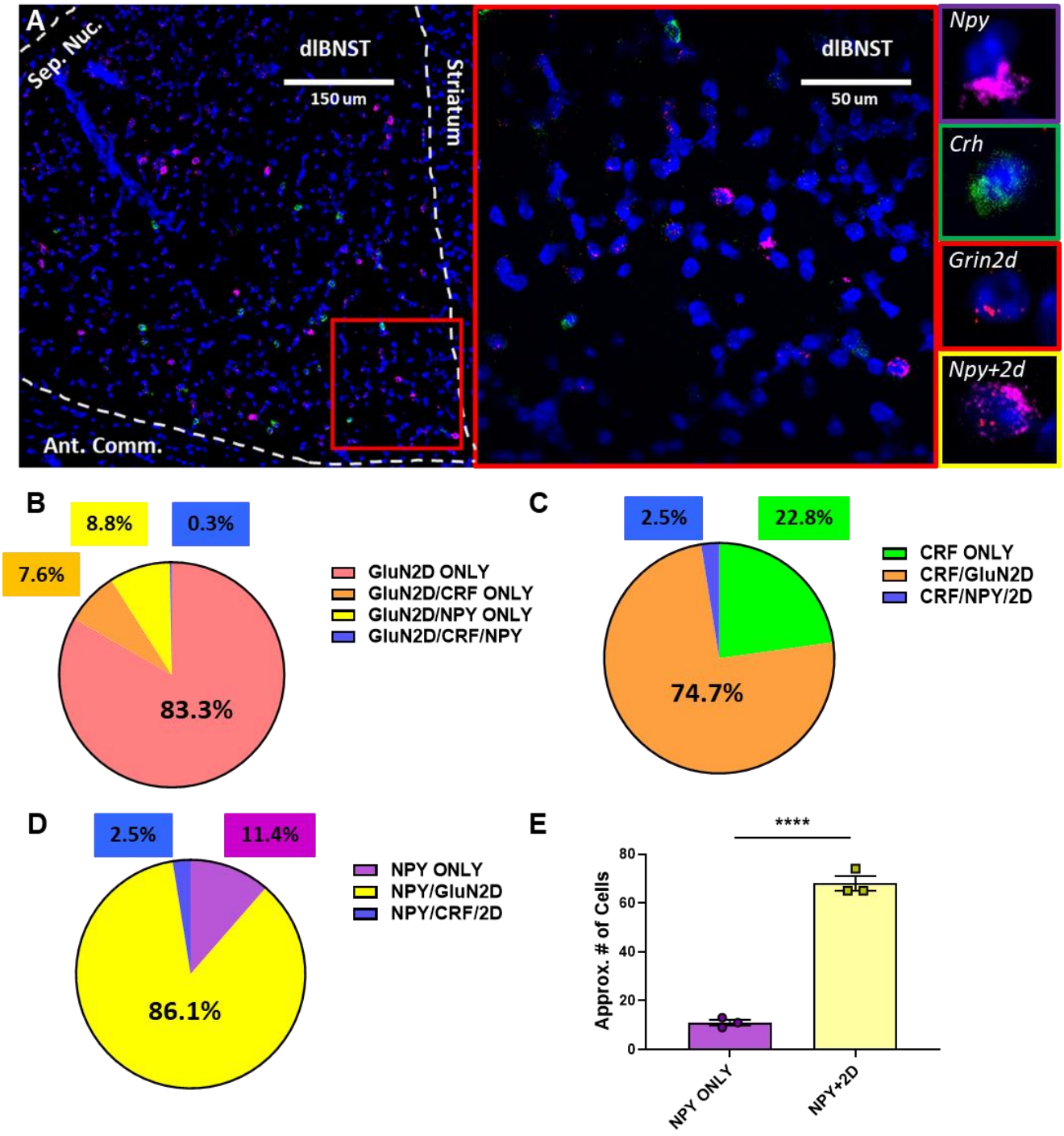


Figure 18. Grin2d mRNA robustly co-localizes with neuropeptide Y (NPY) transcripts in dlBNST.
(A) Representative image of the dlBNST (outlined via dashed white lines) at 20x magnification after undergoing RNAscope® (top left), showing individual cells with DAPI (blue) counterstained nuclei labeled for *Grin2d* (red), *Crh* (green) or *Npy* (magenta) mRNA transcripts. The red boxed area (top right) shows a representative high magnification image of the dlBNST at 63x, along with sample images to the far right of cells labeled for each transcript of interest, and *Npy* and *Grin2d* transcripts co-localized to the same cell. **(B)** Summary graph showing the portion of total counted *Grin2d* (+) dlBNST cells (left & right dlBNST, N = 3 C57BL/6J mice) labeled for the *Crh* or *Npy* transcripts alone or in combination. *Grin2d/Crh* and *Grin2d/Npy* labeled cells comprised a similar,

but low percentage of the total *Grin2d* (+) population within the BNST. **(C)** Summary graph of the *Crh* (+) cell populations in the dlBNST. As a portion of these cells, those co-labeled for *Grin2d* are shown to make up a greater overall percentage once again, while a very low portion of *Crh* labeled cells co-localized with *Npy* transcripts. **(D)** Summary graph of *Npy* (+) cell populations co-labeled for *Grin2d* and *Crh*. *Npy/Grin2d* (+) cells comprised a large majority of total *Npy* (+) cells, while co-labeling of *Npy* and *Crh* was again shown to be very low. **(E)** Additional comparison of cells labeled for the *Npy* transcript alone with those labeled for *Npy* and *Grin2d* in combination shows the number of co-labeled cells to be significantly higher ($p<0.0001$). All data are presented as means ± SEM with individual data points overlain. $^{****}p\leq0.0001$.

The prospect of GluN2D-NMDAR expression on BNST NPY (+) cells is both exciting and highly interesting when considering the oppositional role that the two peptidergic populations appear to play both in regulation BNST excitatory-inhibitory activity and in regards to how this output may influence changes in downstream behavior. While we were interested to follow up on this, and perform similar functional analysis of NPY (+) cells using an approach similar to the one we developed for isolating and recording from BNST CRF (+) cells in both GluN2D$^{+/+}$ and GluN2D$^{-/-}$ mice, we decided to postpone such studies due in part to the burden of generating a new triple transgenic line, as well as conflicting reports from collaborators regarding the integrity/reliability of current commercially available Npy-Cre mouse lines. Despite this, there are still a number of potential future directions that can currently be undertaken to examine the effects of GluN2D manipulation or deletion on BNST NPY (+) cell function without the immediate need for electrophysiological approaches, including the use of combine behavior and post hoc ISH studies for *Fos* labeling in these neurons, as well as further experiments within this same vein that can incorporate an additional level of pharmacology, using Y1 or Y2 agonists or antagonists injected systemic prior to behavior challenge to see if the regulation of NPY signaling alters *Npy* and *Fos* transcript co-localization in the BNST. Additionally, the generation of more robust reporter lines for NPY (+) cells that also allow for the incorporation of optogenetic manipulation of these neurons may open the door to more in depth functional studies within the lab (Daigle et al., 2018). It would also be interesting to add the analysis of BNST CRF (+) function into these studies as well, in order to see if changes in the excitability or excitatory profile on these cells as observed in BNST, GluN2D deleted animals produces compensatory changes in the function of BNST NPY (+) cells as well.

While a significant level of co-expression appeared to be indicated for GluN2D and NPY in this first set of assays, the overlap we observed for between GluN2D subunit and prodynorphin transcripts within the BNST was not as robust (**Fig. 19A**). We did however notice a slight overlap of the two, with ~30-35% of total *Pdyn* (+) cells showing *Grin2d* transcript co-localization (**Fig. 19B**, lower; *Pdyn* [+] cells=157, ~47.6% of total *Pdyn* [+]

cells, *Pdyn+Grin2d* [+] cells=77, ~23.4%, *Pdyn+Crh* [+] cells=55, ~16.6%, *Pdyn+Crh+Grin2d* [+] cells=41, ~12.4%). This appeared to indicate that at least a subset of DYN (+) cells within the BNST may express GluN2D-NMDARs. Interestingly, when we examined co-localization of transcript when looking at the *Grin2d* (+) cells and *Crh* (+) cells in isolation, we noticed several interesting features of how these population percentiles broke down. In regards to the total *Grin2d* (+) cells, *Pdyn+Grin2d* (+) cells were found to represent a similar, but low, proportion of these labeled neurons to *Crh+Grin2d* (+) cells, showing a slight trend for GluN2D transcript distribution across neuropeptidergic populations (**Fig. 19B**, upper; *Grin2d* [+] cells=726, ~81.4% of total *Grin2d* [+] cells, *Grin2d+Crh* [+] cells=48, ~5.4%, *Grin2d+Pdyn* [+] cells=77, ~8.7%, *Grin2d+Pdyn+Crh* [+] cells=41, ~4.6%). For total *Crh* (+) cells, however, we noticed that nearly 50% of these neurons also exhibited co-labeling with *Pdyn* transcripts (**Fig. 19B**, middle; *Crh* [+] cells=42, ~22.9% of total *Crh* [+] cells, *Crh+Grin2d* [+] cells=48, ~25.7%, *Crh+Pdyn* [+] cells=55, ~29.5%, *Crh+Pdyn+Grin2d* [+] cells=41, ~22.0%). Similarly levels of *Crh* and *Grin2d* levels (though slightly lower) were also observed across these metrics, again bolstering this past observation. However, the high overlap of *Crh* and *Pdyn* was intriguing, and perhaps unsurprising, considering the strong link that has been shown between the two signaling systems.

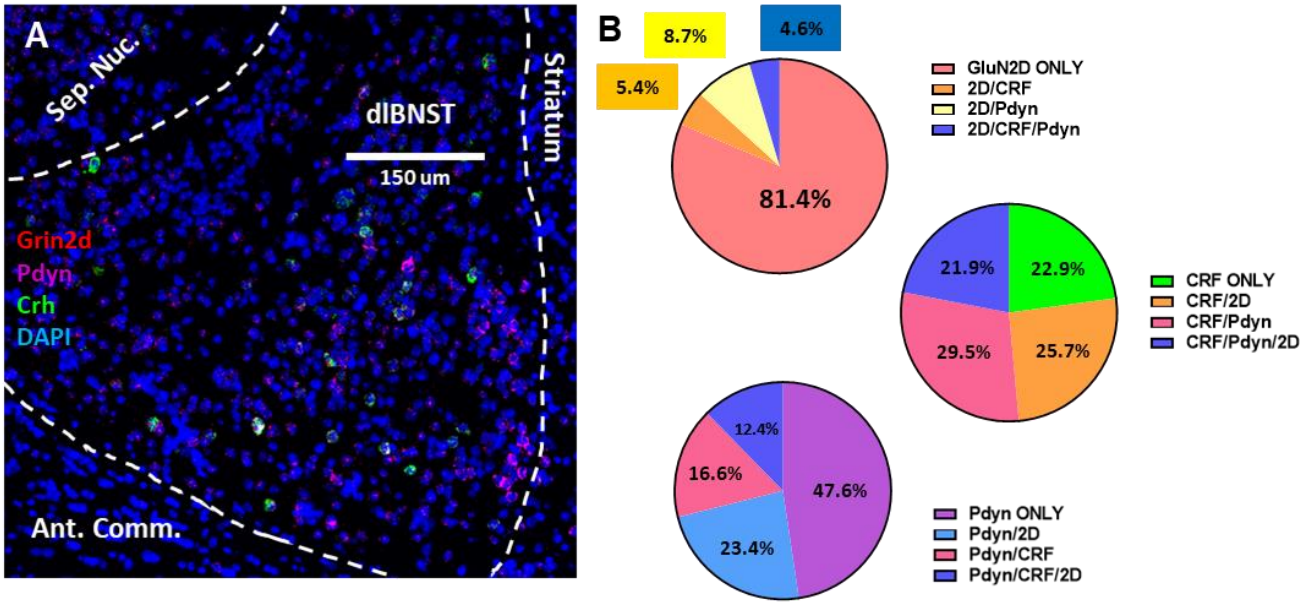


Figure 19. Grin2d mRNA shows modest co-localization with prodynorphin (Pdyn) transcripts in dlBNST. **(A)** Representative image of the dlBNST (outlined via dashed white lines) at 20x magnification after undergoing RNAscope® (top left), showing individual cells with DAPI (blue) counterstained nuclei labeled for *Grin2d* (red), *Crh* (green) or *Pdyn* (magenta) mRNA transcripts. **(B)** Summary graphs showing the portion of total counted *Grin2d* (+) dlBNST cells (left & right dlBNST, N = 3 C57BL/6J mice) labeled for the *Crh* or *Pdyn* transcripts alone or in combination (upper), *Crh* (+) cells labeled for the *Grin2d* or *Pdyn* transcripts alone or in combination (middle), and *Pdyn* (+) cells labeled for the *Grin2d* or *Crh* alone or in combination (bottom).

The prospect of co-release of dynorphin and CRF from the same neuron, is quite interesting and may warrant further investigation. Additionally, although the extent of co-localization we observed for *Pdyn* and *Grin2d* was lower than that observed with both *Crh* and *Npy*, our results here suggest that the effects of GluN2D-NMDAR-mediated excitatory signaling at these cells could be occurring to a limited degree. Examination of whether or not the receptors may be restricted to a subset of DYN (+) BNST neurons that participates in a specific circuit would be potentially exciting to pursue as well, especially when considering the role of the BLA inputs to BNST DYN (+) in regards to the regulation of stress related behavior outlined in Crowley et al. (2016). If GluN2D-NMDARs are more preferentially expressed on this DYN (+) population when compared with others throughout the region, which could be readily tested via anatomical tracing and RNAscope using Pdyn-Cre transgenic mice and stereotaxic injections of Cre-dependent retrograde tracers into the BNST (or anterograde tracers in the BLA of C57 mice), a finding such as this may serve to further implicate the GluN2D-NMDARs as a crucial receptor class for regulating the excitatory function and response to glutamate of other classes of BNST neurons involved in processing stress salient information.

Taken together, the findings from these additional experiment suggest other populations of specialized BNST neurons may also show selective expression of the GluN2D-NMDARs, and suggest that these receptors also be more preferentially sequestered on neuropeptidergic BNST interneurons as well. More extensive qualitative labeling studies of the BNST and its highly heterogenous cell populations will be required to prove this, however. Additionally, studies of the distribution and expression patterns of GluN2D protein and/or *Grin2d* transcripts at other neuropeptidergic cell populations expressed throughout the brain (particularly in relevant extended amygdalar or limbic structures) may also provide additional insights into the potential role of these receptors within the adult brain, particularly into their apparent contribution to the regulation of affective behavior.

APPENDIX B

Examining Changes in Grin2d Gene Expression in Response to Stress

Several preclinical studies have indicated that apart from an apparent elevation in glutamate concentrations that is often seen at the level of both animal models and patients suffering from depression (Chandley et al., 2014; Graybeal et al., 2012; Sanacora et al., 2004), rodent models of depression and/or chronic stress also show alteration in NMDA receptor subunit gene expression. Indeed, GluN1, GluN2A and GluN2B levels (both transcriptionally and translationally) have been found to be elevated in regions such as the ventral hippocampus and prefrontal cortex in rats undergoing chronic stress paradigm (Calabrese et al., 2012), while postmortem studies of the brains of MDD patients have shown similar findings, particularly in regards to GluN2B and GluN2C levels within the locus coeruleus (Karolewicz et al., 2005; Chandley et al., 2014). Treatment with typical antidepressants, interestingly, has been shown to normalized the increased expression for these subunits in rodents (Calabrese et al., 2012), suggesting that alterations in the transcription and translation of NMDAR subunits may occur in response to affective stimuli salient to depression. Few studies however appear to have investigated whether or not these changes can occur at the level of GluN2D gene expression under similar conditions, and indeed, little is known about the dynamics of GluN2D transcriptional/translational regulation both in response to acute stressors or in disease states that may arise from maladaptive responses to chronic stress.

Thus, in this next small subset of experiments, we sought to gain insight into whether or not GluN2D gene expression was impacted at all under such conditions, particularly in several key limbic structures that have been implicated in the stress response, and that are known to shown heightened levels of GluN2D expression overall in the adult brain. Using restraint as our acute stressor, we exposed a small cohort of female mice to 60 minutes of restrain tube stress, which we have previously shown to produce robust changes in gene expression at the level of the BNST (Fetterly et al., 2019), and then waiting an additional 60 minutes afterwards before extracting brain and taking tissue punches of these aforementioned brain regions for processing and RNA extraction (**Fig. 20A**). Samples consisted of two, 0.8mm punches taken bilaterally from each structure per animals (no pooling of tissue was performed), and following extraction and purification, all RNA samples were tested for their overall concentration, and then diluted in preparation for well plate loading and real time

quantitative PCR analysis (qRT-PCR). Samples were loaded in replicates, and custom Taqman cDNA probes designed for Grin2d and Gapdh (internal control/house-keeping gene) were mixed in with each sample prior to amplification. Changes in gene expression were then performed using the ΔΔCt method to compare the normalized expression of our gene of interest (*Grin2d*) in stressed animals to that of control animals that underwent no restraint stress.

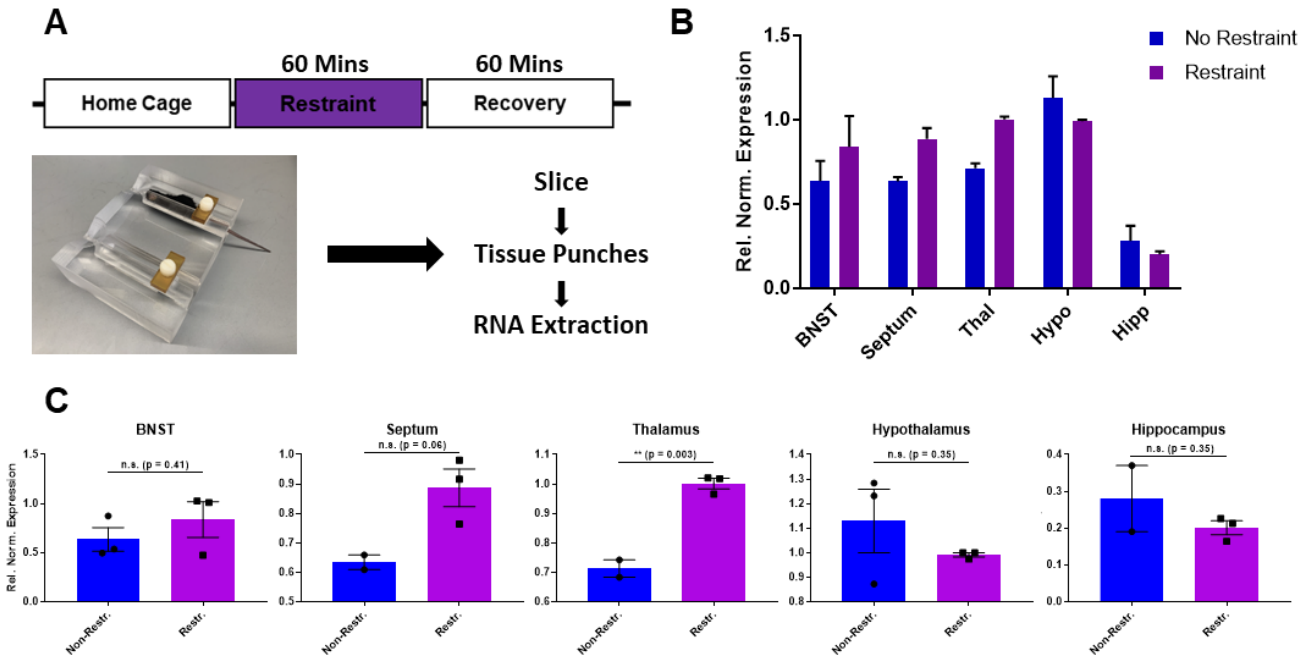


Figure 20. Acute restraint stress may produce alterations in Grin2d gene expression across multiple brain regions. (A) Experimental paradigm for restraint stress procedure, as well as timing for tissue preparation and RNA extraction following stress exposure. An inset showing the restraint stress apparatus is shown on the left. **(B)** Grouped data showing changes in the relative, normalized expression of *Grin2d* across five separate brain regions in female mice following restraint stress exposure (purple), and in control females that underwent no restraint stress (blue). **(C)** Region by region comparisons of changes in relative, normalized *Grin2d* gene expression in restrained (n=3 females) and unrestrained (n=3 females) mice. Slight trends indicative of increased expression of *Grin2d* in response to stress were noted in the BNST (p=0.41) and septum (p=0.06), while a significant increase in expression compared to unstressed conditions were noted in the thalamus (p=0.003). Conversely, trends indicating a decrease in *Grin2d* expression were noted in both the hypothalamus (p=0.35) and hippocampus (p=0.35). All data presented as means ± SEM overlain with individual points. $^{**}p \leq 0.01$, n.s. = not significant.

Even in this smaller cohort of animals, we observed noticeable changes in the expression profile of the *Grin2d* gene across all five regions in response to acute restraint stress (**Fig. 20B**). Indeed, when we broke down the data by region to observe changes in relative normalized expression under either stress or unstressed conditions, both the BNST and the septum revealed non-significant increases in overall *Grin2d* expression when compared with control levels of expression, while expression levels in the thalamus of stressed animals showed

a significant increase relative to unstressed animals (**Fig. 20C**, BNST: Non-Restr.=0.64 rel. norm. expression, Restr.=0.84, t[4]=0.93, p=0.406; Septum: Non-Restr.=0.63, Restr.=0.90, t[3]=2.98, p=0.058; Thal.: Non-Restr.=0.71, Restr.=1.0, t[3]=9.01, p=0.003, unpaired t-tests). By contrast, in samples from both the hypothalamus and hippocampus, we observed trends indicating a potential decrease in *Grin2d* gene expression in response to restraint stress when compared with non-stressed controls (Hypo.: Non-Restr.=1.13, Restr.=0.99, t[4]=1.06, p=0.347; Hipp.: Non-Restr.=0.28, Restr.=0.20, t[3]=1.11, p=0.346, unpaired t-tests). This may suggest that *Grin2d* expression is uniquely regulated across different stress-responsive structures in response to negative stimuli such as restraint stress. However, while promising, these studies are still underpowered, and would require replication in order to more definitively provide substantial data to support such claims. Additionally, while these studies were initially performed using female mice due to availability, given the known differences in stress-responsive behavior and physiological adaptations that is often observed in females when compared with male mice, particularly in regards to restraint stress (Babb et al., 2013), it would also be prudent to replicate these studies in male mice in order to confirm that these alterations in *Grin2d* gene expression is generalized across sexes, and not linked to one sex more preferentially over the other.

Due to the aim of the work presented in this dissertation turning away from any interest in gene expression profile in favor of examining the physiological effect of GluN2D manipulation within the BNST alone, these experiments were not expanded upon much further than what is presented above. However, given previous findings that NMDAR subunits, as well as other synaptic proteins, closely associated with GluN2B are notably altered in their expression profiles within the BNST in response to chronic intermittent ethanol exposure (CIE), a paradigm used to model chronic ethanol abuse and withdrawal, it is possible to hypothesize that the GluN2D subunit may be altered in response to other stressors such as this as well (Wills et al., 2017). Indeed, previous proteomic data from our lab has indicated that the GluN2D protein is associated with the GluN2B subunit in the BNST (**Fig. 21**), and thus may be incorporated into BNST NMDARs along with GluN2B to form a pool of triheteromeric receptors, as discussed in the Conclusions section above. The potential impact of such receptors on the physiology, particularly in regards to synaptic function, within the BNST may thus warrant further study of specific changes in the expression profile of *Grin2d* in response to other stressors modeling aspects of depression that may yield information more germane to the research presented here, or aspects of addictive disorders, particularly ethanol abuse disorders. The prospect of these latter investigations will be

discussed briefly in the final section below, and may hopefully hold some promise for an interesting new direction of research into GluN2D-NMDAR function within the BNST in models of addictive disorders.

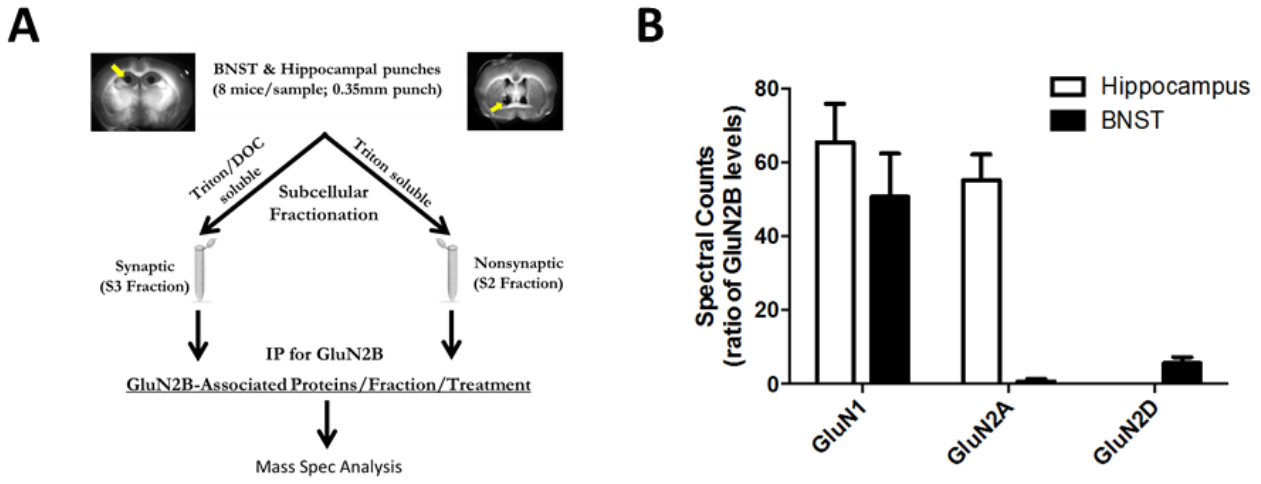


Figure 21. Proteomic analysis suggests GluN2B/2D association in BNST. (A) Outline of workflow for the collection and processing of mouse BNST and hippocampal tissue for fractionation and immunoprecipitation of GluN2B prior to mass spectrometry analysis for other associated proteins. (B) Spectral counts of synaptic fragments, showing relative levels of GluN1, GluN2A and GluN2D associated with GluN2B in mouse BNST and hippocampus; GluN2D is noticeably present in BNST but not hippocampus of adult animals. Unpublished data courtesy of Tiffany Wills and A.J. Baucum.

APPENDIX C

Implications for GluN2D-containing NMDARs in the Physiological Changes in BNST Synaptic Function in Response to Ethanol Exposure and Withdrawal

Several lines of research into the exact stoichiometry of NMDARs containing the GluN2D subunit *in vivo* have all strongly suggested that it is most likely incorporated into triheteromeric receptors that also contain the GluN2B subunit (Brickley et al., 2003; Harney et al., 2008; Brothwell et al., 2008; Engelhardt et al., 2015; DuBois et al. ,2015; Swanger et al. 2015). This appears to be thought to be particularly true in the case of GABAergic interneurons expressing GluN2D, which account for the vast majority of GluN2D positive cell populations in the adult brain (Monyer et al., 1994; Sheng et al., 1994; Zhang et al., 2014; Kotermanski and Johnson, 2009). When considering these finding in regards to the BNST, a region predominantly consisting of GABAergic interneurons, as well as our own data shown above in **Fig. 21**, it seems highly likely that the majority of GluN2D-containing NMDARs within the BNST also contain GluN2B. Indeed, the robust expression profile of GluN2B indicated within the BNST (Monyer et al., 1994; Sheng et al., 1994; Wenzel et al., 1996), along with additional evidence from our lab showing a prominent disruption of regional plasticity and excitatory synaptic function following GluN2B selective deletion or pharmacological inhibition in the BNST indicates that GluN2B-NMDARs play an essential role in maintaining the physiological integrity of the region (Wills et al., 2012). This seems to be particularly true in regards to the BNST's response to ethanol (Wills et al, 2012; Carzoli et al., 2019). Independent of regional expression, the GluN2B-NMDARs seem to display a particular sensitivity to chronic ethanol exposure, with several studies showing the expression of GluN2B to increase in response to such conditions (Follesa and Ticku, 1995; Narita et al., 2000; Wills et al., 2012). Additionally, chronic ethanol use and withdrawal has been noted to produce a recruitment of GluN2B-NMDARs to the synapse from extra-synaptic locations (Clapp et al., 2010; Wills et al., 2012), and it is unsurprising that it has also been demonstrated that such changes in GluN2B-NMDAR clustering at the synapse can have a significant impact on the presentation of multiple different forms of LTD and LTP (Bartlett et al., 2007; Liu et al., 2004). Considering that the current literature on GluN2D-NMDAR localization at the synapse has also suggested that these receptors may show a higher preference to cluster at extra-synaptic sites (Lovovaya et al, 2004; Morris et al., 2018; Brickley et al., 2003; Papouin and Oliet, 2014), while also demonstrating the ability to move into the synapse (Harney et al., 2008; Volianskis et al., 2015), we were interested in investigating whether or not changes in the expression of

GluN2D would produce any significant impact on BNST synaptic function in response to acute withdrawal from chronic ethanol exposure.

To test this, we performed an initial test replicating the parameters and testing conditions for such physiological studies following chronic intermittent ethanol exposure (CIE) in GluN2D$^{+/+}$ and GluN2D$^{-/-}$ male mice as outlined in Wills et al. (2012) and Kash et al. (2009), which developed an effective protocol for interrogating NMDAR-mediated changes in synaptic function following CIE. Mice underwent two round of CIE consisting of four 16 hour bouts of volatilized ethanol vapor exposure (20.3±0.2 mg/L) followed by four 8 hour withdrawal periods back in their home cages, separated by 3 days of complete withdrawal from all ethanol exposure (**Fig. 22A**). To achieve reliably high blood alcohol levels in the mice during CIE, animals were injected with 1 mM/kg of pyrazole to prevent ethanol metabolism, and a priming dose of 0.8 g/kg of ethanol. Control animals were administered pyrazole in a similar manner, but did not receive ethanol priming doses or undergo exposure to volatilized ethanol like the CIE animals, instead being placed in a chamber where they were exposed to volatilized water vapor. On the final day of vapor chamber exposure, animals were sacrificed 4-5 hours following their removal in order to allow for acute withdrawal to set in, and the brain prepared for slice electrophysiology as outlined above in Chapters 2 or 3. Similar protocols were also followed to induce LTP within acute *ex vivo* BNST slices while performing field potential recordings.

For the purposes of these initial experiments, our analysis primarily focused on examining the ethanol exposed group in order to determine any salient changes to BNST plasticity in the GluN2D$^{-/-}$ mice following acute ethanol withdrawal in comparison with the GluN2D$^{+/+}$ mice. Intriguingly, following the application of a tetanizing stimulus (**Fig. 22B**, arrow insert), slices from the ethanol withdrawn GluN2D$^{-/-}$ mice show a robust potentiation of LTP when compared with the responses measured from GluN2D$^{+/+}$ ethanol withdrawn mice (**Fig. 22B**). Indeed, while LTP was still induced within the GluN2D$^{+/+}$ slices, the potentiation observed within GluN2D$^{-/-}$ slices demonstrated a nearly 50% increase relative to baseline over the increase over baseline observed for the wildtypes when examining the amplitude of these responses up to 10 minutes post-tetanus (**Fig. 22C**, [left] % increase over baseline 2D$^{+/+}$: 145.3±7.4%, 2D$^{-/-}$: 185.0±12.9%, t[9]=2.78, p=0.021, unpaired t-test). This effect, however, was only notable early on post-tetanus, as an analysis of the difference in the total amplitude of responses for both the GluN2D$^{+/+}$ and GluN2D$^{-/-}$ during the final 10 minutes of the hour long recording post-tetanus, no significant difference could be observed between either group (**Fig.22**, [right] % increase over

baseline $2D^{+/+}$: 115.7±8.0%, $2D^{-/-}$: 144.6±11.7%, t[9]=2.10, p=0.066, unpaired t-test). The total amplitude of response measured for the $GluN2D^{-/-}$ was elevated at this time point when compared with the $GluN2D^{+/+}$, though, suggesting a potential trend to an overall increase in the strength of potentiation for the duration of the 60 minute experiment that may be pulled out further upon replication.

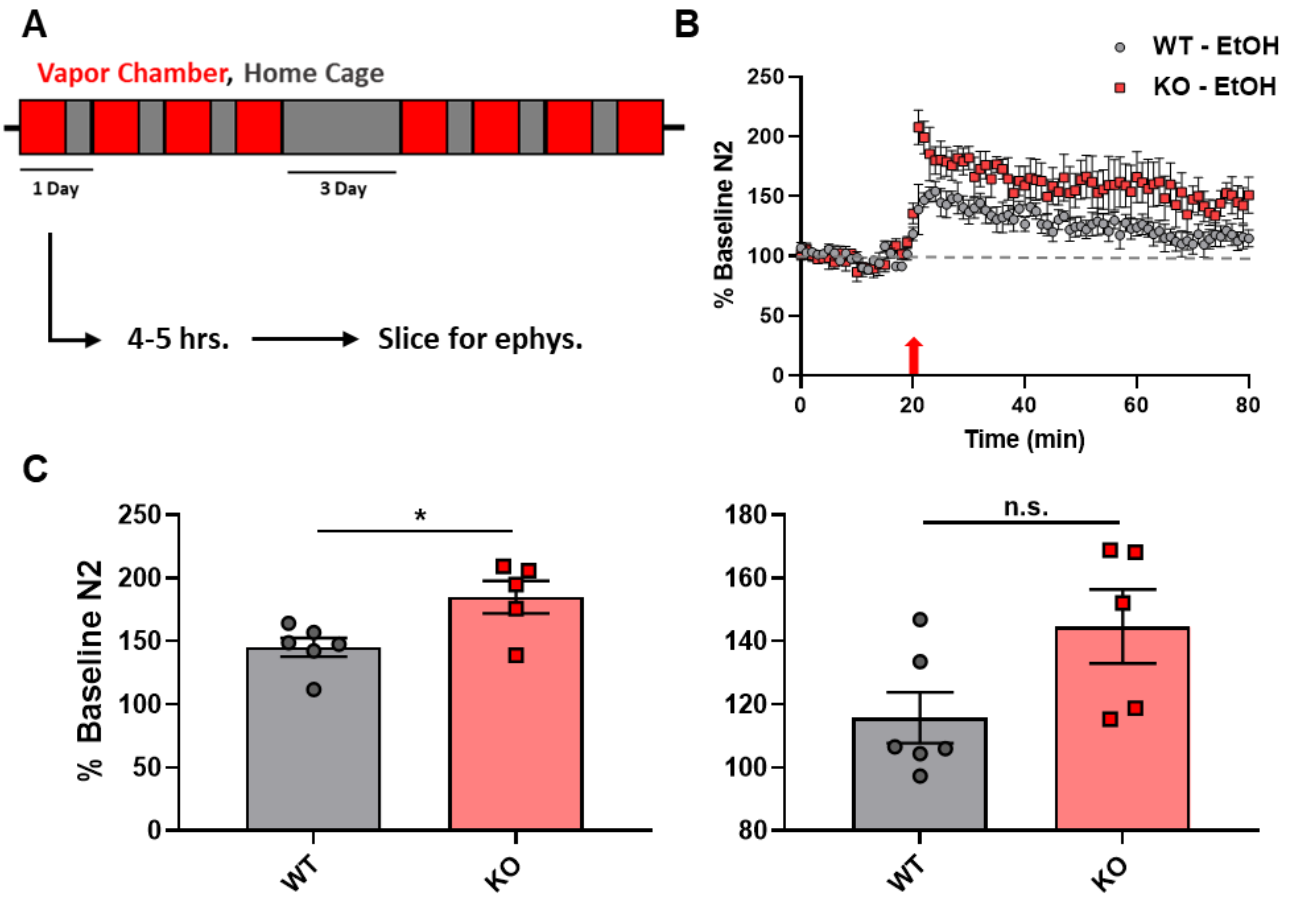


Figure 22. Global deletion of GluN2D may produce a prominent increase in BNST synaptic potentiation in response to withdrawal from chronic ethanol exposure. (A) Schematic of CIE paradigm utilized in these studies, including parameters for slice preparation. **(B)** Averaged time courses of excitatory post-synaptic field potentials recorded from the dlBNST of post-CIE $2D^{+/+}$ and $2D^{-/-}$ acute, *ex vivo* brain slices after high frequency stimulation (arrow; two, 1-s trains at 100 Hz). **(C)** Summary graph of the averaged field potential responses 0-10 mins after tetanus and **(D)** 51-60 mins after tetanus. A significant difference in the amplitude of responses recorded from post-CIE $2D^{-/-}$ slices was noted for up to 10 mins following tetanus when compared with post-CID $2D^{+/+}$ controls (p=0.021), but not during the final 10 mins of recording (p=0.066). Data presented across summary graphs as means ± SEM with individual points overlain ($n_{2D+/+}$ = 6 slices from $N_{2D+/+}$ = 3 mice, $n_{2D-/-}$ = 5 slices, from $N_{2D-/-}$ = 4 mice). $^{*}p \leq 0.05$, n.s. = not significant.

Much as in the case of the data presented in Appendix B, the results of our finding in this small series of experiments show potential promise of a more interesting effect occurring in the BNST of rodents following

acute ethanol withdrawal implicating GluN2D-NMDARs, but additional experiments will be required to flush this effect out more prominently. Indeed, as the effects of ethanol exposure on BNST GluN2D-NMDAR synaptic physiology were not a focus of the dissertation research presented here, these experiments represented an attempt to examine a potentially interested tangential avenue of research related to the primary topic of interest of this work: the role of GluN2D-mediated excitatory signaling in the BNST as related to the pathophysiology of depression. Ethanol abuse disorders, and more specifically the withdrawal from chronic ethanol abuse, often go hand in hand with anxiety-related and depressive mood disorder, with several clinical datasets showing a high comorbidity of depression in patients suffering from chronic alcohol abuse (Grant et al., 2015; Fergusson et al., 2011). Considering the evidence gained from the work presented above indicating a potential role for the GluN2D-NMDARs in modulation BNST function and behavioral responses correlated with depression, as well as other ancillary findings suggesting the possible incorporation of GluN2D subunits into GluN2B/D triheteromers in the BNST, investigating whether or not this receptor population plays an intersectional role in the BNST's response to ethanol use and withdrawal that may factor into the affective changes noted to accompany chronic ethanol abuse may be a fruitful area for future investigations. In addition to attempts to replication the work shown above, a localization of this effect to specifically within the BNST will also be prudent in order to determine whether the effects noted are indeed due to the result of GluN2D deletion alone or the likely compensatory increase in GluN2B expression levels that we have shown to occur in the constitutive knockout line used in these initial CIE experiments (**Fig. 12**). Use of the GluN2D conditional knockout line developed for this dissertation work would readily allow for the performance of these studies, and would also lend itself to additional studies on the effects of chronic ethanol use on BNST function and associated affective behavioral changes upon withdrawal. Apart from CIE, more translational relevant experiments could be performed using the ethanol two bottle choice paradigm, a 6 week long task that presents rodent with a choice between a sipper bottle containing a ramping concentration of ethanol (3%→7%→10%) and a bottle containing water to drink from *ad libitum* used to measure ethanol preference and non-contingent drinking behavior. Animals developing a strong preference for ethanol could be submitted to a forced abstinence period following chronic drinking and then run across several behavioral task examining anxiety and depressive-like behaviors in order to determine whether GluN2D deletion from the BNST of adult mice could contribute to an exacerbation of negative affective (and more specifically, depressive) phenotypes, as observed in our work in Chapter 3. Several reports from our

lab have already established a potential link between changes in BNST function and increases in such behaviors following abstinence (Centanni et al., 2019, Vranjkovic et al., 2018) particularly in regards to changes in CRF signaling and NMDAR-mediated synaptic physiology (Silberman et al., 2013; Silberman and Winder, 2013; Kash et al., 2009; Vranjkovic et al., 2018). These effects as related to BNST NMDAR function following ethanol withdrawal specifically have been correlated with increases in anxiety-like behaviors in rodents (Kash et al., 2009), and demonstrated that the physiological changes observed in the BNST were most likely driven by increased GluN2B-containing NMDAR levels. Interestingly as well, recent reports from the Heilig group have also suggested that GluN2D gene expression is *decreased* significantly in the brains of rats that develop a strong ethanol preference undergoing withdrawal when compared with control or non-ethanol preferring animals (Augier et al., 2018), further indicating a potential link between GluN2D expression/containing-NMDAR function and the effects of ethanol. Collectively, these previous findings suggest that the transposition of our work presented in this dissertation aimed at understanding BNST GluN2D-NMDAR function under basal conditions to future studies under ethanol abuse or withdrawal conditions may very likely show a role for this subunit in these previously reported physiological and behavioral changes. The identification of GluN2D-NMDAR involvement in the ethanol mediated effects at the BNST may also suggest an exciting new means by which NMDAR function can be selectively targeted at a less prominent and more restricted receptor population for the potential treatment of alcohol abuse disorders.

REFERENCES

Aand A, Jones SE, Lowe M, Karne H, Koirala P (2019): Resting state functional connectivity of dorsal raphe nucleus and ventral tegmental area in medication-free young adults with major depression. *Front. Psychiatry.* 9: 765.

Admon R, Kaiser RH, Dillon DG, Beltzer M, Goer F, Olson DP, Vitaliano G, Pizzagalli DA (2017): Dopaminergic enhancement of striatal response to reward in major depression. *Am. J. Psychiatry.* 174: 378-386.

Akazawa C, Shigemoto R, Bessho Y, Nakanishi S, Mizuno N (1994): Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J. Comp. Neurol.* 347: 150–160.

Albert PR and Benkelfat C (2013): The neurobiology of depression – revisiting the serotonin hypothesis: genetic, epigenetic and clinical studies. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368: 20120535.

Alexander B, Warner-Schmidt J, Eriksson T, Tamminga C, Arango-Lievano M, Ghose S et al (2010): Reversal of depressed behaviors in mice by p11 gene therapy in the nucleus accumbens. *Sci. Transl. Med.* 2: 54ra76.

Alheid GF (2003): Extended amygdala and basal forebrain. *Ann. N.Y. Acad. Sci.* 985: 185–205.

American Psychiatric Association (2013): *Diagnostic and Statistical Manual of Mental Disorders (5th ed.)*. Arlington, VA: American Psychiatric Association.

Amico JA, Mantella RC, Vollmer RR, Li X (2004): Anxiety and stress responses in female oxytocin deficient mice. *J. Neuroendocrinol.* 16: 319-324.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999): The role of corticotropin-releasing factor in depression and anxiety disorders. *J. Endocrinol.* 160: 1-12.

Asberg M, Betilsson L, Martensson B, Scalia-Tomba GP, Ythoren P, Traskman L (1984): CSF monoamine metabolites in melancholia. *Acta. Psychiatr. Scand.* 69: 201-219.

Asok A, Draper A, Hoffman AF, Schulkin J, Lupica CR, Rosen JB (2018): Optogenetic silencing of a corticotropin-releasing factor pathway from the central amygdala to the bed nucleus of the stria terminalis disrupts sustained fear. *Mol. Psychiatry.* 23: 914-922.

Augier E, Barbier E, Dulman RS, Licheri V, Augier G, Domi E et al (2018): A molecular mechanism for choosing alcohol over an alternative reward. *Science.* 360: 1321-1326.

Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF et al (2011): NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature.* 475: 91–95.

Autry AE, Monteggia LM (2012): Brain-derived neurotrophic factor and neuropsychiatric disorders. *Pharmacol. Rev.* 64: 238–258.

Avery SN, Clauss JA, Blackford JU (2016): The Human BNST: Functional Role in Anxiety and Addiction. *Neuropsychopharmacology.* 41: 126–141.

Avery SN, Clauss JA, Winder DG, Woodward N, Heckers S, Blackford JU (2014): BNST neurocircuitry in humans. *Neuroimage.* 91: 311–323.

Babb JA, Masini CV, Day HEW, Campeau S (2013): Sex differences in activated corticotropin-releasing factor neurons within stress-related neurocircuitry and hypothalamic-pituitary-adrenocortical axis hormones following restrain in rats. *Neuroscience.* 234: 40-52.

Badhan A, Sareen H, Trivedi J (2008): Endocrine dysfunctions and psychiatric disorders: understanding an interface. *Ind. J. Behav. Sci.* 18: 50-64.

Bagot RC, Cates HM, Purushothaman I, Vialou V, Heller EA, Yieh L et al (2017): Ketamine and Imipramine Reverse Transcriptional Signatures of Susceptibility and Induce Resilience-Specific Gene Expression Profiles. *Biol. Psychiatry.* 81: 285–295.

Bagot RC, Parise EM, Peña CJ, Zhang HX, Maze I, Chaudhury D et al (2015): Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression. *Nat. Commun.* 6: 7062.

Bale TL and Vale WW (2003): Increased depression-like behaviors in corticotropin-releasing factor receptor-2-deficient mice: sexually dichotomous responses. *J. Neurosci.* 23: 5295-5301.

Bale TL, Vale WW (2004): CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol.* 44: 525-57.

Balu DT, Coyle JT (2011): Glutamate receptor composition of the post-synaptic density is altered in genetic mouse models of NMDA receptor hypo- and hyperfunction. *Brain Res.* 1392: 1-7.

Banihashemi L and Rinaman L (2006): Noradrenergic inputs to the bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus underlie hypothalamic-pituitary-adrenal axis but not hypophagic or conditioned avoidance responses to systemic yohimbine. *J. Neurosci.* 26: 11442-11453.

Bartlett TE, Bannister NJ, Collett VJ, Dargan SL, Massey PV, Bortolotto ZA et al (2007): Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology.* 52: 60-70.

Baxter LR Jr, Schwartz JM, Phelps ME, Mazziotta JC, Guze BH, Selin CE (1989): Reduction of prefrontal cortex glucose metabolism common to three types of depression. *Arch. Gen. Psychiatry.* 46: 243–250.

Beaulieu JM and Gainetdinov RR (2011): The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63: 182-217.

Beck AT, Steer RA, Kovacs M, Garrison B (1985): Hopelessness and eventual suicide: a 10-year prospective study of patients hospitalized with suicidal ideation. *Am. J. Psychiatry*. 142: 559-563.

Beckerman MA, Van Kempen TA, Justice NJ, Milner TA, Glass MJ (2013): Corticotropin-releasing factor in the mouse central nucleus of the amygdala: ultrastructural distribution in NMDA-NR1 receptor subunit expressing neurons as well as projection neurons to the bed nucleus of the stria terminalis. *Exp. Neurol.* 239: 120-132.

Belujon P and Grace AA (2014): Restoring mood balance in depression: ketamine reverses deficit in dopamine-dependent synaptic plasticity. *Biol. Psychiatry.* 76: 927-936.

Belujon P and Grace AA (2017): Dopamine System Dysregulation in Major Depressive Disorders. *Int. J. Neuropsychopharmacol.* 20: 1036–1046.

Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH (2000): Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry*. 47: 351-354.

Bernard R, Kerman IA, Thompson RC, Jones EG, Bunney WE, Barchas JD et al (2011): Altered expression of glutamate signaling, growth factor and glia genes in the locus coeruleus of patients with major depression. *Mol. Psychiatry.* 16: 634-646.

Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ (2004): Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. *Neuropsychopharmacology.* 29: 483-493.

Biler P and Ward NM (2003): Is there a role for 5-HT1A agonists in the treatment of depression? *Biol. Psychiatry.* 53: 193-203.

Bilkei-Gorzo A, Racz I, Michel K, Zimmer A (2002): Diminished anxiety- and depression-related behaviors in mice with selective deletion of the *Tac1* gene. *J. Neurosci.* 22: 10046-10052.

Binneman B, Feltner D, Kolluri S, Shi Y, Qiu R, Stiger T (2008): A 6-week randomized, placebo-controlled trial of CP-316,311 (a selective CRH1 antagonist) in the treatment of major depression. *Am. J. Psychiatry*. 165: 617-620.

Biver F, Goldman S, Delvenne V, Luxen A, De Maertelaer V, Hubain P et al (1994): Frontal and parietal metabolic disturbances in unipolar depression. *Biol. Psychiatry*. 36: 381–388.

Blanchard RJ and Blanchard DC (1977): Aggressive behavior in the rat. *Behav. Biol.* 21: 197-224.

Blier P, Zigman D, Blier J (2012): On the safety and benefits of repeated intravenous injections of ketamine for depression. *Biol. Psychiatry.* 72: e11-2.

Blomstedt P, Naesström M, Bodlund O (2017): Deep brain stimulation in the bed nucleus of the stria terminalis and medial forebrain bundle in a patient with major depressive disorder and anorexia nervosa. *Clin. Case Rep.* 5: 679–684.

Blume A, Bosch OJ, Miklos S, Torner L, Wales L, Waldherr M, Neumann ID (2008): Oxytocin reduces anxiety via ERK1/2 activation: local effect within the rat hypothalamic paraventricular nucleus. *Eur. J. Neurosci.* 27: 1947-1956.

Bodnoff SR, Suranyi-Cadotte B, Aitken DH, Quirion R, Meaney MJ (1988): The effects of chronic antidepressant treatment in an animal model of anxiety. *Psychopharmacology.* 95: 298-302.

Boku S, Nakagawa S, Toda H, Hishimoto A (2017): Neural basis of major depressive disorder: beyond monoamine hypothesis. *Psych. Clin. Neurosci.* 72: 3-12.

Borodovitsyna O, Joshi N, Chandler D (2018): Persistent stress-induced neuroplastic changes in the locus coeruleus/norepinephrine system. *Neural Plasticity.* 2018: 1892570.

Borsini F and Meli A (1988): Is the forced swimming test a suitable model for revealing antidepressant activity? *Psychopharmacology.* 94: 147-160.

Boschert U, Amara DA, Segu L, Hen R (1994): The mouse 5-hydroxytryptamine 1B receptor is localized predominantly on axon terminals. *Neuroscience.* 58: 167.

Bota M, Sporns O, Swanson LW (2012): Neuroinformatics analysis of molecular expression patterns and neuron populations in gray matter regions: the rat BST as a rich exemplar. *Brain Res.* 1450: 174–193.

Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JC, Izumi Y, Muglia LJ (2005): Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proc. Natl. Acad. Sci.* 102: 473-478.

Brickley SG, Misra C, Selina Mok MH, Mishina M, Cull-Candy SG (2003): NR2B and NR2D Subunits Coassemble in Cerebellar Golgi Cells to Form a Distinct NMDA Receptor Subtype Restricted to Extrasynaptic Sites. *J. Neurosci.* 23: 4958-4966.

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

Brothers SP and Wahlestedt C (2010): Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* 2: 429-439.

Brothwell, SL, Barber JL, Monaghan DT, Jane DE, Gibb AJ, Jones S (2008): NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurons. *J. Physiol.* 586: 739-750.

Brunello N, Blier P, Judd LL, Mendlewicz J, Nelson CJ, Souery D, Zohar J, Racagni G (2003): Noradrenaline in mood and anxiety disorders: basic and clinical studies. *Int. Clin. Psychopharmacol.* 18: 191-202.

Burnell ES, Irvine M, Fang G, Sapkota K, Jane DE, Monaghan DT (2019): Positive and negative allosteric modulators of N-methyl-d-aspartate (NMDA) receptors; structure-activity relationships and mechanisms of action. *J. Med. Chem.* 62: 3-23.

Butler RK, Oliver EM, Sharko AC, Parilla-Carrero J, Kaigler KF, Fadel JR, Wilson MA (2016): Activation of corticotropin releasing factorcontaining neurons in the rat central amygdala and bed nucleus of the stria terminalis following exposure to two different anxiogenic stressors. *Behav. Brain Res.* 304:92–101.

Calabrese F, Guidotti G, Molteni R, Racagni G, Mancini M, Riva MA (2012): Stress-induced changes of hippocampal NMDA receptors: modulation by duloxetine treatment. *PLoS ONE*. 7: e37916.

Calogero AE, Gallucci WT, Gold PW, Chrousos GP (1988): Multiple feedback regulatory loops upon rat hypothalamic corticotropin-releasing hormone secretion: potential clinical implications. *J. Clin. Invest.* 82: 767-774.

Carmignoto G, Vicini S (1992): Activity-dependent decrease in NMDA receptor responses during development of the visual-cortex. *Science*. 258:1007–1011.

Carr GV and Lucki I (2011): The role of serotonin receptor subtypes in treating depression: a review of animal studies. *Psychopharmacology.* 213: 265-287.

Carzoli KL, Sharfman NM, Lerner MR, Miller MC, Holmgren EB, Wills TA (2019): Regulation of NMDA receptor plasticity in the BNST following adolescent alcohol exposure. *Front. Cell. Neurosci.* 13: 440.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IV, Harrington H et al (2003): Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science.* 301: 386-389.

Cassell MD, Freedman LJ, Shi C (1999): The intrinsic organization of the central extended amygdala. *Ann. N.Y. Acad.* Sci. 877: 217–241.

Castanheira L, Silva C, Cheniaux E, Telles-Correia D (2019): Neuroimaging correlates of depression – implications to clinical practice. *Front. Psychiatry.* 10: 703.

Castillo PE, Chiu CQ, Carroll RC (2011): Long-term plasticity at inhibitory synapses. *Curr. Opin. Neurobiol.* 21: 328-338.

Centanni SW, Morris BD, Luchsinger JR, Bedse G, Fetterly TL, Patel S, Winder DG (2019): Endocannabinoid control of the insular-bed nucleus of the stria terminalis circuit regulates negative affective behavior associated with alcohol abstinence. *Neuropsychopharmacology.* 44: 526-37.

Center for Behavioral Health Statistics and Quality (2018): *2017 National Survey on Drug Use and Health: Methodological summary and definitions*. Rockville, MD: Substance Abuse and Mental Health Services Administration.

Ch'ng S, Fu J, Brown RM, McDougall SJ, Lawrence AJ (2018): The intersection of stress and reward: BNST modulation of aversive and appetitive states. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* http://doi.org/10.1016/j.pnpbp.2018.01.005.

Chalmers DT, Lovenberg TW, De Souza EB (1995): Localization of novel corticotropin-releasing factor receptor (CRF_2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF_1 receptor mRNA expression. *J. Neurosci.* 15: 6340-6350.

Champagne D, Beaulieu J, Drolet G (1998): CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. *J. Neuroendocrinol.* 10: 119-131.

Chandley MJ and Ordway GA (2012): *The Neurobiological Basis of Suicide.* Boca Raton, FL

Chandley MJ, Szebeni A, Szebeni K, Crawford JD, Stockmeier CA, Turecki G et al (2014): Elevated gene expression of glutamate receptors in noradrenergic neurons from the locus coeruleus in major depression. *Int. J. Neuropsychopharmcol.* 17: 1569-1578.

Chandley MJ, Szebeni K, Szebeni A, Crawford J, Stockmeier CA, Tureki G et al (2013): Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder. *J. Psychiatry Neurosci.* 38: 276-284.

Chang CH and Gean PW (2019): The ventral hippocampus controls stress-provoked impulsive aggression through the ventromedial hypothalamus in post-weaning social isolation mice. *Cell Reports.* 28: 1195-1205.

Chang CH and Grace AA (2014): Amygdala-ventral pallidum pathway decreases dopamine activity after chronic mild stress in rats. *Biol. Psychiatry.* 76: 223-230.

Chaudhury D, Liu H, Han MH (2015): Neuronal correlates of depression. *Cell. Mol. Life Sci.* 72 :4825–4848.

Chen L, Li S, Cai J, Wei TJ, Liu LY, Zhao HY et al (2018): Activation of CRF/CRFR1 signaling in the basolateral nucleus of the amygdala contributes to chronic forced swim-induced depressive-like behaviors in rats. *Behav. Brain Res.* 338: 134-142.

Chen X, Shu S, Bayliss DA (2009): HCN1 channel subunits are a molecular substrate for hypnotic actions of ketamine. *J. Neurosci.* 29: 600-9.

Chen Y, Molet J, Gunn BG, Ressler K, Baram TZ (2015): Diversity of Reporter Expression Patterns in Transgenic Mouse Lines Targeting Corticotropin-Releasing Hormone-Expressing Neurons. *Endocrinology*. 156: 4769-4780.

Choi DC, Furay AR, Evanson KN, Ostrander MM, Ulrich-Lai YM, Herman JP (2007): Bed Nucleus of the Stria Terminalis Subregions Differentially Regulate Hypothalamic – Pituitary – Adrenal Axis Activity : Implications for the Integration of Limbic Inputs. *J. Neurosci.* 27: 2025–2034.

Christoffel DJ, Golden SA, Dumitriu D, Robinson SJ, Janssen WG, Ahn HF et al (2011): IκB Kinase Regulates Social Defeat Stress-Induced Synaptic and Behavioral Plasticity. *J. Neurosci.* 31: 314–321.

Chrousos GP (2009): Stress and disorders of the stress system. *Nat. Rev. Endocrinol.* 5: 374-381.

Chrousos GP and Gold PW (1992): The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. *JAMA.* 267: 1244-1252.

Chung S, Kim HJ, Kim HJ, Choi SH, Cho JH, Cho YH et al (2014): Desipramine and citalopram attenuate pretest swim-induced increases in prodynorphin immunoreactivity in the dorsal bed nucleus of the stria terminalis and the lateral division of the central nucleus of the amygdala in the forced swim test. *Neuropeptides.* 48: 273-280.

Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y et al. (2018): Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systemic review and network meta-analysis. *Lancet.* 10128: 1357-1366.

Clapp P, Gibson ES, Dell'acqua ML, Hoffman PL (2010): Phosphorylation regulates removal of synaptic N-methyl-D-aspartate receptors after withdrawal from chronic ethanol exposure. *J. Pharmacol. Exp. Ther.* 332: 720-729.

Clark MS, Sexton TJ, McClain M, Root D, Kohen R, Neumaier JF (2002): Overexpression of 5-HT1B receptor in dorsal raphe nucleus using Herpes Simplex Virus gene transfer increases anxiety behavior after inescapable stress. *J. Neurosci.* 22: 4550-4562.

Clarkson AN, Overman JJ, Zhong S, Mueller R, Lynch G, Carmichael ST (2011): AMPA receptor-induced local brain-derived neurotrophic factor signaling mediates motor recovery after stroke. *J. Neurosci.* 31: 3766–3775.

Clevenger SS, Malhotra D, Dang J, Vanle B, IsHak WW (2018): The role of selective serotonin reuptake inhibitors in preventing relapse of major depressive disorder. *Ther. Adv. Psychopharmacol.* 8: 49-58.

Conrad KL, Louderback KM, Gessner CP, Winder DG (2011a): Stress-induced alterations in anxiety-like behavior and adaptations in plasticity in the bed nucleus of the stria terminalis. *Physiol. Behav.* 104: 248-256.

Conrad KL, Winder DG (2011b): Altered Anxiety-like Behavior and Long-term Potentiation in the Bed Nucleus of the Stria Terminalis in Adult Mice Exposed to Chronic Social Isolation, Unpredictable Stress and Ethanol Beginning in Adolescence. *Alcohol*. 45: 585-593.

Cooper SJ, Kelly JG, King DJ (1985): Adrenergic receptors in depression. Effects of electroconvulsive therapy. *Br. J. Psychiatry.* 147: 23-29.

Coric V, Feldmann HH, Oren DA, Shekhar A, Pultz J, Dockens RC, Wu X, Gentile KA, Huang SP, Emison E, Delmonte T, D'Souza BB, Zimbroff DL, Grebb JA, Goddard AW, Stock EG (2010): Multicenter, randomized, double-blind, active comparator and placebo-controlled trial of a corticotropin-releasing factor receptor-1 antagonist in generalized anxiety disorder. *Depress. Anxiety.* 27: 417-425.

Costa BM, Feng B, Tsintsadze TS, Morley RM, Irvine MW, Tsintsadze V, Lozovaya NA, Jane DE, Monaghan DT (2009): N-methyl-D-aspartate (NMDA) receptor NR2 subunit selectivity of a series of novel piperazine- 2,3-dicarboxylate derivatives: preferential blockade of extrasynaptic NMDA receptors in the rat hippocampal CA3-CA1 synapse. *J. Pharmacol. Exp. Ther.* 331:618–626.

Cottingham C and Wang Q (2012): α2 adrenergic receptor dysregulation in depressive disorders: implications for the neurobiology of depression and antidepressant therapy. *Neurosci. Biobehav. Rev.* 36: 2214-2225.

Covington HE 3rd, Lobo MK, Maze I, Vialou V, Hyman JM, Zaman S, LaPlant Q, Mouzon E, Ghose S, Tamminga CA, Neve RL, Deisseroth K, Nestler EJ (2010): Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J. Neurosci.* 30: 16082-16090.

Crawley J, Goodwin FK (1980): Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol. Biochem. Behav.* 13: 167-70.

Crestani CC, Alves FHF, Correa FMA, Guimaraes FS, Joca SRL (2010): Acute reversible inactivation of the bed nucleus of stria terminalis induces antidepressant-like effect in the rat forced swimming test. *Behav. Brain Funct.* 6: 30.

Crowley NA, Bloodgood DW, Hardaway JA, Kendra A, McCall JG, Al-Hesani R et al (2016): Dynorphin controls the gain of an amygdalar anxiety circuit. *Cell Rep.* 14: 2774-2783.

Cryan JF, Mombereau C, Vassout A (2005): The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* 29: 571-625.

Cull-Candy S, Brickley S, Farrant M (2001): NMDA receptor subunits: diversity, development and disease. *Curr. Opin. Neurobiol.* 11: 327-35.

Cull-Candy SG and Leszkiewicz DN (2004): Role of distinct NMDA receptor subtypes at central synapses. *Sci. STKE.* re16.

Cullinan WE, Herman JP, Watson SJ. (1993): Ventral subicular interaction with the hypothalamic paraventricular nucleus: Evidence for a relay in the bed nucleus of the stria terminalis. *J. Comp. Neurol.* 332: 1-20.

Curran HV and Monaghan L (2001): In and out of the K-hole: a comparison of the acute and residual effects of ketamine in frequent and infrequent ketamine users. *Addiction.* 96: 749–760.

D'haenen HA and Bossuyt A (1994): Dopamine D2 receptors in depression measured with single photon emission computed tomography. *Biol. Psychiatry.* 35: 128-132.

Dabrowska J, Hazra R, Guo JD, DeWitt S, Rainnie DG (2013): Central CRF neurons are not created equal: Phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. *Front. Neurosci.* 7: 1–14.

Daigle TL, Madisen L, Hage TA, Valley MT, Knoblich U, Larsen RS et al (2018): A suite of transgenic driver and reporter mouse lines with enhanced brain-cell type targeting and functionality. *Cell.* 174: 465-480.

Danysz W and Parsons CG (2003): The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *Int. J. Geriatr. Psychiatry.* 18: 523-532.

Davidson C and Stamford JA (2000): Effects of chronic paroxetine treatment on 5-HT1B and 5-HT1D autoreceptors in rat dorsal raphe nucleus. *Neurochem. Int.* 36: 91-96.

Davis M (1997): Neurobiology of fear responses: the role of the amygdala. *J.Neuropsychiatry Clin.Neurosci.* 9: 382–402.

Davis M, Walker DL, Miles L, Grillon C (2010): Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology.* 35: 105–135.

Davis, M., Walker, D. L., Miles, L. & Grillon, C. Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology 35, 105–35 (2010).

de Bellis MD, Gold, PW, Geracioti TD, Listwak SJ, Kling MA (1993): Association of fluoxetine treatment with reductions in CSF concentrations of corticotropin-releasing hormone and arginine vasopressin in patients with major depression. *Am. J. Psychiatry.* 150: 656-657.

de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat. Rev. Neurosci.* 6: 463-475.

Debrowska J, Martinon D, Moaddab M, Rainnie DG (2016): Targeting corticotropin-releasing factor projections from the oval nucleus of the bed nucleus of the stria terminalis using cell-type specific neuronal tracing studies in mouse and rat brain. *J. Neuroendcrinol.* 28: 10.1111/jne.12442.

Del Arco A and Mora F (2009): Neurotransmitters and prefrontal cortex-limbic system interactions: implications for plasticity and psychiatric disorders. *J. Neural Transm.* 116: 941–952.

Delgado PL (2000): Depression: The case for a monoamine deficiency. *J. Clin. Psych.* 6: 7-11.

Denenberg VH (1969): Open-field behavior in the rat: what does it mean? *Ann. N.Y. Acad. Sci.* 159: 852-859.

Der-Avakian A and Markou A (2012): The neurobiology of anhedonia and other reward-related deficits. *Trends Neurosci.* 35: 68–77.

Desai SJ, Borkar CD, Nakhate KT, Subhedar NK, Kokare DM (2014): Neuropeptide Y attenuates anxiety- and depression-like effects of cholecystokinin-4 in mice. *Neuroscience.* 277: 818-830.

Di Chiara G and Tanda G (1997): Blunting of reactivity of dopamine transmission to palatable food: a biochemical marker of anhedonia in the CMS model? *Psychopharmacology.* 134: 351-353.

DiazGranados N, Ibrahim LA, Brutsche NE, Ameli R, Henter ID, Luckenbaugh DA et al (2010): Rapid resolution of suicidal ideation after a single infusion of an N-methyl-D-aspartate antagonist in patients with treatment-resistant major depressive disorder. *J. Clin. Psychiatry.* 71: 1605–1611.

Dobolyi A, Irwin S, Makara G, Usdin TB, Palkovits M (2005): Calcitonin gene-related peptide-containing pathways in the rat forebrain. *J. Comp. Neurol.* 489: 92–119.

Donaldson ZR, le Francois B, Santos TL, Almli LM, Boldrini M, Champagne FA et al (2016): The functional serotonin 1a receptor promoter polymorphism, rs6295, is associated with psychiatric illness and differences in transcription. *Translational Psychiatry.* 6: e746.

Dong HW and Swanson LW (2004a): Organization of axonal projections from the anterolateral area of the bed nuclei of the stria terminalis. *J. Comp. Neurol.*468: 277-298.

Dong HW and Swanson LW (2004b): Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors. *J. Comp. Neurol.* 471: 396-433.

Dong HW and Swanson LW (2006): Projections from bed nuclei of the stria terminalis, anteromedial area: Cerebral hemisphere integration of neuroendocrine, autonomic, and behavioral aspects of energy balance. *J. Comp. Neurol.* 494: 142–178.

Dong HW, Petrovich GD, Swanson LW (2001b): Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res. Rev.* 38: 192–246.

Dong HW, Petrovich GD, Watts AG, Swanson LW (2001a): Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain*. J. Comp. Neurol.* 436:430–55.

Drevets WC (2001): Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Curr. Opin. Neurobiol.* 2: 240-249.

Dubois CJ, Lachamp PM, Sun L, Mishina M, Liu SJ (2016): Presynaptic GluN2D receptors detect glutamate spillover and regulate cerebellar GABA release. *J. Neurophysiol.* 115: 271-285.

Duclot F, Hollis F, Darcy MJ, Kabbaj M (2011): Individual differences in novelty-seeking behaviors in rats as a model for psychological stress-related mood disorders. *Physiol. Behav.* 104: 296-305.

Duguid IC and Smart TG (2004): Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nat. Neurosci.* 7: 525-533.

Dulawa SC and Hen R (2005): Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. *Neurosci. Biobehav. Rev.* 29: 771-783.

Dulawa SC, Hen R (2005): Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. *Neurosci Biobehav Rev.* 29:771–783.

Dunah AW, Luo J, Wang YH, Yasuda RP, Wolfe BB (1998): Subunit composition of N-methyl-D-aspartate receptors in the central nervous system that contain the NR2D subunit. *Mol. Pharmacol.* 53: 429-437.

Dwyer JM, Platt BJ, Rizzo SJ, Pulicichio CM, Wantuch C, Zhang MY et al (2010): Preclinical characterization of BRL 44408: antidepressant- and analgesic-like activity through selective alpha2A-adrenoceptor antagonism. *Int. J. Neuropsychopharmacol.* 13: 1193-1205.

Dziedzicka-Wasylewska M, Wilnder P, Papp M (1997): Changes in dopamine receptor mRNA expression following chronic mild stress and chronic antidepressant treatment. *Behav. Pharmacol.* 8: 607-618.

Ebner K, Bosch OJ, Kromer SA, Singewald N, Neumann ID (2005): Release of oxytocin in the rat central amygdala modulates stress-coping behavior and the release of excitatory amino acids. *Neuropsychopharmacology.* 30: 223-230.

Ebner K, Wotjak CT, Landgraf R, Engelmann M 2002): Forced swimming triggers vasopressin release within the amygdala to modulate stress-coping strategies in rats. *Eur. J. Neurosci.* 15: 384-388.

Edmonds B, Gibb AJ, Colquhoun D (1995): Mechanisms of activation of glutamate receptors and the time-course of excitatory synaptic currents. *Annu. Rev. Physiol.* 57:495–519.

Erreger K, Dravid SM, Banke, TG, Wyllie DJA, Traynelis SF (2005): Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signaling profiles. *J. Physiol.* 536: 345-358.

Erreger K, Geballe MT, Kristensen A, Chen PE, Hansen KB, Lee CJ, Yuan H, Le P, Lyuboslavsky PN, Micale N, Jørgensen L, Clausen RP, Wyllie DJ, Snyder JP, Traynelis SF (2007): Subunit-specific agonist activity at NR2A-, NR2B-, NR2C-, and NR2D-containing N-methyl-D-aspartate glutamate receptors. *Mol. Pharmacol.* 72:907–920.

Escriba PV, Ozaita A, Garcia-Sevilla JA (2004): Increased mRNA expression of alpha2A-adrenoreceptors, serotonin receptors and mu-opioid receptors in the brains of suicide victims. *Neuropsychopharmacology*. 29: 1512-1521.

Evans RC, Morera-Herreras T, Cui Y, Du K, Sheehan T, Kotaleski JH, Venance L, Blackwell KT (2019): The effects of NMDA subunit composition on calcium influx and spike timing-dependent plasticity in striatal medium spiny neurons. *PLoS Comput. Biol.* 8: e1002493.

Fanselow MS and Dong HW (2010): Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron.* 65: 7–19.

Fava M (2003): Diagnosis and definition of treatment-resistant depression. *Biol. Psychiatry*. 53: 649-659.

Fergusson DM, Boden JM, Horwood LJ (2011): Structural models of the comorbidity of internalizing disorders and substance use disorders in a longitudinal birth cohort. *Soc. Psychiatry Psychiatr. Epidemiol.* 46: 933-942.

Fetterly TL, Basu A, Nabit BP, Awad E, Williford KM, Centanni SW, Matthews RT, Silberman Y, Winder DG (2019): α2A-Adrenergic Receptor Activation Decreases Parabrachial Nucleus Excitatory Drive onto BNST CRF Neurons and Reduces Their Activity *In Vivo*. *J. Neurosci.* 39: 472-484.

Flavin SA, Matthews RT, Wang Q, Muly EC, Winder DG (2014): α(2A)-adrenergic receptors filter parabrachial inputs to the bed nucleus of the stria terminalis. *J. Neurosci.* 34: 9319–9331.

Flores BH, Kenna H, Keller J, Solvason HB, Schatzberg AF (2006): Clinical and biological effects of mifepristone treatment for psychotic depression. *Neuropsychopharmacology.* 31: 628-636.

Follesa P and Ticku MK (1995): Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in rat brain. *Brain Res. Mol. Brain Res.* 29: 99-106.

Forbes EE, Hairi AR, Martin SL, Silk JS, Moyles DL, Fisher PM et al (2009): Altered striatal activation prediciting real-world positive affect in adolescent major depressive disorder. *Am. J. Psychiatry.* 166: 64-73.

Forray MI and Gysling K (2004): Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis. *Brain Res. Brain Res. Rev.* 47: 145–160.

France G, Fernandez-Fernandez D, Burnell ES, Irvine MW, Monaghan DT, Jane DE et al (2017): Multiple roles of GluN2B-containing NMDA receptors in synaptic plasticity in juvenile hippocampus. *Neuropharmacology.*112: 76-83.

Francis TC, Chandra R, Friend DM, Finkel E, Dayrit G, Miranda J et al (2015): Nucleus accumbens medium spiny neuron subtypes mediate depression-related outcomes to social defeat stress. *Biol. Psychiatry.* 77: 212–222.

Friedman A, Friedman Y, Dremencov E, Yadid G (2008): VTA dopamine neuron bursting is altered in an animal model of depression and corrected by desipramine. *J. Mol. Neurosci.* 34: 201-209.

Fu Y, Pollandt S, Liu J, Krishnan B, Genzer K, Orozco-Cabal L et al (2007): Long-term potentiation (LTP) in the central amygdala (CeA) is enhanced after prolonged withdrawal from chronic cocaine and requires CRF1 receptors. *J. Neurophysiol.* 97: 937–941.

Funk D and Li Z, Lê AD (2006): Effects of environmental and pharmacological stressors on c-fos and corticotropin-releasing factor mRNA in rat brain: relationship to the reinstatement of alcohol seeking. *Neuroscience.* 138: 235–243.

Garcia-Sevilla JA, Escriba PV, Ozaita A, La Harpe R, Walzer C, Eytan A et al (1999): Up-regulation of immunolabeled alpha2A-adrenoceptors, Gi coupling proteins, and regulator receptor kinases in prefrontal cortex of depressed suicides. *J. Neurochem.* 72: 282-291.

Garcia-Sevilla JA, Ventayol P, Perez V, Rubovszky G, Puigdemont D, Ferrer-Alcon M et al (2004): Regulation of platelet alpha 2A-adrenoceptors, Gi proteins and receptor kinases in major depression: effects of mirtazapine treatment. *Neuropsychopharmacology*. 29: 580-588.

Georges F and Aston-Jones G (2001): Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *J. Neurosci.* 21: RC160.

Georges F and Aston-Jones G (2002): Activation of ventral tegmental area cells by the bed nucleus of the stria terminalis: a novel excitatory amino acid input to midbrain dopamine neurons. *J. Neurosci.* 22: 5173–87.

Geugies H, Mocking RJT, Figueroa CA, Groot PFC, Marsman JBC, Servaas MN et al (2019): Impaired reward-related signaling in remitted unmedicated patients with recurrent depression. *Brain.* 142: 2510-2522.

Ghamari-Langroudi M, Digby GJ, Sebag JA, Millhauser GL, Palomino R, Matthews R, Gillyard T, Panaro BL, Tough IR, Cox HM, Denton JS, Cone RD (2015): G-protein-independent coupling of MC4R to Kir7.1 in hypothalamic neurons. *Nature.* 520: 94-98.

Ghasemi M, Phillips C, Trillo L, De Miguel Z, Das D, Salehi A (2014): The role of NMDA receptors in the pathophysiology and treatment of mood disorders. *Neuroscience and Biobehavioral Reviews.* 47: 336-358.

Ghavami A, Stark KL, Jareb M, Ramboz S, Segu L, Hen R (1999): Differential addressing of 5-HT1A and 5-HT1B receptors in epithelial cells and neurons. *J. Cell. Sci.* 112: 967-976.

Giardino WJ, Eban-Rothschild A, Christoffel DJ, Li S, Malenka RC, de Lecea L (2018): Parallel circuits from the bed nucleus of the stria terminalis to the lateral hypothalamus drive opposing emotional states. *Nat. Neurosci.* 21: 1084-1095.

Gjerris A, Sorensen AS, Rafaelsen OJ, Werdelin L, Alling C, Linnoila M (1987): 5-HT and 5-HIAA in cerebrospinal fluid in depression. *J. Affective Disorders.* 12: 13-22.

Glangetas C, Massi L, Fois GR, Jalabert M, Girard D, Diana M, Yonehara K, Roska B, Xu C, Luthi A, Caille S, Georges F (2017): NMDA-receptor dependent plasticity in the bed nucleus of the stria terminalis triggers long-term anxiolysis. *Nat. Commun.* 8: 14456.

Glasgow SD, McPhedrain R, Madranges JF, Kennedy TE, Ruthazer ES (2019): Approaches and limitations in the investigation of synaptic transmission and plasticity. *Front. Synaptic Neurosci.* 11: 20.

Goddard AW, Ball SG, Martinez J, Robinson MJ, Yang CR, Russell JM, Shekhar A (2010): Current perspectives of the roles of central norepinephrine system in anxiety and depression. *Depress. Anxiety.* 27: 339-350.

Golden SA, Christoffel DJ, Hodes GE, Hashmati M, Magida J, Davis K et al (2013): Epigenetic regulation of synaptic remodeling in stress disorders. 19: 337–344.

Gracy KN and Pickel VM (1995): Comparative ultrastructural localization of the NMDAR1 glutamate receptor in the rat basolateral amygdala and bed nucleus of the stria terminalis. *J. Comp. Neurol.* 362: 71-85.

Graef JD, Newberry K, Newton A, Pieschl R, Shields E, Luan FN et al (2015): Effect of acute NR2B antagonist treatment on long-term potentiation in the rat hippocampus. *Brain Res.* 1609:31–39.

Grammatopoulos DK, Randeva HS, Levine MA, Kanellopoulou KA, Hillhouse EW (2001): Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. *J. Neurochem.* 76: 509-519.

Grant BF, Goldstein RB, Saha TD, Chou SP, Jung J, Zhang H et al (2015): Epidemiology of DSM-5 Alcohol Use Disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions III. *JAMA Psychiatry.*

Graybeal C, Kiselycznyk C, Holmes A (2012): Stress-Induced Deficits in Cognition and Emotionality: A Role for Glutamate. *Curr. Top. Behav. Neurosci.* 12: 189-207.

Griebel G and Holsboer F (2012): Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? *Nat. Rev. Drug Discov.* 11: 462-478.

Griebel G, Beeske S, Stahl SM (2012): The vasopressin V(1b) receptor antagonist SSR149415 in the treatment of major depressive and generalized anxiety disorders: results from 4 randomized, double-blind, placebo-controlled studies. *J. Clin. Psychiatry.* 73: 1403-1411.

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B et al (2002): Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V_{1b} receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders*. Proc Natl Acad Sci.* 99:6370–6375.

Griebel G, Simiand J, Stemmelin J, Gal Serradeil-Le, Steiberg R. (2003): The vasopressin V_{1b} receptor as a therapeutic target in stress-related disorders. *Curr Drug Target CNS Neurol Disord.* 2:191–200.

Grienberger C and Konnerth A (2012): Imaging calcium in neurons. *Neuron.* 73: 862-885.

Gulyaeva NV (2018): Functional neurochemistry of the ventral and dorsal hippocampus: stress, depression, dementia and remote hippocampal damage. *Neurochem. Res.* 44: 1306-1322.

Hamilton JP, Siemer M, Gotlib IH (2008): Amygdala volume in major depressive disorder: a meta-analysis of magnetic resonance imaging studies. *Mol. Psychiatry.* 13 :993–1000.

Hammack SE, Cheung J, Rhodes KM, Schutz KC, Falls WA, Braas KM, May V (2009): Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology.* 34: 833–843.

Hammack SE, Richey KJ, Watkins LR, Maier SF (2004: Chemical lesion of the bed nucleus of the stria terminalis blocks the behavioral consequences of uncontrollable stress. *Behav. Neurosci.* 118: 443–448.

Hammack SE, Todd TP, Kocho-Schellenberg M, Bouton ME (2015): Role of the bed nucleus of the stria terminalis in the acquisition of contextual fear at long or short context-shock intervals. *Behav. Neurosci.* 129: 673–678.

Hansen KB, Yi F, Perszyk RE, Furukawa H, Wollmuth LP, Gibb AJ, Traynelis SF (2018): Structure, function and allosteric modulation of NMDA receptors. *J. Gen. Physiol.* 150: 1081-1105.

Hanson E, Armbruster M, Lau LA, Sommer ME, Klaft Z, Swanger SA, Traynelis SF, Moss SJ, Noubary F, Chadchankar J, Dulla CG (2019): Tonic Activation of GluN2C/GluN2D-Containing NMDA Receptors by Ambient Glutamate Facilitates Cortical Interneuron Maturation. *J. Neurosci.* 39: 3611-26.

Harney SC and Anwyl R (2012): Plasticity of NMDA receptor-mediated excitatory postsynaptic currents at perforant path inputs to dendrite-targeting interneurons. *J. Physiol.* 590: 3771-3786.

Harney SC, Jane DE, Anwyl R (2008): Extrasynaptic NR2D-Containing NMDARs Are Recruited to the Synapse during LTP of NMDAR-EPSCs. *J. Neurosci.* 28: 11685-11694.

Harris NA and Winder DG (2018): Synaptic Plasticity in the Bed Nucleus of the Stria Terminalis: Underlying Mechanisms and Potential Ramifications for Reinstatement of Drug- and Alcohol-Seeking Behaviors. *ACS Chem. Neurosci.* 9: 2173–2187.

Harris NA, Isaac AT, Gunther A, Merkel K, Melchior J, Xu M et al (2018): Dorsal BNST α2A-Adrenergic Receptors Produce HCN-Dependent Excitatory Actions That Initiate Anxiogenic Behaviors. *J. Neurosci.* 38: 8922-8942.

Hashimoto H, Onishi H, Koide S, Kai T, Yamagami S. (1996): Plasma neuropeptide Y in patients with major depressive disorder. *Neurosci. Lett.* 216: 57-60.

Hasue RH and Shammah-Lagnado SJ (2002): Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *J. Comp. Neurol.* 454: 15–33.

Hauger R, Risbrough V, Oakley R, Olivares-Reyes J, Dautzenberg F (2009): Role of CRF Receptor Signaling in Stress Vulnerability, Anxiety, and Depression. *Ann. N. Y. Acad. Sci.* 1179: 120–143.

Hawley DF, Bardi M, Everette AM, Higgins TJ, Tu KM, Kinsely CH, Lamber KG (2010): Neurobiological constituents of active, passive, and variable coping strategies in rats: integration of regional brain neuropeptide Y levels and cardiovascular responses. *Stress.* 13: 172–183.

Heilig M, Koob GF, Ekman R, Britton KT (1994): Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. *Trends Neurosci.* 17: 80–85.

Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH (2007): Serotonin 5-HT(2C) receptors regulate anxiety-like behavior. *Genes Brain Behav.* 6: 491–496.

Helmreich DL, Watkins LR, Deak T, Maier SF, Akil H, Watson SJ (1999): The effect of stressor controllability on stress-induced neuropeptide mRNA expression within the paraventricular nucleus of the hypothalamus. *J. Neuroendocrinol.* 11: 121–128.

Henley JM, Wilkinson KA (2016): Synaptic AMPA receptor composition in development, plasticity and disease. *Nat. Rev. Neurosci.* 17: 337–350.

Herman JP and Cullinan WE (1997): Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci.* 20: 78–84.

Herman JP, Cullinan WE, Watson SJ (1994): Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. *J. Neuroendocrinol.* 6: 433–442.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003): Central mechanisms of stress integration: Hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front. Neuroendocrinol.* 24: 151–180.

Herpfer I and Lieb K (2005): Substance P receptor antagonists in psychiatry: rationale for development and therapeutic potential. *CNS Drugs.* 19: 275-293.

Hess SD, Daggett LP, Deal C, Lu CC, Johnson EC, Velicelebi G (1998): Functional characterization of human N-methyl-d-aspartate subtype 1A/2D receptors. *J. Neurochem.* 70: 1269-1279.

Hildebrand ME, Pitcher GM, Harding EK, Li H, Beggs S, Salter MW (2014): GluN2B and GluN2D NMDARs dominate synaptic responses in the adult spinal cord. *Sci. Rep.* 4: 4094.

Hoffman GE, Simth MS, Verbalis JG (1993): c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front. Neruoendocrinol.* 14: 173-213.

Holleran KM, Wilson HH, Fetterly TL, Bluett RJ, Centanni SW, Gilfarb RA, Rocco LE, Patel S, Winder DG (2016) Ketamine and MAG lipase inhibitor-dependent reversal of evolving depressive-like behavior during forced abstinence from alcohol drinking. Neuropsychopharmacology 41: 2062–2071.

Hollis F, Wang H, Dietz D, Gunjan A, Kabbaj M (2010): The effects of repeated social defeat on long-term depressive-like behavior an short-term histone modifications in the hippocampus in male Sprague-Dawley rats. *Psychopharmacology.* 211: 69-77.

Holsboer F (2001): Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J. Affect. Dis.* 62: 77-91.

Holsboer F and Barden N (1996): Antidepressants and hypothalamic-pituitary-adrenocortical regulation. *Endo. Rev.* 17: 187-205.

Homayoun H, Moghaddam B (2007): NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J. Neurosci*. 27: 11496–11500.

Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE et al (2000): Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. *J. Neurosci.* 20: RC81.

Iadarola ND, Niciu MJ, Richards EM, Vande Voort JL, Ballard ED, Lundin NB, Nugent AC, Machado-Vieira R, Zarate Jr. CA (2015): Ketamine and other N-methyl-d-aspartate receptor antagonists in the treatment of depression: a perspective review. *Ther. Adv. Chronic. Dis.* 6: 97-114.

Ibrahim L, Diaz Granados N, Jolkovsky L, Brutsche N, Luckenbaugh DA, Herring WJ et al (2012): A randomized, placebo-controlled, crossover pilot trial of the oral selective NR2B antagonist MK-0657 in patients with treatment-resistant major depressive disorder. *J. Clin. Psychopharmacol.* 32: 551–557.

Ide S, Hara T, Ohno A, Tamano R, Koseki K, Naka T et al (2013): Opposing roles of corticotropin-releasing factor and neuropeptide Y within the dorsolateral bed nucleus of the stria terminalis in the negative affective component of pain in rats. *J. Neurosci.* 33: 5881-5894.

Ide S, Ikekubo Y, Mishina M, Hashimoto K, Ikeda K (2017): Role of NMDA receptor GluN2D subunit in the antidepressant effects of enantiomers of ketamine. *J. Pharma. Sci.* 135: 138-140.

Ide S, Ikekubo Y, Mishina M, Hashimoto K, Ikeda K (2019): Cognitive Impairment That Is Induced by (R)-Ketamine Is Abolished in NMDA GluN2D Receptor Subunit Knockout Mice*. Int. J. Neuropsychopharmacol.* 22 :449–452.

Ieraci A, Mallei A, Popoli M (2016): Social isolation stress induces anxious-depressive-like behavior and alterations of neuroplasticity-related genes in adult male mice. *Neural Plast.* 2016: 6212983.

Ikeda K, Araki K, Takayama C, Inoue Y, Yagi T, Aizawa S, Mishina M (1995): Reduced spontaneous activity of mice defective in the ε4 subunit of the NMDA receptor channel. *Mol. Brain Res.* 33: 61-71.

Insel TR (2010): The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. *Neuron*. 65: 768-779.

Ishida H, Shirayama Y, Iwata M, Katayama S, Yamamoto A, Kawahara R, Nakagome K (2007): Infusion of Neuropeptide Y into CA3 region of hippocampus produces antidepressant-like effect via Y1 receptor. *Hippocampus.* 17: 271-280.

Ising M, Zimmermann US, Kunzel HE, Uhr M, Foster AC, Learned-Coughlin SM et al (2007): High-affinity CRF1 receptor antagonist NBI-34041: preclinical and clinical data suggest safety and efficacy in attenuating elevated stress response. *Neuropsychopharmacology.* 32: 1941-1949.

Jasnow AM, Davis M, Huhman KL (2004): Involvement of central amygdalar and bed nucleus of the stria terminalis corticotropin-releasing factor in behavioral responses to social defeat. *Behav. Neurosci.* 118: 1052-1061.

Javitt DC, Schoepp D, Kalivas PW, Volkow ND, Zarate C, Merchant K, Bear MF, Umbricht D, Hajos M, Potter WZ, Lee C (2011): Translating Glutamate: From Pathophysiology to Treatment. *Science Translational Medicine.* 102: 102mr2 1-13.

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

Jeong J-W, McCall JG, Shin G, Zhang Y, Al-Hasani R, Kim M et al (2015): Wireless optofluidic systems for programmable in vivo pharmacology and optogenetics. *Cell.* 162: 662-674.

Jones S and Gibb AJ (2005): Functional NR2B- and NR2D-containing NMDA receptor channels in rat substantia nigra dopaminergic neurons. *J. Physiol.* 569: 209-221.

Jourdi H, Hsu YT, Zhou M, Qin Q, Bi X, Baudry M (2009): Positive AMPA receptor modulation rapidly stimulates BDNF release and increases dendritic mRNA translation. *J. Neurosci.* 29: 8688–8697.

Kalsner S and Abdali SA (2001): Rate-independent inhibition by norepinephrine of 5-HT release from the somadendritic region of serotonergic neurons. *Brain Res. Bull.* 55: 761-765.

Karege F, Widmer J, Bovier P, Gaillard JM (1994): Platelet serotonin and plasma tryptophan in depressed patients: effects of drug treatment and clinical outcome. *Neuropsychopharmacology.* 10: 207-214.

Karolewicz B, Stockmeier CA, Ordway GA (2005): Elevated levels of the NR2C subunit of the NMDA receptor in the locus coeruleus in depression. *Neuropsychopharmacology.* 30: 1557-1567.

Kash TL and Winder DG (2006): Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis. *Neuropharmacology.* 51: 1013-1022.

Kash TL, Baucum AJ, Conrad KL, Colbran RJ, Winder DG (2009): Alcohol exposure alters NMDAR function in the bed nucleus of the stria terminalis. *Neuropsychopharmacology*. 34: 2420-2429.

Kash TL, Pleil KE, Marcinkiewcz CA, Lowery-Gionta EG, Crowley N, Mazzone C, Sugam J, Hardaway JA, McElligott ZA (2015): Neuropeptide Regulation of Signaling and Behavior in the BNST. *Mol. Cells.* 38: 1-13.

Katz DA, Locke C, Greco N, Liu W, Tracy KA (2017): Hypothalamic-pituitary-adrenal axis and depression symptom effects of arginine vasopressin type 1B receptor antagonist in a one-week randomized Phase 1b trial. *Brain. Behav.* 7: e00628.

Keck ME, Holsboer F (2001): Hyperactivity of CRH neuronal circuits as a target for therapeutic interventions in affective disorders. *Peptides*. 22: 835-44.

Kempton MJ (2011): Structural neuroimaging studies in major depressive disorder. *Arch. Gen. Psychiatry.* 68: 675.

Kennett G, Marcou M, Dourish C, Curzon G (1987): Single administration of 5-HT1A agonist decreases 5-HT1A presynaptic, but not post-synaptic receptor mediated responses: relationship to antidepressant-like action. *Eur. J. Pharmacol.* 138: 53-60.

Khlestova E, Johnson JW, Krystal JH, Lisman J (2016): The Role of GluN2C-Containing NMDA Receptors in Ketamine's Psychotogenic Action and in Schizophrenia Models. *J. Neurosci.* 36 :11151–11157.

Kim J, Lee S, Fang YY, Shin A, Park S, Hashikawa K et al (2019a): Rapid, biphasic CRF neuronal responses encode positive and negative valence. *Nat. Neurosci.* 22: 576-585.

Kim JS, Han SY, Iremonger KJ (2019b): Stress experience and hormone feedback tune distinct components of hypothalamic CRH neuron activity. *Nat. Commun.* 10: 5696.

Kim SY, Adhikari A, Lee SY, Marshel JH, Kim CK, Mallory CS, Lo M, Pak S, Mattis J, Lim BK, Malenka RC, Warden MR, Neve R, Tye KM, Deisseroth K (2013): Diverging neural pathways assemble a behavioral state from separable features in anxiety. *Nature.* 496: 219-233.

Kiselycznyk C, Jury NJ, Halladay LR, Nakazawa K, Mishina M, Sprengel R et al (2015): NMDA receptor subunits and associated signaling molecules mediating antidepressant-related effects of NMDA-GluN2B antagonism. *Behav. Brain Res.* 287: 89–95.

Knobloch HS, Charlet A, Hoffmann LC, Eliava M, Khrulev S, Cetin AH et al (2012): Evoked axonal oxytocin release in the central amygdala attenuates fear response. *Neuron.* 73: 553-566.

Knowland D and Lim BK (2018): Circuit-based frameworks of depressive behaviors: the role of reward circuitry and beyond. *Pharmacol., Biochem. & Behav.* 174: 42-52.

Kocsis B (2012): Differential role of NR2A and NR2B subunits in NMDA receptor antagonist-induced aberrant cortical gamma oscillations. *Biol. Psychiatry.* 71: 987–995.

Kohrs R, Durieux ME (1998): Ketamine: teaching an old drug new tricks. *Anesth. Analg.* 87: 1186–1193.

Koob GF and Volkow ND (2010): Neurocircuitry of addiction. *Neuropsychopharmacology*. 35:217–238.

Kotermanski SE and Johnson JW (2009): Mg^{2+} imparts NMDA receptor subtype selectivity to the Alzheimer's drug memantine. *J. Neurosci.* 29: 2774-2779.

Kram ML, Kramer GL, Ronan PJ, Steciuk M, Petty F (2002): Dopamine receptors and learned helplessness in the rat: an autoradiographic study. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. 41: 591-599.

Krishnan V and Nestler EJ (2008): The molecular neurobiology of depression. *Nature.* 455: 894-902.

Krishnan V and Nestler EJ (2011): Animal models of depression: molecular perspectives. *Curr. Top. Behav. Neurosci.* 7: 121-147.

Krystal JH, Abdallah CG, Sanacora G, Charney DS, Duman RS (2019): Ketamine: a paradigm shift for depression research and treatment. *Neuron.* 101: 774-778.

Kuhne C, Puk O, Graw J, Hrabe de Angelis M, Schutz G, Wurst W, Deussing JM (2012): Visualizing corticotropin-releasing hormone receptor type 1 expression and neuronal connectivities in the mouse using a novel multifunctional allele. *J. Comp. Neurol.* 520: 3150-3180.

Lachamp PM, Liu Y, Liu SJ (2009): Glutamatergic modulation of cerebellar interneuron activity is mediated by an enhancement of GABA release and requires protein kinase A/RIM1alpha signaling. *J. Neurosci.* 29: 381-392.

Lam MP, Gianoulakis C (2011): Effects of acute ethanol on corticotropin-releasing hormone and β-endorphin systems at the level of the rat central amygdala. *Psychopharmacology.* 218: 229-239.

Lambert G, Johansson M, Argen H, Friberg P (2000): Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness. *Arch. Gen. Psychiatry.* 57: 787-793.

Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C (2008): The dysphoric component of stress is encoded by activation of the dynorphin K-opioid system. *J. Neurosci.* 28: 407-414.

Lebow M, Neufeld-Cohen A, Kuperman Y, Tsoory M, Gil S, Chen A (2012): Susceptibility to PTSD-like behavior is mediated by corticotropin-releasing factor receptor type 2 levels in the bed nucleus of the stria terminalis. *J. Neurosci.* 32:6906–16.

Lebow MA, Chen A (2016): Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatry*. 21: 450-463.

LeDoux J (2007): The amygdala. *Curr. Biol.* 17: R868–R874.

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A et al (2007): Genome-wide atlas of gene expression in the adult mouse brain. *Nature.* 445: 168–176.

Lemberger L, Fuller RW, Zerbe RL (1985): Use of specific serotonin uptake inhibitors as antidepressants. *Clin. Neuropharmacol.* 8:299–317.

Lezak KR, Roelke E, Harris OM, Choi I, Edwards S, Gick N et al (2014): Pituitary adenylate cyclase-activating polypeptide (PACAP) in the bed nucleus of the stria terminalis (BNST) increases corticosterone in male and female rats*. Psychoneuroendocrinology.* 45: 11–20.

Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M et al. (2010): mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists*. Science.* 329: 959–964.

Li N, Lee BY, Liu RJ, Banasar M, Dwyer J, Iwata M et al (2010): mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science.* 329:959-964.

Li N, Liu RO-J, Dwyer J, Banasar M, Lee B, Son J et al (2011): Glutamate N-methyl-d-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol. Psychiatry.* 69: 754-761.

Liao Y, Tang J, Corlett PR, Wang X, Yang YM, Chen H et al(2011): Reduced dorsal prefrontal gray matter after chronic ketamine use. *Biol. Psychiatry.* 69: 42–48.

Lien CC, Mu Y, Vargas-Caballero M, Poo M (2006): Visual stimuli-induced LTD of GABAergic synapses mediated by presynaptic NMDA receptors. *Nat. Neurosci.* 9: 372-380.

Lim BK, Huang KW, Grueter BA, Rothwell PE, Malenka RC (2015): Anhedonia requires MC4 receptor-mediated synaptic adaptations in nucleus accumbens. *Nature.* 487: 183–189.

Lin X, Itoga CA, Taha S, Li MH, Chen R, Sami K, Berton F, Francesconi W, Xu X (2018): c-fos mapping of brain regions activated by multi-modal and electric foot shock stress. *Neurobiol Stress.* 8:92–102.

Lin Z and Madras BK (2006): Human genetics and pharmacology of neurotransmitter transporters. *Handb. Exp. Pharmacol.* 175: 327-371.

Liotti M, Mayberg HS (2001): The role of functional neuroimaging in the neuropsychology of depression. *J. Clin. Exp. Neuropsychol.* 23: 121-136.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M et al (2004): Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science.* 304: 1021-1024.

Liu MY, Yin CY, Zhu LJ, Zhu XH, Zu C, Luo CX et al (2018): Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nat. Prot.* 13: 1686-1698.

Liu Y, Zhang LI, Tao HW (2007): Heterosynaptic scaling of developing GABAergic synapses: dependence on glutamatergic input and developmental stage. *J. Neurosci.* 27: 5301-5312.

Liu Z, Zhu F, Wang G, Xiao Z, Wang H, Tang J et al (2006): Association of corticotropin-releasing hormone receptor 1 gene SNP and haplotype with major depression. *Neurosci. Lett.* 404: 358-362.

Lopez-Rubalcava C and Lucki I (2000): Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. *Neuropsychopharmacology.* 22: 191-199.

Louderback KM, Wills TA, Muglia LJ, Winder DG (2013): Knockdown of BNST GluN2B-containing NMDA receptors mimics the actions of ketamine on novelty-induced hypophagia. *Transl. Psychiatry*. 3: e331.

Lozovaya N, Gataullina S, Tsintsadze T, Tsintsadze V, Pallsei-Pocachard E, Minlebaev M, Goriounova NA et al (2014): Selective suppression of excessive GluN2C expression rescues early epilepsy in a tuberous sclerosis murine model. *Nat. Commun.* 5: 4563.

Lozovaya NA, Grebenyuk SE, Tsintsadze T, Feng B, Monaghan DT, Krishtal OA (2004): Extrasynaptic NR2B and NR2D subunits of NMDA receptors shape 'superslow' afterburst EPSC in rat hippocampus. *J. Physiol.* 558: 451-463.

Lucki I, Dalvi A, Mayorga AJ (2001): Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology.* 155: 315-322.

Maeng S, Zarate CA Jr, Du J, Schloesser RJ, McCammon J, Chen G, Manji HK (2008): Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biol. Psychiatry.* 63: 349–352.

Maeng S, Zarate CA Jr., Du J, Schloesser RJ, McCammon J, Chen G et al (2008): Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biol. Psychiatry*. 63: 349–352.

Maier S (1993): Learned helplessness: relationships with fear and anxiety. Stress: from synapse to syndrome, eds Stanford SC, Salmon P (Academic, London), pp 207–243.

Mak P, Broussard C, Vacy K, Broadbear JH (2012): Modulation of anxiety behavior in the elevated plus maze using peptidic oxytocin and vasopressin receptor ligands in the rat. *J. Psychopharmacol.* 26: 532-542.

Malatynska E and Knapp RJ (2005): Dominant-submissive behavior as models of mania and depression. *Neuroscience and Behavioral Reviews.* 29: 715-737.

Malenka RC (1994): Synaptic plasticity in the hippocampus: LTP and LTD. *Cell.* 78: 535-8.

Malenka RC and Bear MF (2004): LTP and LTD: an embarrassment of riches. *Neuron.* 44: 5-21.

Malenka RC, Nestler EJ, Hyman SE (2009): Widely Projecting Systems: Monoamines, Acetylcholine, and Orexin". In Sydor A, Brown RY (eds.). *Molecular Neuropharmacology: A Foundation for Clinical Neuroscience* (2nd ed.). New York: McGraw-Hill Medical. pp. 147–148, 154–157.

Manji HK, Drevets WC, Charney DS (2001): The cellular neurobiology of depression. *Nat. Med.* 7: 541-547.

Marcinkiewcz CA, Mazzone CM, D'Agostino G, Halladay LR, Hardaway JA, DiBerto JF et al (2016): Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala. *Nature*. 537: 97-101.

Maroteaux L, Saudou F,Amlaiky N, Boschert U, Plassat JL, Hen R (1992): Mouse 5HT1B serotonin receptor: cloning, functional expression, and localization in motor control centers. *Proc. Natl. Acad. Sci.* 89: 3020-3024.

Marrocco J, Mairesse J, Ngomba RT, Silletti V, Van Camp G, Bouwalerh H et al (2012): Anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus. *J. Neurosci.* 32: 17143-17154.

Martella G, Bonsi P, Johnson SW, Quartarone A (2018): Synaptic Plasticity Changes: Hallmark for Neurological and Psychiatric Disorders. *Neural Plast.* 9230704.

Martinez M, Calvo-Torrent A, Pico-Alfonso MA (1998): Social defeat and subordination as models of social stress in laboratory rodents: a review. *Aggress. Behav.* 24: 241-256.

Maue RA (2013): Understanding ion channel biology using epitope tags: progress, pitfalls, and promise. *J. Cell. Physiol.* 213: 618–625.

McBain CJ and Kauer JA (2009): Presynaptic plasticity: targeted control of inhibitory networks. *Curr. Opin. Neurobiol.* 19: 254-262.

McDonald AJ (1998): Cortical pathways to the mammalian amygdala. *Prog. Neurobiol.* 55: 257–332.

McDonald AJ, Shammah-Lagnado SJ, Shi C, Davis M (1999): Cortical afferents to the extended amygdala. *Ann. N. Y. Acad. Sci.* 877: 309–338.

McElligott ZA, Winder DG (2009): Modulation of glutamatergic synaptic transmission in the bed nucleus of the stria terminalis. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* 33: 1329-35.

McEwen B (1999): Stress and hippocampal plasticity. *Ann. Rev. Neurosci.* 22: 105-122.

McReynolds JR, Christianson JP, Blacktop JM, Mantsch JR (2018): What does the Fos say? Using Fos-based approaches to understand the contribution of stress to substance use disorders. *Neurbiol. Stress.* 9: 271-285.

Merali Z, Kent P, Du L, Hrdina P, Palkovits M, Faludi G et al (2006): Corticotropin-releasing hormone, arginine vasopressin, gastrin-releasing peptide, and neuromedin B alterations in stress-relevant brain regions of suicides and control subjects. *Biol. Psychiatry.* 59 594–602.

Meyer AH, Katona I, Blatow M, Rozov A, Monyer, H (2002): In vivo labeling of parvalbumin-positive interneurons and analysis of electrical coupling in identified neurons. *J. Neurosci.* 22: 7055–7064.

Meyer JH, Kruger S, Wilson AA, Christensen BK, Goulding VS, Schaffer A et al (2001): Lower dopamine transporter binding potential in striatum during depression. *Neuroreport.* 12: 4121-4125.

Miller OH, Yang L, Wang CC, Hargroder EA, Zhang Y, Delpire E, Hall BJ (2014): GluN2B-containing NMDA receptors regulate depression-like behavior and are critical for the rapid antidepressant actions of ketamine. *eLife.* 3: e03581.

Milne AMB, MacQueen GM, Hall GBC (2012): Abnormal hippocampal activation in patients with extensive history of major depression: an fMRI study. *J. Psychiatry Neurosci.* 37: 28-36.

Misra C, Brickley SG, Wyllie DJ, Cull-Candy SG (2000): Slow deactivation kinetics of NMDA receptors containing NR1 and NR2D subunits in rat cerebellar Purkinje cells. *J. Physiol.* 525: 299-305.

Miyamoto Y, Yamada K, Noda Y, Mori H, Mishina M, Nabeshima T (2002): Lower sensitivity to stress and altered monoaminergic neuronal function in mice lacking the NMDA receptor epsilon 4 subunit. *J. Neurosci.* 22: 2335-2342.

Mobbs D, Yu R, Rowe JB, Eich H, FeldmanHall O, Dalgleish T (2010): Neural activity associated with monitoring the oscillating threat value of a tarantula. *Proc. Natl. Acad. Sci.* 107: 20582-20586.

Moghaddam B, Adams B, Verma A, Daly D (1997): Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. *J. Neurosci.* 17: 2921–2927.

Molendijk ML and de Kloet ER (2019): Coping with the forced swim stressor: current state-of-the-art. *Behav. Brain Res.* 364: 1-10.

Momiyama A (2000): Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurons of the adult rat spinal cord. *J. Physiol.* 523: 621-628.

Momiyama A, Feldmeyer D, Cull-Candy SG (1996): Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg2+ in rat central neurons. *J. Physiol.* 494: 479-492.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994): Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron.* 12: 529-540.

Morgan CJ, Mofeez A, Brandner B, Bromley L, Curran HV (2004): Acute effects of ketamine on memory systems and psychotic symptoms in healthy volunteers. *Neuropsychopharmacology.* 29 :208–218.

Morilak DA, Frazer A (2004): Antidepressants and brain monoaminergic systems: a dimensional approach to understanding their behavioral effects in depression and anxiety disorders. *Int. J. Neuropsychopharmacol.* 7: 193-218.

Morris PG, Mishina M, Jones S (2018): Altered Synaptic and Extrasynaptic NMDA Receptor Properties in Substantia Nigra Dopaminergic Neurons From Mice Lacking the GluN2D Subunit. *Front. Cell. Neurosci.* 12: 354.

Mullasseril P, Hansen KB, Vance KM, Ogden KK, Yuan H, Kurtkaya NL et al (2010): A subunit-selective potentiator of NR2C- and NR2D-containing NMDA receptors. *Nat. Commun.* 90: 10.1038.

Murrough JW, Iacoviello B, Neumeister A, Charney DS, Iosifescu DV (2011): Cognitive dysfunction in depression: neurocircuitry and new therapeutic strategies. *Neurbiol. Learn Mem.* 96: 553-563.

Murrough JW, Iosifescu DV, Chang LC, Al Jurdi RK, Green CE, Perez AM et al (2013): Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial. *Am. J. Psychiatry.* 170: 1134–1142.

Nackenoff A, Moussa-Tooks AB, McMeekin AM, Veenstra-VanderWeele J, Blakely RD (2016): Essential contributions of serotonin transporter inhibition to the acute and chronic actions of fluoxetine and citalopram in the SERT Met172 mouse. *Neuropsychopharmacology.* 41: 1733-1741.

Narita M, Soma M, Mizoguchi H, Tseng LF, Suzuki T (2000): Implications of the NR2B subunit-containing NMDA receptor localized in mouse limbic forebrain in ethanol dependence. *Eur. J. Pharmacol.*401: 191-195.

Nascimento Hackl LP and Carobrez AP (2007): Distinct ventral and dorsal hippocampus AP5 anxiolytic effects revealed in the elevated plus-maze task in rats. *Neurobiol. Learn. Mem.* 88: 177-185.

Nemeroff CB, Krishnan KR, Reed D, Leder R, Beam C, Dunnick NR (1992): Adrenal gland enlargement in major depression. A computed tomographic study. *Arch. Gen. Psychiatry.* 49: 384-387.

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K et al (1984): Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. *Science.* 226: 1342-1344.

Nestler EJ (2015): Role of the brain's reward circuitry in depression: translational mechanisms. *Int. Rev. Neurobiol.* 124: 151-170.

Nestler EJ and Hyman SE (2010): Animal models of neuropsychiatric disorders. *Nat. Neurosci.* 13: 1161-1169.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002): Neurobiology of depression. *Neuron.* 34: 13-25.

Neumaier JF, Petty F, Kramer GL, Szot P, Hamblin MW (1997): Learned helplessness increase 5-hydroxytryptamine 1B receptor mRNA levels in the rat dorsal raphe nucleus. *Biol. Psychiatry.* 41: 668-674.

Neumann ID (2008): Brain oxytocin: a key regulator of emotional and social behaviors in both females and males. *J. Neuroendocrinol.* 20: 858-865.

Neumann ID and Landgraf R (2012): Balance of oxytocin and vasopressin: implications for anxiety, depression, and social behaviors. *Trends Neurosci.* 35: 649-659.

Newman-Tancredi A, Martel JC, Assie MB, Buritova J, Lauressergues E, Cosi C et al (2009): Signal transduction and functional selectivity of F15599, a preferential post-synaptic 5-HT1A receptor agonist. *Br. J. Pharmacol.* 156: 338-353.

Nguyen AQ, Dela Cruz JAD, Sun Y, Holmes TC, Xu X (2016): Genetic cell targeting uncovers specific neuronal types and distinct subregions in the bed nucleus of the stria terminalis. *J. Comp. Neurol.* 524: 2379-2399.

Nichols DE and Nichols CD (2008): Serotonin receptors. *Chem. Rev.* 108: 1614-1641.

Nosyreva E, Szabla K, Autry AE, Ryazanov AG, Monteggia LM, Kavalali ET (2013): Acute suppression of spontaneous neurotransmission drives synaptic potentiation. *J. Neurosci.* 33: 6990–7002.

Nowak C, Ordway CA, Paul IA (1995): Alterations in the N-methyl-D-aspartate (NMDA) receptor complex in the frontal cortex of suicide victims. *Brain Res.* 675: 157 – 164

Nowak G, Trullas R, Layer RT, Skolnick P, Paul IA (1993): Adaptive changes in the N-methyl-D-aspartate receptor complex after chronic treatment with imipramine and 1-aminocyclopropanecarboxylic acid. *J. Pharmacol. Exp. Ther.* 265: 1380-1386

O'Neill MF and Conway MW (2001): Role of 5-HT(1A) and 5-HT(1B) receptors in the mediation of behavior in the forced swim test in mice. *Neuropsychopharmacology.* 24: 391-398.

Obiang P, Macrez R, Jullienne A, Bertrand T, Lesept F, Ali C, Maubert E, Vivien D, Agin V (2012): GluN2D subunit-containing NMDA receptors control tissue plasminogen activator-mediated spatial memory. *J. Neurosci.* 32: 12776-34.

Ogawa S, Fujii T, Koga N, Hori H, Teraishi T, Hattori K et al (2014): Plasma L-tryptophan concentration in major depressive disorder: new data and meta-analysis. *J. Clin. Psychiatry.* 75: e906-15.

Ogden KK, Khatri A, Traynelis SF, Heldt SA (2014): Potentiation of GluN2C/D NMDA receptor subtypes in the amygdala facilitates the retention of fear and extinction learning in mice. *Neuropsychopharmacology.* 39: 625-637.

Oh SW, Harris JA, Ng L, Winslow G, Cain N, Mihalas S et al (2014): A mesoscale connectome of the mouse brain. *Nature.* 508: 207–214.

Olsen CM, Winder DG (2010): Operant sensation seeking in the mouse. *JoVE.* 45.

Oomen CA, Meyer JL, de Kloet ER, Joels M, Lucassen PJ (2007): Brief treatment with the glucocorticoid receptor antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress. *Eur. J. Neurosci.* 26: 3395-3401.

Ordway GA and Klimek V (2001): Noradrenergic pathology in psychiatric disorders: postmortem studies. *CNS Spectr.* 6: 697-703.

Ortiz S, Latsko MS, Fouty JL, Dutta S, Adkins JM, Jasnow AM (2019): Anterior cingulate cortex and ventral hippocampal inputs to the basolateral amygdala selectively control generalized fear. *J. Neurosci.* 39: 6526-6539.

Otte C, Gold SM, Penninx BW, Pariante CM, Etkin A, Fava M et al (2016): Major depressive disorder. *Nat. Rev. Dis. Primers.* 2: 16065.

Ozsoy S, Olguner Eker O, Abdulrezzak U (2016): The effects of antidepressants on Neuropeptide Y in patients with depression and anxiety. *Pharmacopsychiatry*. 49: 26-31.

Painsipp E, Herzog H, Holzer P (2008): Implication of neuropeptide-Y Y2 receptors in the effects of immune stress on emotional, locomotor and social behavior of mice. *Neuropharmacology*. 55: 117–126.

Paoletti P, Bellone C, Zhou Q (2013): NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience.* 14: 383-400.

Papolos DF, Edwards E, Marmur R, Lachman HM, Henn FA (1993): Effects of the antiglucocorticoid RU 38486 on the induction of learned helplessness behavior in Sprague-Dawley rats. *Brain Res.* 615: 304-309.

Papouin T and Oliet SH (2014): Organization, control and function of extrasynaptic NMDA receptors. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 369: 20130601.

Papp M and Moryl E (1994): Antidepressant activity of non-competitive and competitive NMDA receptor antagonists in a chronic mild stress model of depression. *Eur. J. Pharmacol.* 263: 1-7.

Papp M, Gruca P, Boyer PA, Mocaer E (2003): Effect of agomelatine in the chronic mild stress model of depression in the rat. *Neuropsychopharmacology.* 28: 694-703.

Paquet M and Smith Y (2000): Presynaptic NMDA receptor subunit immunoreactivity in GABAergic terminals in rat brain. *J. Comp. Neurol.* 423: 330-47.

Pare CM, Yeung DP, Price K, Stacey RS (1969): 5-hydroxytryptamine, noradrenaline, and dopamine in brainstem, hypothalamus, and caudate nucleus of controls and of patients committing suicide by coal-gas poisoning. *Lancet.* 2: 133-135.

Pariante CM (2009): Risk factors for development of depression and psychosis. Glucocorticoid receptors and pituitary implications for treatment with antidepressant and glucocorticoids. *Ann. N.Y. Acad. Sci.* 1179: 144-152.

Parker KJ, Kenna HA, Zeitzer JM, Keller J, Blasey CM, Amico JA, Schatzberg AJ (2010): Preliminary evidence that plasma oxytocin levels are elevated in major depression. *Psychiatry Res.* 178: 359-362.

Parsey RV, Ogden RT, Miller JM, Tin A, Hesselgrave N, Goldstein E et al (2010): Higher serotonin 1A binding in a second major depression cohort: modeling and reference region considerations. *Biol. Psychiatry*. 68: 170-178.

Partridge JG, Forcelli PA, Luo R, Cashdan JM, Schulkin J, Valentino RJ, Vicini S (2016): Stress increases GABAergic neurotransmission in CRF neurons of the central amygdala and bed nucleus stria terminalis. *Neuropsychopharamcology.* 107: 239-250.

Paul IA, Layer RT, Skolnick, P, Nowak G (1993): Adaptation of the N- methyl-D-aspartate receptor complex in rat front cortex following chronic treatment with electroconvulsive shock or imipramine. *Eur. J. Pharmacol.* 247: 305-312.

Paul IA, Nowak G, Layer RT, Popik P, Skolnick P (1994): Adaptation of N-methyl-D-aspartate receptor complex following chronic antidepressant treatments. *J. Pharmcol. Exp. Therapeut.* 269: 95-102.

Paxinos G, Franklin B (2004) The mouse brain in stereotaxic coordinates, 3rd edition. Cambridge, Massachusetts: Academic Press.

Pellow S, Chopin P, File SE, Briley M (1985): Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods.* 14: 149-167.

Pepin MC, Govindan MV, Barden N (1992): Increased glucocorticoid receptor gene promoter activity after antidepressant treatment. *Mol. Pharmacol.* 41: 1016-1022.

Perszyk R, Katzman BM, Kusumoto H, Kell SA, Epplin MP, Thariovic YA et al (2018): An NMDAR positive and negative allosteric modulator series share a binding site and are interconverted by methyl groups. *eLife.* 7: e34711.

Perszyk RE, DiRaddo JO, Strong KL, Low CM, Ogden KK, Khatri A et al (2016): GluN2D-containing N-methyl-d-aspartate receptors mediate synaptic transmission in hippocampal interneurons and regulate interneuron activity. *Mol. Pharmacol.* 90: 689-702.

Perszyk RE, Swanger SA, Shelley C, Kharti A, Fernandez-Cuervo G, Epplin MP et al (2020): Biased modulators of NMDA receptors control channel opening and ion selectivity. *Nat. Chem. Biol.* 16: 188-196.

Petit-Demouliere B, Chenu F, Bourin M (2005): Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology.* 177: 245-255.

Phelix CF, Liposits Z, Paull WK (1992): Monoamine innervation of bed nucleus of stria terminalis: an electron microscopic investigation. *Brain Res. Bull.* 28: 949–965.

Pina-Crespo, JC and Gibb AJ (2002): Subtypes of NMDA receptors in new-born rat hippocampal granula cells. *J. Physiol.* 541: 41-64.

Pizzagalli DA, Berretta S, Wooten D, Goer F, Pilobello KT, Kumar P et al (2019): Assessment of striatal dopamine transporter binding in individuals with major depressive disorder: in vivo positron emission tomography and postmortem evidence. *JAMA Psychiatry*. 76: 854-861.

Planchez B, Surget A, Belzung C (2019): Animal models of major depression: drawbacks and challenges. *Journal of Neural Transmission*. 126: 1383-1408.

Pleil KE, Rinker JA, Lowery-Gionta EG, Mazzone CM, McCall NM, Kendra AM, Olson DP, Lowell BB, Grant KA, Thiele TE, Kash TL (2015): NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. *Nat. Neurosci.* 18:545–52.

Pleil KE, Rinker JA, Lowery-Gionta EG, Mazzone CM, McCall NM, Kendra AM et al (2015): NPY signaling inhibits extended amygdala CRF neurons to suppress binge drinking. *Nat. Neurosci.* 18: 545-552.

Poleszak E, Stasiuk W, Szopa A, Wyska E, Serefko A, Oniszczuk A et al (2016): Traxoprodil, a selective antagonist of the NR2B subunit of the NMDA receptor, potentiates the antidepressant-like effects of certain antidepressant drugs in the forced swim test in mice. *Metab. Brain Dis.* 31: 803–814.

Pompeiano M, Palacios JM, Mengod G (1992): Distribution and cellular localization of mRNA coding for 5-HT1A receptor in the rat brain: correlations with receptor binding. *J. Neurosci.* 12: 440-453.

Pomrenze MB, Fetterly TL, Winder DG, Messing RO (2017): The Corticotropin Releasing Factor Receptor 1 in Alcohol Use Disorder: Still a Valid Drug Target? *Alcohol Clin. Exp. Res.* 41: 1986-99.

Porsolt RD, Bertin A, Jalfre M (1977): Behavioral despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.* 229: 327-336.

Preskorn SH, Baker B, Kolluri S, Menniti FS, Krams M, Landen JW (2008): An Innovative Design to Establish Proof of Concept of the Antidepressant Effects of the NR2B Subunit Selective N-Methyl-D-Aspartate Antagonist, CP-101,606, in Patients With Treatment-Refractory Major Depressive Disorder. *J. Clinical Psychopharmacology.* 28: 631-637.

Pruessner JC, Champagne F, Meaney MJ, Dagher A (2004): Dopamine release in response to a psychological stress in humans and its relationship to early life maternal care: a positron emission tomography study using [11C] raclopride. *J. Neurosci.* 24: 2825-2831.

Qian A, Buller AL, Johnson JW (2005): NR2 subunit dependence of NMDA receptor channel block by external Mg2+. *J. Physiol.* 562: 319-331.

Raadsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJ, Tilders FJ et al (1995): Corticortropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. *Am. J. Psychiatry.* 152: 1372-1376.

Rajkowska, G (2000): Histopathology of the prefrontal cortex in major depression: what does it tell us about dysfunctional monoamine circuits? *Prog. Brain Res.* 126: 397-412.

Reagan LP, Reznikov LR, Evans AN, Gabriel C, Mocaër E, Fadel JR (2012): The antidepressant agomelatine inhibits stress-mediated changes in amino acid efflux in the rat hippocampus and amygdala. *Brain Res.* 1466: 91–98.

Redrobe JP, Dumont Y, Herzog H, Quirion R (2003): Neuropeptide Y (NPY) Y2 receptors mediate behaviour in two animal models of anxiety: evidence from Y2 receptor knockout mice. *Behav Brain Res.* 141: 251-5.

Redrobe JP, Dumont Y, Quirion R (2002): Neuropeptide Y (NPY) and depression: from animal studies to the human condition. *Life Sci.* 71: 2921-2937.

Redrobe JP, Dumont Y, Fournier A, Quirion R (2002): The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test *Neuropsychopharmacology*. 26: 615-624.

Redrode JP and Bourin M (1999): The effects of lithium administration in animal models of depression: a short review. *Fund. & Clin. Pharmacol.* 13: 293-299.

Regev L, Neufeld-Cohen A, Tsoory M, Kuperman Y, Getselter D, Gil S, Chen A (2011): Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation. *Mol. Psychiatry.* 16: 714–728.

Reul JM and Holsboer F (2002): Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr. Opin. Pharmacol.* 2: 23-33.

Reznikov LR, Grillo CA, Piroli G, Pasumarthi RK, Reagan LP, Fadel JR (2007): Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: Differential effects of antidepressant treatment. *Eur. J. Neurosci.* 25: 3109–3114.

Riad M, Garcia S, Watkins KC, Jodoin N, Langlois X, El Mestikawy S et al (2000): Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. *J. Comp. Neurol.* 417: 181-194.

Richardson-Jones JW, Craige CP, Nguyen TH, Kung HF, Gardier AM, Dranovsky A et al (2011): Serotonin-1A autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. *J. Neurosci.* 31: 6008-6018.

Rigby M, O'Donnell R, Rupniak NM (2005): Species differences in tachykinin receptor distribution: further evidence that the substance P (NK1) receptor predominates in human brain. *J. Comp. Neurol.* 490: 335-353.

Ring RH, Schechter LE, Leonard SK, Dwyer JM, Platt BJ, Graf R et al (2010): Receptor and behavioral pharmacology of WAY-267464, a non-peptide oxytocin receptor agonist. *Neuropharmacology.* 58: 69-77.

Robinson LA, Berman JS, Neimeyer RA (1990): Psychotherapy for the treatment of depression: a comprehensive review of controlled outcome research. *Psychol. Bull.* 108: 30-49.

Rodaros D, Caruana DA, Amir S, Stewart J (2007): Corticotropin-releasing factor projections from limbic forebrain and paraventricular nucleus of the hypothalamus to the region of the ventral tegmental area. *Neuroscience.* 150: 8-13.

Roman CW, Lezak KR, Hartsock MJ, Falls MA, Braas KM, Howard AB et al (2014): PAC1 receptor antagonism in the bed nucleus of the stria terminalis (BNST) attenuates the endocrine and behavioral consequences of chronic stress. *Psychoneuroendocrinology.* 47: 151–165.

Ronan PJ, Summers CH (2011): Molecular Signaling and Translational Significance of the Corticotropin Releasing Factor System. In: The Brain as a Drug Target: Progress in Molecular Biology and Translational Science (Rahman S, ed), pp235-92. San Diego: Academic Press.

Russo SJ and Nestler EJ (2013): The brain reward circuitry in mood disorders. *Nat. Rev. Neurosci.* 14: 10.0138.

Sacher J, Neumann J, Fünfstück T, Soliman A, Villringer A, Schroeter ML (2012): Mapping the depressed brain: a meta-analysis of structural and functional alterations in major depressive disorder. *J. Affect. Disord.* 140: 142–148.

Sahli ZT, Banerjee P, Tarazi FI (2016): The preclinical and clinical effects of vilazodone for the treatment of major depressive disorder. *Expert Opin. Drug Discov.* 11: 515-523.

Sahuque LL, Kullberg EF, Mcgeehan AJ, Kinder JR, Hicks MP, Blanton MG et al (2006): Anxiogenic and aversive effects of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis in the rat: role of CRF receptor subtypes. *Psychopharmacology.* 186: 122–132.

Sakanaka M, Shibasaki T, Lederis K (1986): Distrivution and efferent projections of corticotropin-releasing factor-like immunoreactivity in the rat amygdaloid complex. *Brain Res.* 382: 213-238.

Sanacora G, Gueorguieva R, Epperson CN, Wu YT, Appel M, Rothman DL, Krystal KH, Mason GF (2004): Subtype-specific alterations of gamma-aminobutyric acid and glutamate in patients with major depression. *Arch. Gen. Psychiatry.* 61: 705-713.

Sanacora G, Smith MA, Pathak S, Su HL, Boeijinga PH, McCarthy DJ et al (2014): Lanicemine: a low-trapping NMDA channel blocker produces sustained antidepressant efficacy with minimal psychotomimetic adverse effects. *Mol. Psychiatry.* 19: 978–985.

Sanacora G, Treccani G, Popoli M (2012): Towards a glutamate hypothesis of depression – An emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology.* 62: 63-77.

Sanacora G, Zarate CA, Krystal JH, Manji HK (2008): Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nature Reviews Drug Discovery.* 7: 426-437.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S et al (2003): Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science.* 301: 805-809.

Sapkota K, Mao Z, Synowicki P, Lieber D, Liu M, Ikezu T, Gautam V, Monaghan DT (2016): GluN2D N-methyl-D-aspartate receptor subunit contribution to the stimulation of brain activity and gamma oscillations by ketamine: implications for schizophrenia. *J. Pharmcol. Exp. Ther.* 356: 702-711.

Sarchiapone M, Carli V, Camardese G, Cuomo D, Di Giuda D, Calcagni ML, Focacci C, De Risio S (2006): Dopamine transporter binding in depressed patients with anhedonia. *Psychiatry Res.* 147: 243-248.

Scheggi S, De Montis MG, Gambarana C (2018): Making Sense of Rodent Models of Anhedonia. *Int. J. Neuropsychopharmacol.* 21 :1049–1065.

Schoemaker H, Claustre Y, Fage D, Rouquier L, Chergui K, Curet O et al (1997): Neurochemical characteristics of amisulpride, an atypical dopamine D2/D3 receptor antagonist with both presynaptic and limbic selectivity. *J. Pharmacol. Exp. Ther.* 280: 83-97.

Schramm NL, McDonald PM, Limbird LE (2001): The α_{2a}-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. *J. Neurosci.* 21: 4875-4882.

Sesack SR and Grace AA (2010): Cortico-Basal Ganglia reward network: microcircuitry. *Neuropsychopharmacology.* 35: 27–47.

Shelkar GP, Pavuluri R, Gandhi PJ, Ravikrishnan A, Gawande DY, Liu J, Stairs DJ, Ugale RR, Dravid SM (2019): Differential effect of NMDA receptor GluN2C and GluN2D subunit ablation on behavior and channel blocker-induced schizophrenia phenotypes. *Sci. Rep.* 9: 7572.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994): Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature.* 368: 144-147.

Shors TJ, Seib TB, Levine S, Thompson RF (1989): Inescapable versus escapable shock modulates long-term potentiation in the rat hippocampus. *Science.* 244: 224-226.

Shulman KI, Herrmann N, Walker SE (2013): Current place od monoamine oxidase inhibitors in the treatment of depression. *CNS Drugs.* 27: 789-797.

Silberman Y and Winder DG (2013): Emerging role for corticotropin releasing factor signaling in the bed nucleus of the stria terminalis at the intersection of stress and reward. *Front. Psychiatry.* 4: 42.

Silberman Y, Matthews RT, Winder DG (2013): A corticotropin releasing factor pathway for ethanol regulation of the ventral tegmental area in the bed nucleus of the stria terminalis. *J. Neurosci.* 33:950-66.

Singewald GM, Rjabokon A, Singewald N, Ebner K (2011): The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses. *Neuropsychopharmacology.* 36: 793–804.

Singh A, Kar SK (2017): How electroconvulsive therapy works: understanding the neurobiological mechanisms. *Clin. Psychopharmacol. Neurosci.* 15: 210-221.

Sink KS, Walker DL, Freeman SM, Flandreau EI, Ressler KJ, Davis M (2013): Effects of continuously enhanced corticotropin releasing factor expression within the bed nucleus of the stria terminalis on conditioned and unconditioned anxiety. *Mol Psychiatry.* 18:308–19.

Slattery DA and Neumann ID (2010): Oxytocin and major depressive disorder: experimental and clinical evidence for links to aetiology and possible treatment. *Pharmaceuticals.* 3: 702-724.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH et al (1998): Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron.* 20: 1093-1102.

Smith KS, Berridge KC, Aldridge JW (2011): Disentangling pleasure from incentive salience and learning signals in brain reward circuitry. *Proc. Natl. Acad. Sci.* 108: E255–64.

Smith RJ and Aston-Jones G (2008): Noradrenergic transmission in the extended amygdala: role in increased drug-seeking and relapse during protracted drug abstinence. *Brain Struct. Funct.* 213: 43–61.

Sos P, Klirova M, Novak T, Kohutova B, Horacek J, Palenicek T (2013): Relationship of ketamine's antidepressant and psychotomimetic effects in unipolar depression. *Neuro. Endocrinol. Lett.* 34: 287–293.

Stahl, SM (2003): Neurotransmission of cognition, part 3. Mechanism of action of selective NRIs: both dopamine and norepinephrine increase in prefrontal cortex. *J. Clin. Psychiatry*. 64: 230-1

Stamatakis AM, Sparta DR, Jennings JH, McElligott ZA, Decot H, Stuber GD (2014): Amygdala and bed nucleus of the stria terminalis circuitry: implications for addiction-related behaviors. *Neuropharmacology.* 76: 320-328.

Standaert DG, Landwehrmeyer GB, Kerner JA, Penney JB Jr, Young AB (1996): Expression of NMDAR2D glutamate receptor subunit mRNA in neurochemically identified interneurons in the rat neostriatum, neocortex and hippocampus. *Brain Res. Mol. Brain Res.* 42: 89–102.

Steru L, Chermat R, Thierry B, Simon P (1985): The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology.* 85: 367-370.

Stockmeier CA, Shapiro LA, Dilley GE, Kolli TN, Friedman L, Rajkowska G (1998): Increases in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression – postmortem evidence for decreased serotonin activity. *J. Neurosci.* 18: 7394-7401.

Stout SC, Mortas P, Owens MJ, Nemeroff CB, Moreau J (2000): Increased corticotropin-releasing factor concentrations in the bed nucleus of the stria terminalis of anhedonic rats. *Eur. J. Pharmacol.* 401: 39–46.

Straube T, Mentzel HJ, Miltner WH (2007): Waiting for spiders: brain activation during anticipatory anxiety in spider phobics. *Neuroimage.* 37: 1427–1436.

Strekalova T, Gorenkova N, Schunk E, Dolgov O, Bartsch D (2006): Selective efecs of citalopram in a mouse model of stress-induced anhedonia with a control for chronic stress. *Behav. Pharmacol.* 17: 271-287.

Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P (2004): Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology.* 29: 2007-2017.

Strosberg AD (1993): Structure, function, and regulation of adrenergic receptors. *Protein Sci.* 2: 1198-1209.

Suárez F, Zhao Q, Monaghan DT, Jane DE, Jones S, Gibb AJ (2010): Functional heterogeneity of NMDA receptors in rat substantia nigra pars compacta and reticulata neurones. *Eur. J. Neurosci.* 32: 359–367.

Suryavanski PS, Ugale RR, Yilmazer-Hanke D, Stairs DJ, Dravid SM (2013): GluN2C/GluN2D subunit-selective NMDA receptor potentiator CIQ reverses MK-801-induced impairment in prepulse inhibition and working memory in Y-maze test in mice. *Brit. J. Pharmacol.* 171: 799-809.

Sutton MA, Taylor AM, Ito HT, Pham A, Schuman EM (2007): Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. *Neuron*. 55: 648–661.

Svenningsson P, Chergui K, Rachleff I, Flajolet M, Zhang X, El Yacoubi M (2006): Alterations in 5-HT1B receptor function by p11 in depression-like states. *Science.* 311: 77-80.

Swanger SA, Vance KM, Acker TM, Zimmerman S, DiRaddo JO, Myers S et al (2018): A Novel Negative Allosteric Modulator Selective for GluN2C/2D-Containing NMDA Receptors Inhibits Synaptic Transmission in Hippocampal Interneurons. *ACS Chem. Neurosci.* 9 :306–319.

Swanger SA, Vance KM, Pare JF, Sotty F, Fog K, Smith Y, Traynelis SF (2015): NMDA Receptors Containing the GluN2D Subunit Control Neuronal Function in the Subthalamic Nucleus. *J. Neurosci.* 35: 15971-15983.

Swanson LW, Sawchenko PE, Rivier J, Vale WW (1983): Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology.* 36: 165–186.

Tamagno G and Epelbaum J (2015): Editorial: neurological and psychiatric disorders in endocrine diseases. *Front. Endocrinol.* 6: 75.

Tanoue A, Ito S, Honda K, Oshikawa S, Kitagawa Y, Koshimizu TA (2004): The vasopressin V1b receptor critically regulates hypothalamic–pituitary–adrenal axis activity under both stress and resting conditions. *Journal of Clinical Investigation.* 113: 302–309.

Tasan RO, Lin S, Hetzenauer A, Singewald N, Herzog H, Sperk G (2009): Increased novelty-induced motor activity and reduced depression-like behavior in neuropeptide Y (NPY)-Y4 receptor knockout mice. *Neuroscience.* 158: 1717–1730.

Tasan RO, Nguyen NK, Weger S, Sartori SB, Singewald N, Heilbronn R et al (2010): The central and basolateral amygdala are critical sites of neuropeptide Y/Y2 receptor-mediated regulation of anxiety and depression. *J. Neurosci.* 30: 6282-6290.

Tatarczynska E, Klodzinska A, Stachowicz K, Chojnacka-Wojcik E (2004): Effects of a selective 5-HT1B receptor agonist and antagonists in animal models of anxiety and depression. *Behav. Pharmacol.* 15: 523-534.

Thompson CL, Drewery DL, Atkins HD, Stephenson FA, Chazot PL (2000): Immunohistochemical localization of N-methyl-D-aspartate receptor NR1, NR2A, NR2B and NR2C/D subunits in the adult mammalian cerebellum. *Neurosci. Lett.* 283: 85–88.

Thompson SM, Kallarackal AJ, Kvarta MD, Van Dyke AM, LeGates TA, Cai X (2015): An excitatory synapse hypothesis of depression. *Trends Neurosci.* 38: 279-294.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK et al (1998): Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat. Genet.* 19: 162-166.

Tome MB, Isaac MT, Harte R, Holland C (1997): Paroxetine and pindolol: a randomized trial of serotonergic autoreceptor blockade in the reduction of antidepressant latency. *Int. Clin. Psychopharmacol.* 12: 81-89.

Tornatzky W and Miczek KA (1994): Behavioral and autonomic responses to intermittent social stress: differential protection by clonidine and metoprolol. *Psychopharmacology.* 116: 346-356.

Tran L, Schulkin J, Greenwood-Van Meerveld B (2014): Importance of CRF receptor-mediated mechanisms of the bed nucleus of the stria terminalis in the processing of anxiety and pain. *Neuropsychopharmacology.* 39: 2633–2645.

Tran PV, Bymaster FP, McNamara RK, Potter WZ (2003): Dual monoamine modulation for improved treatment of major depressive disorder. *J. Clin. Psychopharmacol.* 23: 78-86.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK et al (2010): Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* 62: 405–496.

Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L et al. (2006): Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. *Am. J. Psychiatry*. 163: 28-40.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC et al (1999): Distribution of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat. Genet.* 23: 99-103.

Trullas R and Skolnick P (1990): Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *Eur. J. Pharmacol.* 185: 1-10.

Tschenett A, Singewald N, Carli M, Balducci C, Salchner P, Vezzani A et al (2003): Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. *Eur. J. Neurosci.* 18: 143–148.

Turesson HK, Rodriguez-Sierra OE, Pare D (2013): Intrinsic connections in the anterior part of the bed nucleus of the stria terminalis. *J. Neurophysiol.* 109: 2438-50.

Tye KM, Mirzabekov JJ, Warden MR, Ferenczi EA, Tsai HC, Finklestein J et al (2013): Dopamine neurons modulate neural encoding and expression of depression-related behavior. *Nature.* 493: 537-541.

Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003): Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron.* 39: 401–407.

van Londen L, Goekoop JG, van Kempen GM, Frankhuijzen-Sierevogel AC, Wiegant VM, van der Velde EA, De Wied D (1997): Plasma levels of arginine vasopressin elevated in patients with major depression. *Neuropsychopharmacology.* 17: 284-292.

Van Pett K, Viau K, Bittencourt JC, Chan RK, Li HY, Arias C (2000): Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J. Comp. Neurol.* 428: 191-212.

van Praag HM, de Haan S (1979): Central serotonin metabolism and frequency of depression. *Psychiatry Res.* 1: 219-224.

Vance KM, Hansen KB, Traynelis SF (2012): GluN1 splice variant control of GluN1/GluN2D NMDA receptors. *J. Physiol.* 590: 3857–3875.

Venzala E, Garcia-Garica AL, Elizalde N, Delagrange P, Tordera RM (2012): Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. *Psychopharmacology.* 224: 313-325.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998): Functional and pharmacological differences between recombinant N-methyl-d-aspartate receptors. *J. Neurophysiol.* 79: 555-566.

Volianskis A, Bannister N, Collett VJ, Irvine MW, Monaghan DT, Fitzjohn SM, Jensen MS, Jane DE, Collingridge GL (2013): Different NMDA receptor subtypes mediate induction of long-term potentiation and two forms of short-term potentiation at CA1 synapses in rat hippocampus in vitro. *J. Physiol.* 591:955–972.

Volianskis A, France G, Jensen MS, Bortolotto ZA, Jane DE, Collingridge GL (2015): Long-term potentiation and the role of N-methyl-d-aspartate receptors. *Brain Res.* 1621: 5-16.

von Engelhardt J, Bocklisch C, Tonges L, Herb A, Mishina M, Monyer H (2015): GluN2D-containing NMDA receptors-mediate synaptic currents in hippocampal interneurons and pyramidal cells in juvenile mice. *Front. Cell. Neurosci.* 9: 95.

Vranjkovic O, Gasser PJ, Gerndt CH, Baker DA, Mantsch JR (2014): Stress-induced cocaine seeking requires a beta-2 adrenergic receptor-regulated pathway from the ventral bed nucleus of the stria terminalis that regulates CRF actions in the ventral tegmental area. *J. Neurosci.* 34: 12504-12514.

Vranjkovic O, Winkler G, Winder DG (2018): Ketamine administration during a critical period after forced ethanol abstinence inhibits the development of time-dependent affective disturbances. *Neuropsychopharmacology.* 43: 1915-1923.

Vyklicky V, Korinek M, Smejkalova T, Balik A, Krausova B, Kaniakova M et al (2014): Structure, function, and pharmacology of NMDA receptor channels. *Physiol. Res.* 63, Suppl 1: S191–S203.

Walker DL and Davis M (2008): Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Struct. Funct.* 213: 29–42.

Wallace DL, Han MH, Graham DL, Green TA, Vialou V, Iniguez SD et al (2009): CREB regulation of nucleus accumbens excitability mediates social isolation-induced behavioral deficits. *Nat. Neurosic.* 12: 200-209.

Wang Q, Timberlake MA, Prall K, Dwivedi Y (2018): The recent progress in animal models of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* 77: 99-109.

Wasserman D, Sokolowski M, Rozanov V, Wasserman J (2008): The CRHR1 gene: a marker for suicidality in depressed males exposed to low stress. *Genes Brain Behav.* 7: 14-19.

Watanabe M, Inou Y, Sakimura K, Mishina M (1993): Distinct Spatio-temporal Distributions of the NMDA Receptor Channel Subunit mRNAs in the Brain. *Ann. N.Y. Acad. Sci.* 707: 463-66.

Watanabe M, Inoue Y, Sakimura K, Mishina M (1992): Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Dev. Neurosci.* 3: 1138-40.

Weed MR, Bookbinder M, Polino J, Keavy D, Cardinal RN, Simmermacher-Mayer J, Cometa FL, King D, Thangathirupathy S, Macor JE, Bristow LJ (2016): Negative Allosteric Modulators Selective for The NR2B Subtype of The NMDA Receptor Impair Cognition in Multiple Domains. *Neuropsychopharmacology.* 41: 568-577.

Weitlauf C, Egli RE, Grueter BA, Winder DG (2004): High-frequency stimulation induces ethanol-sensitive long-term potentiation at glutamatergic synapses in the dorsolateral bed nucleus of the stria terminalis. *J. Neurosci.* 24: 5741–5747.

Weitlauf C, Honse Y, Auberson YP, Mishina M, Lovinger DM, Winder DG (2005): Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. *J. Neurosci.* 25: 8386–8390.

Wenzel A, Schurer L, Kunzi R, Fritschy JM, Mohler H, Benke D (1995): Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. *Neuroreport*. 7: 45-48.

Wenzel A, Villa M, Mohler H, Benke D (1996): Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. *J Neurochem.* 66: 1240 –1248.

Werstiuk ES, Coote M, Griffith L, Shannon H, Steiner M (1996): Effects of electroconvulsive therapy on peripheral adrenoceptors, plasma, noradrenaline, MHPG and cortisol in depressed patients. *Br. J. Psychiatry.* 169: 758-765.

Widerlov E, Lindstrom LH, Wahlestedt C, Ekman R (1988): Neuropeptide Y and peptide YY as possible cerebrospinal markers for major depression and scizophrenia respectively. *J. Psychiat. Res*. 22: 69-79.

Wilkinson MB, Xiao G, Kumar A, LaPlant Q, Renthal W, Sikder D et al (2009): Imipramine treatment and resiliency exhibit similar chromatin regulation in the mouse nucleus accumbens in depression models. *J. Neurosci.* 29: 7820-7832.

Willard SS and Koochekpour S (2013): Glutamate, glutamate receptors, and downstream signaling pathways. *Int. J. Biol. Sci.* 9: 948-959.

Willner P (1991): Animal models as simulations of depression. *Trends Pharmacol.* 12: 131-136.

Willner P (2005): Chronic mild stress (CMS) revisited: consistency and behavioral-neurobehavioral concordance in the effects of CMS. *Neuropsychobiology.* 52: 90-110.

Wills TA, Baucum AJ, Louderback KM, Chen Y, Pasek JG, Delpire E et al (2017): Chronic intermittent alcohol disrupts the GluN2B-associated proteome and specifically regulates Group I mGlu receptor dependent long-term depression. *Addict. Biol.* 22: 275-290.

Wills TA, Klug JR, Silberman Y, Baucum AJ, Weitlauf C, Colbran RJ, Delpire E, Winder DG (2012): GluN2B subunit deletion reveals key role in acute and chronic ethanol sensitivity of glutamate synapses in bed nucleus of the stria terminalis. *Proc. Nat. Acad. Sci.* 109: E278-287.

Windle RJ, Kershaw YM, Shanks N, Wood SA, Lightman SL, Ingram CD (2004): Oxytocin attenuates stress-induced c-fos mRNA expression in specific forebrain regions associated with modulation of hypothalamo-pituitary-adrenal activity. *J. Neurosci.* 24: 2974-2982.

Winter C, von Rumohr A, Mundt A, Petrus D, Klein J, Lee T, Morgenstern R, Kupsch A, Juckel G (2007): Lesions of dopaminergic neurons in the substantia nigra pars compacta and in the ventral tegmental area enhance depressive-like behavior in rats. *Behav. Brain Res.* 184: 133-141.

Woulfe JM, Hrycyshyn AW, Flumerfelt BA (1988): Collateral axonal projections from the A1 noradrenergic cell group to the paraventricular nucleus and bed nucleus of the stria terminalis in the rat. *Exp. Neurol.* 102: 121–124.

Wyllie DJA, Livesey MR, Hardingham GE (2013): Influence of GluN2 subunit identity on NMDA receptor function. *Neuropharmacology.* 74: 4-17.

Yamamoto H, Kamegaya E, Hagino Y, Takamatsu Y, Sawada W, Matsuzawa M, Ide S, Yamamoto T, Mishina M, Ikeda K (2017): Loss of GluN2D subunit results in social recognition deficit, social stress, 5-HT2C receptor dysfunction, and anhedonia in mice. *Neuropharmacology*. 112: 188-197.

Yamasaki M, Okada R, Takasaki C, Toki S, Fukaya M, Natsume R, Sakimura K, Mishina M, Shirakawa T, Watanabe M (2014): Opposing Role of NMDA Receptor GluN2B and GluN2D in Somatosensory Development and Maturation. *J. Neurosci.* 34: 11534-11548.

Yang Y, Ju W, Zhang H, Sun L (2018): Effect of ketamine on LTP and NMDAR EPSC in hippocampus of the chronic social defeat stress mice model of depression. *Front. Behav. Neurosci.* 12: 229.

Yi F, Bhattacharya S, Thompson CM, Traynelis SF, Hansen KB (2019): Functional and pharmacological properties of triheteromeric GluN1/2B/2D NMDA receptors. *J. Physiol.* 597: 5495–5514.

Yi F, Rouzbeh N, Hansen KB, Xu Y, Fanger CM, Gordon E et al (2020): PTC-174, a positive allosteric modulator of NMDA receptors containing GluN2C or GluN2D subunits. *Neuropharmacology.* 107971.

Young AH, Gallagher P, Watson S, Del-Estal D, Owen BM, Ferrier IN (2004): Improvements in neurocognitive function and mood following adjunctive treatment with mifepristone (RU-486) in bipolar depression. *Neuropsychopharmacology.* 29: 1538-1545.

Yuan H, Myers SJ, Wells G, Nicholson KL, Swanger SA, Lyuboslavsky P et al (2015): Context-dependent GluN2B-selective inhibitors of NMDA receptor function are neuroprotective with minimal side effects. *Neuron.* 85: 1305–1318.

Zanos P and Gould TD (2018): Mechanisms of ketamine action as an antidepressant. *Mol. Psychiatry.* 23: 801-811.

Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI et al (2016): NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature*. 533: 481–486.

Zarate CA Jr., Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA et al (2006): A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch. Gen. Psychiatry.* 63: 856–864.

Zeng J, Sun F, Wan J, Feng J, Li Y (2019): New optical methods for detecting monoamine neuromodulators. *Curr. Opin. Biomed. Eng.* 12: 68-74.

Zhang W, Hetzel A, Shah B, Atchley D, Blume SR, Padival MA, Rosenkranz JA (2014): Greater physiological and behavioral effects of interrupted stress patterns compared to daily restraint stress in rats. *PLoS One.* 9: e102247.

Zhang X and Chergui K (2015): Dopamine depletion of the striatum causes a cell-type specific reorganization of GluN2B- and GluN2D-containing NMDA receptors. *Neuropharmacology.* 92: 108-115.

Zhang X, Feng, ZJ, Chergui K (2014): GluN2D-containing NMDA receptors inhibit neurotransmission in the mouse striatum through a cholinergic mechanism: implication for Parkinson's disease. *J. Neurochem.* 129: 581-590.

Zhang X, Ge TT, Yin G, Cui R, Zhao G, Yang W (2018): Stress-Induced Functional Alterations in Amygdala: Implications for Neuropsychiatric Diseases. *Front. Neurosci.* 12: 367.

Zhang Z, Wu Y, Wang Z, Dunning FM, Rehfuss J, Ramanan D, Chapman ER, Jackson MB (2011): Release mode of large and small dense-core vesicles specified by different synaptotagmin isoforms in PC12 cells. *Mol. Biol. Cell.* 22: 2324-2336.

Zhu MY, Klimek V, Dilley GE, et al. (1999): Elevated levels of tyrosine hydroxylase in the locus coeruleus in major depression. *Biol. Psychiatry.* 46: 1275-1286.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M et al (2000): Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J. Psychiatr. Res.* 34: 171-181.

Zorrilla EP, Valdez GR, Nozulak J, Koob GF, Markou A (2002): Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. *Brain Res.* 952: 188-199.