MOLECULAR MECHANISMS UNDERLYING DEPRESSION-RELATED BEHAVIOR AND RAPID ANTIDEPRESSANT ACTION

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Dedicated to my mom and dad
Nick and Rita Autry,
To my husband
John Dixon,
And to my family and friends
For their unconditional love and support.

EXPLORING MECHANISMS OF DEPRESSION-RELATED BEHAVIOR AND RAPID ANTIDEPRESSANT ACTION

By

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EXPLORING MECHANISMS OF DEPRESSION-RELATED BEHAVIOR AND RAPID ANTIDEPRESSANT ACTION

Anita Ellen Autry, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2011

Supervising Professor: Lisa Monteggia, Ph.D.

Major Depressive Disorder is a serious mental disorder with a profound disease burden, particularly in the United States. Intriguingly, this disease is almost twice as prevalent in females compared to males. Presently, antidepressant treatment for patients with Major Depressive Disorder requires chronic use and first-line treatment is often ineffective. The neurotrophic hypothesis of depression suggests that a) neurotrophins, in particular brain-derived neurotrophic factor, are necessary for maintaining normal mood states and that b) increases in neurotrophin signaling mediate therapeutic effects of clinical antidepressants. In the laboratory, we have explored aspects of the neurotrophic hypothesis of depression and made progress toward understanding the role of brain-derived neurotrophic factor in depression-related animal models as well as its role in the cellular mechanisms underlying antidepressant efficacy. First, we examined whether loss of brain-derived neurotrophic factor in forebrain

neurons impacted susceptibility to chronic stress, an animal model of depression, in a gender-specific manner. Next we examined the contribution of dorsal raphe nucleus brain-derived neurotrophic factor signaling on traditional antidepressant efficacy. Finally, we uncovered a novel role for brain-derived neurotrophic factor in mediating effects of rapid antidepressant efficacy. In the course of my studies, we have found that brain-derived neurotrophic factor expression may be more important for protecting females from negative behavioral effects of chronic stress; that brain-derived neurotrophic factor receptor activation in dorsal raphe is essential for traditional antidepressant efficacy; and finally that brain-derived neurotrophic factor is required for the action of novel rapid antidepressant ketamine.

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PRIOR PUBLICATIONS

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LIST OF FIGURES

LIST OF ABBREVIATIONS

NT-neurotrophin

NGF- nerve growth factor

BDNF- brain-derived neurotrophic factor

TrkB- tropomysin-related kinase B

PLC γ - phospholipase C γ

PI3K- phosphoinositol 3 kinase

MAPK- mitogen activated protein kinase

GABA- γ-Aminobutyric acid

AMPA- α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

NMDA- *n*-methyl-*d*-aspartate

NMDAR- *n*-methyl-*d*-aspartate receptor

TRPC- transient receptor potential channel C

LTP- long-term potentiation

CREB- cyclic adenosine monophosphate response element binding

MDD- Major Depressive Disorder

SSRI- selective serotonin reuptake inhibitior

TCA- tricyclic antidepressant

MAOI- monoamine oxidase inhibitor

NRI- norepinephrine reuptake inhibitor

mRNA- messenger ribonucleic acid

VTA- ventro-tegmental area

NAc- nucleus accumbens

CA1- *cornu ammonis* 1

IGF- insulin growth factor

ECT- electroconvulsive shock therapy

SNP- single nucleotide polymorphism

BD- Bipolar Disorder

CUS- chronic unpredictable stress

CTL- control

KO- knockout

OF- open field

SCT- sucrose consumption test

FST- forced swim test

TST- tail suspension test

CORT- corticosterone

EIA- enzyme immunosorbent assay

PCR- polymerase chain reaction

DRN- dorsal raphe nucleus

Flox- flanked by loxP sites

fBDNF- floxed BDNF mouse line

fTrkB- floxed TrkB mouse line

CamK- calmodulin dependent kinase

eEF2- eukaryotic elongation factor

LH- learned helplessness

MK801-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate

CPP-3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid

AD- antidepressant

ERK- extracellular related kinase

ActD- actinomycin D

18S- 18 kilodalton subunit of ribosome

ELISA- enzyme-linked immunosorbent assay

CHAPTER 1

INTRODUCTION

Neurotrophins are an important class of signaling molecules in the brain responsible for axon targeting, neuron growth, and maturation of synapses during development. This family of molecules includes neurotrophins (NTs), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). Of these, BDNF is the best characterized in terms of its role in adult brain plasticity and its potential role in the disease pathology or treatment of many psychiatric diseases.

Neurotrophins in the brain are translated into proneurotrophins which are cleaved into mature secreted proteins. ProBDNF signals through the p75 receptor while mature BDNF signals through its high-affinity tropomysin-related kinase B (TrkB) receptor. When TrkB is bound to BDNF, the receptor tyrosine kinase is autophosphorylated leading to activation of phospholipase C gamma (PLC gamma), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase MAPK) pathways. Each of these signaling pathways gives rise to various intracellular signaling cascades which confer the unique function of BDNF on cells, for review see (Mattson, 2008; Yoshii and Constantine-Paton). Briefly, rapid synaptic and ion channel effects are thought to depend on PLC gamma-mediated release in intracellular calcium stores, and longer-lasting effects involving downstream transcription are thought to be downstream of PI3K and MAPK pathway.

Throughout development, BDNF acts as a signal for proper guidance of growing axons. BDNF is secreted from target tissues and TrkB

receptors internalize upon ligand binding and signal to the nucleus of the cell to stimulate neurite outgrowth (Yoshii and Constantine-Paton). BDNF is known to be required for proper development and survival of dopaminergic, GABA-ergic, cholinergic, and serotongeric neurons (Pillai, 2008).

BDNF also serves essential functions in mature brain in synaptic plasiticity (Poo, 2001) and is crucial for learning and memory processes (Lu et al., 2008). BDNF and TrkB are localized at pre-and post-synaptic sites, where BDNF can be released in an activity-dependent manner (Waterhouse and Xu, 2009). Presynaptically, BDNF signaling promotes neurotransmitter release while postsynaptically BDNF is involved in enhancing various ion channels' function including AMPA, NMDA, TRPC, sodium, and potassium channels for detailed review see (Rose et al., 2004). BDNF acts at both excitatory and inhibitory synapses (Kovalchuk et al., 2004) and experimental evidence suggests that BDNF may modulate both spontaneous and stimulated neuronal activity (Schuman, 1999). The action of BDNF signaling on synapses occurs within seconds of stimulation or application/release of the factor (Kovalchuk et al., 2004) and may support long-term potentiation (LTP) processes via sustained TrkB activation due to dendritic protein translation or transcription of BDNF through TrkB-mediated cAMP response element binding protein (CREB) activation (Lu et al., 2008).

Long-term potentiation (LTP) is the lasting enhancement of synaptic strength that is initiated by strong post-synaptic responses to activity that persist in the absence of further stimulation; and this phenomenon is thought to be a cellular model for associational learning and memory processes. BDNF is known

to facilitate LTP by converting early LTP to late LTP and by potentiating sub-threshold activation to elicit LTP (Nagappan and Lu, 2005). Given this essential role of BDNF in LTP facilitation, it is not surprising that experimental loss of BDNF signaling through genetic models or pharmacological manipulation leads to decreased learning and memory in behavioral paradigms (Lu et al., 2008).

Further studies of loss of BDNF signaling in adult brain have led to the discovery of many more roles for BDNF in the modulation of behavior. In particular, BDNF's role in mood-related behaviors is emerging in addition to those understood in regards to memory and cognition, presumably related to the key function of BDNF in rapid synaptic plasticity. For this reason, BDNF is widely studied in relation to neuropsychiatric diseases, particularly Major Depressive Disorder.

BDNF and Major Depressive Disorder

Major Depressive Disorder (MDD) is a leading cause of disability worldwide and is therefore currently the focus of research to determine the causes of the disease and effective treatments for patients. The clinical presentation of MDD consists of a spectrum of neuropsychiatric symptoms including anxiety, feelings of inappropriate guilt, loss of pleasure, appetite changes, and sleep disturbances (Shelton, 2007). Notably, the incidence of MDD is twice as prevalent in women compared to men, though the reason for this difference is currently unknown. Pharmacological therapies for treatment of MDD include serotonin reuptake inhibitors (SSRIs), trycyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and norepinephrine reuptake inhibitors (NRIs). These drugs target neurotransmitter systems to increase receptor signaling thought to be involved in mood regulation. However, given that these drugs have rapid synaptic effects but are associated with delayed onset of clinical efficacy, it is thought that a molecular target downstream of serotonergic or noradrenergic signaling is responsible for relief from MDD symptoms.

For this reason, researchers are interested in identifying a direct target for MDD therapy in order to treat patients more effectively. MDD has approximately a 50% genetic component indicating that environmental effects are a significant contribution to disease onset (Shelton, 2007). Stressful events including early life trauma as well as acute stressors correlate strongly with the occurrence of MDD (Charney and Manji, 2004). Intriguingly, BDNF appears to be a molecular substrate of stress, an important risk factor for MDD (Martinowich et al., 2007).

Furthermore, antidepressant treatment has opposite effects on BDNF levels compared to stress or depression (Castren and Rantamaki, 2010). Given that BDNF expression is decreased by stress and increased by antidepressants, many investigators have focused on BDNF as a biomarker and also a potential target for treatment of MDD.

Anatomically, stress-related disorders including MDD correspond to a reduction in hippocampal volume in patients (Bremner et al., 2000). More strikingly, BDNF levels are decreased in regions of the hippocampus in postmortem tissue taken from suicide victims or in the serum of depressed patients (Castren and Rantamaki, 2010; Castren et al., 2007). Because we know that the hippocampus is a feedback structure of the hypothalamic-pituitary-adrenal (HPA) axis that modulates stress responses and that BDNF expression is decreased by stress, it appears that structural changes in hippocampus related to MDD may in part be attributed to the reductions in BDNF. These findings strongly suggest that both stress and MDD share aspects of molecular mechanisms and also both directly impact BDNF expression, providing the basis for the neurotrophin hypothesis of depression which asserts that BDNF underlies depression pathology and antidepressant efficacy (Duman and Monteggia, 2006).

Intriguingly, BDNF is oppositely regulated by antidepressant treatment in humans. Postmortem tissue studies demonstrate that BDNF levels are increased in hippocampus and cortex after long-term antidepressant use. In addition, studies in live patients indicate that serum levels of BDNF expression are normalized in depressed patients after chronic antidepressant treatment (Duman

and Monteggia, 2006), and these findings have stood up to meta-analyses (Sen et al., 2008). However, studies of BDNF in postmortem tissue are correlative and the exact origin and function of serum-derived BDNF remain unclear. Still, these data support the neurotrophin hypothesis of depression which suggests the following predictions: BDNF is required for normal depression-related behavior; and BDNF is necessary and sufficient for antidepressant efficacy. Some of these assumptions have been tested preclinically in models of reduced BDNF signaling or in animal models of depression.

In rodents, chronic stress is a generally accepted model of depression because it leads to neurochemcial and behavioral alterations that are analogous to those observed in depressed human patients including increases in stress hormones, hippocampal atrophy, increased anxiety- and depression-related behaviors, and cognitive impairments(Willner, 1997; Willner, 2005). However, while many preclinical models have predictive and construct validity, no single model of depression or test has a high face validity in terms of modeling symptoms of human depression in rodents(Duman and Monteggia, 2006; Willner, 1997). Studies have repeatedly shown that chronic restraint stress or unpredictable stress can lead to decreases in hippocampal mRNA and protein levels of BDNF in mice and rats (Autry et al., 2009; van Donkelaar et al., 2009). On a related note, chronic administration of corticosterone, the rodent stress hormone, is sufficient to produce decreases in BDNF expression as well suggesting that the mechanism for these molecular alterations is stress (Jacobsen and Mork, 2006).

In order to determine whether BDNF is required for normal depression-like behavior, multiple animal models of deficient BDNF signaling have been produced. BDNF constituitive knockout models have severe developmental abnormalities in the brain and die early postnatally, therefore investigators have turned to heterozygous, conditional, and region-specific knockout or knockdown models to study behavior associated with depression in adult mice. BDNF heterozygous mice display ~50% reduction of mRNA and protein throughout the brain. Studies demonstrate the BDNF heterozygous mice do not show compelling evidence of alterations in depression-like behavior (Advani et al., 2009; Ibarguen-Vargas et al., 2009). However, baseline behaviors in this line of mice may be altered due to a constituitive lack of BDNF throughout development or that 50% expression of BDNF is enough to compensate in behavioral tests. Therefore, investigators have used conditional and inducible genetic models to remove BDNF postnatally. Again, in lines removing BDNF from forebrain neurons later in development, there are not consistent changes in depression-related behavior (Duman and Monteggia, 2006), though one study suggests that females lacking BDNF may display alterations in depression behavior in certain assays (Adachi et al., 2007). A potential issue with this type of deletion is that BDNF may be acting in depression-related behavior in discrete circuitry rather than in broad regions of the cortico-limbic system. However, all of these lines of mice consistently display an inability to respond to antidepressant treatment revealing an essential role for BDNF in the manifestation of behavioral antidepressant responses (Hu and Russek, 2008; Malberg and Blendy, 2005; Tardito et al., 2006).

To more accurately target particular brain regions, researchers are utilizing viral-mediated deletion techniques to remove BDNF from spatially restricted brain regions. These studies have added more complexity to our understanding of how BDNF operates in circuitry because studies have revealed conflicting evidence on the contribution of particular subregions of hippocampal BDNF to depression-related behavior and have further indicated that BDNF activity in the mesolimbic dopamine neurons of the ventrotegmental area (VTA) and nucleus accumbens (NAc) may exert opposing forces on depression-like behavior. It appears that localized deletion of BDNF in the CA1 or dentate gyrus of hippocampus does not alter baseline depression behavior though dentate gyrus expression of BDNF specifically is required for antidepressant efficacy (Adachi et al., 2008). However, region-specific deletion of BDNF in the VTA produces a behavioral antidepressant-like response (Krishnan and Nestler, 2008). Theories in the field explain this discrepancy as an illustration of the complex circuitry associated with mood-related behaviors (Castren et al., 2007) or that the deletion of BDNF and its precursor proBDNF results in distinct behavioral alterations in different brain areas (Martinowich et al., 2007), but sufficient experimental evaluation of these proposals has not yet been undertaken. Further examination of the region-specific contribution of BDNF to antidepressant-efficacy will be important to determine which brain circuits may underlie mood-related disorders. Taken together, examination of BDNF mouse models in depression-related behavior suggests that BDNF alone is not responsible for symptoms of MDD.

BDNF may not alone be sufficient to explain depression-related behaviors, but it remains an important risk factor for depression. Therefore, researchers have also examined the role that BDNF plays in the susceptibility to developing stress-related mood disorders; but preclinical investigations have not unequivocally demonstrated how loss of BDNF alters vulnerability to stress. Differences in observations likely arise due to variations in type of stressors, duration of stress, choice of behavioral assay or endpoint, mouse strain, and brain pathway examined. Some studies illustrate that BDNF heterozygous mice have altered depression-related behavior after acute or subchronic stress (Advani et al., 2009), though research on conditional or inducible BDNF mutants suggest that depression behavior in these lines is indistinguishable in males from control mice after chronic mild stress (Ibarguen-Vargas et al., 2009) . After chronic social stress, BDNF deletion from the ventrotegmental area reduces depression-related behavior (Berton et al., 2006), again suggesting differences in circuitry or BDNF/proBDNF functions. Further investigation of how BDNF is related to development of stress-related mood disorders is required, but current research indicates that BDNF and stress may interact only to a limited to degree, though further investigation is required.

BDNF and the antidepressant response

While the role of BDNF in depression symptoms is complex, research clearly demonstrates that BDNF is essential for antidepressant efficacy. In addition to human studies illustrating a correlation between BDNF levels and antidepressant use, preclinical research has repeatedly shown that BDNF is both necessary and sufficient for behavioral antidepressant-like effects. It is well documented that, similar to findings in human tissue, BDNF mRNA or protein shows correlative increases in animal studies in response to chronic antidepressant therapies such as electroconvulsive therapy and many drugs including SSRI, NRI, TCA, and atypical compounds in many cortico-limbic brain areas, for review see (Tardito et al., 2006). Furthermore, several studies have shown that infusion of BDNF into midbrain, ventricles, or regions of the hippocampus results in increased antidepressant-like behavior (Hu and Russek, 2008). While many growth factors such as NGF and insulin growth factor (IGF) are also known to increase after antidepressant treatment and to cause similar behavioral effect after infusion as BDNF (for review see (Duman and Monteggia, 2006)), a preponderance of data suggests that animal models lacking BDNF are unable to respond to antidepressants as previously mentioned. Intriguingly, direct infusion of BDNF, but not NT-3 or NGF into hippocampus leads to antidepressant-like effects (Shirayama et al., 2002). Taken together, these data indicate that BDNF is both necessary and sufficient for antidepressant behavioral responses. However, the mechanism by which BDNF confers these behavioral effects is still not known.

Because of the delay in clinical efficacy of antidepressants, investigators have searched for a downstream process that may confer therapeutic effects. One process of interest in addition to BDNF-mediated synaptic plasticity enhancement is augmented hippocampal neurogenesis which occurs on approximately the same time-scale as therapeutic effects of antidepressants (3-4 weeks). Studies have demonstrated that increased cell proliferation in hippocampus is associated with antidepressant treatments like ECT and chemical therapies, for review see (Malberg and Blendy, 2005). However, evidence that increased proliferation is necessary for behavioral responses to these treatments has not been unequivocal; some treatments that increase neurogenesis have no effect on depression-related behavior suggesting that the effect is independent of antidepressant efficacy. Intriguingly, BDNF heterozygous mice do not show a significant reduction in hippocampal cell proliferation but are unable to respond to antidepressants (Duman and Monteggia, 2006; Malberg and Blendy, 2005). Furthermore, recent clinical studies have demonstrated that novel drugs like ketamine (Berman et al., 2000; Zarate et al., 2006) and scopolamine (Drevets and Furey, 2010; Furey and Drevets, 2006) may have antidepressant effects within hours or days, calling in to question the contribution of neurogenesis in the mechanism of rapid alteration in mood states. Taken together, these observations suggest that neurogenesis may not be an essential mechanism by which antidepressants exert behavioral effects and that the link to BDNF, a *bona fide* target of antidepressants, is tenuous.

Because BDNF expression is stress-responsive, researchers have examined the gene for single nucleotide polymorphisms (SNP) that may be linked

to MDD. The most common SNP in humans is at codon 66 resulting in the Val66Met protein variant which prevents the activity-dependent release of BDNF (Duman and Monteggia, 2006). While this polymorphism does appear to impact human cognition, studies have not definitively shown that this mutation affects MDD pathology or suicidality in humans (Dwivedi) and has not been thoroughly examined in mouse models (Martinowich et al., 2007). At present it is not yet clear whether genetic alterations in BDNF may contribute to the expression of MDD symptoms so further work will be necessary to examine this possibility.

CHAPTER 2

GENDER-SPECIFIC IMPACT OF BDNF SIGNALING ON STRESS-INDUCED DEPRESSION-LIKE BEHAVIOR

Introduction

Major depressive disorder (MDD) is a leading debilitating disease in the U.S. and affects about 14.8 million Americans over 18 each year. The clinical presentation of MDD has a spectrum of symptoms including anxiety, anhedonia, loss of appetite, and sleep disturbances as set forth in the Diagnostic and Statistical Manual (2000). Notably, MDD occurs twice as often in women than in men though the cause is currently unknown (Desai and Jann, 2000; Kessler et al., 2003).

Recent work suggests an important role for neurotrophins in psychiatric diseases including MDD (Castren et al., 2007; Duman and Monteggia, 2006). Brain-derived neurotrophic factor (BDNF), the most prevalent growth factor in the brain, may underlie depression-related behavior and mediate the therapeutic action of antidepressants. The neurotrophic hypothesis of depression suggests that loss of BDNF from hippocampus contributes to neuroanatomical and functional alterations that underlie aspects of depression-related behavior, while antidepressants may mediate therapeutic effects in part by increasing levels of BDNF in this brain region (Duman et al., 1997). Recent studies demonstrate that BDNF heterozygous mice, and mice with inducible BDNF deletion in forebrain (inducible knockouts), and conditional BDNF knockouts, display attenuated

responses to antidepressants in forced swim test (Monteggia et al., 2004; Monteggia et al., 2007; Saarelainen et al., 2003), a paradigm that predicts antidepressant efficacy and by analogy 'depression-related' behavior (Dalvi and Lucki, 1999; Porsolt et al., 1977b). Indeed, we have recently extended these findings to show that BDNF in dentate gyrus of hippocampus is required for antidepressant efficacy in this paradigm (Adachi et al., 2007).

While these studies demonstrated that loss of BDNF produces alterations in antidepressant responses, BDNF heterozygous mice, inducible BDNF knockouts (KOs), and dentate gyrus specific BDNF KOs were indistinguishable from wild type littermate control mice in 'baseline' depression-related behavior; this suggests that loss of BDNF *per se* is not sufficient to mediate 'depression-like' behavior (Adachi et al., 2007; Bezchlibnyk et al., 2001; Monteggia et al., 2004). However, it is possible that loss of BDNF may increase vulnerability to particular chronic perturbations.

To investigate this possibility, we exposed BDNF inducible KOs to a chronic unpredictable stress (CUS) paradigm that is known to induce alterations in depression-related behaviors in rodents. We examined BDNF inducible KOs, since this line has a regionally restricted forebrain specific deletion of BDNF compared to our conditional line, to gain a direct assessment of the neurotrophic hypothesis of depression. Since previous work demonstrated that loss of BDNF produces gender specific effects, we examined both male and female inducible KO mice in depression-related behavior following CUS.

Materials and Methods

Mice

The inducible BDNF knockout mice were generated from a trigenic cross of NSE-tTA, TetOp-Cre, and floxed BDNF mice as previously described (Monteggia et al., 2004). For all behavior testing, male and female mice were age (three to six months) and weight matched and groups were balanced by genotype. Eight experimental groups of 7-14 animals were tested; male and female BDNF knockouts (KOs) or wild type littermates (CTLs), nonstressed or stressed (Supplementary Figure 1). The order of behavior tests was performed from least to most stressful and blind to group and genotype (Figure 1A). For more information, refer to supplemental methods (Supplementary Figure S4).

Chronic unpredictable stress model

Our CUS model was adapted from Muscat *et al.* (Muscat and Willner, 1992) and Monleon *et al.* (Monleon et al., 1995). Mice were exposed to one or two stressors for a period of 4-12 hours during each 24 hour period over 52 days, though animals were not stressed within eight hours of behavioral testing. Stressors consisted of food or water deprivation, periods of overnight illumination, 45° cage tilt, single housing, and bedding soiled with water or rat feces (Table 1).

Locomotor Activity

Mice were placed in cages and locomotor activity was recorded for 2 hours under red light by photocell beams linked to computer acquisition software (San Diego Instruments, San Diego, CA).

Open field

Mice were assessed for activity in a 72x72 cm open field (OF) arena at 40 lux for 5 minutes. Movement was tracked by video (Ethovision3.0 Noldus, Leesburg, Virginia) for time spent in center (14x14 cm) and peripheral zones (5 cm around perimeter).

Fur state assessment

Mouse fur state was rated on a 4-point scale with another point each for either hunched posture or redness around eyes (6 points total). The fur scoring scale (found in Supplementary figure S4) was adapted from Mineur *et al.* (Mineur et al., 2003).

Sucrose consumption test

Sucrose consumption test (SCT) protocol was adapted from Gourley *et al.* (Gourley et al., 2008). Mice were habituated to 1% sucrose solution and water deprivation periods followed by 1 hour of sucrose access. On test day, mice accessed sucrose solution for 1 hour and the following day accessed water. We measured percent sucrose intake compared to total volume consumed in both

trials. For more information, refer to supplemental methods (Supplementary figure S4).

Novelty suppressed feeding

The novelty suppressed feeding (NSF) task was performed as previously described (Gross et al., 2000). Detailed methods listed in supplemental methods (Supplementary figure S4).

Tail suspension test

The tail suspension test (TST) was performed as previously described (19) and detailed methods are listed in supplement (Supplementary figure S4).

Forced swim test

The forced swim test (FST) was performed as previously described (Porsolt et al., 1977b) and detailed methods are available in supplemental methods (Supplementary figure S4).

Corticosterone measure

Blood serum was isolated from trunk blood samples by centrifugation. A high sensitivity corticosterone (CORT) enzyme immunoassay (EIA) was performed according to manufacturer's instruction (Immunodiagnostic Systems Ltd., Fountain Hills, AZ).

Quantitative RT-PCR

Fresh frozen whole hippocampi were dissected and total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. Conditions for cDNA synthesis, amplification, and primer sequences were described previously (Berton et al., 2006). Fold change in BDNF expression is normalized to GAPDH.

Statistical analysis

Weight and locomotor data were analyzed with repeated measures ANOVA using SAS software to determine statistical significance ($p<0.05$). The fur score data were analyzed by logistical regression analysis followed with a Mantel-Haenszel test for frequency comparison between groups. Anxiety data, SCT, NSF, FST, TST, CORT, and BDNF expression data were analyzed by a 2-way ANOVA followed with multiple comparisons using a Bonferroni t-test to assess the difference among groups. Data are presented as mean ± SEM.

Results

Weight

Mouse weights were monitored during CUS (paradigm shown in Figure 2-1) on days 5, 21 and 40, at an early, mid-, and late timepoint during behavioral assessments (Figure 2-2A). In females, there was a significant stress effect ($p<0.0001$, $F_{1,11}=36.42$), while there was no significant knockout effect ($p=0.2722$,$F_{1,11}=1.34$) or interaction effect ($p=0.2075$,$F_{1,10}=1.82$) (Figure 2-2 B, D, F). For males, there was a significant stress effect ($p=0.0135$, $F_{1,8}=9.96$), while there was no significant knockout effect ($p=0.3016$, $F_{1,7}=1.24$) or interaction effect ($p=0.3256$,$F_{1,7}=1.12$) (Figure 2-2 C, E, G). Inducible BDNF KO mice have normal weight compared to littermate CTLs. Our CUS paradigm was found to significantly impact weight of the animals over the course of the experiment. However, loss of BDNF in either sex did not further contribute to a change in weight following CUS.

Locomotor Activity

Previous studies showed that a reduction in locomotor activity after CUS correlates to depression-like behaviors (Pardon et al., 2000). Examining two hour locomotor activity in females following CUS revealed a significant stress effect ($p=0.0335$,$F_{1,42}=4.83$) and knockout effect ($p=0.0461$,$F_{1,42}=4.22$) while there was no significant interaction effect ($p=0.1104$,$F_{1,42}=2.66$). Multiple comparisons using a Bonferroni t-test indicated that stressed KOs were significantly hypoactive compared to the other groups ($*p<0.05$) (Figure 2-3A, insert). To gain a better

understanding of this difference in females, data were analyzed in 5-minute epochs (Figure 2-3A); there was a significant main effect of stress ($p<0.0001$, $F_{1,11}=62.00$) and a significant main effect of genotype ($p<0.0001$, $F_{1,9}=57.91$) and the number of beam breaks significantly decreased over time ($p<0.0001$, $F_{23,299}=52.56$) with a significant interaction between stress and genotype ($p<0.0001$, $F_{1,9}=50.76$) while there were no other significant interaction effects (* $p<0.05$). Total locomotor activity in males during a two hour period following CUS revealed a significant stress effect ($p=0.0092$,$F_{1,30}=7.75$), with no significant knockout effect ($p=0.1796$,$F_{1,30}=1.89$) or interaction effect ($p=0.9385$,$F_{1,30}=0.01$). Multiple comparisons using a Bonferroni t-test indicated that under nonstressed conditions BDNF KOs are significantly hyperactive and that following stress CTLs show a significant decrease in locomotor activity compared to nonstressed CTLs (*$p<0.05$) (Figure 2-3B, insert). For males, locomotor data were analyzed in 5-minute epochs (Figure 2-3B; there was a significant main effect of stress ($p<0.0001$,$F_{1,8}=86.32$), a significant main effect of genotype ($p=0.0046$,$F_{1,7}=16.73$), and the number of beam breaks significantly decreased over time ($p<0.0001$,$F_{23,208}=50.57$) while there was no significant interaction effect (*$p<0.05$).

Anxiety-related behavior

To determine the effect of CUS on anxiety-like measures, we assessed open field behavior. In this paradigm a decrease in duration of time in the center or a decrease in the number of entries to center is suggestive of an increase in anxiety related behavior (Prut and Belzung, 2003). For females, examining

duration in the center of the open field revealed a significant stress effect ($p=0.0499, F_{1,45}=4.06$) while there is no significant knockout effect ($p=0.5305, F_{1,45}=0.40$) or interaction effect ($p=0.1829, F_{1,45}=1.83$). Multiple comparisons using a Bonferroni t-test indicated that stressed KOs spend significantly less time in the center compared to nonstressed KOs suggestive of an increase in anxiety (* $p<0.05$) (Figure 2-4A). In females, we examined the number of entries to the center and found a significant stress effect ($p=0.0155, F_{1,45}=6.33$) while there was no significant knockout effect ($p=0.3545, F_{1,45}=0.88$) or interaction effect ($p=0.3239, F_{1,45}=0.99$). Multiple comparisons using a Bonferroni t-test indicated that stressed KOs have a significant decrease in the number of entries in the center of the open field compared to nonstressed KOs (*$p<0.05$) (Figure 2-4C).

Measures of duration in the center of the open field for males revealed no significant stress effect ($p=0.7146, F_{1,31}=0.14$), knockout effect ($p=0.0884, F_{1,31}=3.09$) or interaction effect ($p=0.3666, F_{1,31}=0.84$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between groups (*$p>0.05$) (Figure 2-4B). In males, we examined the number of entries in the center and found no significant stress effect ($p=0.4262, F_{1,31}=0.65$), knockout effect ($p=0.0873, F_{1,31}=3.12$) or interaction effect ($p=0.6111, F_{1,31}=0.26$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between the individual animal groups (*$p>0.05$) (Figure 2-4D).

Depression-like behavior

To address the impact of CUS on depression-like measures, we examined fur state, sucrose intake, and feeding in a novel environment. In examining fur state we were interested whether CUS resulted in more of an 'unkept' appearance, as observed by an increase in fur score as a measure of depression-like behavior. There was no significant main stress and main genotype effect and interaction effect from Logistic regression analysis (*$p>0.05$). However, in females we noted significantly poorer fur state in stressed BDNF KO mice as assessed by a higher fur score with respect to the other groups of animals (*$p<0.05$) (Figure 2-5A). In contrast, in males we generally noted a poorer fur state than in females, however there was no significant difference between groups ($p>0.05$) (Figure 2-5B). SCT is a paradigm used to measure an animal's responsiveness to a natural reward (Barrot et al., 2002). A loss of sensitivity to reward has been suggested as measure of anhedonia, an important feature of major depression. In females, there was a significant genotype x stress interaction effect ($p=0.0278, F_{1,45}=5.17$). Multiple comparisons using a Bonferroni t-test indicated that stressed KOs had a significant decrease in percent sucrose intake compared to nonstressed KOs or stressed CTLs (*$p<0.05$) (Figure 2-5C). For males, there was a significant stress effect ($p=0.0199, F_{1,31}=6.02$), while there was no effect of knockout ($p=0.8997, F_{1,31}=0.02$) nor an interaction effect ($p=0.5528, F_{1,31}=0.36$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between groups (*$p>0.05$) (Figure 2-5D). In NSF testing, an increase in the latency to feed suggests an increase in anxiety (Gross et al., 2000). In females, there was a

significant stress effect ($p=0.0049, F_{1,43}=8.79$) while there was no significant knockout effect ($p=0.6509, F_{1,43}=0.21$) or interaction effect ($p=0.5913, F_{1,43}=0.29$). Multiple comparisons using a Bonferroni t-test indicated that stress significantly increased the latency to feed in the CTLs (*$p<0.05$), with a similar trend observed in KOs (Figure 2-5E). For males, there was a significant knockout effect ($p=0.0052, F_{1,29}=9.16$), while there was no effect of stress ($p=.0532, F_{1,29}=4.06$) nor an interaction effect ($p=0.1754, F_{1,29}=1.93$). Multiple comparisons using a Bonferroni t-test indicated that stress significantly increased latency to feed in CTLs compared to nonstressed CTLs and stressed KOs (*$p<0.05$) (Figure 2-5F).

We performed FST and TST, paradigms that are commonly referred to as depression-like tests and which have been shown to increase immobility in mice after CUS in other studies (Mineur et al., 2006). An increase in immobility time is suggestive of an increase in depression-like behavior. For females in FST, there was no significant stress effect ($p=0.9963, F_{1,45}=0.00$), knockout effect ($p=0.4718, F_{1,45}=0.53$) or interaction effect ($p=0.0931, F_{1,45}=2.94$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between groups (*$p>0.05$) (Figure 2-6A). For males in FST, there was no significant stress effect ($p=0.5919, F_{1,29}=0.29$), knockout effect ($p=0.2265, F_{1,29}=1.53$) or interaction effect ($p=0.9603, F_{1,29}=0.00$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between groups (*$p>0.05$) (Figure 2-6B). Examining females in TST, there was no significant stress effect ($p=0.7447, F_{1,31}=0.11$), knockout effect ($p=0.6301, F_{1,31}=0.24$) or interaction effect ($p=0.8980$, $F_{1,31}=0.02$). Multiple comparisons using a Bonferroni t-test indicated

no significant differences between groups (*p>0.05) (Figure 2-6C). Males tested in TST displayed no significant stress effect ($p=0.2959, F_{1,30}=1.13$), knockout effect ($p=0.4904, F_{1,30}=0.49$) or interaction effect ($p=0.2051, F_{1,30}=1.68$). Multiple comparisons using a Bonferroni t-test indicated no significant differences between groups (*p>0.05) (Figure 2-6D).

Corticosterone levels

To assess the effect of stress on CORT levels in CTL and BDNF KO mice, we collected trunk blood 10-15 minutes after FST, an acute swim stress. We analyzed sera from these samples for CORT concentration by EIA. For female CORT concentrations, there is a significant genotype x stress interaction effect ($p=0.0013, F_{1,42}=11.92$). Multiple comparisons using a Bonferroni t-test indicated that nonstressed KOs had a significant decrease in CORT levels compared to nonstressed CTLs and that stress significantly increases CORT levels in BDNF KOs compared to either nonstressed KOs or stressed CTLs (*p<0.05) (Figure 2-7A). For male CORT concentration, there is a significant knockout effect ($p=0.0001$, $F_{1,24}=20.25$) while there is no significant stress effect ($p=0.2152$, $F_{1,24}=1.62$) or interaction effect ($p=0.3988, F_{1,24}=0.74$). Multiple comparisons using a Bonferroni t-test indicated that nonstressed KOs had a significant decrease in CORT levels compared to nonstressed CTLs and that stressed KOs had a significant decrease in CORT levels compared to stressed CTLs (*p<0.05)(Figure 2-7B).

BDNF levels

We quantified expression of BDNF in hippocampus, a region of interest in stress-response and a well-characterized site of BDNF knockdown in this mouse line, to determine the gender-specific impact of stress on BDNF mRNA levels. We collected whole hippocampi from all mice subjected to behavioral testing and used quantitative PCR to analyze BDNF mRNA levels. For female BDNF levels, there is a significant knockout effect ($p=0.0040, F_{1,34}=9.51$) and stress effect ($p=0.0185, F_{1,34}=6.12$) while there is no significant interaction effect ($p=0.4947, F_{1,34}=0.48$). Multiple comparisons using a Bonferroni t-test indicated that nonstressed CTLs are significantly different from other three groups (*$p<0.05$) (Figure 2-7C). For male BDNF levels, there is a significant knockout genotype interaction effect ($p=0.0040, F_{1,25}=10.04$). Multiple comparisons using a Bonferroni t-test indicated that, similarly to females, nonstressed CTLs are significantly different from all other groups (*$p<0.05$) (Figure 2-7D).

Discussion

Results of this study demonstrate that in several behavioral paradigms female mice are more vulnerable to CUS than males. We found that loss of BDNF makes female mice more sensitive to some measures of anxiety and particular features of depression-like behaviors following CUS compared to littermate CTLs. In contrast, loss of BDNF in males fails to increase measures of anxiety, anhedonia and depression-like behavior following CUS compared to wild type CTLs. Collectively, these data suggest that loss of BDNF does not result in greater susceptibility to depression-related behavior *per se*, but rather is linked to expression of these behaviors in a gender-specific manner in response to stress. CUS paradigms have produced alterations in locomotor activity, anxiety-like behavior, fur state, sucrose consumption, forced swim test, tail suspension tests, and corticosterone levels in rodents (Willner, 1997; Willner, 2005). However, most robust CUS effects on these measures are in rats and effects in mice have been more difficult to ascertain suggesting that mice may be more resilient to chronic stress (Willner, 1997). Most CUS studies have relied solely on males and our data would largely support the resiliency of male mice to stress in many of these behavioral paradigms. However, our data with female mice suggests they may have an increased vulnerability in some behavioral measures following CUS.

In females, we found that stress produced a significant decrease in total locomotor activity of inducible KOs compared to nonstressed CTLs, nonstressed KOs, and stressed CTLs suggesting that loss of BDNF in females exacerbated locomotor deficits. In males, we found that nonstressed inducible KOs were

significantly hyperactive compared to nonstressed CTLs. Following CUS, male CTLs displayed significant hypoactivity compared to nonstressed CTLs while a similar trend, although not significant, was observed in BDNF KOs.

We assessed anxiety-related behavior using the OF test. In females, we found that CUS in BDNF KOs significantly reduced the time in the center of the arena and the number of entries to the center, indicative of an increase in anxiety-like behavior, compared to nonstressed KOs. In males, loss of BDNF did not alter anxiety related behavior following CUS. Our data is in contrast to previous findings of anxiolytic like effects of CUS in elevated plus maze (D'Aquila et al., 1994), however other reports utilizing chronic variable stress report anxiogenesis in this test (Zurita et al., 2000) and further studies suggest that these differences may be accounted for by the length of time between stress exposure and testing (Matuszewich et al., 2007).

We examined the effects of CUS in inducible KOs in paradigms that provide measures of depression-like behavior. We examined fur state of animals to assess grooming behavior, SCT as a measure of anhedonia, and latency to feed in the NSF test in CTL and BDNF KO animals following CUS. In females, we found that in nonstressed conditions loss of BDNF did not alter fur score, sucrose intake or latency to feed in the NSF test compared to CTLs. Following CUS, we found that female BDNF KOs had a significantly poorer fur score and were more anhedonic than nonstressed KOs or stressed CTLs and displaying a strong trend towards an increase in latency to feed compared to nonstressed KOs. In male KOs, CUS did not produce significant differences in fur score or sucrose

consumption compared to other groups, and in the NSF test BDNF KOs appeared less anxious following CUS than CTLs. Collectively, this data suggests that loss of BDNF in CUS females may increase anxiety related behavior and some measures of depression related behavior, however these effects are gender specific since similar effects were not observed in males.

We examined mice in the FST and TST, tests that are commonly used to assess antidepressant efficacy and, by extension, depression (Cryan and Slattery, 2007). Surprisingly, we did not observe increased depression-like behavior as assessed by increased immobility in either FST or TST following CUS in either females or males independent of genotype. The FST and TST are often associated with depressive phenotypes in rodents following acute stress (Mineur et al., 2006). Our lack of a change in immobility in these paradigms may be due to the adaptive aspect of stress responses to CUS over time (McEwen, 2007). Recent interest is focused on uncovering the genetic mechanism behind the consistent observation that some animals display resilience to stress (Krishnan et al., 2007; Strekalova et al., 2004). To this end, neither susceptible nor resilient mice display differences in the FST or TST after chronic social defeat stress (Krishnan et al., 2007), although other groups do see significant differences in the FST (Kudryavtseva et al., 1991). It is possible that the lack of a change in depression-like behavior as determined here could be due to the fact these tests are designed to predict antidepressant efficacy rather than as measures indicative of depression-like behavior (Porsolt et al., 1977a; Porsolt et al., 1977b).

As a physiological measure of stress to support our behavioral findings, we assessed CORT concentration. Rather surprisingly, we found that under nonstressed conditions loss of BDNF is associated with decreased CORT levels in both males and females. Furthermore, we found that susceptibility for developing depression-related behaviors in female BDNF KO mice after CUS was correlated to a significant increase in CORT levels compared to nonstressed KOs and stressed CTLs. In contrast, in males we found that CUS did not significantly alter CORT levels compared to baseline levels of CORT in nonstressed CTLs and KOs. These findings suggest that under nonstressed conditions there is an interaction between BDNF and CORT in that loss of BDNF significantly reduced plasma CORT levels although this was not significantly correlated with decreased anxiety or depression like behavior. Following CUS, we found gender specific effects of the interaction between BDNF and CORT. Interestingly, a significant increase in CORT levels following CUS was observed in female BDNF KOs, the animals with the most pronounced anxiety and in some measures depression like behavior here.

We examined BDNF mRNA expression in all female and male groups. We found that under nonstressed conditions, both female and male KOs had a significant reduction in BDNF in hippocampus in agreement with previous data (9). Following CUS, we found that both male and female CTLs showed a significant reduction in the amount of BDNF in hippocampus. Rather surprisingly, CUS did not further reduce BDNF levels in KOs compared to nonstressed conditions. These data suggest that there is a floor effect in the

amount of BDNF reduction in hippocampus. Thus while there were significant behavioral differences in KOs following CUS, this was not directly correlated with the amount of BDNF mRNA.

Interestingly, studies examining the effect of gender on stress responses have shown that BDNF levels in dentate gyrus are reduced in females but not males after restraint stress in rats (Franklin and Perrot-Sinal, 2006), contrary to what we observed. Instead, our data suggests that dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis stress response between males and females may account for the gender differences in agreement with previous literature (Young, 1995; Young, 1998). Recent studies have suggested that depression based gender differences may be the result of alterations in hormonal levels (Angold et al., 1999; Angold et al., 1998; Rubinow et al., 2005) or in neuroanatomical differences between males and females (Raisman and Field, 1971). Intriguingly, a recent study has shown that in conditional BDNF KO mice there is evidence of increased depression-like behavior in females BDNF KOs but not males (Monteggia et al., 2007), however it is difficult to make direct correlations with the data presented here and the previous study as the pattern of BDNF deletion in various BDNF lines is quite different suggesting that regional pattern of BDNF deletion may influence depression based behavior. Future studies will be necessary to examine the mechanistic link between BDNF, gender differences, and the regional effect of BDNF in susceptibility to depression-related behavior.

Our findings suggest that loss of BDNF in forebrain contributes to some aspects of depression-like behavior in a complex manner with gender. The finding that BDNF deletion in males was not sufficient to produce alterations in many behaviors examined suggests that the neurotrophic hypothesis related to depression is more complicated than simply that loss of the gene triggers depression. The loss of forebrain BDNF increased vulnerability in aspects of depression-related behaviors in females after CUS suggesting a role for BDNF in mediating features of depression-related behavior in females.

Week\Day	M	T	W	Th	F	Sa	Su
1	A3	B1, F2	C1, G	D1, B3	E	E	E, G
2	C1	B3, F1	B2	A2	D2	E	B3, E
3	D2, G	B3	C1	A2, B3	D2	E	E
4	A2	C1	F1	C1	F1	A2	G
5	B3	C1, F2	A1, A4	A5, B1	A4, F1	B3	B2
6	C1, G	B3	F2	D2	A4, B1	E	C3, E
7	B1, G	D2	C1, G	D2, F2	B3	A2, D2	C2
8	G	B1, E					

Figure 2-1. CUS paradigm. Stressor type coding is as follows: (A) water deprivation; (B) 45° cage tilt; (C) food deprivation; (D) rat feces in bedding; (E) single housing; (F) soiled bedding; (G) overnight illumination. Stressor period coding is as follow: (1) 4 hours; (2) 7 hours; (3) 12 hours; (4) 14 hours; (5) 17 hours.

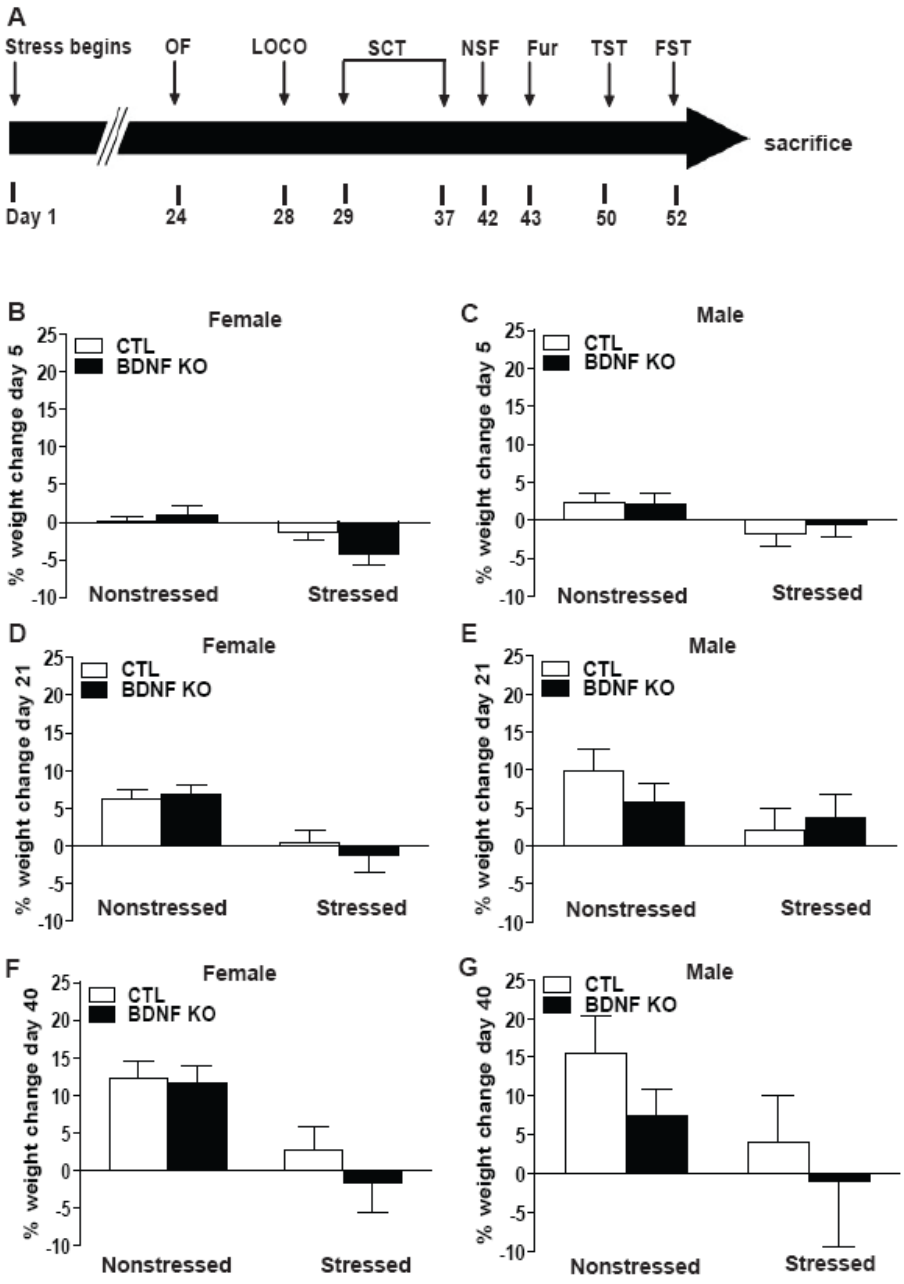


Figure 2-2. CUS produces body weight alterations. (A) The CUS paradigm was conducted over 52 days with behavioral tests performed on days indicated on the timeline (abbreviations listed in methods section). (B, D, F) In females, there was a significant main effects of stress ($F_{1\text{-}11}$=36.42, $p<0.0001$), however there were no interaction or genotype effects. (C, E, G) In males, there was a significant main effect of stress ($F_{1,8}$=9.96, $p<0.05$), but no significant effect of genotype or interaction.

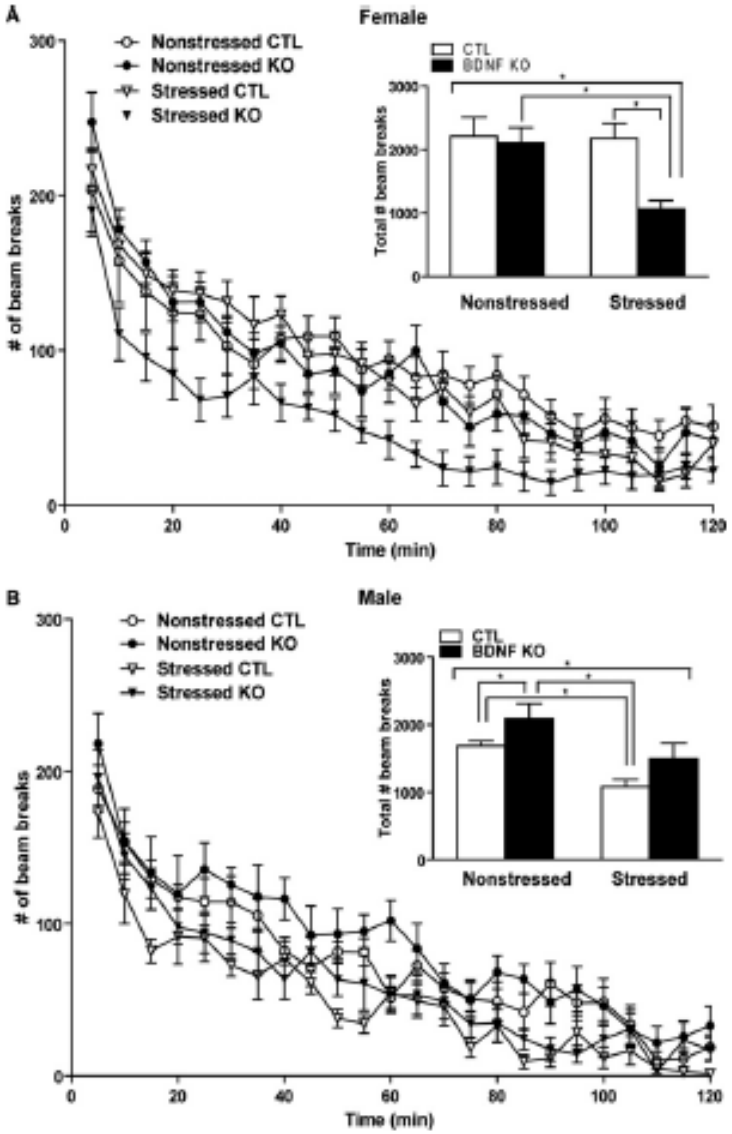


Figure 2-3. CUS produces significant effects on locomotor activity. (A) CUS produced a significant stress ($F_{1,42}$ =4.83, $p<0.05$) and genotype effect ($F_{1,42}$ =4.22, $p<0.05$) on locomotor activity in females. The female stressed BDNF KOs were significant hypoactivity compared to stressed CTLs and nonstressed BDNF KOs (*$p<0.05$). (B) CUS produced a significant stress effect ($F_{1,30}$ =7.75, $p<0.01$) on locomotor activity in males. Male BDNF KO mice were hyperactive compared to CTL mice at baseline (*$p<0.05$). After CUS, male mice (*$p<0.05$) were significantly less active than their nonstressed cohorts while no significant effect was observed in nonstressed KOs compared to stressed KOs ($p>0.05$).

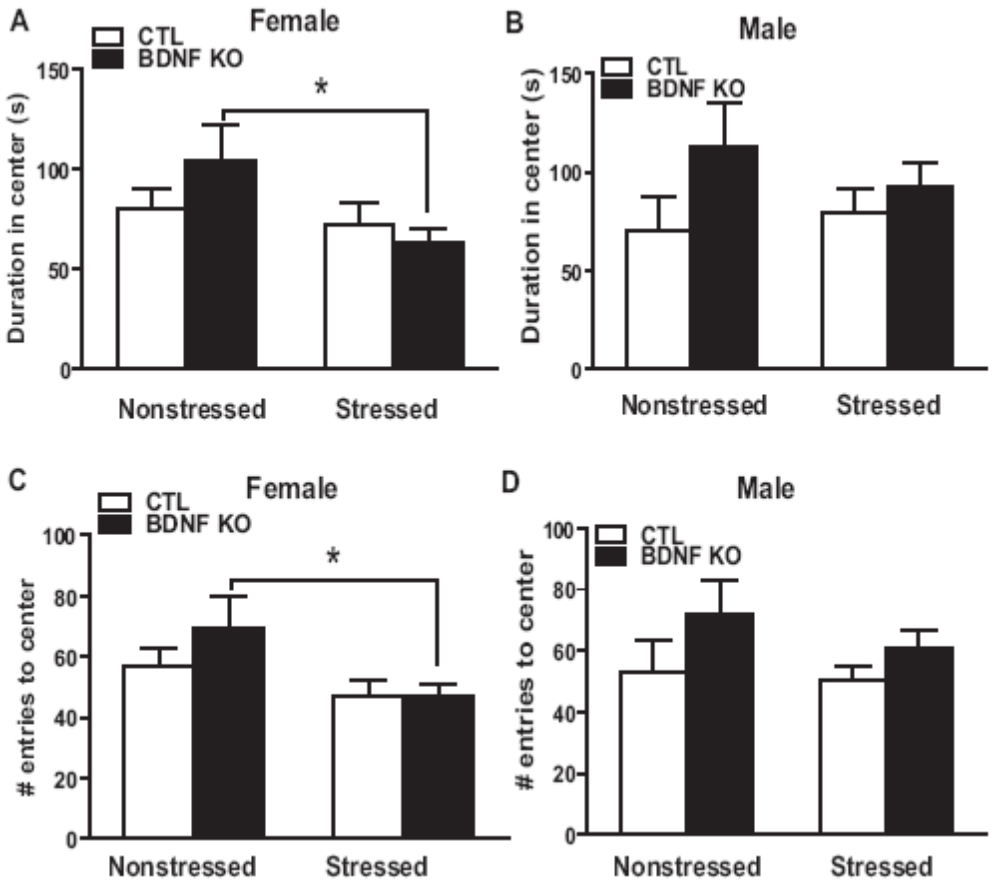


Figure 2-4. Female mice display heightened anxiety following CUS as assessed by open field testing. (A, C) In females, CUS produced a significant stress effect in the duration of time in the open field ($F_{1,45}$ =4.06, $p<0.05$) as well as in the frequency to enter the center area ($F_{1,45}$ =6.33, $p<0.05$). CUS in the BDNF KOs resulted in a significant decrease in duration of time in the center ($p<0.05$) and in the number of entries in the center ($p<0.05$) compared to nonstressed BDNF KOs. (B, D) Male mice did not differ in their anxiety behavior before or after stress regardless of genotype.

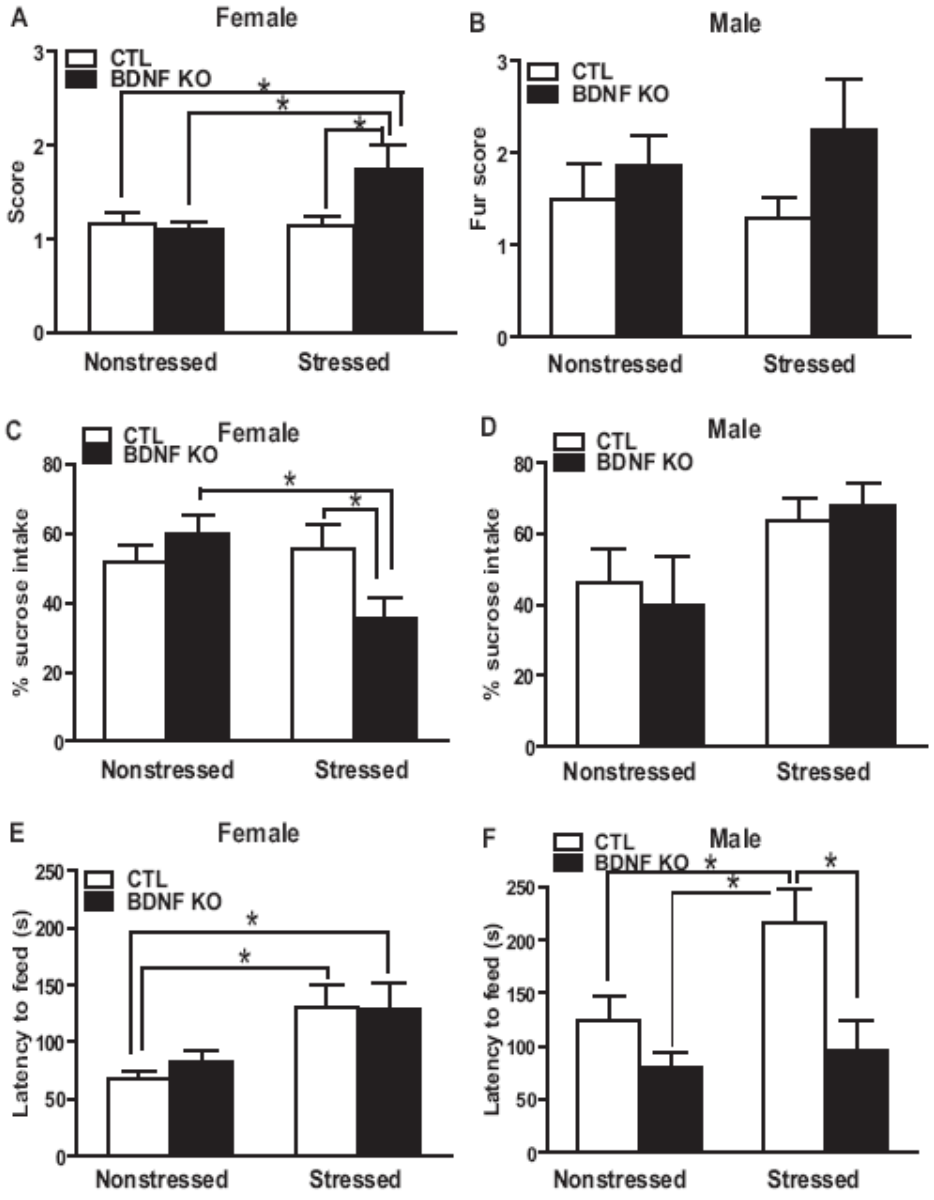


Figure 2-5. CUS increases certain depression-like behaviors in females. (A-B) Females, but not males, show a poorer fur state after CUS and there is an additional effect of genotype. (C-D) CUS results in significant alterations in sucrose consumption in female and male mice. Female stressed KOs consumed significantly less sucrose than both nonstressed KOs and stressed CTLs (* $p<0.05$). (E-F) In the novelty suppressed feeding task, stress significantly increased latency to feed in female mice ($F_{1,43}=8.79$, $p<0.005$) but no effect in male behavior, and both stressed CTL and KO females took significantly longer to feed than their unstressed cohorts ($p<0.05$). However, there was a significant difference in male feeding behavior after stress in CTL mice compared to both nonstressed CTLs and stressed KOs ($p<0.05$).

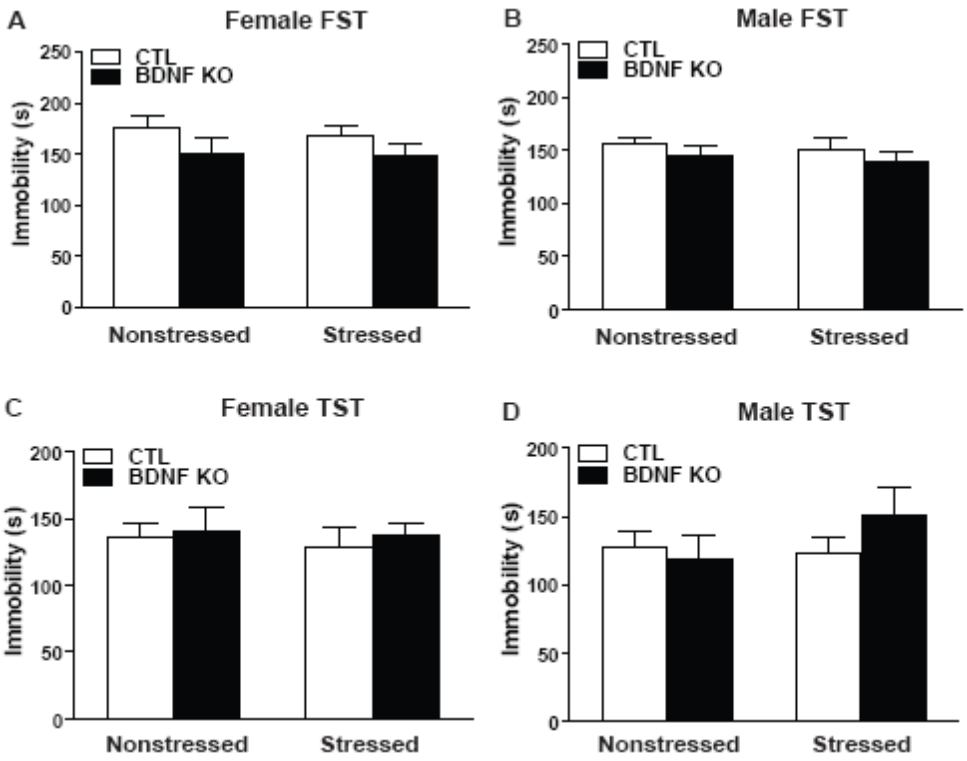


Figure 2-6. Classic measures of depression are not altered by CUS in females or males. (A-B) There was no significant difference in the immobility time in the forced swim test in male or female mice, CTL or KO, after chronic stress. (C-D) Similarly, we observed no significant differences in the tail suspension test between male or female, stressed and nonstressed, mice regardless of genotype.

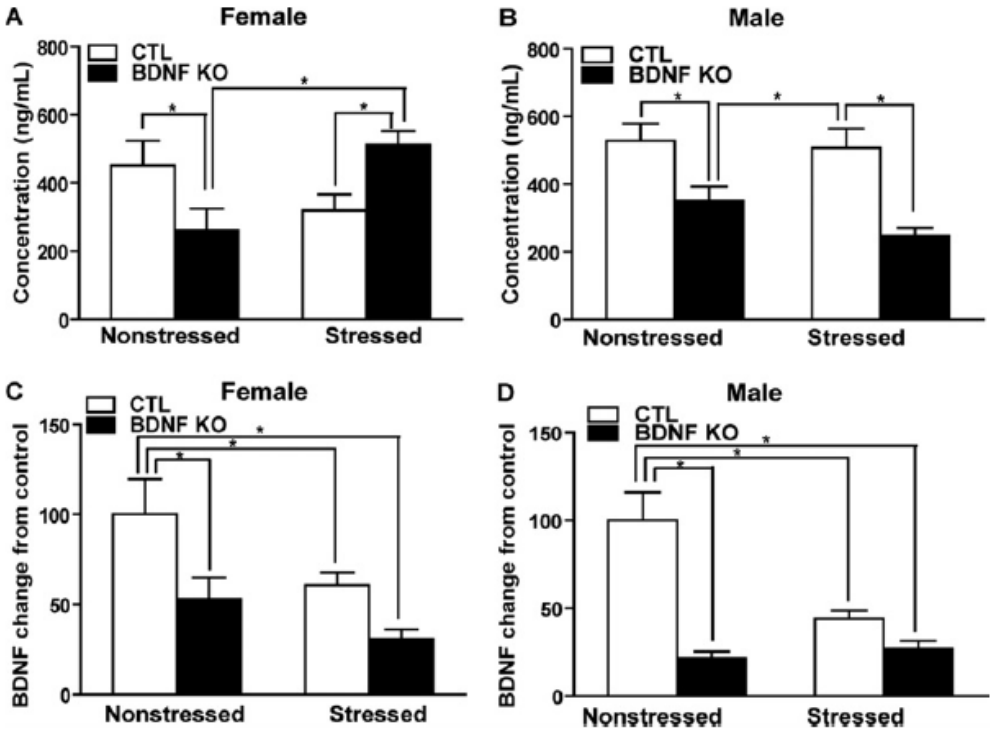


Figure 2-7. Corticosterone and BDNF levels following CUS. (A-B) Stress did not have a main effect on CORT levels in male or female mice. However, in females, stress and genotype have significant interaction on CORT measures ($F_{1,42}$ =11.92, $p<0.05$). In both sexes, CORT levels are significantly lower in BDNF KOs under non stress conditions ($p<0.05$). In females, BDNF KO mice show heightened CORT levels after stress compared both to nonstressed KOs and stressed CTLs ($p<0.05$). However, in males, BDNF KOs displayed significantly lower CORT levels after stress compared to stressed CTLs ($p<0.05$). (C-D) BDNF levels following chronic stress. For female BDNF levels, there is a significant knockout effect ($p=0.0040$,$F_{1,34}=9.51$) and stress effect($p=0.0185$,$F_{1,34}=6.12$) while there is no significant interaction effect ($p=0.4947$,$F_{1,34}=0.48$). Multiple comparisons using a Bonferroni t-test indicated that the nonstressed CTLs are significantly different from other three groups (*$p<0.05$). For male BDNF levels, there is a significant knockout x genotype interaction effect ($p=0.0040$,$F_{1,25}=10.04$). Multiple comparisons using a Bonferroni t-test indicated that, similarly to the females, the nonstressed CTLs are significantly different from the other three groups (* $p<0.05$).

CHAPTER 3

TRKB EXPRESSION IN DORSAL RAPHE NUCLEUS IS ESSENTIAL FOR ANTIDEPRESSANT EFFICACY

Introduction

A potential downstream target of SSRI treatment is brain-derived neurotrophic factor (BDNF). BDNF is the most prevalent neurotrophin in the central nervous system and is essential in neuronal development, survival, and in cellular and behavioral models of learning and memory (Waterhouse and Xu, 2009). BDNF expression is known to be decreased in hippocampus, a brain region important for mood-regulation, in depressed patients (Castren and Rantamaki, 2010). Additionally, BDNF expression is increased in hippocampus after chronic, but not acute, SSRI treatment (Duman and Monteggia, 2006).

Preclinical studies have shown that infusion of BDNF into midbrain (Hoshaw et al., 2005) or hippocampal regions (Shirayama et al., 2002) is sufficient to produce and antidepressant-like effect in rodents. Furthermore, our lab has recently demonstrated that selective deletion of BDNF in dentate gyrus, but not CA1, results in attenuated behavioral antidepressant-like responses, suggesting that BDNF is necessary for antidepressant efficacy (Adachi et al., 2008). However, because BDNF is a secreted factor, it remains unclear if BDNF signaling may be essential for antidepressant responses in other brain regions.

We focused our attention on BDNF signaling in raphe nucleus neurons, where serotonergic projection neurons thought to be essential in mood behavior

originate. Serotonergic neurons from the dorsal raphe nucleus (DRN) project to dentate gyrus, so we asked if selective deletion of BDNF or its high affinity receptor tyrosine related kinase B (TrkB) would impact antidepressant behavioral responses. To address this question, we utilized a local gene deletion strategy combining viral-mediated Cre-expression targeted to dorsal raphe in a floxed BDNF (fBDNF) or floxed TrkB (fTrkB) mouse line.

Materials and Methods

Mice

Mice were housed in a vivarium on a 12/12 light/dark cycle and had access to food and water *ad libitum*. Floxed BDNF and floxed TrkB mice were generated as previously described and maintained as homozygous crosses. Male mice aged 3-5 months (age and weight-matched littermates) were used for experiments. Experiments were approved by the UT Southwestern IACUC committee.

Surgery

Animals were anesthetized by i.p. administration of 100 mg/kg ketamine and 10 mg/kg xylazine and mounted in a sterotaxic apparatus. Holes were drilled above the injection sites on the exposed skull. Coordinates relative to lambda for dorsal raphe nucleus were as follows: AP -0.4; ML $\pm$ 0.6; DV -2.8 with needle at a 14° angle from the AP axis. A Hamilton syringe with a 33 gauge needle delivered 1 µL of either AAV-GFP or AAV-Cre virus over 4 minutes and left an additional 5 minutes for complete diffusion of virus.

Virus

AAV-GFP or AAV-Cre, a fusion construct of GFP and Cre recombinase, were the two constructs utilized in this study. GFP has previously been shown not to interfere with recombinase activity and does not alter neuronal function.

In situ hybridization

After behavioral studies were complete, mice were sacrified and whole brains were dissected and rapidly frozen on dry ice and stored at -80°C. Brains

were sectioned at 14 µm on a cryostat and mounted on superfrost plus slides. Probe sequence and preparation as well as detailed methods were performed as previously described. Briefly, slides were pretreated and hybridized with ^{35}S-labeled anti-sense probes for BDNF, TrkB, or Cre recombinase and exposed to Kodak XOMAT film for 3-6 days.

Quantitative reverse transcription PCR (QRT-PCR)

Brain sections containing DRN (7-8 at 14 µm) were also collected on PEN membrane coated slides, dehydrated in ethanol series, was microdissected using a LMD system (Leica, Bannockburn, IL). DRN neurons that were collected were identified by GFP fluorescence and eight sections (~140µm apart) were pooled per brain, encompassing the majority of the injection site, to extract RNA using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA). The conditions for cDNA construction, amplification, and primer sequences for BDNF, TrkB, Cre, and GAPDH are as described previously. Relative expression is normalized to GAPDH.

Behavior

After surgery, mice recovered for 21 days to ensure viral expression and recombination of the loxP sites. Mouse weight was monitored weekly to determine if gene deletion caused gross health problems. Behavioral assessments were performed in order from least to most stressful. Mice were allowed to habituate to behavior rooms for at least one hour prior to testing.

Locomotor activity

Mice were placed in cages and locomotor activity was recorded for 2 hours under red light by photocell beams linked to computer acquisition software (San Diego Instruments, San Diego, CA).

Sucrose consumption test

Briefly, group housed mice were habituated to a 1% sucrose/tap water solution for 48 hours. The mice were then habituated to water deprivation periods of 4, 14, and 19 hours, followed by a 1 hour exposure to the sucrose solution for three days with intervening access to regular drinking water. To assess individual sucrose intake, the group-housed mice were water-deprived overnight and then housed temporarily in a new cage. Each test mouse was placed in its home cage for one hour with access to the 1% sucrose solution. The bottle of sucrose solution was weighed before and after the test to determine sucrose intake. The following, the same deprivation and liquid access protocol was used to assess water intake. Sucrose consumption is expressed as amount sucrose/total liquid drunk in both trials.

Open field test

Mice were assessed for activity in a 72x72 cm open field (OF) arena at 40 lux for 5 minutes. Movement was tracked by video (Ethovision3.0 Noldus, Leesburg, Virginia) for time spent in center (14x14 cm) and peripheral zones (5 cm around perimeter).

Forced swim test

The forced swim test (FST) was performed as previously described (Porsolt et al., 1977b). Mice received subchronic treatment with three doses of vehicle (saline) or desipramine at 24 hr, 4 hr, and 1 hr prior to behavioral testing at 15 mg/kg, 15mg/kg, and 20 mg/kg respectively. Briefly, mice were placed in 3L of water 22-24°C in 4L beaker and swimming behavior was videotaped by a camera on the side of the beakers for 6 min. Time spent immobile during the last 4 min of the test was recorded in seconds by an observer blind to group assignments.

Resident intruder test

Mice were housed in home cages with bedding that was not changed for 10 days. On the test day, each experimental mouse was presented with a novel adult male mouse in their own home cage for five minutes. Mice were videotaped by a camera on the side of the cages and analyzed for aggressive behavior including latency and number of bite attacks, wrestling, chasing, tail rattling, and submissive posturing of the intruder mouse, for the duration of the encounter with the novel male by an observer blind to group assignments.

Statistical analysis

Data represent the following number of animals in each group: BDNF experiment, AAV-GFP n=19, AAV-Cre n=15; TrkB experiment, AAV-GFP n=13, AAV-GFP n=11. All data are presented as mean ± SEM. Significance was analyzed by Student's t-test or by two-way ANOVA and set at $p<0.05$ (Graphpad Prism).

Results

Localized deletion of BDNF or TrkB in dorsal raphe nucleus

We generated selective deletions of BDNF and TrkB using either a floxed mouse line of BDNF, in which the coding region (exon IX) is flanked by loxP sites, or floxed line of TrkB, in which the start site of the coding region (exon II) is flanked by loxP sites. We bilaterally injected AAV-Cre into the dorsal raphe nucleus (DRN) to induce recombination and delete the gene. We performed *in situ* hybridization to confirm proper placement of the injection sites into DRN.

We further quantitatively assessed the knockdown of BDNF or TrkB as well as Cre expression via QRT-PCR. After visualized infected cells with epifluorescence microscopy, we laser micro-dissected virus infected tissue and extracted RNA for reverse-transcription to obtain cDNA followed by QPCR. In fBDNF mice, we detect ~50% reduction in BDNF expression in AAV-Cre injected tissue compared with AAV-GFP injected mice ($p<0.05$). This decrease in BDNF expression was accompanied by robust expression of Cre (*; $p<0.05$), while mice injected with AAV-GFP show an absence of Cre expression (Figure 3-1A). Similarly, in the fTrkB group, we see ~30% reduction in TrkB expression in AAV-Cre injected tissue compared to AAV-GFP treated mice (*; $p<0.05$). Again, this decrease is due to increased expression of Cre in the DRN (*; $p<0.05$) that is not present in the AAV-GFP group (Figure 3-1B).

Importantly, we use these techniques combined to obtain placement information that is used to verify that viral expression in each mouse is spatially restricted to the DRN. Animals with off-target injections are not included in behavioral data presented here.

Locomotor activity

We assessed the impact of DRN BDNF signaling on psychomotor activity levels by measuring horizontal locomotor activity for 120 minutes. We see that total locomotor activity over the total duration is not distinguishable between fBDNF mice injected with either AAV-GFP or AAV-Cre (Figure 3-2A, inset). However, we do observe increased activity in AAV-Cre injected fBDNF mice during the first five minutes of the task compared to AAV-GFP mice (*; $p<0.05$) (Figure 3-2A). Conversely, when TrkB is knocked-down in DRN, locomotor activity is not different between AAV-GFP and AAV-Cre injected groups (Figure 3-2B).

Sucrose consumption test

To model anhedonia behavior, we utilized the sucrose consumption test to assess contribution of DRN BDNF signaling in this measure. Localized deletion of BDNF in DRN had no impact on sucrose consumption (Figure 3-3A). Similarly, sucrose consumption behavior is not altered by selective loss of TrkB in DRN (Figure 3-3B).

Open field test

We used the open field test to determine if DRN BDNF signaling impinges on anxiety-related behavior. In the open field test, increased time spent in the center of an open field arena is thought to represent reduced anxiety, as this parameter is sensitive to benzodiazepine treatment. We do not observe any alterations in time spent in the center or periphery of the open field arena in AAV-GFP versus AAV-Cre mice on the fBDNF background (Figure 3-3C).

Furthermore, viral-mediated deletion of fTrkB in DRN does not impact open field behavior (Figure 3-3D). Additionally, in each strain, we observed no confounding effect of viral injection on distance traveled in the open field over the duration of the test (data not shown).

Forced swim test

We used a traditional antidepressant screening task, the forced swim test, to assess the impact of DRN BDNF signaling on antidepressant efficacy. We did not observe any baseline alteration in immobility behavior between vehicle-treated fBDNF with AAV-GFP or AAV-Cre virus injections (Figure 3-4A). After sub-chronic administration of desipramine, both AAV-GFP and AAV-Cre injected fBDNF mice displayed an antidepressant response as assessed by two-way ANOVA ($F_{1,30}$=10.34, significant effect of drug with no genotype or interaction effect *; $p<0.05$). Deletion of TrkB from the DRN also did not impact baseline immobility in fTrkB mice treated with vehicle. However, while subchronic administration of desipramine resulted in an antidepressant-like effect in AAV-GFP injected fTrkB mice, this effect was blocked in mice receiving AAV-Cre as assessed by two-way ANOVA ($F_{1,27}$=11.24, significant interaction effect of drug and genotype *; $p<0.05$) (Figure 3-4B).

Discussion

Here, we show for the first time that TrkB signaling in dorsal raphe neurons is critical for antidepressant responses. Deletion of BDNF or TrkB in DRN appears to have specific impact on antidepressant response, leaving other behaviors intact like locomotor activity, sucrose consumption, open-field activity, and baseline depression-like behavior in forced swim test. These data reveal that dorsal raphe nucleus BDNF signaling makes essential contributions to complex behaviors involved in response to antidepressant treatment.

Our targeted deletion of BDNF or TrkB into the DRN appears to be specific and results in significant knockdown of these factors in the targeted region. While the virus injection does not infect every cell in the targeted region, recombination within an infected cell is complete (Adachi et al., 2008). In addition, we found that this viral-mediated deletion technique does not result in cell loss in the injected region (Adachi et al., 2008).

Mice with deletions of BDNF or TrkB in DRN neurons appear to have normal weight gain (data not shown) and locomotor activity with respect to control virus injected mice. These data suggest that localized deletion does not impact the overall health or general psychomotor behaviors of the mice, consistent with our observations of localized BDNF deletions in hippocampal regions (Adachi et al., 2008).

We assessed sucrose consumption to determine the effects of DRN BDNF signaling on anhedonia-like behavior. We did not see any impact of BDNF or TrkB deletion on this measure, consistent with previous data with BDNF

inducible knockouts (Autry et al., 2009; Monteggia et al., 2004). In addition, we did not observe alterations in anxiety-related behavior as assessed by open field behavior, again consistent with previous data with BDNF inducible knockouts (Monteggia et al., 2004).

Localized deletion of BDNF or TrkB from the dorsal raphe nucleus did not impact baseline depression-related behavior in the forced swim test. An effect on baseline depression behavior in genetic models of BDNF deletion have been elusive , and even the impact of a combination of stress in addition to BDNF deletion seems to affect only certain measures in a gender-specific manner (Autry et al., 2009). However, we did observe a loss of antidepressant efficacy in mice with a selective deletion of TrkB, but not BDNF, from dorsal raphe nucleus neurons. These data suggest that cell-autonomous BDNF signaling in dorsal raphe is essential for behavioral antidepressant responses. Taken together with our previous study demonstrating that dentate gyrus BDNF is required for antidepressant behavioral responses (Adachi et al., 2008), we believe that BDNF secreted from dentate gyrus neurons may signal to presynaptic dorsal raphe fibers to potentiate this behavior.

In summary, we demonstrate that dorsal raphe nucleus BDNF signaling has specific impact on aggression behavior and antidepressant efficacy. These data suggest that traditional antidepressant therapies may have efficacy by increasing BDNF in hippocampus which feeds back onto dorsal raphe nucleus neurons via presynaptic TrkB activation in order to confer behavioral antidepressant responses.

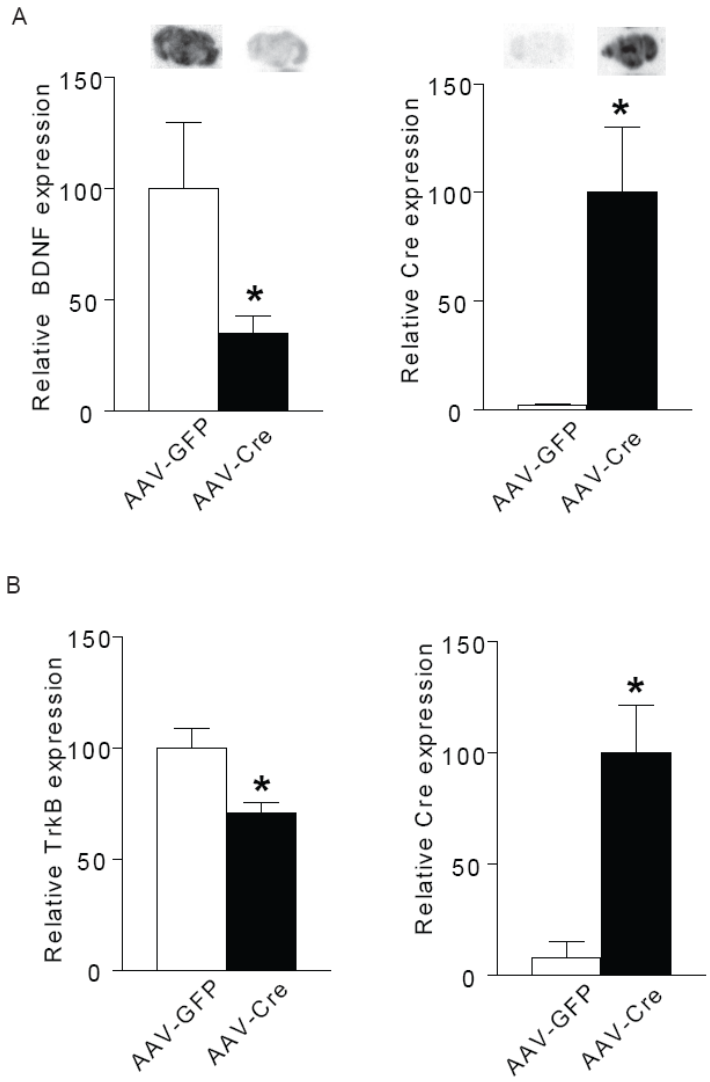


Figure 3-1. Relative knockdown of BDNF signaling in dorsal raphe nucleus. (A, left) Laser capture dissection of dorsal raphe nucleus followed by QPCR reveals ~50% reduction in BDNF expression in fBDNF tissue treated with the AAV-Cre virus relative to a control virus (*P<0.05). (A,right) Similarly, there is almost no expression of Cre in tissue treated with a control virus compared to AAV-Cre virus treated tissue. (B, left) We observed ~30% decrease in expression of TrkB in the dorsal raphe nucleus of fTrkB tissue treated with AAV-Cre with respect to tissue treated with a control virus. (B, right) We observed virtually no Cre expression in control virus treated tissue compared with AAV-Cre virus treated tissue.

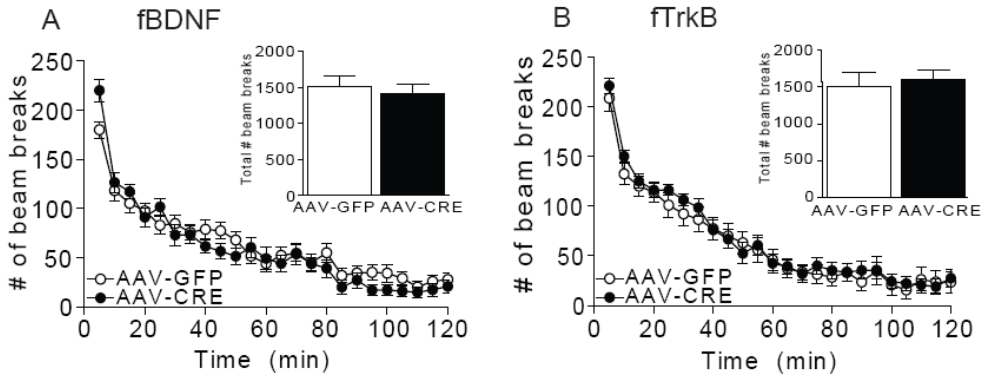


Figure 3-2. Locomotor activity is normal after dorsal raphe nucleus knockdown of BDNF signaling. (A) Locomotor activity is unaltered between control virus and AAV-Cre treated fBDNF mice (P>0.05). (B) Similarly, locomotor activity is indistinguishable between AAV-GFP and AAV-Cre treated fTrkB mice (P>0.05).

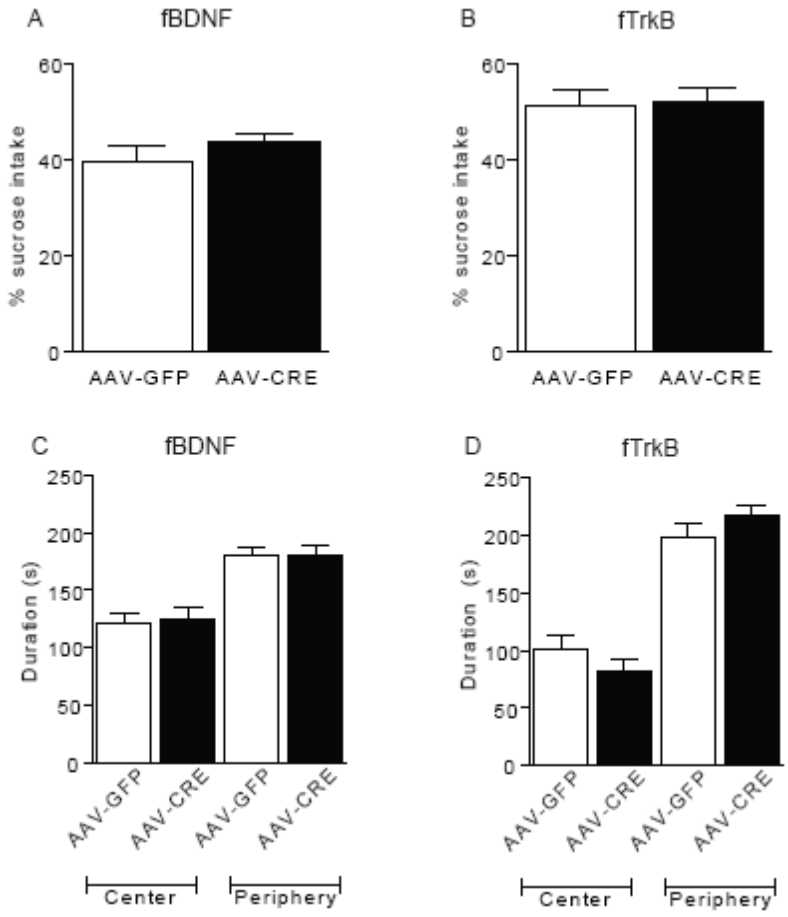


Figure 3-3. Anhedonia- and anxiety- related behaviors are not impacted by dorsal raphe nucleus knockdown of BDNF signaling. (A,B) Knockdown of (A) BDNF or (B) TrkB in dorsal raphe nucleus does not alter sucrose intake ($P>0.05$). (C,D) Similarly, dorsal raphe nucleus knockdown of (C) BDNF or (D) TrkB does not impact open field exploration ($P>0.05$).

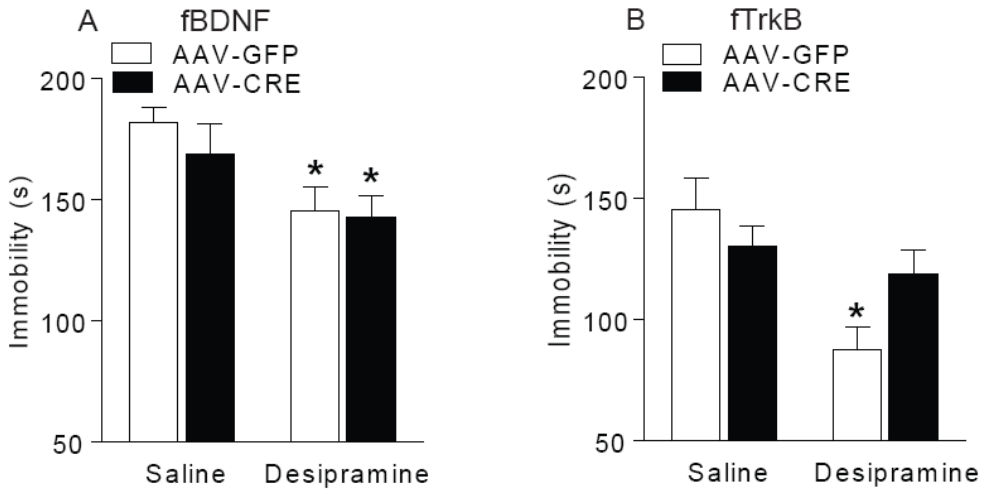


Figure 3-4. Mice lacking dorsal raphe nucleus expression of TrkB do not respond to desipramine. (A) Knockdown of BDNF from dorsal raphe nucleus does not alter the antidepressant-like response in forced swim test after a subchronic desipramine treatment($F_{1,30}$=10.34, significant effect of drug with no genotype or interaction effect *; $p<0.05$). (B) However, selective deletion of TrkB from dorsal raphe nucleus blocks the antidepressant-related effect of desipramine($F_{1,27}$=11.24, significant interaction effect of drug and genotype *; $p<0.05$).

CHAPTER 4

NMDA RECEPTOR BLOCKADE AT REST DESUPPRESSES PROTEIN TRANSLATION AND TRIGGERS RAPID BEHAVIORAL ANTIDEPRESSANT RESPONSES

Introduction

Clinical studies have consistently demonstrated that a single sub-psychomimetic dose of ketamine, an ionotropic glutamatergic *n*-methyl-*d*-aspartate (NMDA) receptor antagonist, produces a fast-acting antidepressant response in patients suffering from major depressive disorder (MDD), though the precise mechanism underlying ketamine's antidepressant response is unclear (Berman et al., 2000; Price et al., 2009; Zarate et al., 2006). Depressed patients report alleviation of core MDD symptoms within two hours of a single low-dose intravenous infusion of ketamine with effects lasting up to two weeks (Berman et al., 2000; Price et al., 2009; Zarate et al., 2006), in contrast to traditional antidepressants such as serotonin reuptake inhibitors (SSRIs), which typically take weeks or months to produce an antidepressant response. This delay in onset is a major drawback to current antidepressant therapies, leaving a crucial need for the development of faster acting antidepressants for treatment of depression as well as suicide-risk patients (Price et al., 2009). The ability of ketamine to produce a rapidly acting and long-lasting antidepressant response in depressed patients provides a unique opportunity for preclinical investigation of cellular mechanisms that mediate these clinically relevant behavioural effects. We show that ketamine, as well as other

NMDA receptor antagonists, produce fast-acting behavioural antidepressant-like effects in mouse models that are dependent on rapid synthesis of brain-derived neurotrophic factor (BDNF). We find that ketamine mediated NMDA receptor blockade at rest deactivates eukaryotic elongation factor 2 (eEF2) kinase (also called CaMKIII) resulting in a reduction of eEF2 phosphorylation and de-suppression of BDNF translation. Furthermore, we find inhibitors of eEF2 kinase trigger fast-acting behavioural antidepressant-like effects. Our findings suggest a behavioural and clinically relevant correlate of protein translational regulation that may serve as a viable therapeutic target for the development of fast-acting antidepressants.

Materials and Methods

Mice

C57BL/6 male mice aged 6-8 weeks old were habituated to animal facilities for one week prior to behavioural testing. Mice were kept on a 12/12 light dark cycle and given access to food and water *ad libitium*. Inducible BDNF knockout mice were generated from a trigenic cross of NSE-tTA, TetOp-Cre, and floxed BDNF mice as previously described (Monteggia et al., 2004). Conditional TrkB knockout mice were made by crossing CamK-cre(93) to floxed TrkB mice. For all behavioural testing, male mice were age- (two to four months) and weight-matched, and groups were balanced by genotype.

Drug

All drugs were injected intraperitoneally (i.p.). Ketamine (Fort Dodge Animal Health) 3.0 mg/kg, MK-801 (Sigma) 0.1 mg/kg, and CPP (Sigma) 0.5 mg/kg were dissolved in saline. Anisomycin (Sigma) was first dissolved in HCl and then subsequently in saline for a final concentration of 100 mg/kg in saline, at pH 7.4. Actinomycin D (Sigma) was administered at 0.5 mg/kg in 5% ethanol. Rottlerin and NH125 (Sigma) were given at 5mg/kg in 20-100% DMSO. SL327 (Sigma) was used at 10mg/kg dissolved in 100% DMSO. NMDA (Sigma) 75 mg/kg, NBQX (Sigma) 10 mg/kg, and PTX (Sigma) 1 mg/kg were dissolved in saline.

Sucrose consumption test

Briefly, group housed mice were habituated to a 1% sucrose/tap water solution for 48 hours. The mice were then habituated to water deprivation periods of 4, 14, and 19 hours, followed by a 1 hour exposure to the sucrose solution for three days with intervening access to regular drinking water. To assess individual sucrose intake, the group-housed mice were water-deprived overnight and then housed temporarily in a new cage. Each test mouse was placed in its home cage for one hour with access to the 1% sucrose solution. The bottle of sucrose solution was weighed before and after the test to determine sucrose intake. A water test was performed in a similar manner the following day. Data are expressed as a percentage of sucrose to total volume consumed in both sucrose and water trials.

Elevated Plus Maze

Mice were placed in the centre of a plus maze (each arm 33 cm x 5 cm) that was elevated 1 meter above the floor with two open arms and two closed arms (25-cm-tall walls on the closed arms) at 40 lux. The exploratory activity was monitored for 5 min with a video tracking system, and the duration in seconds in the closed and open arms was recorded by EthoVision software.

Novelty suppressed feeding

Briefly, group housed animals were food deprived for 24 hours and then placed in a temporary home cage for 30 minutes. For the test, individual mice were placed in a 42 x 42 cm open field arena at 40 lux. A single pellet of the mouse's regular

food chow was placed in the centre of the open field arena. Each animal was placed in a corner of the arena and allowed to explore for up to 10 minutes. The trial ended when the mouse chewed a part of the chow. Amount of food consumed in the home cage was taken as weight of chow consumed in 5 minutes as a control measure for appetite.

Context and Cued Fear Conditioning

Fear conditioning was performed as previously described[5]. Briefly, mice were placed in individual chambers for 2 min followed by a loud tone (90 dB) for 30 s, immediately followed by a 0.5 mA footshock for 2 s. After 1 min, mice received a second pairing of tone and footshock, as described. Mice were placed in home cages until 24 h later, when the mice were placed back in the same boxes without a tone or shock. The amount of time the animal spent freezing was scored by an observer blind to genotype. Freezing behaviour was defined as no movement except for respiration. Four hours later, mice were placed in a novel environment with no tone or shock for 3 min, followed by 3 min of the tone to assess cue-dependent fear conditioning. Again, time spent freezing was recorded as described[5].

Learned Helplessness

Mice were trained on one side of a two-chamber shuttlebox (MedAssociates) with the door closed for 1 hour, receiving 120 variable interval (18-44s average 30 sec) shocks (0.35 mA for 2 sec) on two training days. On the test day, the door was

raised at the onset of shock and the shock ended either when the mouse stepped through to the other side of the shuttlebox or after 25 sec. Latency to step through the door and number of escape failures were recorded for fifteen trials.

Locomotor activity

Mice were placed in cages and locomotor activity was recorded for one hour under red light by photocell beams linked to computer acquisition software (San Diego Instruments).

Forced swim test

The forced swim test (FST) was performed as previously described[11]. The FST is an animal model that is sensitive to conventional AD treatment as well as non-monoaminergic ADs[4]. Mice were placed in a 4000-mL Pyrex glass beaker containing 3000 mL of water 24±1ºC for six minutes. Water was changed between subjects. All test sessions were recorded by a video camera positioned on the side of the beakers. The videotapes were analyzed and scored by an observer blind to group assignment during the last four minutes of the six minute trial. A decrease in immobility time is suggestive of an AD-like response.

Chronic mild stress

Stressed mice were subjected to 2 randomly selected mild stressors/day of variable duration (1-12 hours) for 28 days. Stressors included water deprivation, 45^0 cage tilt, food deprivation, exposure to rat faeces, cage overcrowding, wet

bedding, overnight illumination, dark exposure during normal light cycle, cold bedding, acoustic disturbance (120 dB), strobe lights, and cagemate rotation. Stressors were not applied within 8 hours of behavioural testing.

Time-course experiments

Separate cohorts of C57BL/6 adult male mice were i.p. injected with vehicle or the NMDA antagonists ketamine (3.0 mg/kg), MK-801 (0.1 mg/kg), or CPP (0.5 mg/kg) at 30 minutes, 3 hours, 24 hours, or 1 week prior to FST (n=10 per group).

Anisomycin and actinomycin D experiments

Separate cohorts of C57BL/6 adult male mice were i.p. injected with either vehicle or anisomycin (100mg/kg) or saline or actinomycin D (0.5 mg/kg) one hour prior to FST. Thirty minutes prior to testing, mice received either a saline or ketamine injection (3.0 mg/kg) (n=10 per group). For 24 hour experiments, mice were given anisomycin (100 mg/kg) or saline 30 minutes prior to an injection of ketamine and tested in the FST one day later.

Inducible BDNF KO experiments

Separate cohorts of inducible BDNF KO adult male mice, or wild-type littermate controls, were subjected to FST either 30 minutes or 24 hours after injection with saline, ketamine (3.0 mg/kg), or MK-801 (0.1 mg/kg) (n=7-12 per group).

Quantitative RT-PCR

Fresh frozen anterior hippocampal slices (2/mouse, ~1 mm thick) were dissected and total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. Conditions for cDNA synthesis, amplification, and primer sequences were described previously. Fold change in BDNF expression of the coding exon IX is normalized to GAPDH.

Protein quantification

Anterior hippocampal slices (2/mouse, ~1 mm thick) were dissected from C57BL/6 mice receiving saline vehicle, ketamine (3.0 mg/kg), or MK-801 (0.1 mg/kg) i.p. either 30 minutes or 24 hours post-injection and rapidly frozen. Tissue was lysed in buffer containing protease and phosphatase inhibitors, and total protein concentration was quantified by Bradford analysis. BDNF quantification was determined using SDS-PAGE. Primary antibodies for BDNF (Santa Cruz Biotechnology) and GAPDH (Cell Signaling) were used at dilutions of 1:200 and 1:10000, and anti-rabbit secondary antibodies were used at 1:2000 and 1:50000, respectively. For phosphorylated eEF2 (peEF2) (Thr56) and total eEF2 (teEF2), primary antibodies were used at dilutions of 1:1000, and anti-rabbit secondary antibodies were used at 1:2000. Mouse anti-ARC (C7) (Cell Signaling) was used at primary dilution 1:1000 and secondary dilution at 1:2000. Phospho-mTOR and total mTOR (Cell Signaling) were both used at primary dilution 1:500 and secondary dilution 1:10,000. GluR1 (Chemicon) was used at primary dilution 1:5000 and secondary dilution 1:2000. Pan-Homer antibody

(Cell Signaling) was used at 1:5000 with 1:2000 dilutions for primary and secondary, respectively. Phospho-S6 kinase and total s6 kinase were used at 1:200 and 1:5000 for primary dilutions, respectively, and both had secondary dilutions at 1:5000 (Cell Signaling). ECL developed bands were exposed to film. Films were analyzed by ImageJ. BDNF was normalized to GAPDH bands, and peEF2 and teEF2 bands were taken as a ratio of GAPDH normalized values.

Immunohistochemistry

C57BL/6 mice were treated i.p. with saline, ketamine (3.0 mg/kg), or MK-801 (0.1 mg/kg) and sacrificed 30 minutes later. Protocol is adapted from a previous study[27]. Brains were fresh-dissected and post-fixed for 72 hours in ice-cold 4% paraformaldehyde. Brains were cryoprotected for 2 or more hours in 20% glycerol and sectioned on a freezing microtome at 30 µm and preserved in 1X PBS/0.01% sodium azide. Floating sections were washed in 2X SSC followed by antigen unmasking in 50:50 acetone:methanol performed at 4° C. Sections were rinsed and endogenous peroxidase activity was quenched in 1% H_2O_2 for 30 minutes. Sections were rinsed in 2X SSC/0.05% Tween-20. Tissue was blocked for 30 min in 3% normal goat serum/2X SSC/0.05% Tween followed by primary antibody, rabbit anti-peEF2 (diluted 1:100 in blocking solution; Cell Signaling Technology), incubation for 48 hours at 4° C. After rinsing in 2X SSC, a horseradish peroxidase-labelled secondary antibody at 1:200 was applied and the signal amplified using the tyramide amplification signal (TSA) system (Perkin

Elmer). Slides were counterstained with DAPI, mounted on superfrost plus slides and dried for 2 hours, and coverslipped in DPX mountant.

ELISA

High sensitivity enzyme-linked immunosorbent assay to assess BDNF levels was used per manufacturer's instructions (Promega). Briefly, hippocampal lysates were prepared in the recommended buffer, diluted 1:4 in 1X PBS and acid treated as instructed by the manufacturers. A 96-well plate (Nunc) was coated overnight in carbonate coating buffer, blocked in provided sample buffer for 2 hours at RT, and treated with recombinant human BDNF antibody for 2 hours at RT. Acid-treated samples and provided standards were added to the plate in duplicate. Wells were then treated with anti-IgY conjugated to HRP for 1 hour at RT and colour was developed with provided TMB solution for ten minutes stopped with 1N HCl. Absorbance of wells was measured at 450 nm. BDNF concentration was determined by comparing mean absorbance of the duplicate samples to the standards. BDNF concentration was then normalized to total protein content and expressed as pg BDNF/µg total protein.

Cell Culture

Dissociated hippocampal cultures were prepared as previously described[28].
Briefly, whole hippocampi were dissected from postnatal day 0-3 (P0-3) Sprague-Dawley rats. Tissue was trypsinized (10 mg/ml trypsin) for 10 min at 37^0C, mechanically dissociated by pipetting and plated on Matrigel coated coverslips.

Cytosine arabinoside (4 µM ARAC, Sigma, St. Louis, MO) was added at day 1 *in vitro* (DIV), at 4 DIV ARAC concentration was reduced to 2 µM. All experiments were performed on 14-21 DIV cultures.

Cell culture recordings

Whole-cell patch-clamp recordings were performed on hippocampal pyramidal neurons. Data were acquired using a MultiClamp 700B amplifier and Clampex 9.0 software (Molecular Devices). Recordings were filtered at 2 kHz and sampled at 200 µs. A modified Tyrode's solution containing (in mM): 150 NaCl, 4 KCl, 2 MgCl2, 2 CaCl2, 10 glucose, 10 HEPES, pH 7.4, was used as external bath solution. The pipette internal solution contained (in mM): 115 Cs-MeSO3, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 Tetraethylammonium-Cl, 4 Mg-ATP, 0.3 Na3GTP, pH 7.35, and 10 QX-314 [N-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide], 300 mOsm. Series resistance ranged between 10-30 MΩ. To record and isolate NMDA receptor-mediated miniature EPSCs (NMDA-mEPSCs), MgCl2 concentration was reduced to 0.1 mM and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10µM, Sigma), picrotoxin (PTX; 50 µM; Sigma) were added to bath solution to block α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor mediated excitatory currents and γ-Aminobutyric acid (GABA) receptor mediated inhibitory currents respectively. Baseline for the analysis of NMDA-mEPSCs was automatically determined as the average current level of silent episodes during a recording. The

events were selected at a minimum threshold of 4 pA and the area under current deflection was calculated to quantify charge transfer.

Field recordings

Field recordings were made from hippocampal slices. Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Slices (400 μm) were prepared from 15- to 25-d-old rats. Rats were anesthetized with the Euthasol (50 mg/kg) and decapitated soon after the disappearance of corneal reflexes. The brain was removed, dissected and then sliced using a vibratome (1000 Plus) in ice-cold dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH2PO4, 26 NaHCO3, 0.5 CaCl2, 5 MgCl2, 212 sucrose, and 10 dextrose. Area CA3 was surgically removed from each slice immediately after sectioning. The slices were transferred into a reservoir chamber filled with ACSF containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 2 MgCl2, and 10 dextrose. Slices were allowed to recover for 2–3 h at 30°C. ACSF and dissection buffer were equilibrated with 95% O2 and 5% CO2. For recording, slices were transferred to a submerged recording chamber, maintained at 30°C, and perfused continuously with ASCF at a rate of 2–3 ml/min. Field potentials (FPs) were recorded with extracellular recording electrodes (1 MΩ) filled with ACSF and placed in stratum radiatum of area CA1. Field potentials were evoked by monophasic stimulation (duration, 200 μs) of Schaffer collateral/commissural afferents with a concentric bipolar tungsten stimulating electrode (Frederick Haer Company, Bowdoinham, ME). Stable baseline

responses were collected every 30 s using a stimulation intensity (10–30 μA), yielding 50–60% of the maximal response. After recording 20 min of stable baseline stimulation was stopped and 20 μM of ketamine was applied for 30 min, after this stimulation was resumed. FPs were filtered at 2 kHz, acquired, and digitized at 10 kHz on a personal computer using custom software (LabVIEW; National Instruments, Austin, TX). Synaptic strength was measured as the initial slope (10–40% of the rising phase) of the FP. The group data were analyzed as follows: (1) the initial slopes of the FP were expressed as percentages of the preconditioning baseline average; (2) the time scale in each experiment was converted to time from the end of ketamine application; and (3) the time-matched, normalized data were averaged across experiments.

Results

We examined the acute antidepressant (AD) effects of ketamine in mice and detected a significant behavioural effect suggestive of an AD response in the forced swim test (FST) and the novelty-suppressed feeding test (NSF) behavioural models with predictive value for ADs (Figure 4-1A-E). These data are in agreement with recent data showing that ketamine produces an AD response in these behavioural paradigms (Maeng et al., 2008). We further demonstrate that ketamine has AD effects in sucrose consumption test (SCT), NSF, as well as FST after chronic mild stress, implicating its efficacy in an animal model of depression (Figure 4-1F-I).

To elucidate the mechanisms underlying ketamine's fast-acting AD action we focused our efforts on the FST paradigm, a widely applicable test that can predict effectiveness of non-monoaminergic ADs. To examine the time-course of these behavioural AD effects, and to determine whether it extended to other NMDA receptor antagonists, C57BL/6 mice were examined in FST at specific time-points following treatment with a single low dose of ketamine, MK801 (a use-dependent antagonist), or CPP (a competitive antagonist) (Figure 4-2 A-C). Independent groups of mice were used at each time point for each drug treatment to avoid potential complications of behavioural habituation to FST. At 30 minutes and 3 hours following drug administration, each NMDA receptor antagonist produced a significant reduction in immobility compared to vehicle-injected animals, suggesting that NMDA receptor blockade can produce a fast-acting AD response. Twenty-four hours following drug administration, ketamine

and CPP still produced a significant difference in FST, but not MK-801, suggesting that MK-801 can mediate an acute AD response but not a prolonged response, in agreement with previous data. One week following drug administration, only animals receiving ketamine displayed significant long-lasting AD behavioural responses. Ketamine, CPP, and MK-801 have relatively short half-lives (~2-3 hours) (Kristensen et al., 1995; Schwartz and Wasterlain, 1991; Sinner and Graf, 2008) suggesting that sustained AD responses mediated by NMDA antagonists may be due to sustained changes in neural plasticity rather than persistent blockade of NMDA receptors.

Brain-derived neurotrophic factor (BDNF) is a well-characterized neurotrophin that has been linked to the action of traditional AD drugs; it is known that BDNF expression is increased in the hippocampus by ADs (Chen et al., 2001) and that BDNF deletion in the hippocampus attenuates AD behavioural responses (Adachi et al., 2008; Berton et al., 2006; Monteggia et al., 2004). Moreover, a single intraventricular or intrahippocampal BDNF infusion is known to cause rapid and sustained AD effects in FST lasting 3-6 days (Hoshaw et al., 2005; Shirayama et al., 2002). To examine whether the AD response of ketamine is mediated through BDNF, we administered a single dose of ketamine to inducible BDNF knockout (KO) mice, a line that is behaviourally insensitive to the action of conventional ADs(Monteggia et al., 2004), and tested mice in the FST. Thirty minutes following injection, wild-type littermate controls given ketamine displayed significant reductions in immobility, indicative of an AD-like response, compared to vehicle treated mice (Figure 4-3A). By contrast, ketamine

did not produce an AD-like effect in BDNF KO mice, suggesting that BDNF is required for the fast-acting AD response. Twenty-four hours following injection, in a separate cohort of mice, ketamine produced significant reductions in immobility in wild-type littermates compared to vehicle controls, but did not produce an AD response in BDNF KO mice (Figure 4-3A), indicating that both rapid and sustained effects of ketamine depend on BDNF expression.

To determine if NMDA antagonists alter BDNF expression in the hippocampus, a brain region in which BDNF is necessary for traditional AD response (Adachi et al., 2008), C57BL/6 mice were given an acute dose of vehicle, ketamine, or MK-801. Western blot analysis revealed a significant increase in BDNF protein at 30 minutes that was absent 24 hours following acute ketamine or MK-801 (Figure 4-3C). These data suggest that rapid increases in BDNF protein translation and signalling, but not BDNF mRNA transcription, may be necessary for fast-onset AD responses. However, continued up-regulation of BDNF protein does not appear to underlie the sustained behavioural effects of ketamine.

To examine the involvement of rapid protein translation on AD-like effects of ketamine, we examined FST behaviour with and without anisomycin, an inhibitor of protein synthesis (Lattal and Abel, 2001). In addition, to test whether transcription is also a mediator of the AD-like effect of ketamine, we examined FST with and without the RNA polymerase inhibitor actinomycin D (Capasso et al., 1996). Anisomycin and actinomycin D block translation and transcription, respectively, by approximately 80% within two hours of treatment.

Therefore, to evaluate the effects of these drugs on FST behaviour, we pre-treated mice with anisomycin or actinomycin D prior to ketamine (Figure 4-3 E). Anisomycin prevented ketamine-induced behavioural responses, suggesting that ketamine's effects are mediated through new protein synthesis (Figure 4-3F). Because blocking translation diminished ketamine's rapid AD-like behavioural effects, we next conducted experiments to determine whether the long-lasting effect of ketamine was also dependent on protein synthesis. Using a similar paradigm, anisomycin was given 30 minutes prior to ketamine and the behavioural effects were monitored 24 hours later in FST. Anisomycin prevented ketamine's long-term effect on FST, suggesting that rapid protein translation was involved in mediating sustained AD-like responses (Figure 4-3G). By contrast, actinomycin D did not impact the ability of ketamine to significantly reduce immobility in FST 30 minutes or 24 hours after treatment, suggesting that the behavioural effect of ketamine was not dependent on new gene expression (Figure 4-4B,C). To verify that the lack of a behavioural effect in the actinomycin experiment was not due to its inability to cross the blood-brain barrier, we examined BDNF mRNA expression in these animals and found decreased transcription of BDNF in the hippocampus (Figure 4-4A). Taken together, these findings suggest that rapid, but transient, induction of BDNF translation is required for both the fast-acting and long-lasting AD-like behavioural effects of ketamine. Interestingly, continued increases in BDNF levels were not necessary for sustained behavioural effects of acute ketamine administration, further

indicating that long-lasting AD responses may be due to changes in synaptic plasticity, possibly initiated by this transient increase in BDNF translation.

Several forms of synaptic plasticity and ensuing learning processes are mediated by NMDA receptor activation-driven increases in protein translation. Conversely, our findings suggest that blockade of NMDA receptors produces a rapid increase in protein translation that mediates AD-like effects. To investigate mechanisms that may underlie this paradoxical observation between NMDA receptor antagonism and protein translation, we turned to recent work showing that NMDA receptor blockade by MK-801 or AP5, in the absence of neuronal activity, can augment protein synthesis through de-phosphorylation (activation) of eukaryotic elongation factor-2 (eEF2), a critical catalytic factor for ribosomal translocation during protein synthesis(Sutton et al., 2007). In this model, NMDA receptor activity at rest causes chronic activation of eEF2 kinase (eEF2K; also called CaMKIII), which phosphorylates eEF2, releasing this factor from the translational machinery to halt translation whereas acute NMDA receptor blockade at rest suppresses eEF2 phosphorylation, thereby increasing translation of target transcripts.

To evaluate this model, we first tested whether potential increases in synaptic glutamate levels after NMDA receptor blockade may be responsible for behavioural effects of ketamine. We found that administration of NMDA did not produce a fast acting AD effect in the FST (Figure 4-3B) as previously demonstrated at a dose known to affect behaviour (Poleszak et al., 2007) suggesting that excess glutamate is not involved in the behavioural effect of

ketamine. In order to rule out the contribution of regulated neuronal activity on AD behavioural effects, we tested whether NBQX, an AMPA channel blocker that reduces neuronal activity, or picrotoxin (PTX), a GABA channel blocker that increases activity, had any impact on FST behaviour or BDNF translation at doses previously shown to affect behaviour (Fernandez et al., 2007; Maeng et al., 2008). We found that acute systemic treatment with these drugs had no effect on FST behaviour (Figure 4-3D). However, we saw that, when co-applied with ketamine, NBQX abolished the behavioural AD responses in the FST (Figure 4-3H) as previously demonstrated (Maeng et al., 2008). These data suggest that alterations in evoked neurotransmission are not sufficient to elicit behavioural AD effects, but behavioural AD effects may require ketamine-mediated augmentation of AMPA-receptor activation. Recent evidence suggests that cortical mTOR signalling underlies ketamine-mediated AD responses (Li et al.). We investigated if the rapid component of AD behavioural effects depended on mTOR signalling in hippocampus, and if this signalling was downstream of BDNF. We did not detect any regulation of phosphorylated mTOR thirty minutes after low dose ketamine administration in wild-type or BDNF KO hippocampal tissue contradicting the proposed role of mTOR in this rapid response (Figure 4-3I).

To determine if ketamine, in accordance with other NMDA receptor blockers, inhibits spontaneous miniature NMDA-receptor mediated currents (NMDA-mEPSC) (Atasoy et al., 2008; Espinosa and Kavalali, 2009) that occur at rest and regulates eEF2 phosphorylation, we tested its impact on hippocampal synapses *in vitro*. After we perfused ketamine (1, 5, or 50 µM) for thirty minutes

onto cultured neurons and recorded NMDA-mEPSCs (Figure 4-5C), within several minutes of perfusion we detected a significant decrease in NMDA-mEPSCs similar to that observed with AP5 (Atasoy et al., 2008). Moreover, protein extracts from the same cultured hippocampal neurons treated with ketamine revealed a decrease in phosphorylated eEF2 (peEF2) levels compared to vehicle treated cultures, suggesting that ketamine, in the absence of neuronal activity, dose-dependently leads to de-phosphorylation of eEF2, allowing for de-suppression of protein synthesis (Figure4-5A,B). In addition, we evaluated effects of ketamine on hippocampal field potentials. We noticed that a 30-minute application of ketamine (20 uM at rest) potentiated subsequent evoked synaptic responses in hippocampal slices (Figure4-5E), further suggesting that increased AMPA-mediated neurotransmission underlies ketamine's AD-like behavioural effects in agreement with previous data demonstrating the dependence of synaptic plasticity on BDNF and protein synthesis (Tanaka et al., 2008).

To examine whether ketamine's fast-acting AD response is mediated via an increase in unphosphorylated eEF2, we administered ketamine to C57BL/6 mice and analyzed phosphorylated eEF2 levels in hippocampus by immunostaining. Within thirty minutes, ketamine administration caused a rapid decrease in phosphorylated eEF2 in regions of the CA1 and dentate gyrus of the hippocampus (Figure 4-6 A-C). MK-801 administration caused a similar change in hippocampal phosphorylated eEF2 levels, confirming the specificity of this effect to blockade of NMDA receptors (Figure 4-6 A-C). We also demonstrate

quantitatively a decrease in phosphorylation of eEF2 in hippocampus by Western blot analysis (Figure 4-6D).

To examine whether eEF2K inhibition alters BDNF protein expression *in vivo*, C57BL/6 mice were administered eEF2K inhibitors, rottlerin or NH125, and then sacrificed 30 minutes later. We found that both rottlerin and NH125 produced a significant increase in BDNF protein expression (Figure 4-6E,G), along with a corresponding significant decrease in peEF2 (Figure 4-6F,H) in the hippocampus. To directly assess whether inhibition of eEF2K is sufficient to mediate fast-acting AD-like responses, C57BL/6 mice were administered either rottlerin or NH125 and examined in FST. Both rottlerin and NH125 produced significant decreases in immobility in FST at 30 minutes (Figure 4-6I), a time-scale similar to the AD-like effects of NMDA antagonists, suggesting that the fast-acting behavioural effect is mediated through this signalling pathway. A separate group of C57BL/6 mice were injected with the extracellular-related kinase (ERK) inhibitor SL327, to test whether inactivation of ERK, a regulator of protein translation during activity, impacts depression-related behaviour. This treatment did not reveal a significant difference in immobility compared to vehicle controls (Figure 4-6I). In order to validate that the AD effect following eEF2K inhibition was mediated through BDNF, we administered rottlerin to BDNF KOs and tested them in FST. Similar to the effects of ketamine and MK-801, rottlerin does not produce an AD-like response in BDNF KO mice, demonstrating the requirement for increased BDNF expression upon eEF2K inhibition to produce an AD-like behavioural response (Figure 4-6J).

Discussion

In summary, our data support the hypothesis that ketamine produces rapidly acting AD-like behavioural effects through inhibition of NMDA-mEPSCs activated via spontaneous glutamate release, which leads to decreases in eEF2 kinase activity, permitting a rapid increase in BDNF translation (Figure 4-7). The observation of behavioural effects mediated through spontaneous, but not evoked neurotransmission, provides the first evidence that alterations in tonic resting neurotransmission may mediate behavioural effects, and indeed supports the idea that spontaneous and evoked forms of glutamatergic signalling are segregated in neurons(Atasoy et al., 2008; Sutton and Schuman, 2009; Sutton et al., 2007). Furthermore, we show that the mechanistic basis of this fast acting behavioural effect cannot be attributed to disinhibition of behavioural circuitry, or evoked neurotransmission, but must rely on enhanced neurotransmission following NMDA antagonist-induced plasticity which occurs at rest. These data show that eEF2K inhibition, resulting in de-suppression of protein translation, is sufficient to produce AD-like effects in a behavioural model of depression. Moreover, these findings provide initial support for the efficacy of eEF2K inhibitors as putative novel treatments for MDD with rapid onset of action. Taken together, our results suggest that a behavioural and clinically relevant correlate of synaptic translational machinery may serve as a viable therapeutic target for the development of faster acting antidepressants.

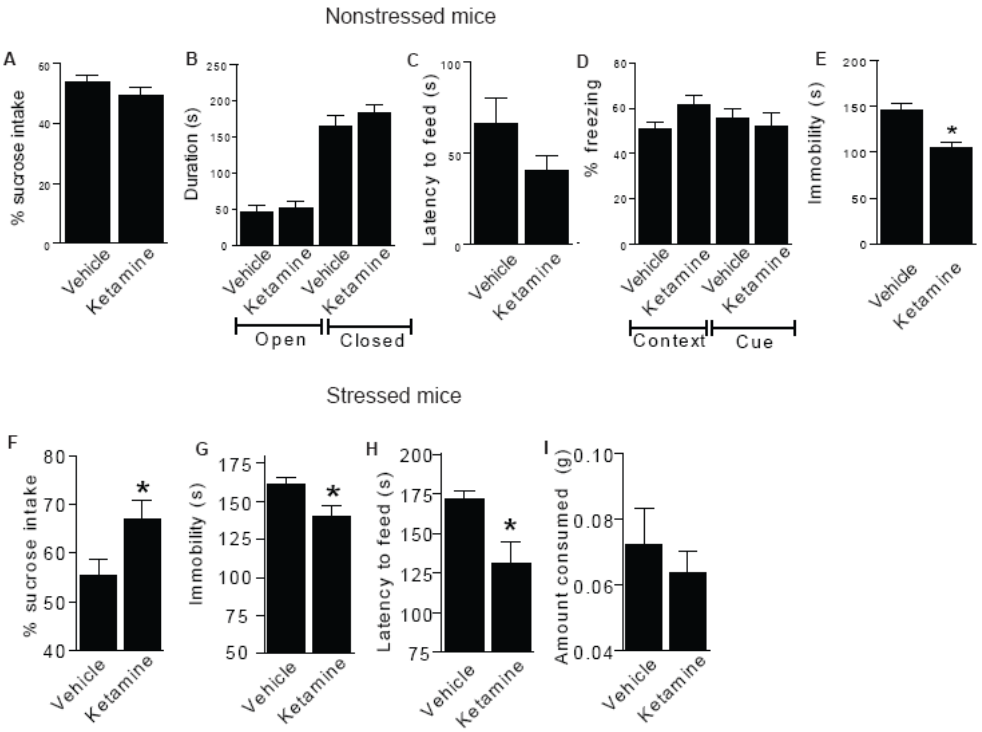


Figure 4-1. Behavioral effects of ketamine in non-stressed and stressed mice. (A) Thirty minutes after injection, ketamine treated mice show no differences in sucrose consumption compared to vehicle injected controls. (B) One day after injection, there is no difference in elevated plus maze behavior between groups. (C) Three days after injection, there is no significant alteration in novelty suppressed feeding behaviour. (D) Mice were trained for fear conditioning on day 5 and tested on day 6 and ketamine did not prevent acquisition of fear conditioning. (E) On day 7 mice, by t-test analysis the ketamine injected mice showed a significant reduction in immobility compared to vehicle controls on the forced swim test (*, $P<0.05$). Next, we assessed AD-related behaviours with ketamine after 28 days of chronic mild stress. (F) After stress, we observed significant AD-like effects with acute ketamine treatment in the SCT (*, $P<0.05$) assessed by t-test. (G) We also observed significant effects in the FST on the following day as demonstrated by t-test analysis. (H,I) Two days after ketamine administration, stressed mice displayed AD-like responses in the NSF test (*, $P<0.05$), with no confounding effects on appetite as shown by t-test analysis.

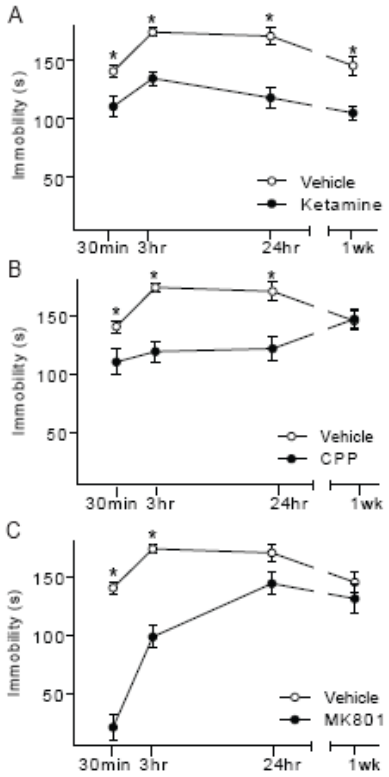
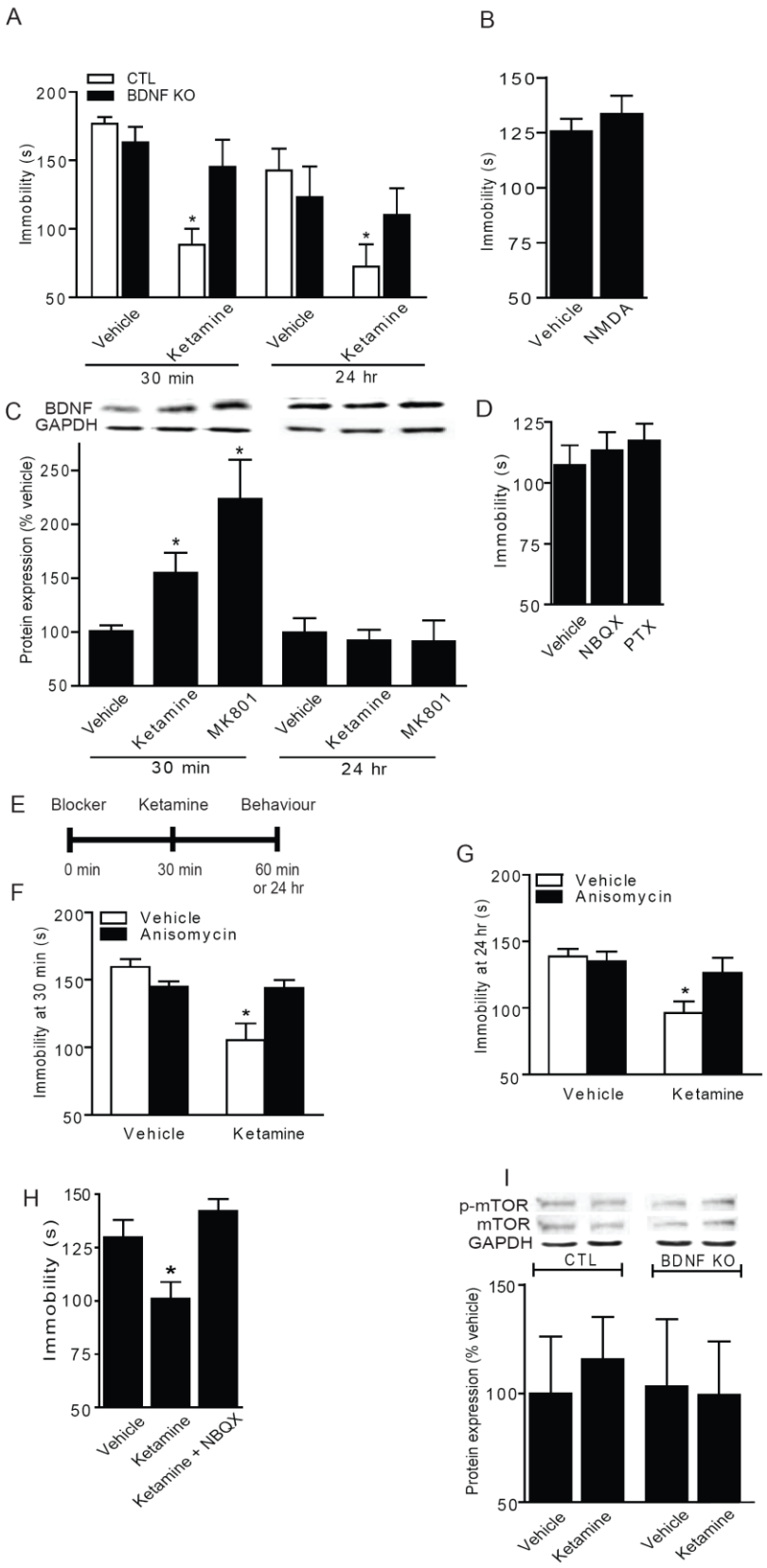


Figure 4-2. Time-course of NMDA receptor antagonist-mediated antidepressant-like behavioral effects. Independent groups of C57BL/6 mice were used at each time point for all drug treatments to avoid potential complications of behavioral habituation to the FST. ANOVA analysis revealed significant differences between the NMDA receptor antagonist treatments ($F_{3,27}$ = 30.31, P < .0001) and duration of response ($F_{3,27}$ = 19.06, P < .0001) with a significant interaction of AD-like effects between treatments and duration of response ($F_{9,81}$ = 9.32, P < .0001); therefore, we examined the treatment effect over time at each time point. (A) A single injection of ketamine (3.0 mg/kg, i.p.) produced a significant fast-acting AD-like response at 30 minutes compared to vehicle treated mice. This was also significant at 3 hrs, 24 hrs, and 1 week following injection (*; P < 0.05). (B) A single injection of CPP (0.5 mg/kg, i.p.) produced a significant rapidly acting AD-like response at 30 minutes compared to vehicle treated mice, as well as at 3 hrs and 24 hrs post injection (*; P < 0.05). The effect of CPP did not persist at the one-week time point. (C) A single injection of MK-801 (0.1 mg/kg, i.p.) produced a significant decrease in immobility at 30 min and at 3 hrs compared to vehicle injected mice (*; P < 0.05), but the AD-like response was absent by 24 hrs. Data represent mean ± SEM, n=10/group/time-point.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BDNF KO
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BDNF
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Protein expression (% vehicle)
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75
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Vehicle
NBQX
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Blocker
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BDNF KO
Protein expression (% vehicle)
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100
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Vehicle
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Vehicle
Ketamine

Figure 4-3. BDNF translation is involved in the antidepressant effects of NMDA receptor antagonists. (A) Thirty minutes following an acute injection of ketamine (3.0 mg/kg, i.p.), ANOVA analysis revealed a significant effect of drug ($F_{1,35} = 17.13$, $P = 0.0002$), as well as an interaction between ketamine and genotype ($F_{1,35} = 7.57$, $P = 0.0093$). Multiple comparisons with t-test showed that wild-type littermate controls injected with ketamine had a significant reduction in immobility at 30 minutes compared to vehicle controls (*; $P < 0.05$), while ketamine did not produce an AD-like effect in BDNF KOs. Twenty-four hours following injection, in separate groups of mice, a significant effect of ketamine was observed by ANOVA analysis ($F_{1,29} = 3.77$, $P = 0.0619$). Further multiple comparisons with t-test show the only significant difference was ketamine producing a significant reduction in immobility compared to vehicle injected littermate controls (*; $P < 0.05$). Ketamine did not produce a significant AD response in the BDNF KO mice after 24 hours (n=7-12/group). (B) C57Bl/6 mice were given vehicle (saline) or NMDA (75 mg/kg) and assessed in FST behaviour 30 minutes later. No significant differences were noted between treatment groups. (C) A single injection of ketamine (3.0 mg/kg, i.p.) or MK-801 (0.1 mg/kg, i.p.) produced a significant treatment effect ($F_{2, 12} = 6.77$, $P = 0.0108$) on BDNF protein levels at 30 minutes after injection; a posthoc test indicates a significant difference between vehicle and MK-801(*; $P < 0.05$) and between vehicle and ketamine (*; $P < 0.05$). ANOVA showed no significant treatment effect on BDNF protein levels 24 hours after injection (n=5-6/group). (D) Mice were treated with NBQX (10 mg/kg; i.p.) or PTX (1.0 mg/kg; i.p.) and tested in FST 30 minutes later. Neither drug altered FST behaviour ($F_{(2,27)}=0.46$, $P = 0.6370$). (E) Time line of anisomycin + ketamine and actinomycin D + ketamine treatments for 30 min and 24 hr FST behaviour. (F) Anisomycin (100 mg/kg, i.p.) was injected 30 min before ketamine (3.0 mg/kg, i.p.), and then mice were tested in FST 30 min later. ANOVA analysis demonstrated a significant difference between ketamine and vehicle injected animals ($F_{1,34}=11.83$, $P=0.0016$) and a significant interaction between groups ($F_{1, 34} = 10.91$, $P = 0.0023$). Focusing on multiple comparisons between groups, there was a significant difference between the ketamine injected group and the other groups (*; $P < 0.05$), with ketamine + anisomysin not altering immobility, indicating that anisomycin prevented the ketamine-induced AD-related response (n=8-10/group). (G) Anisomycin (100 mg/kg, i.p.) was injected 30 min before ketamine (3.0 mg/kg, i.p.), and then mice were tested in FST 24 hrs later. Significant differences between ketamine and vehicle injected animals ($F_{1, 31} = 9.34$, $P = 0.0046$). Multiple comparison analysis shows a significant difference between the ketamine injected group and the other groups (*; $P < 0.05$), with ketamine + anisomysin not altering immobility (n=8-10/group). (H) Mice were given a single injection of vehicle, ketamine (3.0 mg/kg), or ketamine and NBQX (10 mg/kg) and assessed for FST behaviour 30 minutes later. Ketamine produced a significant reduction in immobility but this effect was blocked with NBQX co-application ($F_{2,26}=8.226$, $p< 0.0019$). Bonferroni post-hoc analysis revealed significant differences between vehicle and ketamine treatment as well as ketamine and ketamine + NBQX treatments (*; $P < 0.05$). (I) Hippocampal

tissue from littermate CTL or inducible BDNF KO mice was collected 30 minutes after vehicle or ketamine administration. Phosphorylated and total levels of mTOR did not change after treatment, regardless of genotype ($F_{3,19}$=0.0889, P = 0.9651).

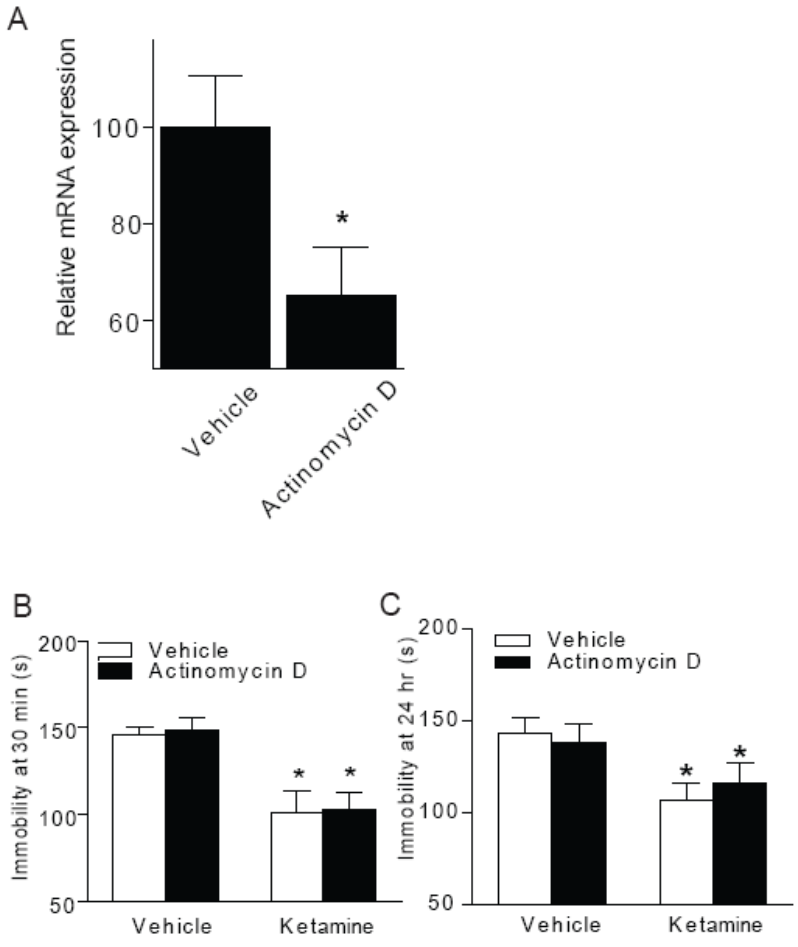
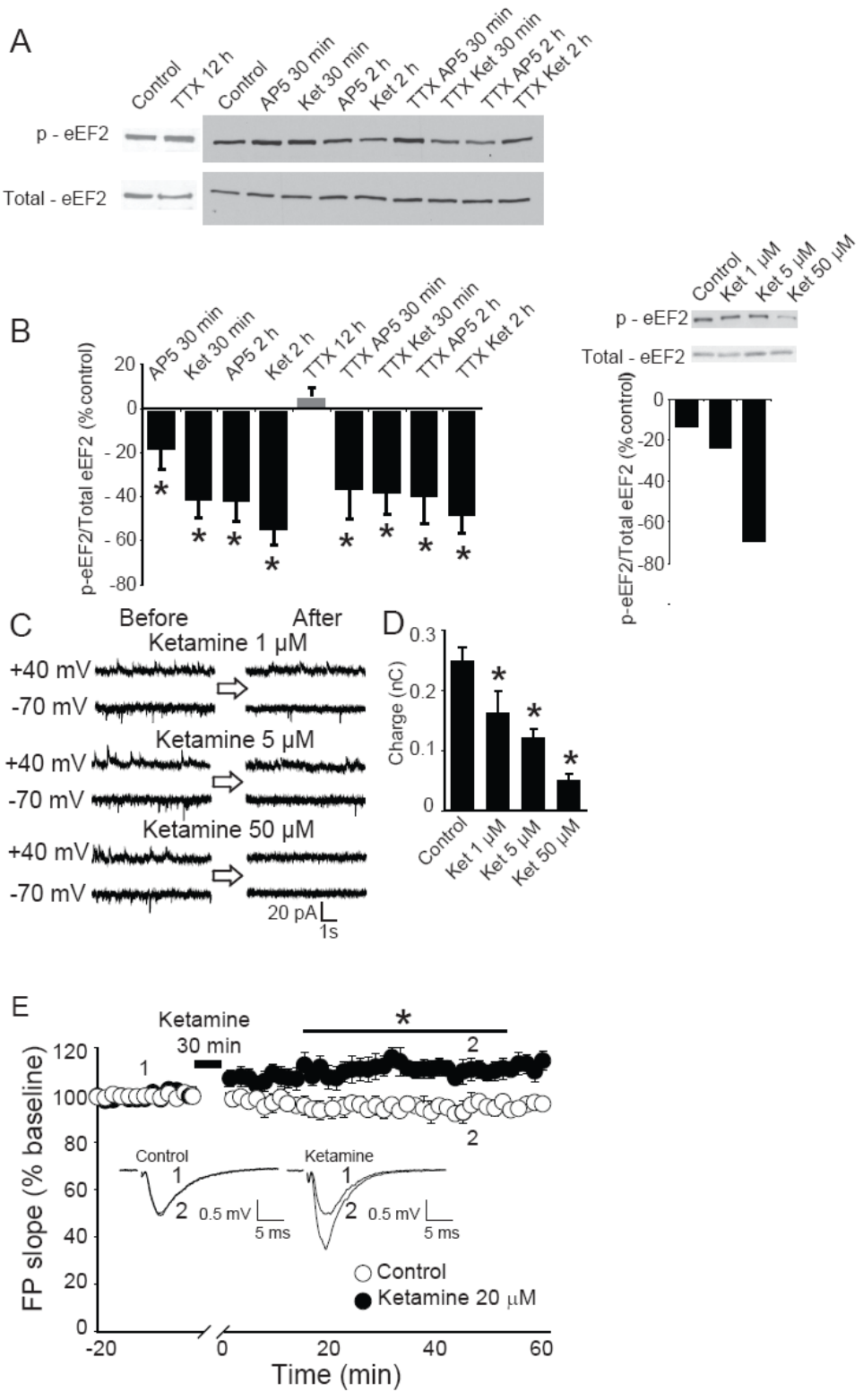


Figure 4-4. Actinomycin D decreases BDNF mRNA levels, but does not impact FST behavior. (A) BDNF expression is normalized to 18S. T-test analysis revealed a significant decrease in BDNF expression in actinomycin D treated samples compared to vehicle (*, P<0.05). (B) Actinomycin D (0.5 mg/kg, i.p.) was injected30 min before ketamine (3.0 mg/kg, i.p.), and then mice were tested in FST 30 min later. There were significant differences between the ketamine and vehicle-treated groups ($F_{(1, 34)} = 23.76$, $P < 0.0001$). T-test used for multiple comparisons between groups shows a significant difference between the ketamine injected and vehicle injected groups (*; $P < 0.05$), as well as a significant difference between ketamine + actinomycin D treatment compared to the actinomycin D treatment group alone (*; $P < 0.05$), demonstrating that actinomycin D does not prevent the ketamine-induced antidepressant-like response (n=8-10/group). (C) Twenty four hours later, ketamine still produced significant differences on immobility compared to the vehicle-treated group ($F(3,36) = 3.06$ $P < 0.0402$). T-test used for multiple comparisons shows a significant effect of ketamine and vehicle injected groups (*; $P < 0.05$).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p-eEF2/Total eEF2 (% control)
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- 20
- 40
- 60
-80
Control
Ket 1 μM
Ket 5 μM
Ket 50 μM
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Total - eEF2
p-eEF2/Total eEF2 (% control)
0
-20
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Ketamine 1 μM
+40 mV
-70 mV
Ketamine 5 μM
+40 mV
-70 mV
Ketamine 50 μM
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20 pA
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Charge (nC)
0.3
0.2
0.1
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Control
Ket 1 μM
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Ketamine
30 min
1
2
2
FP slope (% baseline)
120
100
80
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Ketamine 20 μM
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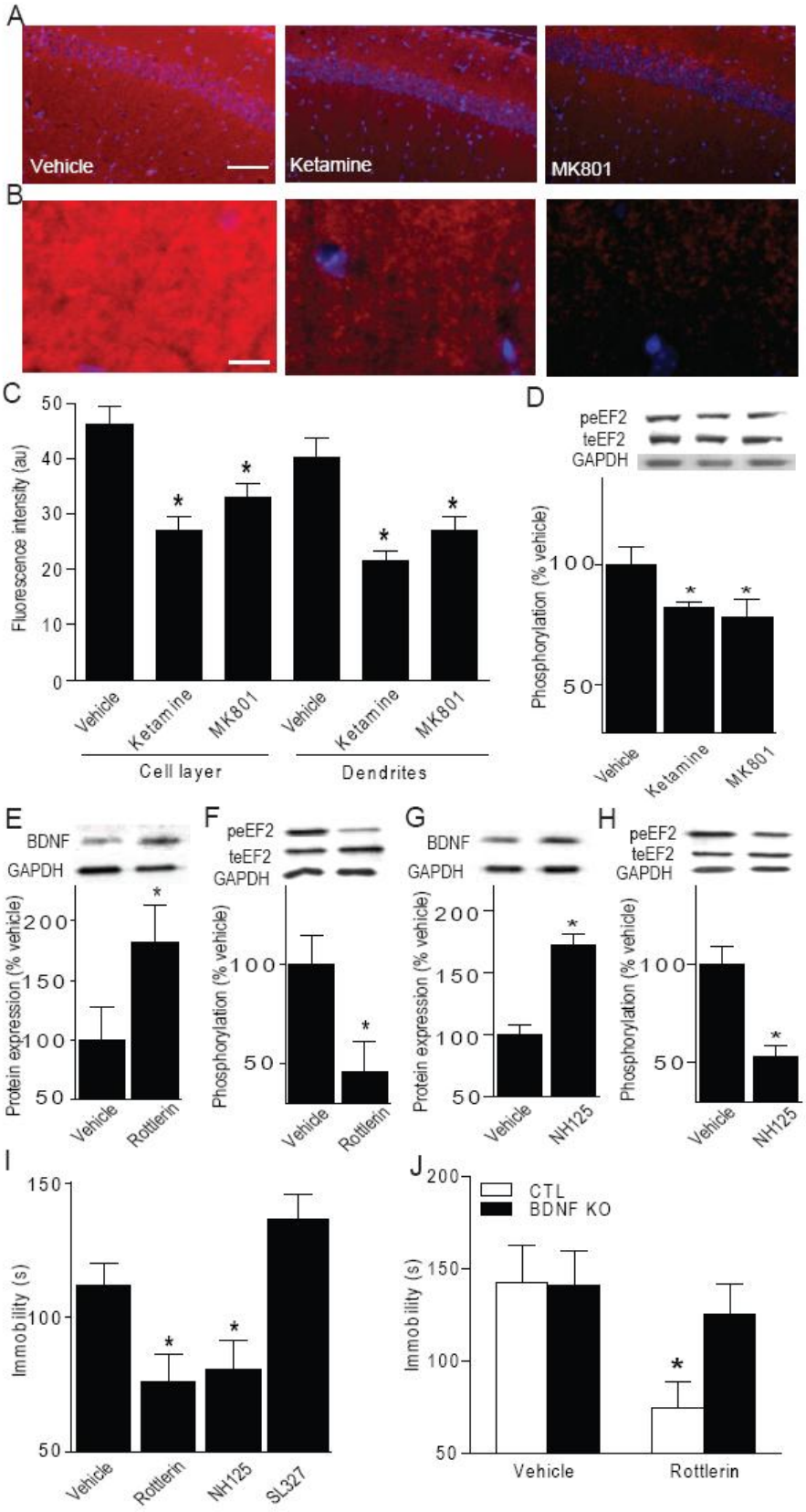
40
60
Time (min)

Figure 4-5. Ketamine blocks NMDAR spontaneous activity, reduces the level of eEF2 phosphorylation, and strengthens synaptic responses. (A) Representative western blots and summary data (B) (left) using antibodies specific to phosphorylated (Thr56) and total eEF2 are shown TTX alone slightly increases levels of p-eEF2 after 12 h of treatment. Application of AP5 or ketamine alone or in presence of TTX decreases the levels of p-eEF2 as assessed by t-test analysis (*; $P < 0.05$). (right) application of 1, 5 and 50 μM of ketamine for 30 in decreases the levels of eEF2 phosphorylation. (C) Application of 1, 5 and50 μM of ketamine blocks NMDAR spontaneous activity. NMDAR spontaneous activity was recorded at holding potentials +40 and -70 mV in modified Tyrode solution with (mM): 2 $CaCl_2$, 0 $MgCl_2$, 0.01 glycine, 0.001 TTX, 0.02 DNQX, 0.05 PTX. NMDA mEPSC's were recorded in modified Tyrode solution and then immediately after perfusion with modified Tyrode solution with different concentrations ofketamine. The charge transfer measured during 10 sec of NMDAR spontaneous activity at -70 mV is significantly reduced by approximately 1.65, 2, and 5 fold after application of 1, 5, 50 μM ketamine respectively. (D) Control n=16, Ket 1 μM n=8, Ket 5 μM n=11, Ket 50 μM n=6. T-test reveals significant effects (*; $P < 0.05$) for each concentration compared to control. (E) Field potential slopes are plotted (mean ± SEM) as a function of time. After 20 min of stable base line 20 μM of ketamine was applied for 30 min in the absence of stimulation, after this field potentials were recorded for 60 min (n=6). In control experiments, stimulation was stopped after 20 min of baseline and resumed after 30 min (n=5). Application of ketamine induced a significant increase in field potentials amplitude and slope compared to control. Representative field potential traces, (average 2 min) are shown for each experiment during baseline (1) and at the time indicated by the numbers on the graph (2). The asterisk refers to significantly different field potentials values ($p < 0.05$). For statistical analysis we used two way repeated measurements with Bonferroni post hoc analysis. The interaction effect (drug X time) was significant $F_{(143,1430)}=6.723$ $p < 0.001$.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MK801
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GAPDH
Phosphorylation (% vehicle)
100
50
Vehicle
Ketamine
MK801
E
BDNF
GAPDH
Protein expression (% vehicle)
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100
50
Vehicle
Rottlerin
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Vehicle
Rottlerin

Figure 4-6. Acute NMDA receptor antagonist treatment and inhibition of eEF2K result in antidepressant-like behavior mediated by a decrease in eEF2 phosphorylation and rapid BDNF translation. (A) Representative images of CA1 pyramidal cell layers and stratum radiatum dendritic layers in hippocampus 30 min after administration of vehicle, ketamine, or MK-801; scale bar represents 100 µM (red: peEF2 immunostaining, blue: DAPI counterstain). NMDA antagonist treatment decreased peEF2 levels. (B) Bottom panels depict 5X magnification of stratum radiatum; scale bar represents 20 µM. (C) Selected regions of the cell layer or dendrite layer of the same size were analyzed by ImageJ and assigned an average fluorescence intensity. ANOVA analysis revealed a significant decrease in peEF2 fluorescence in both cell layers ($F_{2,23}$=13.13, P=0.0002) and dendrites ($F_{2,23}$=14.06, P=0.0001) (n=4/group). (D) Western blot analysis also reveals a significant decrease in phosphorylation of eEF2 (F2,23=3.183, P=0.03) (n=8/group). (E-H) Western blot analysis of BDNF and peEF2 levels with eEF2K inhibitor treatment. (E,G) T-test analysis reveals a significant increase in hippocampal BDNF protein levels in rottlerin (5.0 mg/kg, i.p.) treated mice compared to vehicle treated controls (*; P<0.05), as well as in NH125 (5.0 mg/kg, i.p.) treated mice compared to vehicle controls (*P<0.05). (F,H) T-test analysis shows a significant decrease in peEF2 levels relative to total eEF2 in the hippocampus of rottlerin treated mice compared to vehicle controls (*; P<0.05) and in NH125 treated mice compared to vehicle treated controls (*; P<0.05). (I) A single acute (30 min) injection of rottlerin (5.0 mg/kg, i.p.) or NH125 (5.0 mg/kg, i.p.) in C57BL/6 mice recapitulates the fast-acting AD behavioural effects of NMDA antagonists *in vivo*. Significant differences between the various drug treatments ($F_{3, 44}$ = 8.13, P =0 .0002). T-test analysis shows a significant difference between the rottlerin and vehicle injected groups (*; P < 0.05), as well as between the NH125 and vehicle injected animals (*; P <0.05). The ERK inhibitor, SL 327 (10 mg/kg, i.p.), did not significantly alter AD responses compared to vehicle treated animals. (J) A single injection of rottlerin (5.0 mg/kg, i.p.) was administered to BDNF KO and littermate control mice 30 minutes prior to FST. Significant differences between rottlerin (5.0 mg/kg, i.p.) and vehicle treated groups ($F_{1, 19}$ = 5.77, P =0.0267). T-test analysis shows a significant decrease in immobility between rottlerin treated wild-type controls and vehicle controls (*; P < 0.05), suggestive of an AD-like response. Rottlerin did not alter immobility in BDNF KO mice, suggesting that BDNF is necessary for the AD-like effect (n=5-7/group). Data represent mean ± SEM.

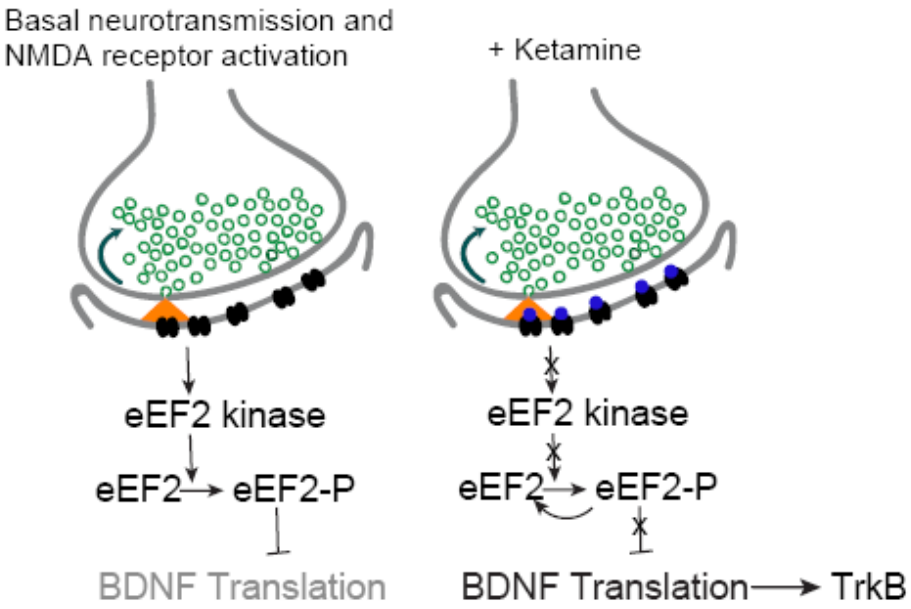

Figure 4-7. Linking glutamatergic signalling at rest and the regulation of BDNF translation. (Left) When neurons are at rest, spontaneous glutamate release and NMDA receptor activation leads to eEF2 kinase activation triggering eEF2 phosphorylation and silencing of BDNF translation. (Right) NMDA receptor blockade at rest in turn does not activate eEF2 kinase and desuppression of BDNF protein translation ultimately triggering TrkB signalling.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The preceding chapters describe our recent observations that define the role of BDNF signaling in depression-related and antidepressant-like behavior and furthermore illustrate a novel role for BDNF signaling in the mechanism underlying rapid antidepressant action. While these data extend our understanding of how BDNF is involved in the pathology of MDD and in the therapeutic effects of antidepressant compounds, there is still much to be discovered on a mechanistic level regarding the nature of MDD and mechanisms of rapid and effective clinical treatments.

We began our investigation by asking whether BDNF might impact susceptibility to depression-related symptoms in a gender-specific manner. In order to pursue this line of inquiry, we exposed inducible BDNF knockout mice, both males and females, to a chronic unpredictable stress paradigm. We observed that both male and female mice lost weight, regardless of genotype, after stress suggesting that the paradigm was stressful and that the mice did not habituate to CUS over time. However, regarding expression of depression-related behaviors subsequent to stress, females appeared to be more susceptible than males, and females with loss of forebrain neuronal BDNF expression appeared more vulnerable than wild-type females. We believe that these data suggest that, in females, BDNF may be an important factor to protect against susceptibility to specific aspects of stress-induced depression related behavior, while males

generally appear to be more resilient to this chronic unpredictable stress paradigm.

In the future, it may be important to determine whether loss of BDNF in males plays a role in susceptibility to other forms of stress. However, preliminary observations from our lab (and others) suggest that BDNF KO mice (and other lines of mutant mice) may not be suitable for chronic social defeat stress, a well-accepted paradigm associated with development of depression-related behaviors. Still, this paradigm of stress may be valuable in terms of dissecting out those stressors which may be more salient for males versus females.

Furthermore, we have replicated our finding that male BDNF KO mice have an increased level of CORT in the bloodstream as part of a separate study. This finding was particularly intriguing given the forebrain neuronal specificity of this BDNF mutation. It may be that administering excess exogenous CORT to BDNF KO male mice may reveal a susceptibility to developing depression-related behaviors.

We have investigated the circuit-level contribution of BDNF and TrkB signaling to the action of antidepressant compounds. Here, using floxed mouse models of BDNF and TrkB, we were able to selectively delete signaling molecule and receptor in the dorsal raphe nucleus. We were interested in the role of BDNF signaling in dorsal raphe nucleus neurons because these neurons are the serotonergic projection neurons into hippocampus. Previous studies in our lab have revealed that BDNF signaling may regulate activity of serotonergic autoreceptors located on dorsal raphe neurons. In our studies, we found that

TrkB, but not BDNF, expression in dorsal raphe neurons is essential for antidepressant efficacy. Deletion of BDNF or TrkB does not appear to grossly affect any other behavioral parameters.

In the future, it will be interesting to determine whether TrkB expression in DRN is necessary for efficacy of fast-acting antidepressants like ketamine or scopolamine. In addition, it is important to explore the relevance of TrkB signaling within hippocampus in order to delineate further the circuitry between dorsal raphe nucleus and hippocampus in terms of cell-autonomous or non-autonomous BDNF/TrkB signaling visa vis antidepressant signaling.

Finally, my studies have focused on determining mechanisms involved in rapid antidepressant efficacy. We studied the relevance of BDNF to the rapid antidepressant action of novel antidepressant ketamine. We have shown that ketamine rapidly induces expression of BDNF in hippocampus through enhanced protein translation. We propose that protein translation is engaged by a decrease in spontaneous NMDA receptor activity which dephosphorylates eEF2 to activate protein translation.

A key question raised by these observations is how the action of MK801 is distinct from the action of ketamine. We originally compared these two compounds because they have a similar mechanism of action, blocking the magnesium site in the pore of open NMDA receptors. However, it is known that MK801 has much higher affinity for the NMDA receptor, and additionally that ketamine has many non-specific effects. We have not thoroughly tested the possibility that off-target effects contribute to ketamine's long-lasting behavioral

antidepressant-like effects, we were able to show that targeting eEF2 kinase with rottlerine has similarly long-lasting effect, suggesting pathway specificity in this aspect of the ketamine response. We hypothesize that homeostatic plasticity may underlie the enhanced AMPA transmission after acute ketamine treatment. To clarify, we believe that during NMDA receptor blockade, homeostatic processes enhance membrane expression of AMPA receptors to compensate for decreased NMDA receptor activity. When NMDA receptor blockade is released, enhanced neurotransmission is unmasked and permits further downstream plasiticity. We believe that because ketamine has lower affinity for the NMDA receptor, this unmasking is rapid whereas the persistent blockade of NMDA receptors by MK801 allows for BDNF-mediated plasticity, but does not allow further plasticity uncovered by enhanced synaptic neurotransmission. This question is intriguing and would be essential to test in order to further define the notion that homeostatic mechanisms are involved in ketamine's behavioral and synaptic plasticity.

We have demonstrated a link between de-phosphorylation of eEF2 and spontaneous neurotransmission. However, we have not yet identified the phosphatase responsible for this de-phosphorylation and whether this factor is regulated by neuronal activity. Biochemical studies have proposed that phosphatase 1 alpha (PP-1 alpha) may be linked to eEF2 de-phosphorylation, but direct evidence is lacking. Experiments to mechanistically link a phosphatase to spontaneous neurotransmission, either PP-1 alpha or a known activity-regulated phosphatase like calcineurin, would be of utmost interest.

We are currently undertaking a behavioral characterization eEF2 kinase knockout mice. We are first planning to determine whether eEF2K KO animals are able to respond to ketamine, in order to determine specificity of ketamine's rapid antidepressant behavioral effects to this pathway. In the future, it will be interesting to discover whether an animal model of stress is associated with enhanced phosphorylation of eEF2 (given that recent evidence suggests that spontaneous neurotransmission is enhanced in the acute learned helplessness model of stress) and whether loss of this kinase confers stress-resilience to animals.

An idea was proposed to us by a reviewer of our paper that we may be able to distinguish the importance of dendritic protein synthesis of BDNF by utilizing a mutant mouse strain in which dendritic transport of BDNF mRNA was deficient. We were unable to obtain this strain in order to address this point, but if we do procure these mice in the future, it may be interesting to observe whether ketamine is effective in this mouse strain. However, this issue may be more tractable in culture or in slices wherein the addition of anisomycin can be selectively applied to dendrites.

Currently, we are not certain whether the effect of BDNF is on pre- or post-synaptic neurons and also whether ketamine's behavioral effect is primarily mediated in hippocampal neurons. We are interested in addressing this question *in vivo*. We believe that by selectively deleting BDNF or TrkB in CA1 or dentate gyrus, we may be able to determine whether one sub-region is essential for

ketamine's behavioral antidepressant effects, and whether this effect occurs cell-autonomously or non-autonomously in pre- or post-synaptic neurons.

Recently, a paper has suggested that the behavioral effects of ketamine are mediated through a protein-synthesis dependent increase in dendritic spines (Li et al., 2010). This study examined a later timepoint, 24 hours, after ketamine treatment to observe activation of the mTOR signaling pathway and enhanced number of dendritic spines, while our study primarily examined the acute 30 minute timepoint after ketamine treatment. We were unable to replicate their findings of mTOR activation at this timepoint as illustrated in Figure 4-3I. We went on to examine whether we could block the ketamine behavioral response with a pre-treatment of rapamycin (1 mg/kg i.p.). Again, we were unable to show alteration of the ketamine behavioral response (Figure 5-1) as did the Li et al. study, though their observations were made using an *icv* injection of rapamycin. Careful examination of the rapamycin effect, particularly keeping in mind that previous studies have demonstrated that rapamycin (4.0 mg/kg, 4 daily injections) acts as an antidepressant (Cleary et al., 2008) is necessary. Therefore, I propose that forced swim test behavior be observed in a time-course and dose-dependent manner in order to parse out whether rapamycin does or does not produce an antidepressant effect in relevant animal models. Additionally, it would be important to examine dendritic spine number both at 24 hours as well as at a longer timepoint, such as one week, in order to determine whether the finding regarding spine number can be replicated and extended to a chronic timepoint at which we know ketamine-mediated behavioral effects persist.

The same study proposed that the PI3K and Akt pathways, which lie downstream of TrkB activation, may be responsible for ketamine's behavioral antidepressant-like effects. However, these authors looked at behavior 24 hours after ketamine treatment, not the rapid timepoint we have examined. Given that we believe that rapid insertion of AMPA receptors may underlie enhance synaptic transmission following acute ketamine treatment, study of activation of phospholipase C gamma that rapidly release intracellular calcium stores which enriches synaptic AMPA receptor content would be very insightful.

In conclusion, the current research has furthered our understanding of the complex role of BDNF/TrkB signaling in depression, traditional antidepressant- and novel rapidly acting antidepressant-responses. These studies open many new exciting avenues of research and pose important questions. I hope that this research will challenge the field to explore these questions further and foster new ideas regarding the role of BDNF and neurotransmission in MDD and MDD therapies.

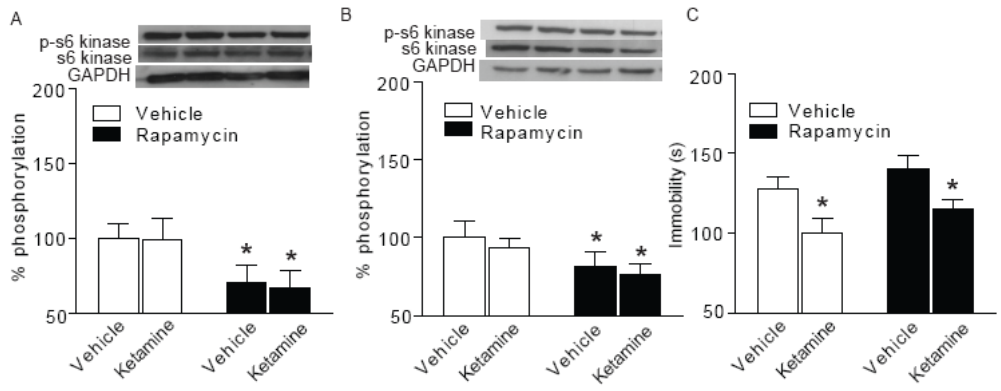

Figure 5-1. Acute rapamycin treatment does not impact ketamine-mediated antidepressant responses. Phosphorylation of mTOR target S6 kinase is unaffected by ketamine treatment, but decreased by rapamycin treatment in both (A) frontal cortex ($F_{3,21}$=6.468, p<0.0189) and (B) hippocampus ($F_{3,26}$=5.071, p<0.0330). (C) Forced swim test analysis reveals that 1.0 mg/kg rapamycin does not impact significant effects of ketamine on immobility time at 30 minutes (F3,37=4.998, p<0.005).

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